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Co-Evolution of Secondary Metabolites

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Co-Evolution of Secondary Metabolites

With 204 Figures and 48 Tables

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Preface

This book is like a dream project putting together metabolic aspect of complex biological processes like pollination, symbiosis, herbivory by insects, and volatiles released by plants in their atmosphere. Most of these processes are very complex and produce very small amount of metabolites, which remains a challenging task to detect and quantify. Though the chemistry and biosynthesis of secondary metabolites is increasingly well studied, less attention is paid to their evolutionary and interactive aspect. Almost all plants are attacked by insect herbivores, pests, and animals and they cannot escape from being non-movable unlike animals. Therefore, they evolve and conserve several defensive traits to combat pests by various types of chemical weapons. Improvement in tools of chemical analysis like GLC, HPLC with sensitive sensors and detectors such as mass spectrometer, and high-throughput screening along with gene expression using transcriptome analysis paved the way for analyzing, detecting, and identifying these molecules, small or large, in quantities unnoticeable with old prevailing technology.

Therefore, this book presents state of information about secondary metabolites produced in plants during interaction with parasites, pollinators, pests, and herbivores. As secondary metabolites are specialized classes of compounds biosynthesized by different pathways involving several genes, this is an interesting evolutionary mechanism to adapt to the changing host or the pests by modifying the secondary metabolites. Secondary metabolites play a crucial fundamental biological role in a plant's life, and genetic changes are required to execute the energy-expensive process.

The book *Co-evolution of Secondary Metabolites* is divided into six parts covering the entire gamut of bioactive molecules present in plants. This includes phenomena like diversity within plant, changes in secondary metabolites during adaptation of plants to life on land, and involvement of secondary metabolites in pollination, allelochemy, abiotic stress, host–parasite interaction, sensory perception, insect–plant interaction, and plant defense. These interactions are vital for survival of plants and their pests and have evolutionary consequence.

This book is planned as a reference work providing state-of-the-art knowledge composed by highly renowned scientists of the field. Well-recognized international specialists in their respective fields of research contributed the chapters. This book will be useful to all those working in the field of botany, evolutionary biology,

phytochemistry, physiology, molecular biology, biotechnology, and plant pathology. This book is arranged in 36 well-illustrated chapters.

We would like to acknowledge the cooperation, patience, and support of our contributors who have put serious efforts to ensure the high scientific quality of this book with up-to-date information. We are thankful to the staff at Springer, namely Dr. S. Blago and N. Clifford, for their professional support in this project.

February 2020

Professor Jean-Michel Mérillon
Professor Kishan Gopal Ramawat

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About the Editors

Jean-Michel Mérillon
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Prof. Dr. Jean-Michel Mérillon received his Pharm.D. (1979), Ph.D. (1984), and HDR (1992) from the University of Tours in France. He joined the same university as assistant professor in 1981 and became associate professor in 1987. In 1993, he moved to the Faculty of Pharmacy, University of Bordeaux, France, accepting a position as full professor. He is currently leading the "study group on biologically active plant substances" at the Institute of Vine and Wine Sciences, which comprises 25 scientists and research students. The group has been working on phenolic compounds from vine and wine for many years, mainly complex stilbenes and their involvement in health. Prof. Mérillon has supervised the doctoral theses of 20 students. He is involved in developing teaching on plant biology, natural bioactive compounds, and biotechnology.

Prof. Mérillon has published more than 150 research papers in internationally recognized journals, resulting in an H index of 48 (documents published between 1996 and 2019). He has co-edited books and reference works on secondary metabolites and biotechnology.

Throughout his career, Prof. Mérillon has traveled widely as a senior professor. Scientists from several countries have been and are working in his laboratory, and his research is supported by funding from the Aquitaine Regional Government, the Ministry of Higher Education and Research, and various private companies. In 2004, he founded the technology transfer unit

"Polyphenols Biotech," providing support for R&D programs for SMEs and major groups from the cosmetic, pharmaceutical, agricultural, and health-nutrition sectors.

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Prof. Dr. Kishan Gopal Ramawat is former professor and head of the Botany Department, M.L. Sukhadia University, Udaipur, India, and can look back on longstanding research experience. He received his Ph. D. in Plant Biotechnology in 1978 from the University of Jodhpur, India, and afterward joined the university as a faculty member. In 1991 he moved to the M.L. Sukhadia University in Udaipur as associate professor and became professor in 2001. He served as the head of the Department of Botany (2001–2004, 2010–2012); was in charge of the Department of Biotechnology (2003–2004); was a member of the task force on medicinal and aromatic plants of the Department of Biotechnology, Government of India, New Delhi (2002–2005); and coordinated UGC-DRS and DST-FIST programs (2002–2012).

Prof. Ramawat had done his postdoctoral studies at the University of Tours, France, from 1983 to 1985, and later returned to Tours as visiting professor (1991). He also visited the University of Bordeaux 2, France, several times as visiting professor (1995, 1999, 2003, 2006, 2010) and Poland in 2005 during an academic exchange program (2005). Through these visits in France, Prof. Ramawat and Prof. Mérillon established a strong connection, which has resulted in productive collaborations and several book and reference work publications.

Prof. Ramawat has published more than 170 well-cited peer-reviewed papers and articles and edited several books and reference works on topics such as the biotechnology of medicinal plants, secondary metabolites, bioactive molecules, herbal drugs, and many other topics. His research was funded by several funding agencies.

In his research group, Prof. Ramawat has supervised doctoral thesis of 25 students. He is an active member of several academic bodies, associations, and editorial boards of journals.

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Part I

Metabolic Co-evolution

Co-evolution of Secondary Metabolites During Biological Competition for Survival and Advantage: An Overview

1

Kishan Gopal Ramawat and Shaily Goyal

Contents

Abstract

Plants produce secondary metabolites which are involved in several biological processes and interactions with other organisms from microbes to insects to higher plants. These processes are variously termed as plant-plant interaction, allelopathy, herbivory, parasitism and mutualism, and induction of plant protection by other microorganisms. Plants are under selection pressure to protect themselves from herbivores/parasites, whereas herbivores/parasites struggle for their survival from plant defense to obtain food and reproduction site. Plants develop defense mechanism from herbivores over a period of 400 million years. Therefore, both develop various strategies to adapt and adjust with changing environment. In this introductory chapter, a brief review of co-evolution of secondary metabolites not only to complete the biological process but also to compete with each other for survival is presented.

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Keywords
Co-evolution · Pollination · Plant defense · Secondary metabolites · Allelopathy · Biotic stress

1 Introduction

Photosynthesis is the largest photochemical reaction on the planet Earth producing carbohydrates. Besides carbohydrates, proteins and oils are other primary metabolites. These metabolites are not only used by humans for nutrients and energy but also by other animals, birds, insects, and a variety of lower animals and microorganisms. Therefore, there is not only competition between plants for obtaining light, water, and nutrients but also for protection from harmful biotic predators. Thus life of the plants is not as simple as it appears and they have to evolve various morphological and physiological characteristics to fight against all odd situations and survive. On the same time, predators have to survive by obtaining nutrition from their host plants and produce progeny by reproduction. How these hosts and their dependents developed various mechanisms to survive is a focus of this book.

Besides primary metabolites, plants produce various classes of secondary metabolites also, generally in lower quantities than primary metabolites. These secondary metabolites are synthesized by various pathways mainly from shikimic acid pathway and mevalonic acid pathway by several steps and from primary metabolites. Three major classes, alkaloids, phenolics, and terpenes, are recognized for secondary metabolites, and these secondary metabolites are considered more like waste products, generated by plants, in the absence of excretory systems [1–4]. Secondary metabolites are present almost in all living organisms, even in bacteria and prominently in immune system-lacking organisms [5]. With the progress of science, now we know that these metabolites are involved in plant defense, deterrent to predators and herbivore, and signaling molecules in pollination, animal and insect attraction, communication between host and pathogen, and various biological events. Various biological processes in which secondary metabolites play important role are presented in Fig. 1. These events are not only important for the host plant but also for the survival of microorganisms, insects, and other organisms (plants and animals both). However, the functions of secondary metabolites are not limited to defense alone. Synthesis of secondary metabolites demands energy and resources. The total available content of a plant sample is the sum of synthesis and degradation/utilization of secondary metabolites. Sometimes during the growth phase of the plants, production of secondary metabolites is minimized, or secondary metabolites are used as substrates [6]. Secondary metabolite content also varies from juvenile to mature state of the plant, particularly during reproductive stage. Intraspecific variation is reported in many plant species which is further influenced by latitude [7].

Interactions among organisms influence their population, phenotypes and genotypes. Often these ecological interactions are termed as co-evolution. Some described co-evolution as perusal of patterns of interaction between two major groups of organisms with a close and evident ecological relationship, such as plants

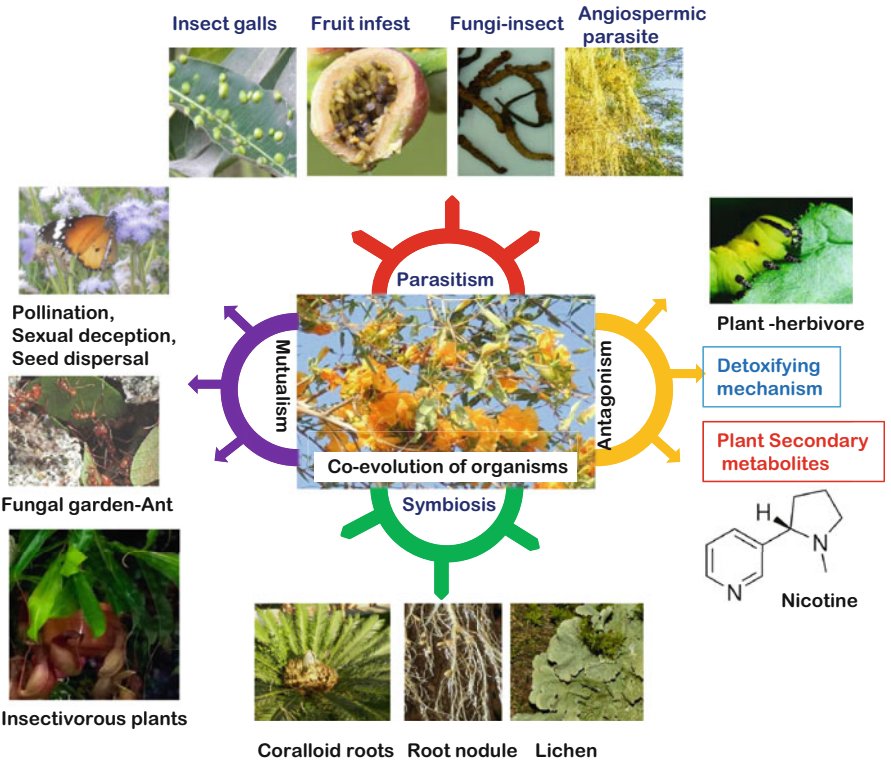


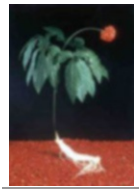
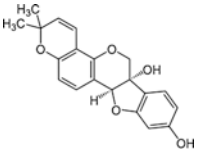
Fig. 1 Overview of biological processes co-evolved in two competitive organisms for better survival in which secondary metabolites are involved

and herbivores [8]. Various interactions involve secondary metabolites of host plants whether it is pollination or plant defense. Interaction of host plants to herbivore insects is evident in fossil records of plants during Paleozoic period (300–400 million years ago) when first vascular plant and arthropods emerged [9, 10]. Since then biological processes involving plants and insects have co-evolved which require rapid adaptation to changed morphological and physiological characters. Involvement of different secondary compounds in various interactions and their functions is presented in Table 1. In this brief overview, we have summarized biological processes influenced by the presence or absence of secondary metabolites and make a win situation for the plant or its predator.

2 Diversity and Adaptation

Intraspecific diversity can increase the community structure and productivity of the species and affects population dynamics and ecosystem functions [16, 17]. It can also modify the properties of associated herbivore communities and plant fitness.

Table 1 Secondary metabolites involved in various biological processes (compiled from references [1, 11–15])

Functions	Compounds	Plant species
Plant defense		
	Glucosinolates	Cabbage, Brassicaceae
	Phytoalexin: capsidiol, allixin	*Glycine max*, garlic
	Stilbenes-resveratrol	Grapevines, mulberry
	Withanolide-withferinA (saponin)	*Withania somnifera*
	Ginsenoside Rg1(saponin)	Ginseng (*Panax ginseng*)
	Bacoside A3(saponin)	*Bacopa monnieri*
Signaling molecules for parasites		
	Strigolactones	For *Striga* from hosts sorghum and maize
	(+)-Orobanchol	For *Orobanche* spp. from red clover
	Alectrol	For *Striga* and *Orobanche* spp. from hosts cowpea and red clover
Antimicrobial and antifungal		
 Glyceollin I	Biochanin A and genistein	Pulses (*Glycine max*), *Pueraria* species
	Quercetin	Red onions, peppers, apples, grapes, black tea, green tea, red wine
	Glabridin	*Glycyrrhiza glabra*
	Glyceollins	*Glycine max*
	Pterostilbene and resveratrol	*Pterocarpus marsupium*, grapevine
Pollination		
	Anabasine, nicotine	*Nicotiana glauca*
	Caffeine	*Citrus* spp., *Coffea* spp.
	Cyanogenic glycoside: amygdalin	*Prunus amygdalus*
	Phenolic: gallic acid	*Fagopyrum esculentum*
Poison and deterrent		
	Alkaloids, terpenoids, and phenolics	*Nicotiana tabaccum*
	Repellents, deterrents, antidigestive (protease inhibitor), induction of SM production by microbes	*Tagetes* spp.; inhibition of protein digestion in insects and Acari, induced resistance in plants
	Herbivore-induced plant volatiles	Attraction of the natural enemies (parasitoids and predators) of the herbivores

The diversity can be genetic diversity or chemical diversity, and both diversities can help in reducing herbivory. This diversification in plants has co-evolved with herbivore populations [18]. Consequently herbivores and pathogens put great

selection pressure on plants. Plant genes involved in defense and virulence genes of the pathogens are polymorphic in their genome and hence change rapidly with selection pressure. This can be termed as gene-to-gene interaction in which genes of both sides evolved (modify) to counteract each other [19]. Such variations play important role in creating the ecological niche differentiation among individual plants. Such intravariations reduce competition and support coexistence [20]. Glucosinolates in cabbage are an excellent example of variation in secondary metabolites in host and protection from herbivores. Glucosinolates are an important group of secondary metabolites mainly present in cabbage and mustard family (Brassicaceae). A recent study on wild cabbage showed that increased variation in glucosinolates among neighboring plants correlated positively with associated insect community and negatively with plant damage [21]. In another study, the dynamics of glucosinolates in the perennial wild cabbage, in response to herbivory by *Pieris rapae* caterpillars, was studied, and it was recorded that herbivore-induced changes in the concentrations of aliphatic glucosinolates were population-specific and their concentrations were found to increase in primarily one population only [22].

Adaptation of plants to land was a major breakthrough toward the requirement to synthesize and diversify secondary metabolites. Colonization of land by phototrophic organisms is confronted with a hostile environment for these organisms as land has low content of mineral nutrition, harmful UV radiation from the sun, high variation in day and night temperature, as well as frequent drought. All of these abiotic factors played important role in adaptations to the terrestrial environment. Mutualism was one of the major strategies to share fight against all odds, and lichens are an example to colonize new habitats (see ► Chap. 9, "Lichen Metabolites: An Overview of Some Secondary Metabolites and Their Biological Potential" in this book). Fossil records show that lichens might have originated even before evolution of vascular plants. On land, plants were exposed to different stress conditions, and in order to survive, they began to synthesize an array of secondary metabolites. These secondary metabolites helped them in attracting pollinators and defending predators and hence adapting to grow successfully in their ecological niche. Probably today's secondary metabolites are the outcome of pre-existing molecules from primary metabolism in algae and bryophytes [23, 24]. Some of the precursors are detected in Charophycean algae, mosses, and hornworts [25–28]. It was concluded from these works that land plants (Bryophytes and vascular plants) are descended from green algae-like ancestors. Present-day green algae and vascular plants might have a common ancestor, bifurcating at initial evolutionary stage (Fig. 2). This conclusion is based on phylogenetic analysis using DNA sequence data of a large number of algal and vascular plants. As a part of evolution, plant lineage continues to synthesize new compounds and limit the synthesis of others. Sometimes plant lineage synthesizes secondary metabolites which are already present in different pedigree to fulfill same type of functions [29].

Plants also started to develop secretory structures like resin ducts and laticifers. These are thought to be first apparent as intracellular oil bodies in liverworts. These secretory structures were also a significant adaptation, as they are being able to sequester secondary metabolites and defense proteins [30]. Further, an important development was biosynthesis of lignin and the origin of lateral meristems, which

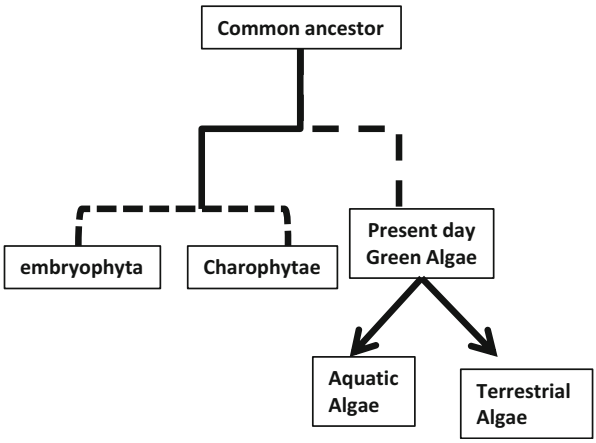


Fig. 2 Common ancestry and phylogenetic relationship of vascular plants based on DNA sequence data. (Based on reference [27])

were important in development of large trees. This hard-to-decay lignified wood is decomposed by lignin-decomposing fungi (*Ascomycetes* and *Basidiomycetes*), the evolution details of which are still not clear [31, 32]. Adaptation of plants to land also gave rise to synthesis of volatile secondary metabolites. Plant volatiles can be produced by both anabolic and catabolic processes.

3 Biology of Survival

Three important aspects of plant discussed in this section are pollination, host-parasite relationship with its angiosperm parasite, and plant-plant interactions. Autotoxins are phenolics from root exudates or decaying plant debris which inhibit the growth of own plants; the phenomenon is known as "autotoxicity" and exhibited by several crop plants such as alfalfa, cucumber, rice, and wheat. Several secondary metabolites including phenolics play important roles in plant-plant interaction by inhibiting root ion and water uptake, membrane permeability and cell cycle, protein biosynthesis, respiration, photosynthesis, and many other physiological processes [33, 34]. These phenolics also influence the quality and quantity of soil microorganism which in turn affects the plant growth and health by mutualistic and pathogenic effect as well as by modulating nutrient cycle [35].

Pollination is an important biological process in plant's life. Some plants totally depend on pollinators for pollination and reward the pollinators by nectar [36, 37]. However, there are many organisms that feed on nectar without helping the plant in pollination. They are called as robbers. These robbers can range from being insects to birds to mammals. Among insects different species of bees are the main robbers, like *Xylocopa* spp., *Bombus* spp., *Trigona* spp., etc. The robbers and pollinators are usually attracted toward a plant by its floral traits and in particular nectar. Nectar is rich in sugars and amino acids and also secondary metabolites, such as alkaloids, phenolics, and nonprotein amino acids. These secondary metabolites

are thought to repel some nectar robbers and sometimes potential pollinators [38]. Flower parts have a strong effect on the fitness of a plant and, thus, are protected by relatively high amounts of defensive secondary metabolites. Plants' secondary metabolites are known to be present in good amount in leaves, but some reports suggest that flowers have more amounts of secondary metabolites in comparison with leaves. Thus, allocation of secondary metabolites should be directed to the most valuable tissues, hence flowers. Similarly, 1,2-saturated pyrrolizidine alkaloids (T-and iso-phalaenopsine) that are produced in every tissue of *Phalaenopsis* orchid hybrids are the highest in the pollinia [39]. Chemical differences between nectar, pollen, and flowers indicate that plants can regulate these compounds in specific tissues [40, 41] as can be seen in *Delphinium* sp. where its nectar, anthers, corollas, stems, and pollen contain similar alkaloids differing only in their concentration, suggesting a similar origin [42].

Pollinators have the ability to find nectar-rich flower and avoid the toxic nectars. It is generally seen that they become proficient in avoiding toxic nectar by their past experiences [43]. There are some examples, like moths learning to avoid quinine by recognizing its odor in food [44] and bumble bees learning to avoid alkaloids gelsemine or quinine [45]. Toxicity of nectar is generally associated with the secondary metabolite concentrations and its combination ratio with the carbohydrate, but still there are some secondary metabolites like cyanogenic glycoside amygdalin, which are non-detectable by the honey bees even in the sucrose solutions [46, 47]. Besides all these examples, it is interesting to know that some alkaloids may optimize pollination service without being beneficial to the pollinator. As shown by Wright and co-workers [48], caffeine in food affects the perpetuation of bees returning to the food source.

The presence of secondary metabolites in nectar, apart from helping in deterring the robbers and herbivores, has other advantages as well. Secondary metabolites prevent the nectar spoilage from microbes [49] and increase the resistance in pollinators against parasites and pathogens [50]. Besides the presence of secondary metabolites in fruits and seeds helps in seed and fruit dispersal by attracting birds and animals. There are many reports showing induced plant responses to herbivory as a process of resistance offered by plants. These inductions effect the plant and pollinator interactions as well. In a study on wild tomato, *Solanum peruvianum*, herbivore-induced emission of volatile organic compounds alters pollinator behavior and consequentially affects plant fitness [51]. Leaf herbivory by *Manduca sexta* induced alkaloids in the nectar of *Nicotiana tobaccum* [52], and leaf damage in *N. sylvestris* increased nicotine concentrations in *N. sylvestris* flowers [53]. It is still unclear that the presence of secondary metabolites in nectar and flowers, and the pollinators acquired adaptations toward SMs has been co-evolved? Some studies suggest that pollinators evolved in response to the secondary metabolites. Furthermore, some studies suggest that pollinators impose selection pressure on plants, especially on floral traits, so it is rational to say that they might have co-evolved in some cases [54].

Angiosperm parasitic weeds *Orobanche* (~28 spp.) and *Striga* (~100 spp.) pose a serious threat to crop plants, while *Cuscuta* is a problem of both trees and crop

plants. Life cycle of these obligate angiosperm parasitic plants depends upon the presence of a suitable host and chemical signal released by the hosts (Table 1) [55]. Therefore, chemical cue plays an important role in life cycle of these parasitic plants and shows co-evolution of chemical biology of host and parasite (see ▶ Chap. 5, "Field Dodder: Life Cycle and Interaction with Host Plants" in this book). More information will be available with development of sensitive tools of molecular biology showing gene expression in parasites.

4 Competition for Survival

Plants, cultivated crop plants and weeds, compete with each other in field for available resources for survival. Rhizosphere is a biologically active soil zone where different plant roots compete for water, nutrients, and space. Here the roots communicate with neighboring plants and symbiotic and pathogenic organisms by the release of root exudates. It is suggested that these exudates play an active role in root-root and root-microbe communication by manipulating the biological and physical interactions between these interacting organisms. These chemicals can change the physical, chemical, and biological properties of the soil and inhibit the growth of competing plant species [56]. Thus, these exudates mostly play a role of phytotoxins in the process of allelopathy.

Allelopathy was defined as influence of one plant on another through releasing of chemicals into the environment [57]. Although the definition of allelopathy is not confined to positive or negative aspect in particular, most of the studies are done on the harmful effect of allelochemicals/phytotoxins. Plants release phytotoxins in decomposing plant tissue, in green leafy volatiles, in leachates from live tissue, and in root exudates [58, 59]. Sometimes these phytotoxins change the chemistry of the soil for quite a long time. For example, the phenolic compounds and constituent diterpenes from *Cistus ladanifer* L. exudates are toxic and harmful to germination and growth of herbaceous plants. These allelochemicals are incorporated into the soil through leaching of leaves and litter, whereas the flavonoids enter the soil through litter degradation. The long retention of these compounds in litter will maintain their phytotoxic levels for prolonged periods of time, without the need for continuous supply of these compounds from the plant [60, 61].

Allelochemicals are mostly a wide variety of secondary metabolites such as phenolics, cyanogenic glycosides, quinones, lactones, organic acids, and volatile terpenes. The rich diversity of secondary metabolites evolves because of selection for improved defense mechanisms against a broad range of microbes, herbivores, and plants. These allelochemicals affect the other organisms by inducing a secondary oxidative stress via producing reactive oxygen species (ROS). This as a result, increases antioxidant enzyme activities and synthesis of molecular antioxidants (glutathione, ascorbate, tocopherol, (−)-catechin). The allelochemical from root exudates of *Centaurea maculosa* triggers a wave of reactive oxygen species (ROS) in *Arabidopsis thaliana*, which leads to a Ca2 + signaling cascade triggering genome-wide changes in gene expression and, ultimately, death of the root system [11, 62]. Similarly, a study on *Lactuca sativa* suggested that β-cembrenediol

Table 2 Examples of well-known invasive plants in India exhibiting strong allelochemical effects on crop plants and surroundings (compiled from [64, 65])

Plant species	Effects known	Native country
Ageratum conyzoides (Asteraceae)	Allelopathic, highly invasive, (Himalayan region); ageratochromene, precocene I, and precocene II have strong insecticidal effects, endo-borneol, farnesol, quercetin, kaempferol, and its glucosides	Tropical America
Argemone mexicana (Papaveraceae)	Harms native flora through allelopathy; salicylic acid, *p*-hydroxybenzoic acid, vanillic acid, cinnamic acid	Tropical America
Eichhornia crassipes	Water hyacinth, N-phenyl-2-napthylamine, anti-algal activity	Tropical region of South America (Brazil)
Lantana camara (Verbenaceae)	Cytotoxic lantadene A and lantadene B, salicylic acid, gentisic acid, coumarin, ferulic acid, p-hydroxybenzoic acid, and 6-methyl coumarin; lantolonic acid, ursolic acid, and oleanolic acid (in roots)	Central and South America
Parthenium hysterophorus (Asteraceae)	Carrot weed, aggressive colonizer, highly allelopathic, causes allergy to animals and human being; phenolics and sesquiterpenes cause threat to crops and other native flora	Tropical Central and South America
Prosopis juliflora (Fabaceae)	Tannins, flavonoids, steroids, hydrocarbons, waxes, and alkaloids	Native to Mexico, South America, and the Caribbean

(an allelochemical from tobacco) causes an oxidative damage through enhanced generation of ROS, as indicated by increased lipid peroxidation, disruption of membrane integrity, and impacted mitosis, and thus ultimately results in growth inhibition of the *L. sativa* plants [63].

It has been observed that allelopathic species are more successful as invaders. Invasion of aggressive allelopathic species causes habitat loss of local species. This is not only a global problem but also has economic, biodiversity, and environmental consequences [64]. A few selected examples of invasive plant species in India are presented in Table 2. Most of these species entered India with imported wheat seeds, whereas *Prosopis juliflora* was introduced for fuel wood, shade, and combating soil erosion in desert. *P. juliflora* has become a major invader in Africa as a whole [65]. Disruption of native communities by the invader's allelochemicals suggest that native plant communities are more tightly knit entities and that invasion disrupts inherent, co-evolved interactions among long-associated native species. Comparing the competitive ability of species of the same genus against other species from the native and non-native regions of invasive species can provide insight into the role of evolutionary experience with different competitors [66]. Plant communities develop this balanced, harmonious, and dynamic stability by acquiring resistance to allelochemicals of the associated allelopathic species. It has also been established that longtime neighbors are more stable in comparison with new associations.

Adverse environmental conditions, biotic or abiotic generates stress on plants; consequently modification in physiological signaling and metabolism results in the production of defense-related metabolites. Biosynthesis, transport, and concentration of metabolites are affected by stress on plants [67]. Abiotic stresses such as drought, salinity, cold, toxic metals/metalloids, ozone, and UV-B radiation are important factors affecting plant growth, development, and reproduction. Plant evolved various strategies to combat these adverse and changing environmental conditions. Several genes, including those for protein kinases and transcription factors, have been identified which are involved in abiotic response of plants [68]. According to John Thompson [69], locations where different abiotic and biotic conditions promote evolution are called "co-evolutionary hotspots," and locations where conditions do not promote co-evolution are called as cold spots. Both plant-plant and plant-environment interactions generate stress on plants. Plants have to evolve and develop mechanism to cope with this situation or perish with time.

5 Role in Plant-Insect Interaction

Plants defend themselves against insects and microorganisms by physical means (e.g., thick cuticle, spines) or physiological actions (the presence of toxic chemicals or antifeedants). However, they cannot protect themselves from large herbivores (mammals and others). Plant-herbivore contact is one of the largest interactions involving from microorganisms to vertebrates. All groups of animals (~25–30%) and phytophagous insects (26%) feed on green plants [70, 71]. Plants have evolved defense mechanism against insect herbivores over a period of 400 million years [72].

Herbivory is an important selection pressure on plants resulting in the development of new chemicals and physical traits for defense. Simultaneously, herbivores develop new detoxifying mechanisms to tolerate or degrade the toxic chemicals of plant defense, e.g., *Argemone* species are equipped with prickles as well as alkaloid-containing resin [7]. There is intra- and interspecific variation in defense chemicals in this plant and its organs. It is not possible to identify modification against a weapon, but process is continuous.

Living organisms, as small as bacteria, protozoa, and fungi to as large as vertebrates, can detect and select useful odor among thousands of odors available in the surrounding. Olfactory receptors are responsible for this property of living things [73]. About 2500 plant species are known to contain cyanogenic glucosides. When plant tissues are damaged by herbivores, β-glycosidase and α-hydroxynitrile lyase enzymes degrade cyanogenic glucosides to toxic hydrogen cyanide. Hydrogen cyanide is a potent mitochondrial respiratory chain inhibitor and thus provides defense against herbivores. Findings with transcriptome analysis of plant showed that the gene for cyanogenic glucoside degradation might have horizontally transferred from bacteria [74]. Herbivore insects maintain a close relationship with their host plants because hosts provide food, mating site, oviposition site, and habitat for whole or part of their life cycle. This requires quick adaptation and possible changes to cope with variation of the host plants. This selection pressure can be noticed in survival of insects with new varieties showing phenotypic and physiological changes

[75]. On the same pattern, soil microorganisms like bacteria and fungi affect growth and health of plants in several ways. These beneficial microbes may provide broad-spectrum resistance to insect herbivores. These beneficial microbes adapt plant defenses against insect herbivores. Beneficial soil microorganisms can regulate hormone signaling including the jasmonic acid, ethylene, and salicylic acid pathways, consequently changing gene expression, biosynthesis of secondary metabolites, plant defensive proteins and different enzymes, and volatile compounds that may induce defenses against leaf-chewing as well as phloem-feeding insects [12].

Synthesis of secondary metabolites is unique feature of plants. Secondary metabolites deter herbivores but on the same time protect herbivores from parasitic infection. However, little is known about the impact of secondary metabolites of nectar on pollinators. Recently, Richardson and colleagues [13] showed that alkaloids, terpenoids, and iridoid glycosides present in secondary metabolites (~61–81% of total secondary metabolites) reduced the parasitic load of bumble bee. Besides, secondary metabolites of plants have other beneficial effects on herbivores such as enhancing memory and foraging efficiency [48, 76], reducing parasite infection [77], and controlling pathogenic fungi [78]. Therefore, there are evidences and possibility that secondary metabolites can play tritrophic interactions among plants, pollinators, and parasites. However, how this affects bee's survival and reproduction in respect of the pros and cons of chemical consumption is yet to be known [13]. Thus there are strong evidences that host plant and its herbivores/pollinators are in continuous process to adapt and evolve to match the counter defense. This process of co-evolution is described in the chapters of this book.

6 Conclusion

Biological processes like pollination, symbiosis, plant damage by herbivore insects, and volatiles released in atmosphere are complex and produce very small amount of metabolites. With the development of new tools of chemical analysis like GLC, HPLC and high-throughput screening along with gene expression using transcriptome analysis paved the way for analyzing, detecting, and identifying these molecules, small or large, in quantities unnoticeable with old prevailing technology. These techniques enable us to detect small changes in cell sap, environment, and even microorganisms and insects. Sensitive image sensors can generate useful but huge data which need to be used for understanding the plant-insect relationship and developing resistant varieties [79, 80]. Gene expression technology is also evolving rapidly to monitor the minute physiological and gene expression changes in plants.

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Plant-Insect Interaction: The Saga of Molecular Coevolution

2

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Contents

Abstract

Plant-insect interaction is a prime and evolutionarily successful association that offers an excellent platform to understand coevolution. These interactions have spurred speciation and are vital players of ecological dominance. The emergence of mutualistic relationship leads to the inception of molecular coevolution and diversification of plants and insects. Here, we have cataloged the various molecular factors that drive the establishment and progression of plant-insect interactions. An imbalance in the mutualistic relationship initiated the molecular arms

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race between plants and insects that resulted in a plethora of defense molecules in both counterparts. We have discussed the molecular events involved in the interaction, plant defense mechanism, and strategies employed by insects to combat plant defense. Furthermore, we have also focused on the coevolution of these molecules and their implications on plant-insect dialogue. We believe that this chapter will provide detailed molecular insights involved in the plant-insect interaction.

Keywords
Plant-insect interactions · Coevolution · Diversification

Abbreviations

CO	Carbon monoxide
DAMPs	Damage-associated molecular patterns
FACs	Fatty acid amino acid conjugates
GLV	Green leaf volatiles
GOX	Glucose oxidase
GRN	Gustatory neurons
GRs	Gustatory receptors
GSH	Glutathione
GST	Glutathione-S-transferase
H2O2	Hydrogen peroxide
HAMPs	Herbivore-associated molecular patterns
HIPVs	Herbivore-inducible plant volatiles
JA	Jasmonic acid
MAMPs	Microbe-associated molecular patterns
MYA	Million years ago
OPDA	12-oxophytodienoic acid
ORN	Olfactory neurons
ORs	Olfactory receptors
P450	P450 monooxygenase
PAMPs	Pathogen-associated molecular patterns
PRR	Plant recognition receptors
SA	Salicylic acid
UGT	UDP glucosyl-transferase
VOCs	Volatile compounds

1 Introduction

After the origin of life, the earth started to flourish with a plethora of life forms. These life forms establish different kinds of interactions among themselves and the surrounding environment. Some groups of organisms have exclusive mutual interactions, which drive their coevolution. Though there is a considerable debate on the timeline of plant-insect interactions, it is one of the most primitive and successful

coevolved systems. An epic allusion can be found in Charles Darwin's book "On the Origin of species." He states that these plant-insect interactions could date back to the Devonian period (420 million years ago [Mya]), as plants established themselves on land. Plant-insect interactions became prominent in the Carboniferous period (250–320 Mya) which is characterized by the appearance of insect pollination and continue to dominate even today [1]. These interactions have resulted in a vast diversification of plant and insect species [2].

The first evidence of plant-insect interaction is that of pollinivory and goes as far back to the early Permian era (298 Mya) [3]. Furthermore, the emergence of angiosperms is followed by an immediate shortfall of insect diversity. This can be attributed to the time taken by insects to adapt and start feeding on seeds and fruits. The advent of terrestrial plants caused insects to develop mouthparts that assisted them to feed on seeds and other plant parts. Nectar-consuming insects (Hymenoptera and Lepidoptera) came into existence in the late Cretaceous period after the appearance of pollen and nectar-producing plants (early Cretaceous period) [2]. Thus, plants and their composition have a great influence on insect evolution.

The key feature in the success of these interactions is the adaptability of plants and insects to generate diverse chemical compounds and utilize them for their survival. The various chemicals synthesized by plants attract insects for pollination or parasites for pest infestation. In addition, the arena of plant chemicals also acted as defense compounds against herbivorous insects [4]. Depending on the level and type of insect attack, plants modulated the production and distribution of these defense metabolites. In response to this, insects developed functional survival tactics like sequestration, detoxification, and repellence to combat negative or toxic effects of defensive secondary metabolites [5]. The stress of toxic metabolites on insects induced selection pressure that led to the emergence of resistance to phytochemicals. Thus, mutualistic coevolution turned into a molecular warfare that resulted in the generation of vast diversity in plant secondary metabolites [6] (Fig. 1).

Here, we are discussing various aspects of chemical coevolution of plants and insects in response to their interactions. We have cataloged plant metabolites, their role in insect response, and the modulation of their interplay due to the evolution of chemicals.

2 Plant Metabolites Involved in Establishment of Interaction with Insects

Plant-insect relationship is a bidirectional process. For instance, upon insect feeding, plants elicit specific defense response against them. In order to trigger insect-specific defense, plants need to differentiate between physical injury and insect feeding. It has been reported that insect's oral secretion or oviposition fluid contain specific active compounds called elicitors. These are sensed by plants and are responsible for the activation of downstream signaling cascades related to defense [7]. Also in contradiction, oral secretions have been seen to suppress plant defense machinery [8].

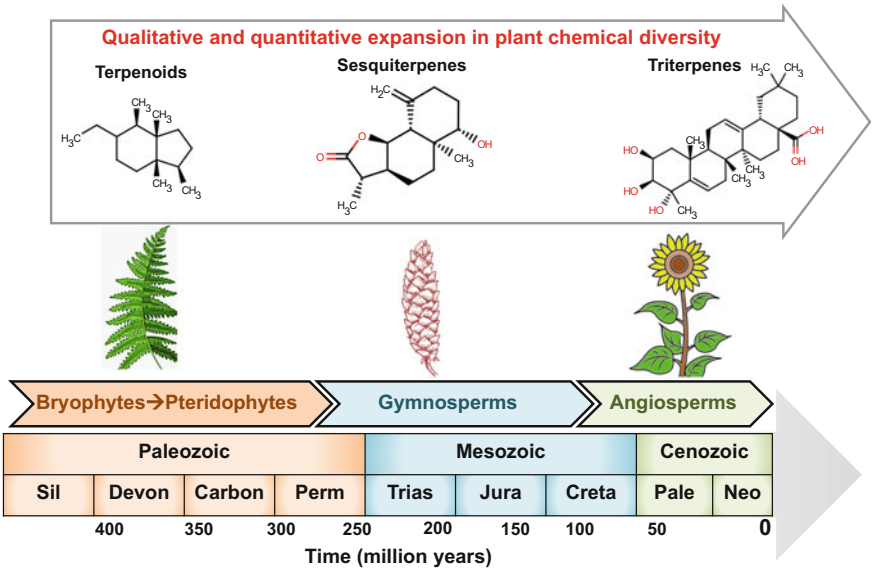

Fig. 1 Evolution of plants and change in the diversity of plant secondary metabolites along the geological timescale. As seen in the figure, an increased complexity of secondary metabolites is observed in higher plants. The evolution of different resistance strategies in insects caused plants to develop complex secondary metabolites that have a larger impact on a wide range of herbivores

On the basis of chemical structure and composition, elicitors are classified into six different groups, namely: enzymes, fatty acid amino acid conjugates (FACs), fatty acids, peptides, esters, and benzyl cyanide (Table 1). Of these, enzymes, fatty acids, FACs, and peptides are present in the oral secretions, whereas esters and benzyl cyanide are secreted in oviposition fluids during egg deposition [7, 9, 10]. Each type of elicitor has a different mode of action and effect. We have discussed the congruity and irregularity of several elicitors in the following sections.

2.1 Elicitors from Oral Secretions

2.1.1 Enzymes

One of the first insect elicitor to be identified and reported was an enzyme elicitor, β-glucosidase, from *Pieris brassicae* regurgitate [11, 12]. This β-glucosidase activates the plant defense and triggers the release of an array of volatiles in cabbage, lima beans, and corn plants [4, 13]. These released volatiles attract the parasitoid *Cotesia glomerata*. Thus, the β-glucosidase in *P. brassicae* induces indirect plant defense [11]. Along with chewing herbivore, this type of elicitor is also found in sucking insects. *Nilaparvata lugens* exhibits β-glucosidase, causing an increase in the levels of Jasmonic acid (JA), hydrogen peroxide (H_2O_2), and ethylene [14]. These induce

Table 1 List of different elicitor molecules and its insect source

Class		Elicitor	Insect	Reference
A	**Oral secretions**			
1.	**Enzymes**	β-glucosidase	*Pieris brassicae*	[12]
		Glucose oxidase	*Helicoverpa zea, H. armigera, H. assulta, Ostrinia nubilalis*	[17, 134, 135]
		Lipase	*Schistocerca gregaria*	[23]
		Alkaline phosphatase	*Bemisia tabaci*	[26]
2.	**FACs**	Volicitin [N-(17-hydroxylinoleoyl)-L-glutamine]	*Spodoptera exigua Teleogryllus taiwanemma and T. emma Drosophila melanogaster Menduca sexta*	[136, 137]
		N-linolenoyl-L-glutamine and N-linoleoyl-L-glutamine	*S. exigua, M. sexta, T. taiwanemma, T. emma, D. melanogaster, B. triannulella*	[30, 31, 137]
		Fatty acid glutamine and glutamic acid conjugates (C14-C18)	*S. exigua, S. frugiperda, S. litoralis, Heliothis virescens, Epirrita autumnata, Operophtera, Chloroclysta truncata*	[138]
		N-(15,16-Dihydroxylinoleoyl)-glutamine and N-(15,16-epoxylinoleoyl)-glutamine	*S. frugiperda, S. exigua*	[139]
		Phosphorylated derivative of N-acyl glutamine	*S. exigua*	[8]
3.	**Peptides**	Inceptin	*S. frugiperda*	[38]
4.	**Fatty acids**	Caeliferins	*Schistocerca americana*	[41]
B	**Oviposition secretions**			
5.	**Esters**	Burchins	*Bruchus pisorum Callosobruchus maculatus*	[140]
6.	**Benzyl cyanide**	Benzyl cyanide	*P. brassicae*	[42]

downstream signaling cascade and release of volatiles like dodecenal, tetradecane, etc., which attract the parasitoid *Anagrus nilaparvatae* [14].

β-glucosidases are compartmentalized away from the inactive, glucosidically bound volatiles i.e., glucosinolates [15]. Upon insect feeding, mixing of these two elements results in β-glucosidase action and release of volatiles – isothiocyanate, thiocyanates, and nitriles [8, 15, 16]. This elicitor is not directly involved in ligand-receptor binding and indirectly induces plant defense [10]. However, the exact mode of action of β-glucosidase is yet unknown.

Furthermore, glucose oxidase (GOX) discerned in the saliva of *Helicoverpa zea,* led to the production of high levels of JA in *Solanum lycopersicum* and outburst of Salicylic acid (SA) in *Nicotiana attenuate* [10, 17]. GOX is an enzyme catalyzing

oxidation of glucose to form gluconic acid and H_2O_2. An increase in endogenous levels of H_2O_2 has shown to levitate ethylene production in tobacco [18]. Thus, it has been postulated that GOX triggers plant defense by increasing H_2O_2 levels. Apart from various Lepidopteran species, GOX has also been determined in *Apis mellifera*, *Myzus persicae*, and *Schistocerca americana* [19–22]. Another enzyme discovered in *Schistocerca gregaria* is lipase, which is found to trigger high levels of cyclopentenone 12-oxophytodienoic acid (OPDA) [23]. OPDA is a substrate of JA and has also shown a direct effect on plant defense activation [24, 25]. Other enzymes like alkaline phosphatase in *Bemisia tabaci*, and digestive enzymes in aphids have also been discovered as elicitors in insect saliva [26]. However, their role in elicitation of plant defense system is yet unexplained.

2.1.2 Fatty Acid Amino Acid Conjugates

Fatty acid amino acid conjugates (FACs) is a broad class of insect elicitors and accord to a majority of insect oral secretions. It comprises of a fatty acid moiety (mostly linoleic or linolenic acid or their hydroxylated and phosphorylated forms) linked to an amino acid [27]. Predominantly in FACs, the fatty acid component is of plant origin, whereas the amino acid is derived from insect gut [12]. An exception to this is volicitin, wherein both the fatty acid and amino acid (glutamine) are plant-derived.

Volicitin (N-(17-hydroxylinolenoyl)-L-glutamine) was the first determined insect elicitor in *Spodoptera exigua* and caused the release of terpenoids [27]. Insects acquire linolenic acid from plants, which is then hydroxylated to glutamine for the production of volicitin [28]. Additionally, other volicitin conjugates, N-(17-hydroxylinoleoyl)-L-glutamine, N-linolenoyl-L-glutamine and N-linoleoyl-L-glutamine were also found in *S. exigua* oral secretions [29]. *N*-linolenoyl-L-glutamine and/or *N*-linoleoyl-L-glutamine were the prime FACs found in *Manduca sexta, Teleogryllus taiwanemma, Teleogryllus emma*, *Drosophila melanogaster,* and primitive Lepidopteran species, *Helcystogramma triannulella* [8, 30, 31]. Thus, it was proposed that they might be the ancestral compounds [31]. Recently, insect N-linolenoyl-L-glutamine induced an outburst of Reactive Oxygen Species (ROS) in *Arabidopsis* [32]. L-glutamine is prevalent in FACs and perhaps plays a crucial role in protein binding and activating plant volatile release. Moreover, a study showed that among the L-linolenic acid conjugates, only L-glutamine exhibited elicitation in *Zea mays* [33].

Volicitin showed strong binding to the plasma membrane fractions of *Z. mays*, suggesting binding of volicitin to plant membrane protein. This further activates MAPK pathway and plant defensive response [24]. Also, FACs are involved in nitrogen metabolism of insects. Any variation in FACs metabolism can lead to various adverse effects in insects. Therefore, insects preferably might not modify FACs.

2.1.3 Peptides

Peptides indirectly allow plants to perceive the feeding damage caused by insects. Due to the action of insect salivary proteases, plant proteins get digested into small

peptides. These salivary peptides from insects are further detected by plants via pattern recognition receptors (PRR). These receptors present on the plasma membrane of plant cells possess an extracellular domain that specifically binds to the herbivore or microbe-associated molecular patterns (HAMPs or MAMPs) [34].

These endogenous peptides (derived from plants) are classified into several families such as systemins, Peps (plant elicitor peptides), HypSys (Hydroxyproline-rich systemin), Inceptin, and Subpep [35, 36]. Peptide elicitors were first discovered in fungus *Trichoderma viride* and were later reported in various insect species [37]. Here, we have discussed about inceptin, as it is the only peptide type that occurs in insects, acting as herbivore-associated molecular patterns (HAMPS). The other four families of peptides are present in plant and get activated on wounding or herbivore attack. Thus, these are recognized as damage-associated molecular patterns (DAMPS) [34].

Inceptin, an active 13 residue protein, is a proteolytic fragment of ATP synthase γ subunit derived from plant chloroplast and is known to trigger the production of ethylene, JA, and SA [38]. In case of *Spodoptera frugiperda,* inceptin was reported to be involved in indirect insect recognition by inducing the release of ethylene in *Vigna unguiculata* [39]. Inceptins bind to the PRRs and commence the downstream defense signaling cascade in plants. As inceptin is specific to chloroplast ATP synthase, stem, root, and pod borer insects might not be perceived by plants through inceptins [34].

2.1.4 Fatty Acids

Fatty acid elicitors dominantly present in insect oral secretions are sulfoxy fatty acids [7, 40]. This class of elicitors typically contains saturated and monounsaturated sulfated α-hydroxy fatty acids. As these fatty acids are commonly found in Caelifera (Orthoptera), they are termed as "caeliferins." They were first determined in *S. americana* and occur in insect regurgitate [41]. In most of the cases, they activate ethylene and JA pathways of plant defense. It is demonstrated that synthetic fatty acids induce the release of ethylene and JA in *Arabidopsis* [10]. Interestingly, these are present in oviposition fluid as well. However, the molecular mechanism of fatty acid elicitation needs to be studied.

2.2 Elicitors from Insect Oviposition Fluid

Along with food and shelter, plants also serve as a site for insect's oviposition. Insects secrete an array of fluids from the ovary and posterior parts of the body during oviposition. These secretions either coat the newly laid eggs or are present at the plant-egg interface and trigger plant defense in both circumstances. Chemically, these elicitors largely encompass esters.

Esters are long-chained α,ω-diols (C_{22} to C_{24}), which are mono- or diesterified by 3-hydroxypropanoic acid. These have been distinguished in bruchid beetles and are also known as "bruchins" [42]. As a result of these bruchins, plants produce a tumor-like structures at oviposition site to obstruct entry of newborn larvae and also induce

plant immunity at an extremely low concentration of 1 fmol [42]. Expression of plant defense genes like pathogen-associated molecular patterns (PAMPs) in *Arabidopsis thaliana* was triggered during *P. brassicae* oviposition [43]. In case of *Diprion pini*, bruchins caused an outburst of plant terpenoids and reduction in ethylene in *Pinus sylvestris* [44].

Along with esters, benzyl cyanide may also be discharged during oviposition. Benzyl cyanide is obtained by female flies from males that acts as antiaphrodisiac and prevents further female mating [45]. It is secreted by the female accessory gland and induces plant defense. In *P. brassicae*, it is known to elevate the ROS levels in *Arabidopsis*. Benzyl cyanide also causes leaf surface aberrations, callose formation, and restricts the entry of *Trichogramma brassicae* newborns [46, 43].

Thus, there are several chemical strategies that plant adapts to perceive insect presence and activity. Further on insect detection, plant activates a spectrum of chemical defense mechanisms to maintain its survival and fitness.

3 Evolution of Chemical Defense in Plant against Herbivore

As biotic stress, herbivores impose a great threat to the plants. To counterattack herbivores, plants produce a pool of toxic, deterrent, and volatile compounds. Plant chemical defense system against herbivores can be direct or indirect. Also, it could be constitutive (phytoanticipins; already present in the tissue) or inducible (phytoalexins; synthesized and released only after the attack of herbivore) (Fig. 2).

Till date, it has been observed that compounds called secondary metabolites released by plants in response to stress have a pivotal role in herbivore defense [47]. They mostly possess a deterrent and toxic activity against the insects. But the role and mode of action of most of the secondary metabolites in insect resistance are enigmatic. Many studies have been carried out to uncover the ancestral metabolite's prime function and their evolution [48]. In recent past, with advancement in analytical methods and sophisticated tools, researchers have elucidated the role of some specific metabolites against the insect.

Depending on the chemical structure, secondary metabolites can be classified into three groups: phenolic- (lignin, tannins, flavonoids, coumarins, ravonaids, and phenolic acids), nitrogen and sulfur-containing compounds (glucosinolates and terpenoids), and nitrogen-containing compounds (alkaloids). They display distinction in terms of their occurrence and abundance throughout plant tissues. This variability in distribution and concentration of phytochemicals could be attributed to their tissue specificity, developmental stage specificity, or stress response. Upon insect feeding, secondary metabolite level upsurge in localized tissue, followed by their increment in systemic tissues. The disparity in feeding behavior could instigate production of specific secondary metabolites. A clear difference is observed in the plant defense response against chewing and sap-sucking insects. Chewing insects (Orthoptera, Coleoptera) elicit a strong plant response similar to that of wounding, whereas mild plant defense response is triggered by sap-sucking insects [7]. For example, *Spodoptera littoralis* (chewing insect) feeding increased JA levels and a

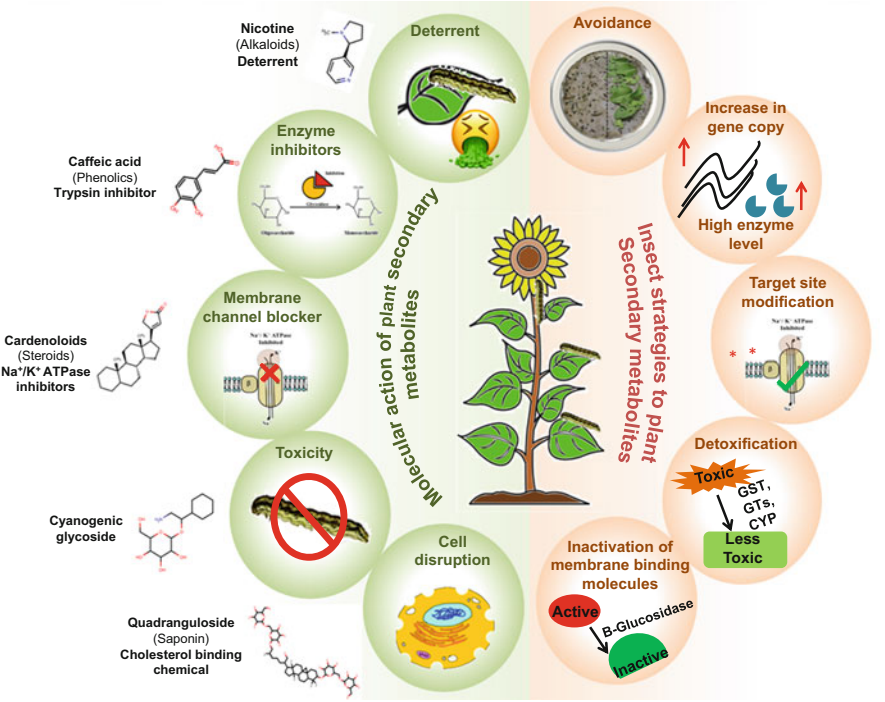

Fig. 2 The mode of action of various plant secondary metabolites on insects and their counter-defense by the insects to these compounds. Plants produce a variety of plant secondary metabolites, which target a range of insect molecules and impose a great threat to the herbivores. As a counter-attack, insects have developed strategies to prevent the adverse effects of plant secondary metabolites. For example, the insect enzymes detoxify the harmful plant allelochemicals and thus prevent their toxic effect

wide range of sesquiterpenes was released. Whereas, in *Tetranychus urticae* (sap-sucking insect), very low diversity of the volatiles were liberated [49].

The class and level of secondary metabolites also play a vital role in the decision of host plant and the scope of herbivory. Any alteration or fluctuation in the levels and composition of plant-derived allelochemicals can greatly influence the plant-insect interaction dynamics. As a specialized behavioral modification, insects subject to minimal exposure of highly toxic tissues [50]. We now discuss in detail about each type of secondary metabolites in order to get an idea about the intricacies of plant defense.

3.1 Phenolics

Phenolics represent a prime class of plant secondary metabolites, profoundly comprising of flavonoids, tannins, and lignins. Flavonoids dominate the class of

phenolics with more than 9000 compounds identified till date [51]. Their basic scaffold moiety is 2-phenyl-benzyl-γ-pyrone derivative. This group of phenolics is synthesized in all the developmental stages, tissues, and stress conditions in the plant. Flavonoids perform various functions on the basis of their localization, abundance, and temporal synthesis. Its substantial significance lies in defense against abiotic or biotic stress, pigmentation, fragrance, UV protection, etc. Anthocyanins, aurones, chalcones, flavonols, and flavanones are generally responsible for floral pigmentation [52]. Pigmentation and fragrance serve as visual and chemical cues for insects to identify and locate host plants. Changes in floral pigmentation due to various secondary metabolites can result in differential host selection. For instance, *Petunia axillaris* (white flowers) are pollinated by bees whereas, *Petunia integrifolia* (violet flowers) are pollinated by moths [52].

The genesis and diversification of various plant secondary metabolites are in consonance with their evolution and interaction with varied abiotic or biotic components. From the fossil records, it has been observed that molecules similar to flavonoid class might have established 500 Mya [48]. Plant-specific distribution of the molecules belonging to this class like chalcones, flavanones, and flavonols were reported in the Silurian period of Paleozoic era (540–250 Mya), the time when the progression and diversification of bryophytes are observed [53, 52]. There are numerous speculations about functions of these ancestral flavonoids but most argued it to be in cytoprotection from either UV radiations or signaling molecules. These flavonoids further evolved to aid plant defense [53]. Pro-anthocyanins, which portray an intermediate link between early nonpigment flavonoids and pigmented anthocyanins, are documented to come into existence in parallel with vascular plants [53]. Following this, anthocyanins, involved in pollination, were reported to have organized along with flower-producing plants – gymnosperms and angiosperms [54].

As per fossil records, lignins are reported to be synthesized after flavonoids and they came into existence along with the tracheophytes (ferns, gymnosperms, and angiosperms) and remain from the Silurian period [5]. These molecules build the woody mesh-like tissue through lignification and provide mechanical strength to the tracheophyte structure. Besides increasing the rigidity of cell walls, lignins are also reported to have antinutritive effects on herbivorous insects [55].

Furthermore, there is major plant phenolic compound tannin, which came into existence post-Carboniferous period [48]. Gymnosperms that appeared in this period used metabolites like tannins for seed protection against various pathogens and pests. Tannins are majorly found in leaves of gymnosperms and angiosperms, specifically the class of woody plants – Fabaceae, Fagaceae, Myrtaceae, and Polygonaceae [56]. As a mode of action, tannins act as pest deterrents and also inhibit insect digestive enzymes, reducing their digestibility [55]. These primitive classes of phenolic secondary metabolites further get diversified in corroboration with plant evolution and serve distinct functions from seed protection to plant-plant communication. In course of evolution, plants also produced various metabolites from other classes to employ more specialized functions.

3.2 Nitrogen-Containing Compounds

After flavonoids, the next largest group of secondary metabolites is alkaloids. These are nitrogen-containing chemicals. Till date, 10,000 different derivatives of alkaloids have been revealed from different plant species [47]. Alkaloids have been classified into three major groups: true-alkaloids (nicotine and atropine), pseudo-alkaloids (caffeine and solanidine), and proto-alkaloids (mescaline). Alkaloids are found in gymnosperms, angiosperms, and in other primitive plant genera like *Lycopodium* [57]. Secondary metabolites, along with terpenes and phenolics, are also present in trichomes of the plants. Trichomes appeared along with the true leaves in late Paleozoic era with the appearance of alkaloids [48]. The insect toxicity arises as a result of interference of these compounds in biological activities like neuronal signal transduction, DNA replication, protein synthesis, and enzyme activity [5, 58, 59].

3.3 Nitrogen and Sulfur-Containing Compounds

Predominant class in this category of compounds is glucosinolates, which are characterized by the presence of nitrogen and sulfur in their basic scaffold. In plants, they are sequestered in compartments to protect them from the action of myrosinase enzyme [4]. On insect attack or wounding, these compounds come in contact of myrosinase and are converted to glucon isothiocyanates, which are found to be toxic to insects [15]. Glucosinolates are found only in the order Capparales and genus *Drypetes* of the Euphorbiaceae [4]. *Brassica napus* is studied extensively for the herbivore-resistance activity of glucosinolates [60, 61]. Due to the similarity in chemical structure and synthesis process, glucosinolates are hypothesized to have been evolved from primitive cyanogenic glucosides [62]. Aldoxime-metabolizing enzyme, belonging to the cytochrome (CPY) family, is involved in the synthesis of both cyanogenic glucosinolates and glucosinolates. It is reported that cyanogenic glucosinolates are present ubiquitously in ferns, gymnosperms, and angiosperms [63]. The evolutionary theory suggests that the mutation in CPY family enzyme of cyanogenic glucosinolates has caused the formation of a toxic compound – glucosinolate [15, 62, 64]. Glucosinate levels are found highest in young leaves and reproductive parts – seeds and siliques.

3.4 Plant-Volatile Compounds

Volatile compounds (VOCs) are phytoalexins that are released by plants on herbivore attack, also called as herbivore-inducible plant volatiles (HIPVs) [40]. Feeding of the herbivore induces downstream cascade in plant cells, releasing specific VOCs. As discussed previously, insect elicitors play an essential role in the release of these VOCs. Though VOCs cause indirect defense, many of these like indoles, (E)-β-caryophyllene are found to be toxic to the herbivores (direct defense). This suggests that the primary function of VOCs might have been toxicity, which evolved further to attract herbivore

predators [65]. Depending on the specific insect species and plant-insect interactions, different volatiles are released by plants. VOCs mainly include terpenes, terpenoids, green leaf volatiles (GLV), methyl salicylate, and others.

Terpenes are the most diverse group of plant-volatile compounds. These are modified unsaturated compounds made up of isoprene units. More than 40,000 terpenes are known to date [66]. Among terpenes, isoprene and monoprenes are dominantly occurring plant volatiles. The levels of biosynthesis of terpenes are dependent on various factors like tissue, developmental stage, and phenological status. Studies conducted in *Malus domestica* and *Prunus avium* show high levels of terpenes in reproductive parts of the flower with the highest levels found during and after flowering [18]. In addition to reproductive parts, they are also extensively synthesized in vegetative parts like leaves. However, young leaves have higher terpene levels compared to the old ones [67].

Terpenoids are synthesized in the epidermal cells of leaves and roots. 1,8-cineole, a monoterpene volatile, and sesquiterpene (E)-β-caryophyllene are examples of terpenoids produced in roots [68]. They are also found in special secretory structures like resin ducts or lactifers in conifers, glandular trichomes seen in *Artemisia annua*, and *Ocimum basilicum*. The concentration of terpenoids also differs within the same tissue depending upon localization [69]. In case of ponderosa pine needles, levels of monoterpene cyclase in the base of the needle were highest which caused the *Arctiinae* larvae to feed on upper part of the needle. It has been observed that terpenoid levels depend on various physiological factors [70]. Several terpenoids are found to be neurotoxic to insects, by inhibiting acetylcholine esterase, causing anoxia and further death.

Besides terpenoids, GLVs (fatty acid derivatives) also play an acute role in host perception and localization of herbivore to its natural enemies. These include C6 aldehydes, alcohols, and esters. They were first identified in braconid *Microplitis croceipes* and *Ichneumon netelia*. The *Liriomyza huidobrensis* larvae elicit the release of GLVs in a number of host and non-host plants. (Z)-3-hexanol is one such GLV that attracts *Opius dissitus*, a parasite of *L. huidobrensis* [71]. Many other GLVs serve as an attractant to an aphid parasitoid, *Aphidius ervi* [72]. Thus, GLVs resist insect indirectly by attracting the parasitoid of the infesting herbivore.

Along with the indirect defense mechanism, GLVs might serve as DAMPs as they cause expression of plant defense genes [73]. They also play an essential role in host location by natural enemies of the herbivore and guide them to the damaged site. The GLVs found in *Solanum tuberosum* cause excitation of olfactory sensilla of Colorado beetle, *Leptinotarsa decemlineata* [74]. In another study, the female braconid parasitoids, *M. croceipes*, were attracted towards the GLVs – Z-3-hexen-l-ol and Z-3-hexenyl acetate released by caterpillar feeding [75]. Thus, GLVs guide the herbivore parasites to the damage site.

Other VOCs found are methyl-salicylate, 6-methyl-5-hepten-2-one, indole, and nitrogenous compounds [76, 77]. Nitrogenous compounds like aldoximes, nitriles, methyl butyronitriles, and benzyl cyanides are synthesized in minor quantities in plants. However, these have shown to be essential for attracting caterpillar parasitoids – *Cotesia glomerata* and *Cotesia rubecula* [78].

In such a way, plants defense system subject insects to multitudinous phytochemicals, with each chemical having a different mode of action. As a result of decades of association of plants and insects, insects have developed numerous resistance mechanisms of circumventing plant defense systems and surviving in this arms race.

4 Insect Resistance to Plant Defense

The various modes of plant defenses have been discussed by us so far. We will now look into the counter strategies employed by insects in response to them. Insects have devised methods to protect themselves from the hazards of the toxic chemicals released by plants using enzymatic detoxification, followed by excretion or sequestration, physiological tolerance or behavioral avoidance. They have evolved novel mechanisms of detoxification through gene recruitment, neofunctionalization, and horizontal gene transfer. The various molecular mechanisms of insect resistance have been discussed in the following section.

4.1 Avoidance

Chemosensation plays a crucial role in insect avoidance of secondary metabolites. Insects possess the ability to avoid ingestion of toxins by detecting them visually, through olfaction or by contact. Chemosensation in insects is facilitated by the transmembrane proteins – gustatory receptors (GRs) and olfactory receptors (ORs) present in gustatory receptor neurons (GRNs) and olfactory receptor neurons (ORNs), respectively. These neurons are present in hair-like projections called "sensilla," which are distributed throughout the insect body surface. GRs are involved in metabolite detection, whereas ORs detect volatile compounds. GRs are further classified as sweet, bitter, umami, salt, and carbon dioxide based on the type of ligand binding. The deterrent secondary metabolites bind to the bitter receptors and activate the downstream cascade. This aversive mechanism is genetically determined or learned. Studies have shown that certain females avoid oviposition on unsuitable plants, due to genetic cues [79].

Phenological shifts are also seen in certain insects to refrain from feeding on toxic compounds. This means, that insects restrict themselves to toxin-free plant organs or they feed on the plant at a stage when the toxin is not produced or is present at low levels [80]. Insects are also aversive to bitter compounds and may sometimes avoid them even if the compounds are non-toxic. For example, *M. sexta* on encountering a non-toxic phenolic compound (salicin) and a toxic alkaloid (caffeine) activate the bitter-signaling pathway [81]. Similarly, grasshoppers and weevils avoid bitter-tasting cyanogenic glucosides even when they are present in non-toxic concentrations [82]. Thus, bitterness as a signal restricts the range of host plants and increases the cost of avoidance since it is not always triggered by a toxic compound.

In another intriguing study conducted by Perkins et al. (2013), it was observed that *H. armigera* larvae avoid elicited and closely connected leaves of *Arabidopsis*

[83]. This suggested that insects are capable of detecting previously elicited response and thus reduce contact with induced plants. *M. persicae*, an aphid, avoids ingesting the toxic nicotine present in the xylem by feeding on the phloem tissue [84]. While feeding on *Solanum sp*., larvae of *Mechanitis isthmia* spin a silk fabric over spines, allowing them to move and feed without being affected by the defensive trichomes [85]. Another way in which insects refrain from plant defenses is by leaf vein severing or cutting trenches across leaves before feeding so as to depressurize the secretary canals and get rid of toxins at the site before feeding [86]. For example, chrysomelid beetles of the *Blepharida* genus, that feed on certain *Bursera* species, puncture leaf veins to stop the flow of terpene-containing resins stored in leaf canals [87]. Therefore, avoidance in insects offers the first line of defense against plant allelochemicals. In spite of this, the insects might sometimes ingest the toxins. Hence, they have adapted other resistance mechanisms like detoxification, which ensures the conversion and elimination of the ingested secondary metabolites.

4.2 Detoxification

Detoxification is an enzyme-mediated conversion of toxic compounds into non-toxic or less toxic forms. Detoxification generally occurs in distinct phases. Phase I involves the hydrolysis or oxidation of secondary metabolites and Phase II conjugates Phase I products with endogenous compounds. The predominant enzymes used for detoxification are cytochrome P450 monooxygenases (P450s), esterases, UDP glucosyl-transferases (UGTs), glutathione S-transferases (GSTs), and ABC transporters [58, 88].

4.2.1 Cytochrome P450

P450s are Phase I detoxifying enzymes. In insects, P450s play an important role in the biosynthesis of hormones, fat metabolism, and insecticide resistance [89]. P450s are extensively found in microsomal membranes and possess different electron-transfer partners. Despite having a common catalytic chemistry, these enzymes exhibit different metabolic capabilities. P450s get their common name since they bind to carbon monoxide (CO) in their reduced state and form a P450:CO complex. The microsomal P450s are heme-dependent, mixed-function oxidases, and use NADPH and/or NADH for reduction [90]. P450s are extremely versatile and thus play a central position in the evolution of interspecies defense strategies. This is because of their biochemical flexibility of multiple substrate recognition.

There are many instances of different classes of P450s assisting detoxification. A cytochrome P450 was detected in *Papilio* species. In this case, exposure to furanocoumarins caused expression of CYP450 from the CYP6B class [89]. An engaging distinction of this enzyme class has been observed in specialist and generalist insects. Molecular modeling studies indicated that CYP6B enzyme from *H. zea* (generalist) had a flexible catalytic pocket and an additional substrate access channel compared to *Papilio polyxenes* (specialist) [91]. These results indicated that generalist P450s can accept structurally diverse compounds as compared to

specialist defense proteins, which draws generalists to a wide host range [88, 90, 92]. Similarly, the CYP321 class of enzymes can accommodate a diversity of structural classes of phytochemicals and are present in generalists, whereas absent in specialist (*Bombyx mori*) [93]. Usually, insects show resistance on coming in contact with the plant secondary metabolites. However, the generalist insect (*H. zea*) has shown to activate the expression of four CYP450s in response to plant phytohormones – JA and SA, that further detoxify furanocoumarins and other plant toxins [94]. Thus, the insect protects itself from toxins by activating the resistance either prior to or concomitantly with the synthesis of allelochemicals [94].

P450 is one of the major players in the plant-insect arms race. Thus, the evolution of plant allelochemicals has effected an increase in P450 genes. Phylogenetic studies on lepidopteran P450s suggest active duplication of gene loci and increase in their copy number. This can be attributed to the fine-tuning of substrate specificity conferring them resistance to P450 inhibitors of plant origin [95, 96].

4.2.2 Esterase

Esterases are also Phase I detoxification enzymes which catalyze the hydrolysis of carboxylic acid esters. They are essential for development, neurogenesis, pheromone degradation, hydrolysis of acetylcholine, and juvenile hormones. Esterases introduce hydrophilic groups into apolar molecules and enhance their water solubility. Carboxylesterases are important multifamily enzymes of the esterase. The mechanism of action of insect esterases for detoxifying plant secondary metabolites has not been extensively studied. However, several studies conducted have shown that there is a high degree of overexpression of esterases in response to plant allelochemicals. Detoxification due to increased activity of esterases in response to *Nicotiana tabacum* was reported in *M. persicae* [97].

Esterases are mainly involved in phenolic glycosides. In the Gypsy moth, *Lymantria dispar*, survival rate while feeding on phenolic glycosides has been positively correlated with esterase activity [98]. Similarly, in *Papilio canadensis* and *Papilio glaucus*, carboxylesterases are induced in response to phenolic glycosides in their salicaceous host plants [99]. Reports suggest that carboxylesterases also perform detoxification against the plant glycoside rutin in *Spodoptera litura*. The enzymes can also detoxify an indole alkaloid: gramine, quercetin, and 2-tridaconone [100, 101].

4.2.3 Glutathione-S-Transferases (GSTs)

Glutathione-S-transferases (GSTs) are found in all aerobic organisms and are responsible for detoxification of endogenous and xenobiotic compounds, intracellular transport, hormone synthesis, and protection against oxidative stress [102, 103]. These enzymes are primarily Phase II enzymes, which metabolize secondary products generated in Phase I by P450s and esterases. Sometimes, they also show Phase I detoxification by directly binding and sequestering the toxins [104]. It is interesting to know the mode of action of GSTs. GSTs exhibit binding sites for glutathione (GSH) and other toxic compounds [105]. In the reaction, the active site residue of the GST interacts with GSH sulfhydryl group (-SH) to generate the catalytically active-

thiolate anion (GS-), which then attacks the electrophilic center of lipophilic compounds (allelochemicals) to form GS-conjugates. This reaction leads to neutralization of reactive sites of these allelochemicals, making them water soluble and nontoxic, which are then excreted from the body [5]. According to where they are located, insect GSTs are either microsomal, mitochondrial, or cytosolic [106]. These enzymes have broad substrate specificity and are thus important in resistance against a wide range of xenobiotics. Studies have provided evidence that GSTs take part in detoxification of glucosinolates in *Scaptomyza flava* and *Trichop lusiani* [66].

4.2.4 UDP Glucosyl Transferases (UGTs)

UDP glucosyl-transferase belongs to the class of Phase II detoxification enzymes functioning in detoxification, olfaction, endobiotic modulation, and sequestration. These enzymes catalyze the transfer of sugar moieties to a wide range of lipophilic plant secondary metabolites [107]. UGTs have wide substrate specificity and act on terpenoids, coumarins, phenols, and flavonoids [108]. UGT conjugates glycoside group to chemicals and facilitates excretion by making them more hydrophilic and minimizing their detrimental effects [58].

UGTs are present in different body parts and are involved in various processes [109]. UGTs which are expressed in the olfactory mucosa are responsible for olfactory processing and detoxification. In an example study from *S. littoralis,* where males were exposed to pheromones and plant odorants, UGTs were downregulated. Whereas, the introduction of insecticide to antennae upregulated the level of UGTs. Thus, this implied that UGTs protect the olfactory organ and play a role in xenobiotic [110]. In a similar study, UGT facilitated odorant inactivation was observed in *D. melanogaster*, as the glucurono-conjugated odorants did not elicit any olfactory signals [110].

Along with this, UGTs were also expressed in the fat body, midgut, Malpighian tubules, and antennae, suggesting the enzyme's role in pheromone deactivation [107, 111]. This was observed in *B. mori*, where the UGT genes were expressed in the fat body, midgut, integument, testis, silk gland, and hemocytes of the fifth instar larvae. The coregulation of UGTs with other detoxification enzymes such as cytochrome P450s has also been reported which may have evolved in the course of the plant-insect warfare [112]. UGTs were also seen to detoxify naturally occurring benzoxazinoid from *Z. mays* in *Ostrinia furnacalis* [113].

After detoxification and further processing so as to decrease hydrophobicity, there exists Phase III detoxification. This includes ATP-binding cassette transporters (ABCs), which aid in the efflux of the detoxified products from the cell. The detoxified plant secondary metabolites can either be thrown out of the body or sequestered for other purposes as discussed below.

4.3 Sequestration

As seen in the above sections, the toxic effects of allelochemicals are overcome by insects with detoxification. These detoxified secondary metabolites are further

excreted as waste, lost during molting or sequestered for the insect's defense [114]. Sequestration aids the uptake, transfer, and concentration of phytochemicals, which may or may not be modified and are stored in tissues or hemolymph. It is a biochemically sophisticated process and has been observed to be independently evolved as plant toxins are chemically diverse. The sequestered secondary metabolites are used for defense against predators, as pigments for adult coloration, as pheromones or for protection against UV radiation and photoactivated phytotoxins like furanocoumarins [115, 116].

The key enzymes involved in sequestration are ABC transporters, as they promote the uptake of toxins and their sequestration. In a research held on poplar beetle larvae (*Chrysomela populi*), defensive glands showed significant expression of salicin-transporting ABCC protein *Cp*ABC35 compared to other tissues [117]. This pointed out the role of ABCs in accumulating salicin in storage compartments of the gland cells to exocytose into the glandular reservoir. In another observation, it was noted that *C. populi* and *Phratora vitellinae* are evolutionarily adapted to transport and sequester host plant glycosides. These glycosides are further delivered to dorsal glandular reservoirs, which are signaled to release defensive secretions on any disturbance or attack [118]. Larvae of a chrysomelid beetle sequester a phenol glucoside, salicin, and hydrolyze it to salicylic acid, which is used as a defensive secretion. The hydrolysis of salicin liberates glucose in the insect body implying that sequestration is not an energy intensive process [119]. A large number of studies have been carried out to understand the sequestration mechanism of insects against an array of plant defense compounds. One such interesting study is that a polyphagous lepidopteran moth, *Estigmene acrea*, which feeds on Asteracea. It sequesters pyrrolizidine alkaloids while detoxifying them by N-oxidation [120].

Other plant secondary metabolites like cyanogenic glycosides are also processed using this resistance strategy. The cyanogenic glycosides are either metabolized by β-cyanoalanine synthase, which converts the cyanide moiety to asparagine or they are sequestered in insect organs [121].

4.4 Mutation of the Target Site of Plant Secondary Metabolites

Most of the plant secondary metabolites act on insects by binding to a specific receptor. Therefore, insects have developed mutations of these target sites to prevent the binding. The most well-documented example of this type of adaptation strategy is Na^+/K^+ ATPase mutations. Cardenolides, a toxic secondary metabolite found in Apocynaceae plant, inhibit Na^+/K^+ ATPase and disrupt the sodium pump [122]. Thus, insects have employed mutations in this receptor to render resistance against the cardenolides. Till date, the widely observed mutation in insect species is N122H in the α-subunit of Na^+/K^+ ATPase and has evolved in very few cardenolide sequestration insects – monarch butterfly (*Danaus* species), milkweed bug (*Oncopeltus fasciatus*), fruit fly (*D. melanogaster*), and beetles (*Chrysochus auratus* and *Chrysochus cobaltinus*) [123, 124]. However, another substitution L111 V has also been found in the Danai species – *D. chrysippu, D. genutia*, and the *Trimala*

species. Along with cardenolides, alteration of Na^+/K^+ ATPase receptor also confers resistance to ouabain in *D. melanogaste*r and *Oncopeltus fasciatus* [123, 125].

Until now, very few insect species have been found to have developed target site mutations against plant secondary metabolites. Although a very high resistance is determined by the target site mutations, it results in high specificity and restricts the ligand binding classes. Contrarily, other strategies like detoxification act against a wide range of chemicals. This might be the leading cause of the metabolic resistance strategies of detoxification and sequestration being evolutionarily more favourable than target site mutations [126].

5 Coevolution in Plant Secondary Metabolites and Insects

The field of plant-insect interactions was revolutionized by the study done in 1964 by Ehrlich and Raven "Butterflies and Plants: A Study in Coevolution". Though the significance of plant-insect interaction was put forth by Darwin, Ehrlich and Raven highlighted the impact of plant-insect arm race in the evolution of plant defense. Thus, the concept of "coevolution" was introduced. Ever since then many studies are being carried out to find the evolutionary cause of plant secondary metabolites and their diversification. Ehrlich and Raven also proposed the "Escape and radiate" theory according to which, insects render new resistance strategies and radiate into a new species [127, 128]. Thus, the enormous speciation and dominance of plants and insects observed today is the consequence of their intercommunication over millions of years.

The arms race between plants and herbivores is long being debated to be the primary root for secondary metabolites escalation. This has not only led to chemical changes in these phytochemicals but also has expanded their complexity in plants. In 2009, Becerra et al. carried out a phylogenetic study of volatile chemicals among 70 *Bursera* species. This study perceived an evolutionary hike in the volatile compounds' complexity. Thus, we can propose that over time, plants tend to adapt to the virulent herbivores by increasing the intricacies of allele chemicals, rather than introducing a new compound. Hence, this makes it difficult for herbivores to develop new resistance strategies [87].

One of the most successful plant-insect interaction examples is that of monarchs and milkweeds. There is a negative relationship between monarchs and milkweed. Monarch caterpillars eat only milkweed plants and butterflies use milkweed to lay eggs. Though the monarchs benefit from the milkweed, they are only pests for the host plant and are ineffective in milkweed pollination. Thus, the coevolution of monarchs and milkweed is not synergistic. The dominance of monarchs on milkweeds has led to the expansion and modification in the plant's defense. One of the coevolution theories predicts that the secondary metabolites might have evolved in response to diversifying herbivores. Therefore, according to this, the newer plant species must exhibit higher production of plant secondary metabolites. However, Agrawal et al. in 2008 discovered chronological decrement in cardenolides and an exclusive rise in phenolic levels in *Asclepias* species. This phenolytical reduction in

plant secondary metabolites can be ascribed to the dominance of monarchs on milkweeds [129]. These events highlight the influence of insects on the development of plant secondary metabolites. Variable cardenolides production in milkweed has been espied with different insect species. The sap-sucking insects (aphids) induce lesser cardenolides production compared to the leaf-chewing insects. Thus, there is disorder in the coevolution of monarchs and milkweeds [130]. Along with herbivores, geographical location has also determined the regulation of cardenolide synthesis in milkweeds. Therefore, resistance profile (Na+/K+ ATPase) of monarchs against milkweeds must deviate according to varied locations. Nevertheless, researchers did not find any discrepancy in an extensive study on six monarch populations [131].

Another example of coevolution is that of parsnip webworm (*Depressaria pastinacella*) and the wild parsnip (*Pastinaca sativa*). In defense to the parsnip webworm, the wild parsnip produces a large amount of furanocoumarins. Parsnip webworms resist the furanocoumarins by detoxifying it by enzyme, P450 (as seen in Sect. 4.2.1). They also pose behavioral defiance by feeding exclusively on parthenocarpic fruits of parsnip, which have lower levels of furanocoumarins [132]. In spite of this phenomenon, in a study conducted, parsnip webworms were unable to distinguish between high and low levels of furanocoumarins in artificial diet. It was further speculated that octyl butyrate (deterrent), present in parallel with furanocoumarins, is responsible for the behavioral alteration of webworms [132]. Here, it can be noted that only one secondary metabolite may not be the cause of a certain modification in insect, rather a medley of chemicals are involved in the interrelation. Also, in this study, unlike the previous monarch-milkweed example, the resistance offered by webworms is comparable to the change in the furanocoumarin levels in parsnips.

From the above instances, it is apparent that the herbivores drive the evolution of plant defenses. Nevertheless, not much is established about the impact of plant defenses on herbivore diversification. A recent phylogenetic analysis of Inga and lepidopteran coevolution suggests that the plant defense drive host selection of herbivore and not plant evolution. Thus, the closely related Lepidopteran insects fed on plants with similar defenses and not evolutionarily closely related Inga species [133]. They further indicate an asymmetry in the coevolution of Inga and Lepidoptera. As seen earlier, many coevolution theories propose the diversification of plants due to the pressure exerted by herbivores. Nonetheless, this might not be true for the insects. They adapt to a new host plant having defenses similar to its own traits. If a host plant changes its defense, the insect might change the host to which it is now resistant.

6 Conclusion

Numerous studies have revealed the molecular complexity in Plant-Insect interactions. It comprises an enumeration of molecules involved in insect perception, plant defense system, and resistance in herbivore insects. Thus, any transition at the

molecular level disturbs the equilibrium of the system. The past research has examined many macromolecules, their function, and mode of action in the plant-insect dialogue. This has simplified the comprehension of plant-insect coevolution. It is illustrious from the reports that plant-insect interrelation knows no bounds and can never cease. There is a continuous competition between these two entities to develop new counter strategies and impose pressure on the other. The repercussion of which is the immense and perpetual diversification of the molecules. Still, the realm in which the plant-insect arms race is occurring is very large and includes various other environmental factors. The impact of these biotic factors on plant-insect molecular interactions needs to be investigated. A better understanding of plant-insect communication at the molecular level accentuates its application for pest control in agriculture.

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Coevolution: Plant-Herbivore Interactions and Secondary Metabolites of Plants

3

Eunice Kariñho-Betancourt

Contents

Abstract

Plant-herbivore interaction has long been a central model to explain the evolutionary success of vascular plants and insects, and the extraordinary diversity of secondary compounds produced by plants. Coevolutionary theory proposes that herbivorism has spur diversification and speciation of host-plants and phytophagous animals through an arms race, which results in a general concordance on their phylogenies, and the evolution of diverse (mostly defensive) chemical compounds by plants and counter-defenses by herbivores. Main assumptions of the micro- and macroevolutionary postulates of the coevolutionary model have

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been extensively tested within populations and along phylogenies. Common patterns found indicate that plants and herbivores constitute a selective context for each other, and that plant secondary metabolites are adaptations that constraint phytophagous insects to use a plant as a host or as a food source. Herbivorism is strongly implicated in the evolution of specialized associations that are usually mediated by a conserved biochemical machinery of host plants. The evolution of specialism appears to be correlated to speciation events and even with adaptive radiations. However, there is little evidence that these correlations reflect a causal relationship. Perhaps the most compelling evidence linking macroevolutionary patterns and mechanisms that produce new species is the matching of the genetic machinery responsible for the evolution of chemical *novelty* on plants and major emergency events of plants and herbivore lineages. This body of work developed from the study of the antagonistic association of plants and herbivores in more than 60 years has evince the great potential of adaptive evolution to generate much of the Earth's biodiversity.

Keywords
Adaptive evolution · Plant defense · Coevolution · Herbivory · Secondary metabolites · Antagonistic interactions · Diversification

1 Introduction

Why is there such a large diversity of secondary metabolites produced by plants? And, why relationship between numerous taxa of plants and animals is constant? Both are questions that, from entomology to chemical ecology, and from evolutionary biology to genetics, have been linked in the effort to be answered for many decades. Already in the late nineteenth century, the botanist Ernst Sthal had documented that secondary metabolites (i.e., compounds not involved in the primary metabolism) produced by plants provide them protection against herbivores [154]. Although some evidence of the ecological role of plant secondary metabolites was collected over the early twentieth century (e.g., [27]), the hypothesis of the *defensive* function of plant compounds was formally introduced by mid 1950s (see [39, 40, 62]), replacing the previous conception of secondary metabolites as "waste products" of the primary metabolism. Framed on this adaptive notion, several models were elaborated, mainly based on the effects of plants' compounds on organisms and the environment, and on the comparison of their distribution and the phylogenetic relationships of plants that produced them. Theory of plant-herbivore coevolution, in particular the model of "escape and radiation" [48], has provided for the last decades an articulated framework for the study of plant and arthropods codiversification. This model has also contributed to the development of hypothesis that attempt to explain both the phytochemical diversity and the longstanding associations of plants and animals which lead in some cases to specialization habits. In essence, the model proposes that sequential responses to selection pressures exerted by plants and their

consumers to each other resulted in the evolution of novel chemical defensive mechanisms by plants and disarming mechanisms by herbivores. This process, hence, would be responsible for the chemical diversity and toxicity of plants, and feeding habits of herbivores. The coevolutionary model placed the plant-herbivore interaction, or more precisely, its reciprocal evolutionary responses, as the ultimate drivers of speciation and diversification [48, 89]. Although the phytochemical coevolution has received some criticism [93, 161], a large body of experimental evidence supports the view that secondary metabolites have diversified as a result of natural selection [61, 65, 139, 186], suggesting that their occurrence reflects the functional (adaptive) response to particular selective contexts mainly impose by plant consumers.

2 History of Vascular Plants, Their Secondary Metabolism and Interaction with Early Arthropods

Current ecological roles of secondary metabolites are diverse but mainly associated to mediation of biotic interactions. Fossil-based evidence and biochemical and molecular analysis suggest that two major evolutionary events are implicated in the evolution of biosynthetic paths of secondary metabolites and their early function: (1) the *great oxygenation* of the Earth and (2) plant vascularization (Box 1). These events laid the foundations for the evolution of terrestrial ecosystems and preclude the emergence of arthropods (Fig. 1) and their interaction with plants that came with it. The paleontological evidence from Paleozoic and Mesozoic eras has been crucial to trace the beginning of the association of plants and arthropods and has contributed to the inference of the resulting evolutionary patterns.

Box 1 Evolutionary Milestones of Photosynthetic Eukaryotes and Function of Early Secondary Metabolites

Communities of cyanobacteria (i.e., photosynthetic prokaryotes able to produce oxygen) lived in freshwater and marine aggregates as early as one billion years ago [207, 208]. Cyanobacterias are strongly implicated in the evolution of photosynthetic eukaryotes (algae and plants) by endosymbiosis with plastids [201, 204] and are presumably responsible for converting the early oxygen-poor, reducing atmosphere, into an oxidizing one, causing the "rusting of the Earth" [206]. This photosynthetic activity would increase the metabolic wastes or excretes. Early functions of secondary metabolites are thought to be related to excretion mechanisms derived from the incomplete cycling of primary compounds soluble in water [200, 201].

(continued)

Box 1 (continued)

With land colonization, early (nonvascular) plants, such as bryophytes (e.g., hornworts and mosses) where exposed to desiccation, the lack of structural support, and damaging UV-B radiation. The phenylpropanoid metabolism was crucial for plant vascularization and the occurrence of tracheophytes in terrestrial ecosystems. Lignin, a phenolic polymer derived from phenylpropanoid metabolism, synthetized from hydroxycinnamyl alcohols, provides structural rigidity and regulates the hydration of the hydrophilic molecules in the cell wall to bear the negative pressure generated during transpiration. In addition, as most of phenolic acids derived from the aromatic amino acids of algae and other photosynthetic eukaryotes, lignin function as absorption agent of UV light [182]. Given these functional properties of lignin, its accumulation in cell walls (i.e., lignification) has been considered a key biosynthetic process for the success of land plants.

2.1 Secondary Metabolites and the Origin of Plant-Insect Interaction

Since the emergence of first embryophyte land plants in mid Ordovician ~470 Mya, to late Devonian and early Carboniferous ~360 Mya, many of the features recognized in land plants today were present, including roots, leaves, and early seeds. The evolutionary innovation from the Carboniferous that still continues today is not only restricted to photosynthetic eukaryotes. Fossil records suggest that ca. 100 Mya after the emergence of vascular plants, evolution of arthropods took place during mid Carboniferous (~300 Mya) [138, 150]. In late Paleozoic and early Mesozoic 250 Mya, important groups of phytophagous insects such as Coleoptera and Lepidoptera appeared. Fossil records of damaged leaves, coprolite dispersion, specialized mouthparts and intestinal contents of orthopterans evince the origins of an antagonistic interaction between plants and first phytophagous arthropods [84, 106, 107]. Interaction of plants and arthropods (especially insects) diversified extensively with the emergence of flowering plants about 160 Mya and became widespread by 120 Mya during the lower Cretaceous. Paleochemical analyses of ancient angiosperms from late Mesozoic and early Cenozoic demonstrate an impressive increase in biochemical diversity, which include lignin derivatives, terpenoids, tannins, and flavonoids, among others [37]. Most of these secondary metabolites derived from phenolic metabolism are known from their toxic and deterrent effect on plant consumers (e.g., flavonoids prevent pathogen invasion and affect the activity of digestive enzymes of animals, [171, 178]). These findings suggest that although metabolites' evolutionary origin may be associated with selective factors other than phytophagy (see Box 1), their diversification might be related to the coexistence of angiosperms and first arthropods. This whole paleontological and biochemical evidence has contribute to the notion that the diversification patterns of plants and arthropods instead of only coincide in time rather reflects the reciprocal evolutionary influence of plants and phytophagous insects, that arose from their interaction.

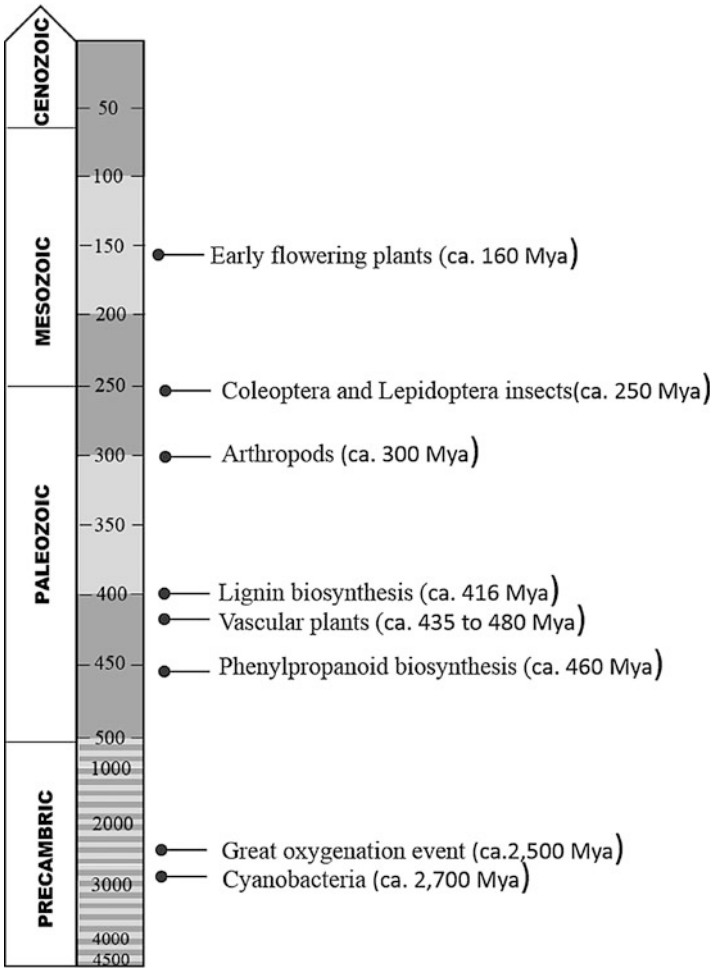


Fig. 1 Timeline of evolutionary events implicated in the interaction of plant and herbivores mediated by secondary metabolites. Based on geological, isotopic, and chemical evidence, hypotheses of (1) the emergence of photosynthetic prokaryotes [12, 29], (2) the oxygenation of Earth resulting from cyanobacteria metabolism [74, 82], (3) the appearance of phenolic biosynthesis [182], and (4) the evolution of vascular plants and arthropods [138, 150] are depicted. (Original artwork by the author)

3 Coevolution: Mechanism and Consequences

Coevolution is the reciprocal evolutionary change (i.e., changes in the allelic frequencies within population) in interacting species driven by natural selection [92]. Coevolution can occur when two species influence each other's evolution (pairwise or specific coevolution) or when several species evolve in reciprocity with another species (guild or diffuse coevolution) [165, 166]. Natural selection acts on the

population's heritable traits: selecting for beneficial alleles and, thus, increasing their frequency in the population, while selecting against deleterious alleles and, thereby, decreasing their frequency [172]. This process is known as adaptive evolution and can result in ecological specialization for particular niches [66] and may eventually result in speciation events [181]. Natural selection can favor a particular phenotype over the others, causing the allele frequency to shift in the direction of such phenotype (directional selection). Selection can also act on intermediate phenotypic variants maintaining multiple alleles in the gene pool of a population at larger frequencies (stabilizing selection) or can increase the variance of traits favoring the extreme values over intermediates (disruptive selection) [50, 110, 142]. Disruptive selection, also called diversifying selection, divides a population into two distinct groups. This process may lead to divergent evolution, which is a likely outcome for coevolving species and for species evolving in sympatry [42, 130]. Evolutionary dynamics between species are often driven by coadaptation process (Box 2). Consequences of the reciprocal fitting between partners of biological associations emerged at different levels of organization (e.g., traits or genes), and as any adaptive dynamic, their evolutionary patterns are dissected at different time, space, and organizational scales [22, 87]. Based on biological complexity, the most contrasting standpoints for the study of coevolution are the phylogenetic and the genetic/genomic perspectives. Tree thinking and gene thinking suppose different patterns that involve specific mechanisms (Table 1). Both perspectives have influenced the study of adaptive evolution in different ways. Whereas tree thinking has helped to understand events that emerged at supraspecific level (e.g., speciation, diversification, and adaptive radiation), in the last two decades, the genomic approach has helped to disentangle the molecular mechanisms behind evolutionary change. In particular, the study of plant chemistry has been a key to discover the mechanisms of evolutionary innovation. Several studies on enzymatic complexes of secondary metabolisms have consistently documented the central role for gene and genome duplication as a common mechanism to achieve *novel* function of traits, during speciation or adaptive radiation events (e.g., [17, 46, 47, 140]). After a long tradition of independent field-growing of phylogenetics and ecology, the development of genomics for the study of biotic interactions has begun to connect intra- and interspecific approaches, unraveling the genetic mechanism linked to macroevolutionary patterns (e.g., [89, 183]). The study of plant-herbivore coevolution provides a good example of how macro- and microevolution begin to approach but at the same time, of how these two angles have been very prolific when develop independently.

Box 2 Coadaptation

Coadaptation appears as a result of interaction with others, which produces a reciprocal adaptation.

Coadaptation generates and allows coevolution [165], which favors the survival of the systems or individuals. The two parts obtained advantages.

(continued)

Box 2 (continued)
Coadaptation involves a series of mutual adjustments between interacting entities determined by the frequency of interaction, the impact on fitness (i.e., survival, mating success, or fecundity), and the relative evolutionary potential (e.g., population size, generation time, genetic variation). Among the interactions that caused strong coadaptation or coevolution in *strictu* sensu are antagonistic associations such as parasite-host and plant-herbivore interactions and mutualistic associations. These relationships are intrinsically dynamic, and may endure for million years, as has happened with flowering plants and pollinating insects [67, 166]. Pollinator hawk-mots and orchids are a classic example of fine reciprocal fitting between traits involved in a specialized association of plants and insects (see Nilsson et al. [128], Boberg et al. [23]).

4 The "Escape and Radiate" Model of Coevolution

Much of what we know about coevolution has come from the study of one of the main antagonistic and widely specialized associations, the plant-herbivore interaction. The conceptual framework for the study of plant-herbivore interaction was constructed based on phylogenetic patterns among host plants and phytophagous insects. Using ecological data from butterflies of the superfamily Papilionoideae and their host plants, Ehrlich and Raven [48] documented a conserved phylogenetic pattern of host use, mediated by secondary metabolites characteristic of the host-plants. This pattern was placed in a theoretical context, known as the "escape and radiate" hypothesis. The authors proposed an "arms race" model of coevolution [38] that describes one of the ways in which coevolution can occur (mainly diffuse, see [166]). The model suggests that in response to reciprocal selection pressures exerted by plants and herbivores: (1) plants evolve novel defensive traits to avoid or reduce herbivory and (2) herbivores evolve counter-defenses to cope with mostly chemical barriers impose by plants. As a consequence, plants can (3) *escape* from herbivores and radiate, and herbivores, by surpassing chemical defenses of plants could thus (4) radiate on diversified host-plants. The "escape and radiate" hypothesis predicts corresponding patterns of speciation and diversification, reflected in taxonomic congruence. Coevolutionary metaphors of the "escape and radiate" by an "arms race," encompass the outcomes of coevolution and the mechanisms behind it, that function at different hierarchical scales. The "arms race" metaphor describes the process (i.e., reciprocal natural selection) behind patterns that emerged either at ecological or phylogenetic scales. Their predictions are usually tested within populations by means of trait-based analyses. The "escape and radiation" metaphor entails the resulting patterns of natural selection. This prediction is only tested across phylogenies and often uses taxon-based analyses [101]. Phylogenetic and ecological evidence supporting particular postulates of the coevolutionary model is discussed below.

Table 1 The approach of tree and gene thinking for the study of coevolution

Rationale of coevolutionary perspectives	Common mechanisms	Coevolutionary outcomes	Example(s)
Genetic. Genes carry sequence information that determines how living organisms inherit phenotypic traits and interact with the environment. Genome comprises the totality of genetic material carried by an organism. Within species, the majority of nucleotides are identical, but genetic diversity is determined by the sampling of several individuals. The comparison of several aspects of genes and genomes such as genome size, CG content, or karyotype, among species, allows to determine what mechanisms produce genome diversity (i.e., species diversity). When interacting species (or interacting entities such as cell organelles) influences each other's evolution, a change in a gene or the entire genome of one interactor stimulates the change in the genetic machinery of the other	Reciprocal mutation Duplication Horizontal gene transfer	Matching genetic features of interacting entities Matching burst of diversification with genetic novelty of interacting species	**Genome/gene duplications involved in the achievement of evolutionary novelty, matching with speciation/ radiation events of interacting species** [45, 47] **Horizontal gene transfer (the special case of plasmids and bacteria)** [78, 80]
Phylogenetic. Living and extinct organisms are the result of organic descent from earlier ancestor. Their evolutionary relationships can be depicted through diagrammatic hypothesis – phylogenetic tree – based on inference methods that evaluate observed heritable traits. Phylogenies recap process occurring within populations. The tips of a phylogenetic tree can be living organisms or fossil, and represent the "end" or the present in an evolutionary lineage. Close interaction between taxa influences tree topology of the interacting groups. Every time a taxa speciate, the other speciate as well. Thus, the phylogenetic trees of closely interacting organism – pathogens, herbivores, parasites, pollinators, and their host – are expected to have same shape and match when laid on top of each other	Cospeciation Synchronous and asynchronous adaptive radiation	Matching tree topologies of antagonistic/ mutualistic interacting taxa	**Phylogenetic congruence of plant–herbivore associations** [100, 123] **Sequential speciation/ radiation of plant and herbivores lineages** [1, 60, 95, 134]

5 Phylogenetic Patterns: Diversification of Interactions and Molecules

Since plants and arthropods were associated thousands of million years ago, both lineages and the secondary compounds produced by plants have been widely diversified. Only green plants (~25–30%) and phytophagous insects (26%) account for nearly half of the known species on the planet [113, 161]. And, known plants' secondary metabolites are close to 200,000 different molecules [79], most of which have important ecological roles [105]. The widespread of plants and herbivorous lineages across diverse ecosystems reflects the dynamic relationship between both groups and the potential of adaptive evolution to shape current biodiversity.

5.1 The Diversity of Associations and Their Specialization Degree

Insects are one of the most diverse group of organisms, most recent estimates of their "current diversity is estimated in 1,053,578 named species" [209], but the estimated number of living species ranges up than five million [76, 127]. A large fraction of these species feed on plants [88]. It has been shown that plant-feeding clades are consistently much more diverse and specialized than their non-phytophagous sister groups [124]. A number of aspects are thought to contribute to host fidelity and thus promote specialization, including interspecific factors such as resistance to predators (e.g., by sequestration of plant defenses; [135]), mate-finding [35], and competition for resources (e.g., reproductive interference; [129]). Habitat-specific adaptations that includes genetically based trade-offs in performance between different habitats [10, 64, 66, 88, 98] and deleterious mutations associated to their utilization [102] are also factors that may favor specialization. On the other hand, factors involved in the evolution of generalism include habitat heterogeneity [97, 161], greater resource availability [21], and physiological constraints for meeting nutritional requirements using a single food type [13, 19].

When herbivores' diet is restricted to feed from only one or a few related plant taxa, often a single genus, herbivores are considered monophagous (or highly specialized). In the opposite end of the diet breath spectrum, insects that feed on species in more than one plant family are designated polyphagous (or highly

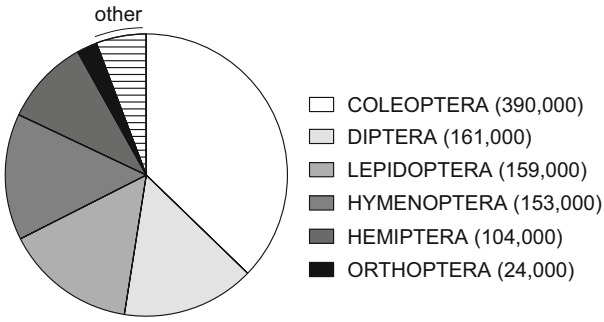


Fig. 2 Relative proportions of major taxonomic orders in the Insecta class. The number of named species is shown in parenthesis. Data from the Catalogue of life [209]

Table 2 Examples of studies reporting supraspecific patterns of the association between angiosperm host plants and specialized insects inferred from phylogenetic analyses

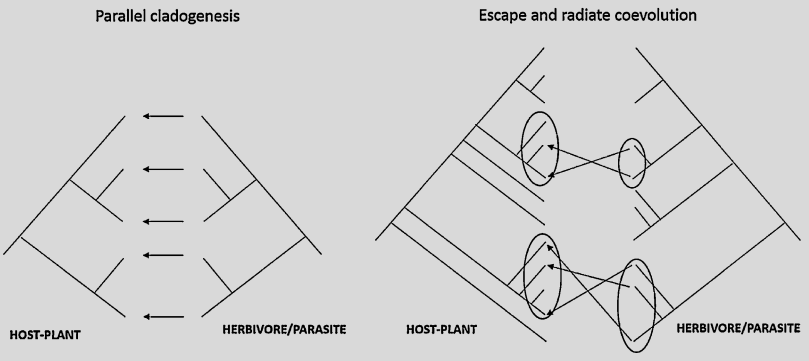
Type of interaction	Association	Phylogenetic relationship	Representative studies
Host plant–parasite	Wasp gallers (Cynipidae: Hymeoptera) Oaks (*Quercus* spp.: Asteraceae)	[1,2]High degree of conservatism mixed with rare shifts between distantly related hosts [1]No evidence of parallel cladogenesis, nor codivergence	[1]Ronquist and Liljeblad [144] [2]Stone et al. [159]
	Aphids (*Uroleucon* spp.: Aphididae) Asteraceous plants (Asteraceae)	Host shifts No evidence of cospeciation	Peccoud et al. [133]
	Gall midges (*Asteromyia* spp.: Cecidomyiidae) Asteraceous plants (Asteraceae)	Conservatism Asynchronous radiation	Stireman et al. [158]
Host plant–herbivore	Skeletonizing and flea leaf beetles (Phyllobrotica: Chrysolmelidae) Skullcaps (*Scutellaria* spp.: Lamiaceae)	Parallel diversification	Farrell and Mitter [53]
	Red milkweed beetle (*Tetraopes* spp.: Cerambycidae) Milkweeds (*Asclepias* spp.: Apocynaceae)	Synchronous diversification	Farrell and Mitter [56] Farrell [55]
	Flea beetles (Blepharida: Chrysomelidae) Burseras (Burseraceae: Rosidae)	Conservatism related to host plant chemistry	Becerra [16]
	Seed beetles (Bruchidae. Coleoptera) Legumes (Fabaceae)	[1]Parallel evolution [2]Conservatism	[1]Kergoat et al. [103] [2]Kergoat et al. [104]
	Leaf beetles (Chrysomelidae: Coleoptera) Flowering plants (Angiosperms)	Asynchronous radiation No support for ancient host associations	Gómez-Zurita et al. [71]

generalized) [11]. Categorization of insects based on arbitrary observations of feeding habits can "hide" the accurate distribution of diet breath across community and lead to inherit limitation in the use of such categories. Nonetheless, some groups of insect herbivores such as leaf miners, leafhoppers, butterflies, and

beetles are clearly dominated (>75%) by monophagous [54, 122, 149]. By contrast, across all phytophagous insects, it is estimated that <10% are polyphagous [11, 20]. Coleoptera, Lepidoptera, Diptera, and Hymenoptera are the richest taxonomic orders of insects (Fig. 2) and at the same time comprise most of the specialized phytophagous (or parasitoids) that entangle long-standing relationships with plants. In Table 2 are shown representative phylogenetic studies of the association of angiosperm host-plants and specialized phytophagous insects. The evolutionary patterns inferred from specialized associations of plants and phytophagous insects indicate that host use is often highly conserved, i.e., closely related herbivores feed on closely related plants (e.g., [53]). Besides coadaptation, the conserved host use depicted by taxonomic congruence can either reflect parallel cladogenesis/cospeciation (Box 3) or sequential evolution (i.e., herbivores follows the evolution of plants while the plant evolution is not affected by herbivores, [94]). Nonetheless, although the inference of the accurate evolutionary path behind matching phylogenies may be challenging, and the extent to which those paths lead to adaptive radiations is not always clear (see [119]), patterns of host use seems to be correlated with speciation.

Box 3 Cospeciation and the "Escape and Radiate" Model of Coevolution

Close ecological relationship between interacting taxa are not sufficient to explain speciation, nor coevolution between them. Taxonomic correspondence (i.e., phylogenetic congruence) between lineages of plants and herbivores may not always be attributed to a coevolutionary process since interacting entities that experience same vicariance events can cospeciate (i.e., be associated by descent) in parallel, having no reciprocal responses [28]. Nonetheless, the "escape and radiate" coevolution should not be expected to involved cospeciation but rather asynchronous radiation of the associated lineages triggered by evolutionary breakthroughs [89, 122]. Thompson [167] compares the "escape and radiate" coevolution with paralleled cladogenesis.



(continued)

Box 3 (continued)

The proposed hypothetical figure shows two bouts of the escape and radiate process, producing starburst of speciation in the plant-host and the herbivore/parasite. Each oval encircles only the initial starburst of speciation in the host lineage resulting from new defenses or the initial starburst of speciation in the parasite lineage resulting from new counter-defenses (Modified from Thompson [167]).

5.2 Host Shift and Speciation

Speciation events of plants and herbivores taxa are not only related to the conserved use of host-plants by phytophagous insects but also to host shifts. In a relative recent compilation of phylogenies of insect herbivores, Winkler and Mitter [187] found that close to 10% of speciation events involve host shifts to a nonrelated plant family. Although the role of host-plant shifts in insect diversification may seems limited, hosts shift has been widely documented in several plant lineages (e.g., [90, 91]) and has important implications for the evolution of host range. Host shifts may occur if the developmental aspects required to colonize/exploit a novel niche (host plant) and the ancestral, overlap at some point. Thus, if the range for overlapping increases, more *plasticity* for using new host is expected. Changes in feeding habits (e.g., polyphagy) should increase shifts by colonization of new hosts. Consequently, the evolution of host plant range appears to be closely linked to the diversification of host use through colonization [89]. Changes in host use by phytophagous insects across plant phylogeny also revealed a key link between diversification of plants and herbivores lineages and plant secondary chemistry. Empirical evidence suggests that host shifts are often constrained by similarity in the chemical profile of host species. For instance, phylogenetic analysis showed that historical patterns of host shift of Blepharida (Coleoptera) into Bursera (Burseraceae) correspond to chemical similarity based on terpenoids of host-plants [16]. The importance of secondary metabolites to drive host use patterns has been also demonstrated at molecular level. Cruciferous plants (Brassicaceae) usually hosts butterflies from the Pierinae family [48], and are known by the production of glucosinolates. Wheat et al. [183] showed that the occurrence of NSP (nitrile-specifying protein) glucosinolate detoxification gene on butterflies matched the occurrence of glucosinolate in their host-plants. The detoxification mechanism likely evolved shortly after the diversification of Brassicales, allowing the colonization of new glucosinolate-base host. The host shift has led to adaptive radiation in the Brassicales-feeding butterflies. Key *innovation* breakthrough of Brassicaceas and butterflies has been recently revisited. Based on gene family analysis, Edger et al. [47] confirmed the molecular fitting between secondary compounds of plants and counter defenses of insects by means of gene and genome duplications. The study documented a repeated escalation of key innovations and burst of diversification on each side of the plant-insect interaction (Fig. 3). These examples show how genetic mechanisms can be linked to macroevolutionary

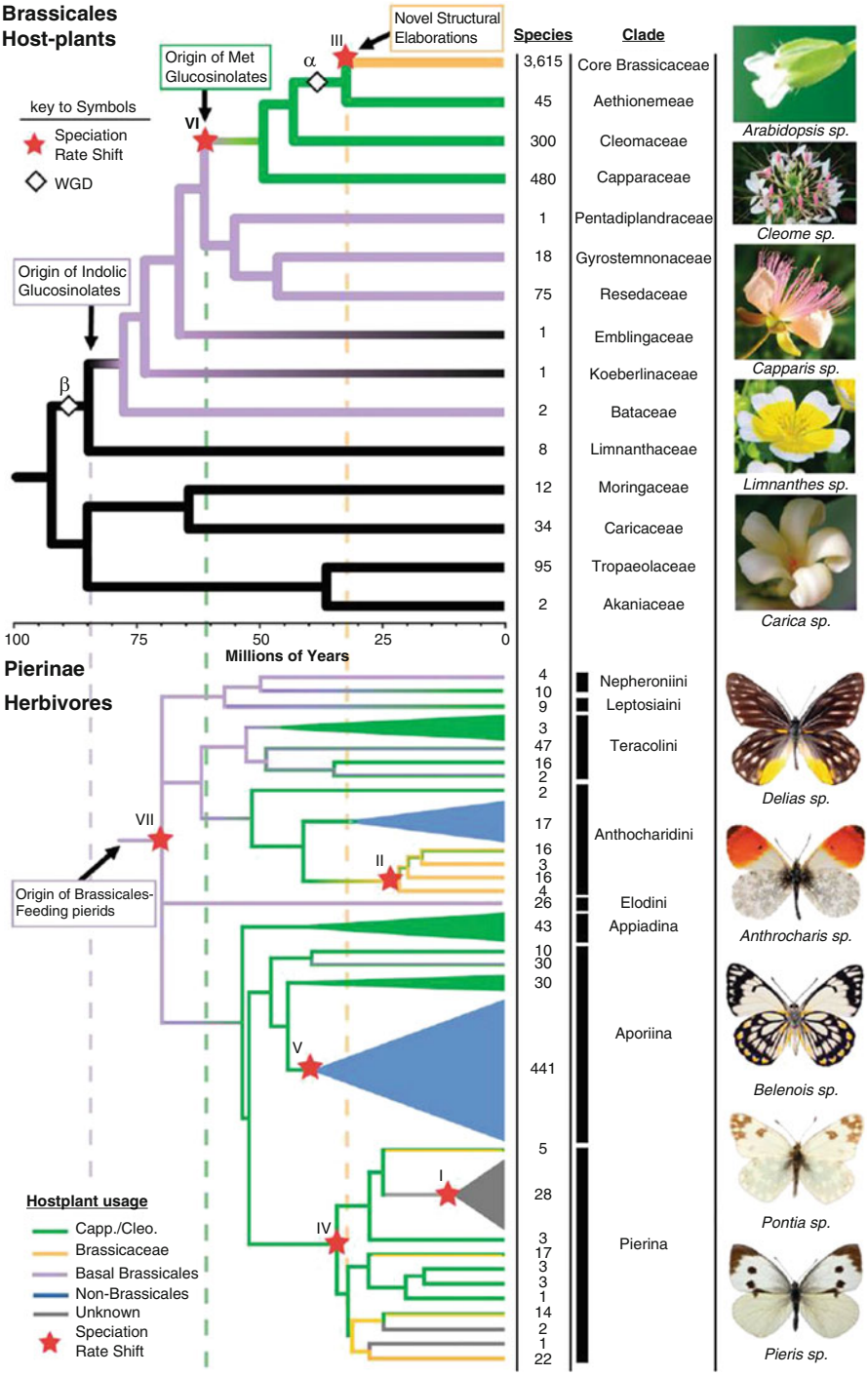


Fig. 3 A chronogram of Brassicales families (upper) and Pierinae butterfly genera (lower), matching the emergence events and adaptive radions of butterflies, host-plants, and their chemical

patterns and contribute to understand the mechanistic bases for cospeciation. The chemical profile of plants linked to host use and the correspondence of plants and herbivores phylogenies documented for certain lineages are perhaps the best evidence supporting the cospeciation and codiversification patterns assumed by the "escape and radiate" model of coevolution.

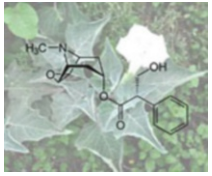
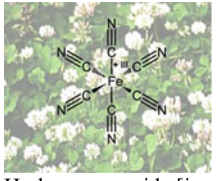
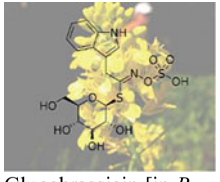
5.3 Biochemical Diversity

Secondary metabolites are functionally and structurally diverse organic molecules not involved in primary metabolic functions of living organisms but involved in more than one important biological process, usually related to survival and interaction with the environment [2, 171, 178]. In plants, secondary metabolites are characterized by having low carbon content (less than 1%, [26]), being the nitrogen:carbon ratio crucial for secondary metabolism modulation (e.g., [63]). Since deamination of amino acid phenylalanine that enabled the accumulation of simple phenylpropanoids in early tracheophytes [182], products of secondary metabolism of plants broadly diversified. Current diversity of phytochemical classes includes steroids, terpenes, alkaloids, phenols, glucosinolates, glycosides, and also nonprotein amino acids and phytohormones [18, 185]. Secondary compounds are distributed in close to 302,211 species including vascular and nonvascular plants, from which angiosperms represent close to 85% [33, 70, 163, 173]. Steroids and terpenes (>30,000), along with phenols (>9000) and (>12,000) alkaloids are the most structurally diverse classes of compounds. The first three have also the most ample distribution in vascular plants [121].

In despite of molecule diversity, most secondary metabolites of plants have a restricted taxonomic distribution, sometimes occurring only in particular genus or botanical family (Table 3). Yet, chemically akin compounds can be found in distantly related plant families (e.g., tropane alkaloids are common in Solanaceae but also occur in Euphorbiaceae, Rhizoporaceae, and Convolvulaceae; [73]). However, the machinery and pathways needed to produce different classes of compounds are highly conserved [4, 186] and much less diverse than their resulting products [179]. Three basic biosynthetic pathways are thought to be responsible for the majority of phytochemicals. (1) The shikimic acid pathway is the biosynthetic

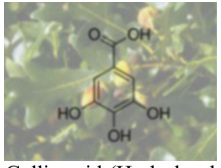
Fig. 3 (continued) defenses. Species numbers and identification of clades are indicated in the adjacent table. The branches in the Brassicales phylogeny are colored to indicate the origin of indolic glucosinolates (purple), methionine-derived glucosinolates (green), and novel structural elaborations to glucosinolates unique to the core Brassicaceae lineage (orange). Vertical dashed lines indicate the origin of these novel chemical groups. Primary host-plant associations of several Pierinae lineages are colored: orange (Brassicaceae), green (Capparaceae or Cleomaceae), orange-green (mixture of previous), purple (more basal Brassicales that synthesize indolic glucosinolates), blue (non-Brassicales feeding), and gray (unknown). The phylogenetic positions for the At-α and At-β WGDs are depicted with white diamond symbols and significant net diversification rate shifts with red star symbols. (Modified from Ref. [47])

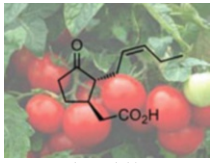
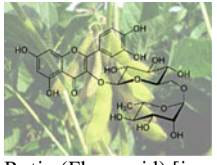
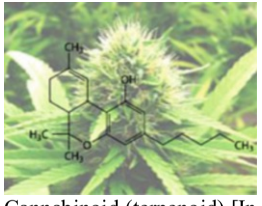
Table 3 Main classes of secondary metabolites of plants and the mode of action of representative compounds

Biosynthetic pathway	Class of secondary metabolites	Taxonomic distribution	Example	Effect on animals
Shikimic acid	**Alkaloid**	• In ~20% of angiosperms (commonly found in Leguminosae, Liliaceae, Solanaceae, Papaveraceae, Apocynaceae, Amaryllidaceae y Rununculaceae), rarely in gymnosperms and some algae [57, 77, 180, 184].	 Hyoscine [in genus *Datura*]	**1. Toxic effect** • Inhibits enzyme activity [34, 126] • Competes for muscarinic receptors of acetyl-choline [143] • Reduces levels of cerebral acetylcholine in mammals [69] **2. Positive effect on fitness** • *In Datura stramonium,* hyoscine was positively correlated with leaf damage inflected by flea beetle *Lema trilineata* [152]
	Cyanogenic glycoside	• In most vascular plants; Gymnosperms, Angiosperms (monoctodilenoeas and dicotyledons) [151, 175]	 Hydrogen cyanide [in *Trifolium repens*]	**1. Toxic effect** • Affects cellular respiration by inhibiting oxygen from binding with the enzyme cytochrome-c-oxidase [111] • Reduces grazing of clover *(Trifolium* spp.) by terrestrial mollusks [96]
	Glucosinolate	• In dicotyledonous angiosperms. Mainly distributed in Brassicales/ Capparales (larger presence in Brassicaceae, Capparidaceae, and Tropaolaceae) [49, 114]	 Glucobrassicin [in *Brassica* spp.]	**1. Toxic and deterrent effect** • As for other glucosinolates, degradation of glucobrassicin by the enzyme myrosinase is expected to produce isothiocyanates. Isothiocyanates reduce herbivore survival and growth, and increase development time of specialist herbivore *Pieris rapae* in a dose-dependent manner [7]

(*continued*)

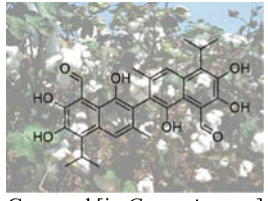
Table 3 (continued)

Biosynthetic pathway	Class of secondary metabolites	Taxonomic distribution	Example	Effect on animals
				2. Positive effect on fitness • Stimulates egg-laying of specialist cabbage white butterflies *Pieris rapae* and *Pieris brassicae* [9, 170]
Shikimic acid	**Phenol-phenyl propanoid derivatives (C6-C3)-**	• Widely distributed in vascular plants. * Tannins and lignin are not found in animals [75, 188]	 Gallic acid (Hydrolysable tannin) [in *Quercus* sp.]	**1. Toxic and deterrent effect** • In vertebrate herbivores, tannins can decrease protein digestion. Tannins are especially prone to oxidize in insects with high pH guts, forming semiquinone radicals and quinones, as well as other reactive oxygen species. Tannin toxicity in insects is thought to result from the production of high levels of reactive oxygen species. [14] • Hydrolysable tannins can reduce feeding by mammals at relatively high concentrations in artificial diets [99] • In vitro, gallic acid showed antibacterial activity [15] **2. Positive effect on fitness** • In natural populations of oaks, leaf and gall concentration of hydrolysable tannins positively correlate with wasp parasite diversity and density [162]
	Phytohormone ***Mixed biosynthetic path**	• Widely distributed in angiosperms and gymnosperms. [18]		**1. Negative effect on plants' consumers by the eliciting of plant inducible defenses** • Hormonal regulator of plants' induced responses to herbivores attack

			 Jasmonic acid/Jasmonate [in Solanaceae]	(plant wounding). Jasmonic acid is involved in the production of diverse plant secondary compounds such as glucosinolates, oxidative enzymes, and alkaloids. [83] • Involved in the display of wound-inducible proteinase inhibitors (Pis) in *Lycopersicon* sp., which function as antinutritive agents by blocking digestive proteases in the herbivore gut. [52, 112, 147]
Acetate-malonate	**Phenol-side-chain-elongated phenyl propanoids-**	• Widely distributed in vascular plants *Except for marine coral and fungi, only plants possess the biosynthetic ability for flavonoids [24, 85]	 Rutin (Flavonoid) [in *Glycine max*]	**1. Negative effect on fitness** • Genotypes of soybean *(Glycine max)* containing rutin and other related flavonoids caused higher mortality, negatively influenced initial larval and pupal weight, and elongated larval cycle of the velvetbean caterpillar *(Anticarsia gemmatalis)*. [136]
Acetate-mevalonate	**Phenol-aromatic terpenoids-**	• Widely distributed in vascular plants. *Diterpenoids are originated mainly from conifers, while triterpenoids occur in angiosperms [131]	 Cannabinoid (terpenoid) [In genus *Cannabis*]	**1. Toxic/deterrent effect** • In mammals alters neurotransmitter release in the brain. [132, 155] • In arthropods, specific cannabinoids can have toxic or deterrent effects. Extracts of *Cannabis* rich in Δ^9 tetrahydrocannabinol (THC) can cause paralysis in larvae of nonbiting midges *(Chironomous samoensis)*. [146] • Pure THC can deter butterflies from laying eggs. [145]

(*continued*)

Table 3 (continued)

Biosynthetic pathway	Class of secondary metabolites	Taxonomic distribution	Example	Effect on animals
				2. Indirect negative effect on fitness • The release of certain volatile organic compounds (VOCs) such as terpenoids can attract natural enemies of herbivores. Many VOC are released after wounding by herbivores or egg deposition [121] • Both bacterial and fungal pathogens and phytophagous insects have been implicated in the modulation of cannabinoid biosynthesis in cannabis. [72]
	Steroid and ispoprenoid	• Widely distributed in angiosperms, steroids commonly found in Caryophyllaceae, Leguminosaceae, Sapindaceae, Liliaceae, and Dioscoreaceae [68, 77]	 Gossypol [in *Gossypium* sp.]	**1. Toxic/deterrent effect** • Inhibits enzymatic activity [31, 43] • Reduces survival of the corn earworm larvae *(Helicoverpa zea),* cotton aphids *(Aphis gossypii),* lygus bugs *(Lygus hesperus),* salt-marsh caterpillars *(Estigmene acrea),* and turberia weevils *(Anthonomus grandis thurberia),* among others. [25, 157]

route of the aromatic amino acids phenylalanine, tyrosine, and tryptophan. This route is restricted to microorganisms and plants and is responsible for the synthesis of alkaloids and glycosides, among others [81]. (2) The mevalonate or the isoprenoid pathway is the route by which terpenes and steroids are synthetized. This pathway is present in eukaryotes, archaea, and some bacteria. Through this route, cholesterol, vitamin K, and all steroid hormones are synthetized. (3) The acetate-malonate pathway is the route of fatty acids and polyketides. This pathway is found in bacteria, fungi and plants, and is responsible for the synthesis of aromatic and aliphatic compounds, prostaglandins and some flavonoids, among many others [41]. Each of these classes of phytochemicals that often characterize botanical families and even a single genus have specific effects on animals (Table 3). Related compounds tend to share a similar range of effects on plants' consumers, affecting specialized cells and tissues. For instance, alkaloids such as cocaine (found in *Erythroxylum spp.*: Erythroxylaceae), atropine (found in *Datura spp.*: Solanaceae), and nicotine (found in *Nicotiana spp.*: Solanaecea and other members of the nightshade family of plants) affect nervous cells by inhibiting the reuptake of neurotransmitters and by competing for the muscarinic receptors of acetylcholine [137, 143]. Also alkaloids, in particular those derived from tropane, may affect the enzymatic activity of plants' consumers inhibiting phosphodiesterase (e.g., [126]) and α-mannosidase enzymes (e.g., [34]). The pharmacological and phytochemical study of secondary metabolites have helped to understand their nature and the extent in to which they can affect plants' consumers either causing sickness or death by poisoning. For instance, quantification of the lethal median dose (LD50) or median lethal concentration (LC50) allows to determine the required dose to kill half the members of a tested population after a specified test duration. For nicotine, the LC50 of humans ranges from 6.5 to 13 mg/kg [118], whereas the LD50 of neonicotinoids (a class of neuroactive insecticide chemically similar to nicotine) for bees typical ranges from 0.03 to 3.6 μg/bee [148]. Nicotine and neonicotinoids cause hyperactivity and death in insects. Their extreme toxicity to insects (but not for specialized ones, see [176]) contrasts with their relative low toxicity to vertebrate taxa [116]. This selectivity is due to a different kind of nicotinic acetylcholine receptors (nACh-R) found in vertebrates [148, 169]. Work derived from bioassays and study cases of herbivores' sensitivity to plants' phytochemicals at cellular and molecular level exhibits the fine-tuning mechanisms of plants when faced consumers. These evidences support the coevolutionary assumption of the reciprocal adaptive adjustments between plants and consumers, mainly driven by plant chemistry.

6 Ecological Patterns: The Defensive Role of Secondary Metabolites

In accordance with macroevolutionary patterns, many studies within species have shown that current associations of plants and herbivores are often related to the presence of a particular kind of phytochemical (e.g., [36, 164]). Unlike the phylogenetic approach, the ecological approach allows to directly measure natural selection,

fitness, and heritability of putative defensive/counter defensive traits. From this, two major ecological patterns have been extensively documented. (1) Secondary metabolites of plants have an adaptive role, mainly related to defense against herbivores (but see [30]), and (2) plants and herbivores impose selection to each other.

6.1 Toxins and Digestibility Reducers

Across natural populations, many studies have tested the defensive *quality* of chemical traits, estimating the strength to which it can ward off herbivores' attack (e.g., by reducing leaf damage or herbivores' survival) and/or increase plant fitness. A good example of a chemical trait holding back herbivores is plant's latex. The sticky white latex produced by milkweeds (*Asclepias spp*.: Apocynaceae) is delivered via specialized canals (laticifers) to most plant parts and can be copiously exuded upon tissue damage [6], suffusing small herbivores (Fig. 4). By addressing the effect of notched versus undamaged leaves of milkweeds on first-instar monarch butterfly larvae (*Danaus plexippus:* Lepidoptera), Zalucki et al. [189] documented a negative impact of sticky latex on larvae survival. Mortality due to miring in the latex was 27% on the notched leaves compared with 2% on the intact leaves. Besides of the physical impediment to herbivores, latex also have a toxic effect on consumers. Latex produced by milkweeds contents cardenolides, which are bitter tasting steroids that act by disrupting the sodium and potassium flux in cells, and have toxic effect on most animals [115]. Latex is an interesting multifaceted defensive trait that can impact herbivores both mechanically and physiologically. However, most chemical traits that function as defensive mechanisms do not mechanically prevent herbivores to freely feed on plants but dissuade them through a toxic or deterrent effect (see the Plant Apparency Hypothesis, [59, 141]). Toxins or *qualitative* defenses are small molecules, peptides or proteins that by interacting with biological macromolecules causing disease or death when consumed or absorbed by animal tissues [44]. Toxins are predicted to occur in plants that do not have a foreseeable distribution (i.e., unapparent plants, such as short-lived plants of early successional stages, sensu [59]), affecting only

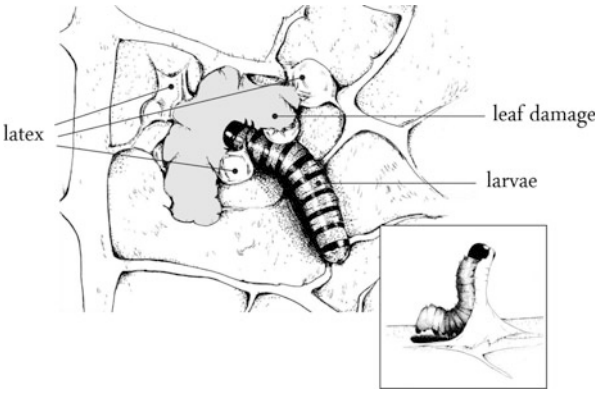


Fig. 4 Plant's latex exuded while the larvae feeds. Sticky latex impede larvae from freely feed and can cause death by miring or poisoning. (Original illustration by the author)

nonspecialized herbivores. By contrast, digestibility reducers or *quantitative* defenses are large molecules (accounting for 5–40% of dry weight, [164]) that would occur in plants with a predictable distribution (i.e., apparent plants like long-living trees, shrubs, and perennial grasses), having a deterrent effect on specialized herbivores [59, 141]. A classic example of deterrent compounds are tannins from oaks. Tannins are the most abundant secondary metabolites made by plants, ranging from 5% to 10% dry weight of leaves. In vertebrate herbivores, tannins can decrease protein digestion. In phytophagous insects, tannins are especially prone to oxidize with high pH guts, forming semiquinone radicals and quinones [14]. Condensed tannins of *Quercus robur* have shown to negative correlate with growth and survival of specialized butterfly *Operophtera brumata* (Lepidoptera: Geometridae) larvae [58, 168]. Several experimental studies have shown the contrasting effect of plant defenses (especially toxins), as a function of the degree of specialization of herbivores. For instance, in *Arabidopsis*, the larvae of the generalist lepidopteran *Helicoverpa armigera*, "cotton worm," avoids feeding on rosette leaves with a high content of glucosinolates [153], while other specialist lepidopteran, the butterfly *Pieris rapae,* successfully feed on plants containing glucosinolates. After ingesting leaf tissue, *P. rapae* synthesizes a protein in the intestine which prevents the formation of isothiocyanates by reorienting the hydrolysis of glucosinolates toward the formation of nitriles which are excreted with feces. Some insects are capable not only of disabling the glucosinolato-myrosinase system but can even use glucosinolates as a cue to locate their host plants [177]. These evidences show how a single chemical attribute can have multiple effects (e.g., toxic, repellent, or attractant, [86]) on the diverse consumers that plants faced along their life. On this regard, contrasting patterns of selection can be expected.

6.2 Natural Selection and Herbivores' Community

Empirical evidence suggests that herbivores act as selecting agents for secondary compounds of plants on natural and experimental populations [17, 32]. Using quantitative genetics or measuring natural selection, studies have documented that plant's compounds that reduce the impacts of herbivores are favored by (positive) directional selection (e.g., [3, 152]) or by balancing or disruptive selection (e.g., [117, 120]). Studies have demonstrated that several chemical compounds of plants, such as glucosinolates have significant phenotypic and genetic variance [5, 125]. Detection of genetic variance for particular compounds indicates that those traits are likely to evolve. However, given that once selection acts on a population, the variance and heritability in the progeny is reduced [51], high genetic variation may suggests that selection is weak or constrained by trade-offs among traits when facing multiple selective forces simultaneously [6, 156, 174].

The effect of multiple phytophagous animals on fitness of a shared host plant may differ between herbivores (e.g., [160]). Specialized and generalist herbivores can exert opposite selective pressures on chemical defensive traits. In experimental populations of *Brassica nigra* (Brassicaceae), Lankau [108] manipulated the presence of the generalist slug (*Agriliomax reticulate*), and the specialist aphid (*Brevicoryne brassicae*), to test

their effect on sinigrin glucosinolate. The author found that generalist damage was negatively correlated with concentration of sinigrin, whereas the specialist herbivore was positively correlated with increasing sinigrin concentrations. At the same time, sinigrin concentration was favored when specialists were removed, disfavored when generalists were removed and selectively neutral when plants were expose to both generalists and specialist herbivores. When plants are simultaneously exposed to the attack of herbivores with different degree of specialization, the evolutionary outcome would depend not only from the strength or direction of selection that each herbivore can impose but also from the dynamics between them. In the same Brassicacea, *B. nigra*, Lankau and Strauss [109] analyzed the effect of inter- and intraspecific competition of two phytophagous species (a generalist folivore mollusk and a specialist aphid) on sinigrin concentration. They found that only interspecific competition favored the investment in chemical defense and that this pattern of selection was dependent on the presence of both specialist and generalist herbivores (diffuse selection). This study shows that selection acting on chemical traits is highly sensitive to community composition of herbivores (for a review of the impact of ecological heterogeneity on selection gradients, see [8]). These ecological genetics studies that assess traits mediating plant-herbivore interactions have exhibit that selection impose by herbivores play a central role in shaping chemical phenotypes of plants.

7 Conclusions

Codiversification of vascular plants and arthropod groups is a common outcome of the antagonistic interaction of host-plants and herbivores mediated by plant chemistry. The reciprocal fitting between host-plants and phytophagous insects that includes species, traits and genomes draws major adaptive patterns. Either from a phylogenetic or ecological approach several studies have documented that (1) secondary metabolites of plants function mainly as defenses to prevent herbivory, but their reach is constricted of herbivores' specialization. And, (2) the evolution of herbivorism is linked to diversification and speciation of plant and insect lineages. The match of the genetic mechanisms responsible of evolutionary *novelty*, such as gen and genome duplications with codiversification and speciation, is the best piece of evidence of how coadaptation driven by diffuse coevolution over long periods of evolutionary time is a major source of biodiversity.

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Differential Response of Herbivores to Plant Defence

4

Martin Volf

Contents

Abstract

The differential response of insect herbivores to plant traits is one of the mechanisms promoting diversity and specificity of insect-plant interactions. The response differs mainly among generalist insects on the one hand and specialized or adapted insects on the other hand. While generalists are often strongly affected by toxic defences of their hosts, specialists have evolved various adaptations to overcome such defences. These adaptations include tolerance, detoxification, or sequestration of secondary metabolites of the host. In addition, behavioral adaptations help herbivores to avoid particularly potent defences. The response of herbivores is also tightly linked to their feeding mode (i.e., herbivore guild), physiology, metabolism, or size. The resulting specificity of interactions gives rise to diversification of host defences as no single trait can provide an efficient defence against diverse communities of insects. The diversification of host defences then seems to be one of the key factors promoting diversity of insects in a reciprocal way.

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Adaptation · Diversity · Evolution · Generalists · Secondary metabolites · Specialists · Specialization

1 Introduction

Herbivorous insects and vascular plants represent two of the most numerous groups of multicellular organisms, driving major ecological processes in many terrestrial habitats [1–3]. They owe their diversity largely to a long-shared history and to the specificity of their interactions [4–6]. Various insect herbivores often show specific responses to host plant defences, which reciprocally support diversification of plant defensive strategies and in turn the diversity of plants and insects themselves [4, 5, 7, 8]. In this chapter, I summarize how the specificity of insect responses arises and discuss the consequences for insect-plant coevolution. I focus on the role of insect specialization and mode of feeding (i.e., a feeding guild) as two major factors governing their response to host plant defences.

The first insects began utilizing plants as a food source in the Early Devonian period, only several million years after vascular plants colonized terrestrial habitats [9, 10]. This led to a progressive radiation in the diversity of herbivorous insects and their feeding guilds. The pioneer insect herbivores were sap-suckers, stem-borers, and consumers of spores [11]. Thalli, which evolved into leaves some time later, only started to be consumed by chewing herbivores shortly after that in the Middle Devonian [10]. All modern herbivore guilds, with the possible exception of leaf-miners, were present by the Late Carboniferous, more than 300 million years ago [10].

The proliferation of herbivore lineages and guilds has increased and diversified herbivory pressure on plants and led to an arms-race between plants and insects [4, 5] (Box 1). This generally required plants to employ a broad suite of defences in order to maintain efficient protection against diverse communities of herbivores [12, 13].

Box 1
In their seminal paper, Ehrlich and Raven [4] proposed the so-called *escape-and-radiate* scenario of insect-plant coevolution. According to the *escape-and-radiate* scenario, the genesis of novel defensive traits allows plants to escape herbivory, leading to speciation of the respective plant lineage. However, after some evolutionary time, herbivores adapt to the novel defence and overcome it. This allows herbivores to colonize that plant lineage, opening a novel niche to them. The adapted herbivores speciate, and the process starts over. This should, on the one hand, lead to diversification or escalation of host plant defences over evolutionary time and on the other hand to co-diversification of the plant and insect lineages involved.

Recent studies suggest that clear cases of co-diversification are relatively scarce [128, 129], with *sequential radiation* and *phylogenetic tracking* being

(continued)

Box 1 (continued)
more prevalent [22]. In such a situation, herbivores colonize an existing lineage of plants well after its divergence, often specializing on hosts with similar defences [5].

Reciprocally, the resulting diversification of host plant defences probably supported further proliferation of feeding strategies in insect herbivores, in many cases leading to their specialization. Although there are numerous exceptions, the specialization of insect herbivores remains one of the main factors governing their response to host plant defences in virtually all the systems where this was examined (e.g., [7, 13–15]).

2 Roles of Insect Specialization in Their Response to Host Plant Defences

Insect herbivores show a broad range of host specialization (Box 2). Specialization to a limited set of hosts should allow herbivores to become well adapted to host defences and *vice versa* feeding on multiple host species should come at a cost of being maladapted to host defensive traits (e.g., [16]). Unspecialized insects are often excluded from strongly defended plants [17–19]. For example, there are few generalist herbivores found on milkweeds, which are strongly defended by cardenolides [17]. Generalist herbivores also strongly respond to unique or rare secondary metabolites as predicted by *feeding specialization* and the *biochemical barrier* hypotheses [20]. Indeed, plant lineages with a specialized defence often have highly specialized herbivore fauna [18, 19].

Box 2
Herbivore specialization, defined as the number or diversity of host plant species used, is one of the most widely used concepts in insect-plant ecology [14, 130]. Insect herbivores are traditionally classified as specialists or generalists. But there is a broad continuum between the two – from herbivores feeding on a single host (monophagy), through herbivores feeding on a limited set of hosts (oligophagy), to herbivores feeding on multiple hosts (polyphagy). The definition of oligophagy and polyphagy is sometimes ambiguous. Most definitions of specialization derive from simple counts of host species. However, most herbivores show some level of specialization when examined in detail. For example, most insect herbivores feed on closely related hosts [131]. The majority of herbivores in lowland tropical forests in Papua New Guinea seem to prefer to feed on congeneric or confamilial tree hosts [132], for example. A similar trend is apparent on a global scale and among several herbivore guilds [24].

(continued)

Box 2 (continued)

Recent studies have suggested that indices measuring herbivore specialization should comprise affinities among resources as well as their co-occurrence with consumers (e.g., [130]). Such indices allow to define specialists as herbivores using significantly clustered sets of resources, feeding on related or otherwise similar hosts. For example, specialization can be measured as phylogenetic or chemical relatedness of the used resources. This allows distinctions to be made between lineage specialists, herbivores tracking a lineage of hosts, and trait specialists, herbivores tracking certain host defences [130]. On the other hand, generalists can be defined as herbivores using overdispersed resources. Intermediate species are classed as indiscriminate consumers.

There are some notable exceptions, and some herbivores feeding on multiple hosts, such as tiger moth *Grammia geneura*, are able to feed on plants with highly toxic defences. *G. geneura* is sometimes considered as a generalist as it feeds on several unrelated hosts high in pyrrolizidine alkaloids [21]. But even such herbivores are rarely indiscriminate consumers. In this particular case, *G. geneura* feeds on a pool of locally available alkaloidal hosts, making it a pyrrolizidine alkaloid specialist (Box 2). This may be rather common under *sequential radiation* and *phylogenetic tracking* scenarios [22], when herbivores colonize already existing plant lineages. In such a situation, host shifts can track similarities in host defences rather than strictly follow host phylogeny. For example, host shifts in *Melitaeini* nymphalid butterflies feeding on 16 plant families have been shown to follow the presence of iridoid glycosides [23].

Highly polyphagous insect species feeding on large number of hosts from various lineages disregarding their traits are relatively rare even among herbivores considered to be generalists [24]. In many terrestrial ecosystems, such highly polyphagous herbivores represent only a small portion of the herbivore community [25]. When forced to feed on toxic diet, consisting of a narrow set of toxic hosts, these herbivores generally perform poorly. This is because instead of employing elaborate detoxification mechanisms, some of these herbivores rather mix different diets to achieve optimal quality and dilute toxins [26, 27]. Their diet thus often includes a diverse set of plants belonging to different functional groups under natural conditions [28].

3 Tolerance and Adaptations of Specialized Insect Herbivores

Insect specialists have repeatedly evolved adaptations to overcome toxic or deterrent effects of host plant defences. For example, specialized sawflies on birch are able to detoxify flavonoid aglycones by glycosylation [29, 30]. One of the most iconic examples of secondary metabolite detoxification by insects involves specialized

herbivores on Brassicaceae. Brassicaceae possess a strong chemical defence, known as the glucosinolate-myrosinase system, generally maintaining an efficient protection of *Brassicaceae* hosts to generalist herbivores [31, 32]. When the host tissue is damaged, formerly compartmentalized myrosinase enzyme gets into contact with glucosinolates. These are then hydrolyzed into isothiocyanates, which are toxic to herbivores [32]. Specialized herbivores, such as *Pieris* butterflies or *Plutella xylostella* diamondback moths, have evolved mechanisms to detoxify this defence [32, 33]. Larvae of *Pieris rapae* express a nitrile-specifier protein in their midgut, promoting the formation of nitrile breakdown products instead of toxic isothiocyanates [32]. *Plutella xylostella* diamondback moths employ glucosinolate sulfatase to desulfate glucosinolates, producing metabolites that no longer act as substrates for isothiocyanate production by myrosinases [33]. This illustrates that detoxification mechanisms have evolved several times independently in insect specialists even within a single insect-plant system. Although such detoxification mechanisms facilitate a similar response to host plant defences, their background is often different and crucial for understanding how tolerance to plant defences in particular cases is maintained.

Furthermore, some specialized herbivores were able to adapt to host defences to such an extent that they can use them for their own benefit. In these cases, specialized herbivores often respond positively to host plant chemical defences. This is also the case for several specialized herbivores adapted to salicylates in Salicaceae hosts. Salicylates are phenolic glycosides typical for willows and poplars. Although various derivatives of salicyl alcohol can be found in many plant lineages (e.g., [34]), in Salicaceae they reach the highest diversity with many compounds being unique and novel for this family [35]. Salicylates have been reported to serve an anti-herbivorous function primarily and their documented impact on generalist herbivores involves deterrent effects, retarded larval growth, and increased mortality [36, 37]. However, certain specialist herbivores show preference for willow hosts with high salicylate content, using salicylates as feeding cues [38]. This is probably because they are able to sequester salicylates and even use them to their own benefit. Such an ability was best documented in *Phratora* and *Chrysomela* leaf beetles. Larvae of these beetles use salicylates as a precursor for salicylaldehyde, a metabolite deterring invertebrate predators including ants and lady beetles [39, 40]. They secrete salicylaldehyde from specialized abdominal glands (Fig. 1). In addition, larval growth in several specialized leaf beetle and sawfly species has been shown to be promoted on hosts with high salicylate content, providing them with an additional advantage [41].

Another example of specialized herbivores showing a strong preference for highly toxic hosts are *Asota* tiger moths. *Asota* tiger moths are broadly distributed in Africa, south Asia, and tropical parts of the Australian region. Both adults and larvae are brightly colored and usually feed on alkaloidal hosts (Fig. 2). In lowland tropical forests in Papua New Guinea, *Asota* moths are largely specialized on *Ficus*. The abundance of the larvae shows a strong positive correlation with the content of phenantroidolizidine alkaloids in the host leaf tissue [7]. Other tiger moth species have been reported to be able to sequester host alkaloids, convert them into their

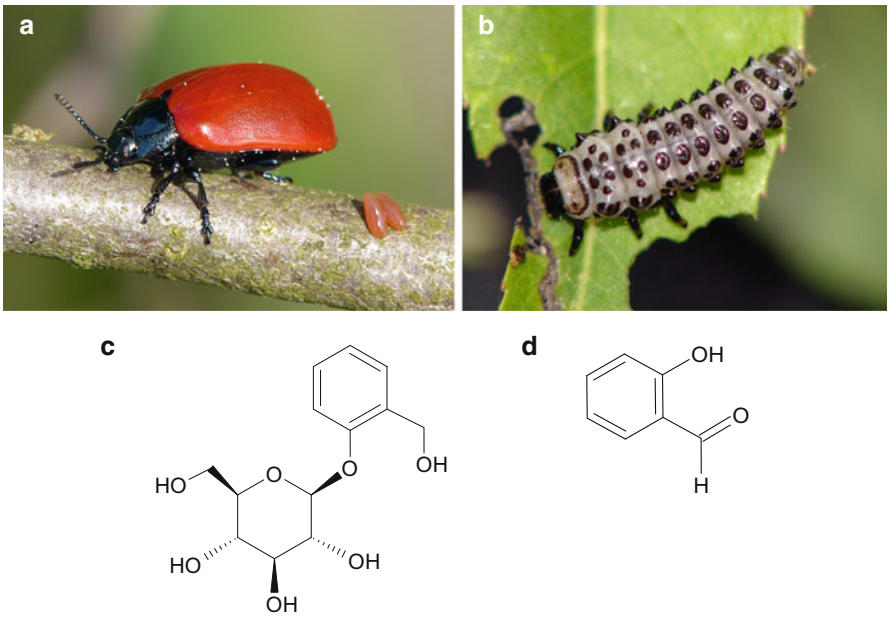

Fig. 1 A female of *Chrysomela populi* laying eggs on a poplar twig (**a**) and a larva of *Chrysomela vigintipunctata* feeding on a leaf of *Salix fragilis* (**b**). These leaf beetle species are specialized on feeding on Salicaceae hosts and often prefer species with a high content of salicylates, such as salicin (**c**). Their larvae use salicylates as precursors for production of salicyladehyde (**d**). They secrete salicyladehyde from abdominal glands as a protection against invertebrate predators

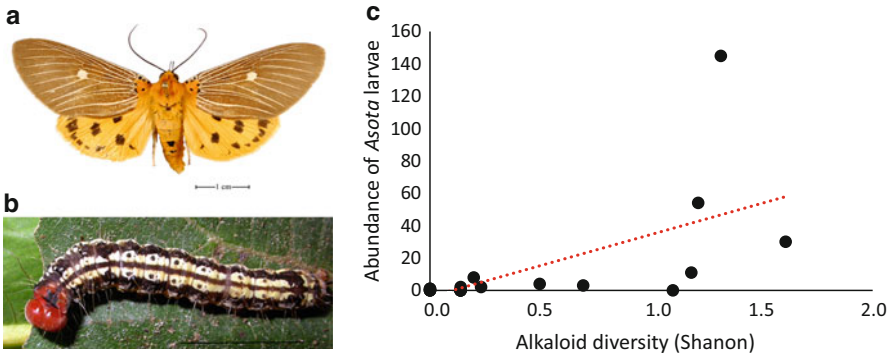

Fig. 2 An adult *Asota* moth (**a**) and its caterpillar (**b**). These brightly colored moths use highly alkaloidal host plant species. In lowland forests of Papua New Guinea, they specialize on *Ficus* species containing high concentration of phenantroindolizidine alkaloids, with abundance of larvae being strongly positively correlated to alkaloid content in host plant leaves (**c**). (Data taken from on Novotny et al. [42] and Volf et al. [7]. Insect photos were downloaded from "Caterpillars feeding on New Guinea plants" database [43] curated by the New Guinea Binatang Research Center)

nontoxic N-oxides, and store them in metabolically inactive tissues [21]. Although the specific mechanism of alkaloid sequestration remains unknown in the case of *Asota* moths, it has been suggested that they can use host alkaloids for their protection [44]. Indeed, both *Asota* adults and caterpillars are highly toxic to predators. They also show high toxicity to humans to such an extent that there were cases of mass lepidopterism fever outbreaks during an *Asota* population explosion in India [45].

In addition, herbivores have evolved behavioral adaptations and mechanisms to avoid defences of their hosts. *Passiflora lobata* has its leaves protected by dense hook-shaped trichomes, which have strong negative effects on non-adapted herbivores. However, larvae of specialized *Heliconius charithonia* butterfly are able to release themselves from the hooked trichomes if entrapped. Moreover, they produce silk mats and bite off the hooked tips of the trichomes in order to be able to move around the leaf. Such adaptations significantly improve their feeding efficiency. While trichomes deter generalist herbivores, specialists thus do not seem to be strongly affected by them in this particular case [46].

Another behavioral adaptation includes herbivores able to cope with hosts producing latex. Production of latex has independently evolved in multiple plant lineages, including around 10% of flowering plants species [47]. Latex serves as a mechanical protection directly interfering with insect feeding. In addition, latex serves as a vessel for various defensive compounds. In *Ficus*, latex contains high concentrations of cysteine proteases, interfering with processes in the insect mid-gut and being among the traits with the most pronounced effects on *Ficus* herbivores [7, 48]. In milkweeds, latex contains cardenolides, inhibiting the Na^+/K^+-ATPase enzyme and showing high toxicity to most animals [49]. As such, latex is usually an efficient form of defence, which probably supported diversification of plant lineages possessing latex [6]. To avoid latex, specialized herbivores have evolved behavioral adaptations including cutting leaf veins, impairing latex transportation and outflow. On hosts with non-articulated venation, herbivores cut the main vein only. Such a behavior can be observed in later instars of monarch butterfly caterpillars (*Danaus plexippus*), for example. On hosts with articulated venation, herbivores have to cut multiple veins by creating trenches over large parts of the leaf blade (Fig. 3) [47].

4 Nutrients, Natural Enemies, and Induced Defences

Host-plant defences can increase herbivore mortality directly (e.g., by intoxication) or indirectly through enhanced risk of predation or parasitism [50]. Negative effects of host defences may prolong the time herbivores need for feeding. Such a prolonged period of feeding exposes herbivores to higher risks of being predated or parasitized. A caterpillar has ca. 100× higher risk of being predated or parasitized when active and feeding [3, 51]. Therefore, the effects of host traits can be modulated by natural enemies of herbivores – by predators and parasitoids. There is some evidence that high predation can even facilitate host-shifts to novel hosts. For example,

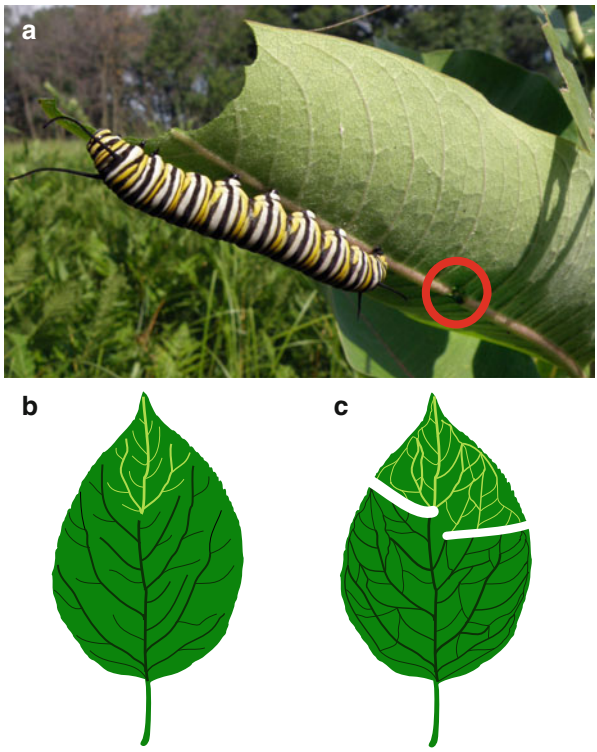

Fig. 3 A caterpillar of monarch butterfly (*Danaus plexippus*) feeding on a milkweed (**a**). Note the vein cut by the caterpillar (marked by a *red circle*). Specialized herbivores feeding on plants high in latex often cut the veins in order to limit the latex outflow during their feeding. On leaves with a non-articulated venation, herbivores can cut only the main vein to disrupt a latex outflow in a given part of leaf (**b**). On leaves with an articulated venation, herbivores cut the leaf by wide trenches (**c**) (Adapted from Agrawal and Konno 47). Leaf areas with disrupted latex outflow are in *yellow*

swallowtail butterflies in Alaska started to use novel hosts, despite being well adapted to their ancestral Apiaceae hosts, probably as the new hosts represent an enemy free space [52].

All insect herbivores are trying to achieve optimal feeding efficiency and growth rates. They pursue maximal nutrient value of their diet, while balancing other risks [3, 53]. However, generalist insects often cannot fully benefit from high nitrogen and nutrient content as they are not able to fully overcome toxic metabolites of their hosts, as mentioned above. Therefore, they often show lower growth rates and nitrogen content is a weaker predictor of their community composition than secondary chemistry of the host [54]. On the other hand, specialists are usually able to cope with toxins of the host and can pursue high nutrients more efficiently, making nitrogen an important factor structuring their communities [15]. As such, the abovementioned adaptations help specialized insects to avoid predation and parasitism not only by using secondary metabolites of their hosts [55] but also by fast growth, avoiding predators and parasitoids in time (if generalists are directly defended, they seem to rely on physical defences, such as spines or shelters [54]).

Many herbivores use behavioral adaptations, such as staying cryptic, to avoid their natural enemies in space [56]. Locating insect herbivores is thus not an easy task for predators and parasitoids that often have to rely on cues provided by host plants. Plants have evolved mechanisms to attract predators and parasitoids in order

to facilitate their own protection against herbivores. These defences rely largely on volatile infochemicals [57, 58]. While many volatiles are produced by plants even when not attacked by herbivores [3], the true complexity of these interactions is revealed after a herbivore attack and an induction of the host defences. The previous examples in this chapter largely focused on constitutive defences, which are more or less steadily present in the plant tissue and their level is not directly governed by external stimuli, such as herbivory. Induced defences are deployed after a herbivore attack and represent an alternative form of plant defence with possibly differential effects on various insects.

Induced defences are based on several complementary mechanisms and often show a high degree of specificity, which makes them an efficient protection against a variety of herbivores. When induced, plants can upregulate defences (secondary metabolites, trichomes, leaf thickness, etc.) that target the herbivore [59, 60]. These defences directly affect herbivore preference and performance. Herbivores should be able to cope with the induction of direct defences in a largely similar way to constitutive defences, although it may require them to habituate to increased defence levels (e.g., they need to increase the efficiency of their detoxification mechanisms [61]).

However, plants can also employ elaborate indirect defences which help them to attract natural enemies of herbivores through the production of herbivore induced plant volatiles (HIPVs) [57, 58, 62]. HIPVs, such as shikimic acid derivatives, terpenoids, or alcohols, are generally well detectable even in complex environments, unlike the scents emitted directly by herbivores themselves, and help predators and parasitoids to navigate efficiently toward their prey [63]. Importantly, the induced responses in HIPV production seem to differ between herbivores, showing a large degree of specificity [64]. In a greenhouse experiment, Danner et al. [65] demonstrated that the responses in indirect induced defences differed among herbivores from different feeding guilds (see also below). Leaf-chewing herbivores induced a strong response in HIPVs, while sap-sucking herbivores were able to suppress their production. Induced responses may also differ between specialist and generalist herbivores, but these differences seem to be much more subtle and vary among systems [65, 66].

So far, the relative importance of different forms of induced defences in plant defence and their effects on specialist and generalist herbivores remain largely unknown [62]. For example, the attraction of predators is likely to benefit plants through the immediate removal of herbivores [59]. On the other hand, parasitism does not lead to an immediate termination of herbivory and in some cases it can even prolong the feeding period of parasitized larvae [59]. Several common direct defences, such as some phenolic secondary metabolites, have only limited effects on immediate insect mortality on their own, though they retard larval growth [60]. Their main defensive value can possibly result from an interplay with indirect defences as they can prolong larval growth and increase the exposure of herbivores to predators or parasitoids attracted by HIPVs. The effect of defensive traits is thus highly dependent on the third trophic level context.

HIPVs have been long known to attract insect parasitoids or predators [67, 68]. Recent results suggest that these volatiles can also be perceived by birds [58]. For

example, Amo et al. [58] showed that birds were attracted to trees infested by lepidopteran larvae, even if the larvae and their damage was removed just before the experiment. This largely rules out the possibility that the birds used visual cues for locating the attacked tree in this case.

5 Feeding Guilds and Their Response to Host Plant Defences

The previous sections of this chapter mainly focused on leaf-chewing herbivores (Fig. 4). Indeed, leaf-chewing herbivores represent one of the main herbivore groups in terms of diversity, abundance, and the amount of damage they do to their host plants [3]. However, there are many other herbivore guilds, some of them displaying quite different responses to host plant traits compared with leaf chewers. The levels of specialization differ among herbivore guilds, ranging from polyphagous root-chewing larvae typically feeding on hosts from multiple plant families, through leaf-chewing larvae feeding on several congeneric or confamilial hosts, to gallers and miners with very limited host spectra [24, 42]. The variation in feeding modes provides herbivores from different feeding guilds various options to avoid host plant defences, largely shaping their response to host plant traits. For example, highly specialized endophytic herbivores evolved mechanisms to manipulate hosts, while some sucking herbivores specialized on feeding on resources with relatively low defensive metabolite content.

Endophytic herbivores such as leaf miners and gallers (Fig. 4) belong among herbivores with the most intimate interactions with their host plants. Their diet usually includes only a couple of closely related congeneric hosts or even a single

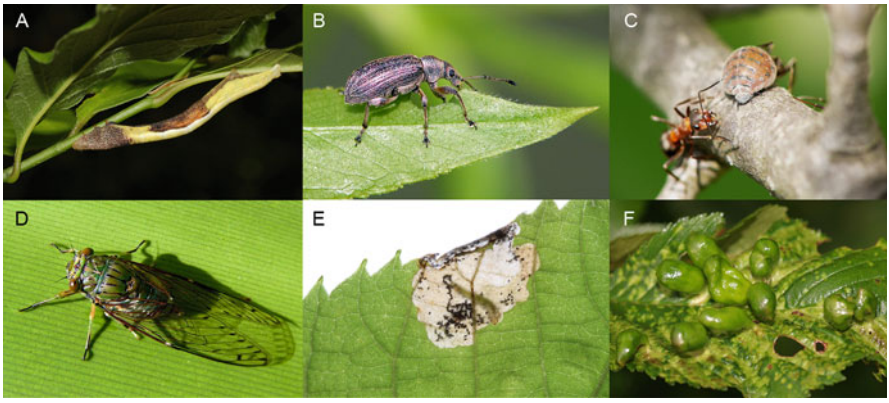

Fig. 4 Herbivores from various feeding guilds mentioned in this chapter: (**a**) a larval leaf chewer (a geometrid caterpillar feeding on *Magnolia kobus*, Japan), (**b**) an adult leaf chewer (*Phyllobius* sp. weevil feeding on *Salix alba*, Czech Republic), (**c**) a phloem sucker (Hemiptera feeding on *Salix aurita*, Czech Republic; note the ants guarding the phloem sucker), (**d**) a xylem sucker (Cicadidae, Papua New Guinea), (**e**) a miner (*Parna kamijoi* sawfly feeding on *Tilia maximowicziana*, Japan), (**f**) gallers (galls of *Tetraneura ulmi* on *Ulmus glabra*)

host species [42, 69]. These herbivores live inside the plant tissue, either inside the leaf lamina or in induced galls. Their endophytic lifestyle provides them with some protection against biotic and abiotic factors such as UV irradiation and drought, and in some cases probably also with protection against predaors or generalist parasitoids [3, 70, 71] (but there are many specialized parasitoids of mining or galling herbivores (e.g., [70, 72])). The highly specialized nature of miners and gallers also allows them to escape negative effects of certain defences of their hosts.

First, a miner larva living inside the leaf lamina does not have to deal with a tough leaf surface and can preferentially feed on cells with high nutrition value [73]. Indeed, leaf toughness is an important predictor of food choice in leaf-chewing insects, which cannot avoid chewing on the tough cuticle of the leaves [3, 74]. Some plant groups such as palms or grasses, which contain high levels of silica-based physical defences have especially tough leaves, which erode mandibular jaws of chewing herbivores, significantly lowering their feeding efficiency [75, 76].

Second, their highly adapted nature allows gallers to manipulate their hosts to form galls by using metabolites closely resembling or identical to phytohormones [77, 78]. The ability of host manipulation was probably a key innovation in several taxa of gall-forming herbivores because there have been repeated and often dramatic radiations of gall-forming Arthropods including various insect orders and mites [79]. The radiations of several galler taxa have been characterized by associations with key plant genera – e.g., radiation of Cynipidae wasps on *Quercus*, Pemphigidae aphids on *Pistacia*, and Tenthredinidae sawflies on *Salix* [79]. A gall itself can have a significantly different chemical profile than the rest of the plant tissue, and gallers can control host plant defences, such as secondary metabolites [80]. Galls thus can have lower content of defensive secondary metabolites, while they have higher nutrient values than normal plant tissue (but note that increase of certain defensive metabolites in galls is also possible). Downregulation of host defences has been recorded in many plant-galler systems as reviewed by Giron et al. [78] and include downregulation of various phenolics, proteases, or volatile compounds (e.g., [80–83]). As suggested by Stone et al. [84] gallers "represent examples of an alternative coevolutionary arms race paradigm, not between toxins and detoxification systems as in the Ehrlich and Raven model, but between host plant susceptibility and gall inducer virulence." This may lead to a situation when abundance or diversity of gallers does not respond to host chemical defences [15]. This is quite different from leaf chewers, which often show negative or positive correlation to the secondary metabolite content of the host [13, 19]. Indeed, in some cases host selection in gallers seems to focus on plant species with high nitrogen content disregarding their defences, possibly as such hosts may be more easily manipulated to contain even higher nutrient concentration [15]. Relatively recently, host manipulation toward higher nutrients and lower defences was also recorded in the case of miners [78, 85], suggesting that some members of this guild can, to some extent, ignore host defences as well.

Another example of a herbivore guild with a response to host plant defences largely different from leaf-chewers are sucking herbivores. Sucking herbivores have evolved specialized, so-called haustellate mouthparts. Such mouthparts evolved

independently in Thysanoptera, Lepidoptera, and mainly in Hemiptera, which include most sucking herbivores [3]. The haustellate mouthparts are formed from several components, including maxillary and mandibular stylets, which serve for piercing plant surface. There are three main guilds of sucking herbivores feeding on aboveground plant parts – phloem suckers, xylem suckers, and leaf suckers [42] (Fig. 4). The different food source influences their level of specialization and host preference.

Phloem suckers feed on phloem fluids. This guild includes mainly aphids, scale insects, and most leaf hoppers. Their diet is high in primary metabolites such a sugars, while it is relatively low in nitrogen. Excess sugars are often excreted to attract ants, which protect the phloem sucker from predators. Phloem is also low in secondary metabolites. Hence, when the relative ratio of nutrients and defensive secondary metabolites are compared, phloem may be a more favorable food source than leaf tissue in some cases [3]. Xylem suckers include mainly cicadas and cercopoid froghoppers. These herbivores have specialized on an even poorer diet, the xylem fluids. Xylem is ca. 200× lower in nitrogen than Angiosperm leaf tissue [3, 86]. However, it is almost devoid of defensive secondary metabolites. Due to the avoidance of chemical defences, both phloem and xylem suckers are often primarily affected by nutrient content and physical characteristics of the host, such as thickness of the surface cuticle [3, 87]. Although there are some notable exceptions, both guilds show relatively low levels of specialization on the whole [42]. This is in sharp contrast to leaf suckers. Leaf suckers include specialized Heteroptera [88] and some cicadellids, which evolved from their phloem-feeding ancestors [89]. These herbivores suck on the content of individual leaf cells. Although they can avoid some compartmentalized secondary metabolites, they are probably still exposed to much higher content of defensive compounds than phloem- and xylem-sucking herbivores. This may be one explanation as to why this guild includes mainly highly specialized herbivores [42, 89].

In addition, sucking herbivores, such as aphids or some leaf hoppers, can suppress plant defences [65]. Aphids inject saliva containing suppressor proteins in the host tissue while feeding [90]. This has been shown to affect mainly Ca^{2+} signaling in the attacked tissue [91]. Suppressing Ca^{2+} signaling has pronounced cascading effects on induced responses to damage. Indeed, sucking herbivores have been shown to induce a weaker response of their hosts than leaf-chewing insects [65], probably allowing them to partially avoid indirect induced defences.

6 Roles of Insect Morphology and Physiology in the Response to Host Plant Traits

The response to host plant defences is also driven by morphologic and metabolic characteristics of insect herbivores, some of them being unrelated to their specialization. For example, small herbivores tend to respond to host defences differently than large ones. As outlined above, xylem suckers, which can feed on a diet low in

defensive chemicals, include mainly large froghoppers or cicadas. This is not a coincidence as small herbivores would not be able to efficiently suck on xylem due to its negative pressure. On average, xylem suckers are thus larger than phloem or leaf suckers [89]. Small herbivores also often respond more strongly to physical defences. This is well illustrated by small, freshly hatched caterpillars, which sometime have troubles with chewing on tough, mature leaves. Small chewing herbivores also have problems cutting through sclerophyllous veins of grasses [76]. Other physical traits which probably also affect small herbivores more than large ones are trichomes [17]. Trichomes possess various functions and show high morphological variability. Glandular trichomes may serve for secreting defensive secondary metabolites [3]. Simpler, nonglandular trichomes serve mainly as mechanical protection. They prevent small herbivores from reaching the surface of the plant, make their movement more difficult, and increase their chance of falling. This makes herbivore feeding less efficient and increases predation risk [37, 92]. Trichomes also prevent females of small herbivores from ovipositing their eggs on the leaf surface [93]. On the other hand, females of some specialized herbivores can use trichomes to get a better grip on the plant, enhancing oviposition efficiency [94].

Different herbivores can also have different conditions in their guts, which largely affects how they process their diet and what traits of the host affect them. One such example is the response of caterpillars to tannin content and activity. Tannins represent a diverse group of phenolic compounds that are broadly distributed among plants [95]. It was proposed that one of the main defensive values of tannins in terms of anti-herbivore protection results from their ability to precipitate proteins in guts of herbivores under low pH. Such a mechanism is known in the case of mammalian herbivores, in which some tannin groups reduce apparent N digestibility [96]. The protein precipitation activity is especially high in procyanidins (condensed tannins) [97], which have been shown to affect food selection in beavers, for example [98]. Many of the previous studies on insect-plant interactions focused primarily on this group of tannins when interpreting herbivory by insects. However, most caterpillars tend to have alkaline mid-guts [99]. Several studies have shown that ability of procyanidins to precipitate proteins is limited in such conditions [95, 100, 101]. Condensed tannins thus probably serve simply as indigestible matter, lowering overall feeding efficiency in caterpillars [59, 102]. From the perspective of anti-caterpillar protection, groups of tannins other than procyanidins may be more important. These include ellagitannins, which show high oxidative activity. Recent results suggest that tannin oxidative activity tends to have much more pronounced effects on caterpillar community composition and diversity than does tannin protein precipitation capacity [7, 103]. The oxidation of ellagitannins in caterpillars' mid-gut can facilitate nucleophilic reactions with proteins and the formation of highly reactive hydroxyl radicals. In other words, the products of tannin oxidation can damage nutrients in the gut lumens of insect herbivores or produce cytotoxic effects in their tissues [95, 104]. The nutritional stress may be especially important as a form of defence against some herbivores specialized on high tannin content, such as

Lymantria dispar, in which the oxidative stress itself cannot reduce growth rates on its own [59, 105].

7 Implications for Evolution of Host Plant Defences and Insect Diversity

The differential response of herbivores has important implications for evolution of host plant defences. Mainly, it restricts plants from developing a universal anti-herbivore defence [12, 13]. Specialists have been shown to prefer or tolerate hosts with high levels of specific defensive compounds in the case of multiple plant genera (e.g., [7, 13, 106, 107]). This might have been one reason for the decline in specific defences in *Asclepias* [107]. Similarly, specialized insects were able to adapt to salicylates and reach high densities on salicylate-rich willow hosts as outlined above [19]. Although salicylates play a significant role in structuring insect communities, their protective value against specialized herbivores appears to be low. Maintaining an efficient defence thus probably requires several defensive mechanisms, such as chemical defence and trichomes, which affect both generalists and specialists on willows [13]. As a result, defensive traits are often mutually independent or positively correlated, forming suites of complementary defences or so-called defensive syndromes [7, 108, 109]. Trade-offs between individual defensive traits may be expected only under specific conditions, such as low nutrients or in the case of negative dependence in metabolic pathways (e.g., a competition for a specific precursor) [110, 111]. Furthermore, some recent results suggest that defensive syndromes can consist of traits following different evolutionary trajectories, possibly making adaptation even harder for herbivores [7]. This seems to shape the evolution of plant defensive traits into a dynamic system, with traits undergoing periods of diversification, divergence, and sometimes decline [5].

Indeed, the differential response of insect herbivores can shape evolutionary trajectories in individual defensive traits. Ehrlich and Raven [4] proposed escalation of host plant defences over evolutionary time, allowing plants to escape herbivory by unadapted generalist herbivores. An escalation of host plant defences has been found in several plant genera, with *Asclepias* and *Bursera* being the most iconic examples [107, 112]. Divergent, rather than escalating, defences (Box 3) have been found in sympatric communities of closely related hosts. Such a divergence in defences between sympatric congeners appears to lower the risk of sharing specialized herbivores [113, 114]. As such, the ability to employ divergent defensive traits, which are harder to follow for specialized herbivores, may be beneficial and facilitate coexistence of closely related hosts [5, 115]. For example, divergence and a character displacement in leaf shapes help closely related *Passiflora* hosts to avoid herbivory by impairing host recognition by ovipositing butterfly females [116]. Similarly, a divergence in chemistry among closely related species growing in sympatry have been recently found in many plant genera such as *Bursera*, *Eugenia*, *Ficus*, *Inga*, *Ocotea*, and *Psychotria* [7, 113–115].

Box 3
Escalation of defences refers to the macroevolutionary increase in host plant defensive traits. Under this scenario, derived plant lineages should possess more diverse or escalated (potent) defences than their less derived counterparts [4, 133]. As such, their trait values are a function of their phylogenetic distance, and closely related species should possess similar defensive traits. On the other hand, divergent defences are those which show high disparity between closely related hosts. Divergent traits are more dissimilar between close relatives than expected under a conserved model of evolution.

On the global scale, Becerra [117] found a strong correlation between herbivory by specialized insects and chemical variability in the local communities. Plant communities exposed predominantly to herbivory by specialized insects were much more chemically diverse than those exposed predominantly to generalist mammalian herbivores. The composition of insect communities attacking the host largely forms its defences – assemblages of specialists should select mainly for divergent traits, whereas assemblages of generalists, sensitive to specialized defences, should impose selection for escalating and highly toxic defences [7, 113]. Therefore, plant lineages harboring diverse insect communities consisting of herbivores with various levels of specialization are expected to possess both escalating and divergent defences (Fig. 5).

Both escalation and divergence of defensive traits may contribute to diversity of host plant defences as suggested by recent evidence (e.g., [7, 106, 112, 115]). Escalation of host plant defences should promote α-diversity of defences within a given plant lineage [4]. Divergence in host plant defences promotes β-diversity of defences between closely related hosts [113, 117]. Divergence in defences seems to be especially prominent in speciose and dominant tree genera. These genera often represent a large proportion in the local forest communities and form so-called species swarms [115, 118]. For example, the five most speciose tree genera represent ca 25% of the local tree diversity in Barro Colorado Island, Panama [119]. Divergence in their defences can thus significantly increase defensive and chemical diversity on the community level [117]. This has important reciprocal effects on diversity of associated insects due to the specificity of their response. In turn, host plant chemical diversity has been shown to be an important driver of insect diversity almost invariably in all the systems where this was studied (e.g., [7, 115, 117, 120, 121]) (but see Salazar et al. [122]). For example, there is a strong positive correlation between the number of caterpillar species associated with *Ficus* species and host plant polyphenol and triterpene diversities (Fig. 6) [7]. High diversity of defences can probably lower the dominance of the abundant insect species, preventing any single herbivore from dominating the community and opening niches for the less dominant ones. Indeed, many insect herbivores appear at low densities in tropical forests [123], where both insect and chemical diversities reach their peak.

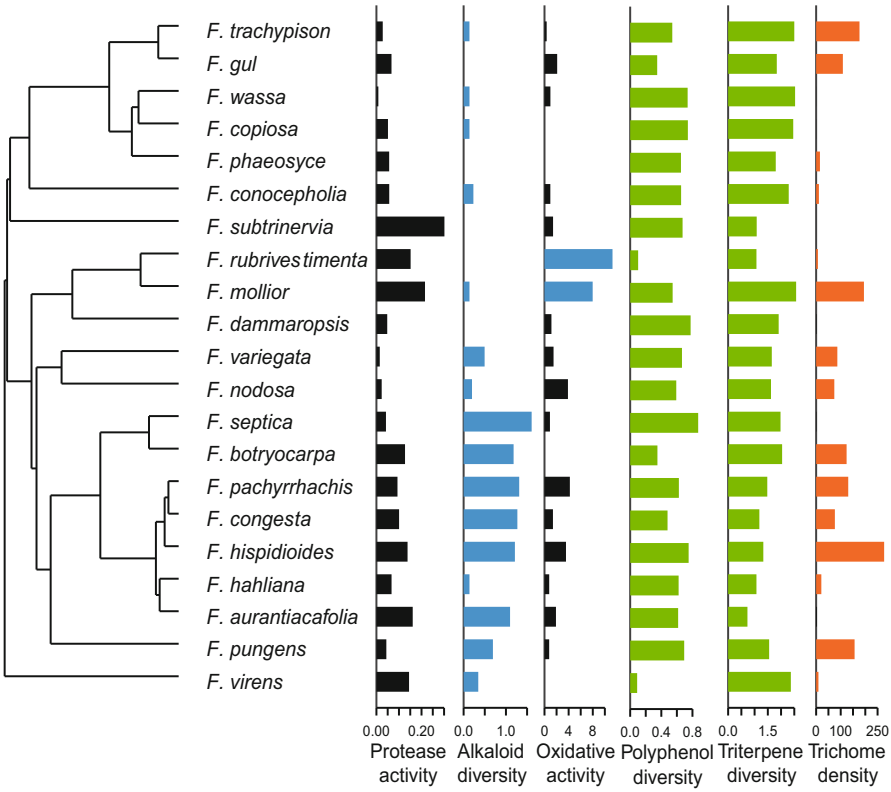

Fig. 5 Distribution of selected defences across *Ficus* phylogeny showing various trends in their evolution. *Ficus* harbors diverse communities of herbivores. Traits with various evolutionary histories probably help *Ficus* to maintain an efficient protection against herbivores with various levels of specialization and life histories [10]. Conserved traits are in *black*, escalating traits are in *blue* (traits showing only a local escalation within one *Ficus* lineage are only partly colored), traits showing divergence at deeper levels of *Ficus* phylogeny are in *green*, and traits showing high divergence at terminal levels of *Ficus* phylogeny are in *orange*. The shown *Ficus* traits include protease activity in latex (ΔA280), alkaloid diversity (Shannon), polyphenol diversity (Shannon), polyphenol oxidative activity (mg/g), triterpene diversity (Shannon), and trichome density (number of trichomes per 10 mm^2). (Adapted from Volf et al. [7])

8 Conclusions

Here I have tried to illustrate that the differential response of insect herbivores to plant defences is one of the mechanisms promoting high specificity of insect-plant interactions. The resulting specificity of interactions gives rise to diversification of host defences as no single trait can provide an efficient defence against diverse communities of insects. The diversification of host defences then seems to be one of the key factors promoting the diversity of insects in a reciprocal way. This chapter

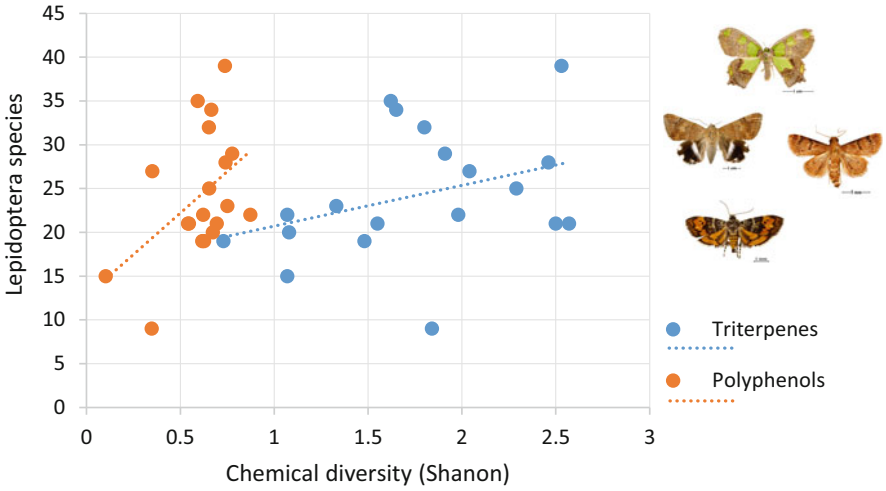

Fig. 6 Positive correlations between diversity of caterpillar communities and polyphenol and triterpene diversity of their *Ficus* hosts. The positive correlation remained significant in case of polyphenols when analyzed in the phylogenetical context using *Phylogenetic Least Squares* ($F_{(1, 17)} = 6.39$, $P = 0.022$) while the effect of triterpenes turned nonsignificant ($F_{(1, 17)} = 1.87$, $P = 0.189$). (The insect and chemical data were taken from Novotny et al. [42] and Volf et al. [7]. Insect photos were downloaded from "Caterpillars feeding on New Guinea plants" database [43] curated by the New Guinea Binatang Research Center)

primarily focused on the response of insect herbivores to host plant defences and nutrient content. There are also other plant traits such as growth-form, architecture, distribution, or abundance, which significantly affect insect herbivores [124, 125]. In some cases, these traits may modulate or drive responses of insect herbivores to host plant defences, often being tightly linked to them. In addition, insect herbivores strongly respond to host phylogeny, usually because of its covariation with host traits. The relative importance of host plant phylogeny and defensive traits is highly dependent on the phylogenetic scale and identity of the traits one considers [126]. For example, secondary metabolites often exhibit a weak phylogenetic signal among congeneric plant species, as outlined above, but the major differences in secondary metabolite presence or absence may be generally conserved when comparing hosts at the family level [7, 113, 114, 127]. The composition of herbivore communities, therefore, usually arises from the interplay between host phylogeny and functional traits, both with differential effects on insects with various levels of specialization and life histories [7, 13, 126].

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Field Dodder: Life Cycle and Interaction with Host Plants

5

Marija Sarić-Krsmanović

Contents

Abstract

Cuscuta as a generalist type of holoparasitic plant interacts with various host plants in different manners, and all *Cuscuta* species depend (absolutely) on host plants to complete their life cycle. Field dodder is a parasitic plant that attaches to stems and leaves of broadleaf plants, including weeds, field crops, vegetables, and ornamentals, across most agricultural regions of the world. Most hosts of *Cuscuta* plants are passive, only a few hosts are known to show clear resistance (e.g., *Ipomoea* sp.). Unlike other weeds occurring in anthropogenic habitats that have been well-studied in their taxonomic, biological, and ecological aspects, as well as their anatomical and physiological properties to some extent, the parasitic flowering species of the genus *Cuscuta* have been examined very scarcely despite the great damage that they are able to cause. More extensive research is required

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in order to develop new means for parasitic weed control. A basic research should identify new targets for control within the life cycle of the parasites and among their metabolic activities.

Keywords
Field dodder · Host plant · Life cycle · Metabolic activities

Abbreviations

chl *a*/*b*	Ratio of chlorophyll *a* to *b*
DAI	Days after infestation
*F*m	Maximal fluorescence
*F*o	Minimum fluorescence
*F*v	Variable fluorescence
*F*v/*F*m	Maximum quantum efficiency of photosystem II
HLR	Hypersensitive-like response
IF	Intensity of fluorescence
RCC	Relative chlorophyll content
TCC	Total chlorophyll content
Φ_{PSII}	Effective fluorescence yield of photosystem II

1 Introduction

Plants of the genus *Cuscuta* (common name: dodder) are obligate holoparasitic species. Dodders are the most important group of parasitic weeds in the world, inhabiting virtually every continent and causing sweeping damage to both crop and non-crop species [1]. Agriculturally, the most important *Cuscuta* species are *C. campestris* and *C. pentagona*, which show an almost worldwide distribution and have a wide host spectrum. Field dodder (*C. campestris*) parasitizes many different plants, inducing negative impacts on the growth and yield of infested hosts, and has significant effects on the structure and function of plant communities that are infested by these holoparasites [2, 3]. Parasitic plants fuse to host vascular systems (xylem and phloem) via a specified organ present in all parasitic plants, the haustorium. This organ serves as the structural and physiological bridge for the parasites to withdraw water, minerals and organic molecules, and solutes from host plant conductive systems, leading to severe host growth and yield reduction [4]. Parasitic plants of the genus *Cuscuta* either have no chlorophyll at all, or merely low amounts of it, or usually do not have a photosynthetic activity [5, 6]. However, all *Cuscuta* species fully depend on host plants to complete their life cycle and therefore are considered as obligate holoparasites.

Plants are sessile organisms that have evolved unique strategies for interacting with various environmental changes as well as dealing with the biological influence of other living organisms. These can roughly be divided into abiotic stress responses and biotic responses [7, 8]. Pathogenic responses are typical examples of biological interactions in plants. These include interactions with bacteria, virus, fungi, and animals (e.g., parasitic nematodes and herbivorous insects). In contrast, less is known about plant-plant

interactions. Especially, although the morphology and anatomy of *Cuscuta* spp. are well-studied, the cellular mechanisms of the interactions between parasitic plants and their susceptible hosts are not well understood.

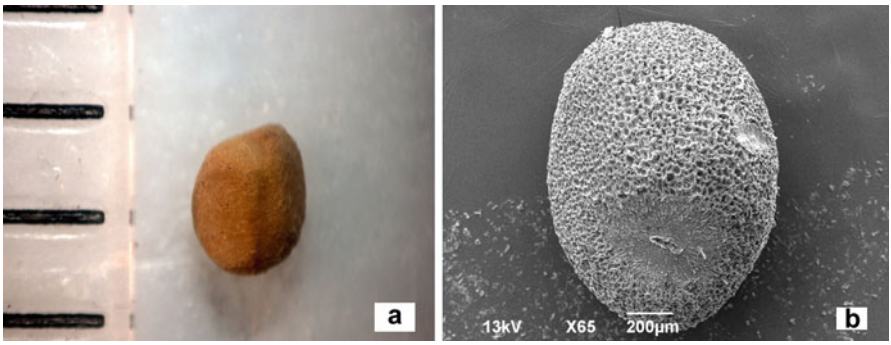
Cuscuta can serve as a key model plant for deciphering the mechanism of parasitism as well as for examining host plant-parasite plant interactions [9]. Most studies used isotope labels and observed carbon or nitrogen flux between *Cuscuta* and the host plant [10, 11]. Some studies compared metabolites (e.g., plant hormones) in *Cuscuta* seedlings (haustorium-induced and/or non-induced seedlings) with *Cuscuta* attached to host plants [12, 13]. Documented host plant responses to attack by *Cuscuta* spp. include a hypersensitive-like response (HLR) and phytoalexin production by a non-host tropical liana in response to *C. reflexa* [14] and the expression of a PR gene by *Cuscuta*-infested alfalfa [15]. Best studied among host plant defenses against *Cuscuta* spp. are the responses of resistant tomato varieties to *C. reflexa*, in which elongation of hypodermal host cells, a subsequent HLR, and accumulation of phenolics and peroxidases at the attachment site create a mechanical barrier that can block haustorial formation [16, 17].

Effective field dodder control is extremely difficult to achieve due to the nature of attachment and close association between the host and the parasite, which requires a highly effective and selective herbicide to destroy the parasite without damaging its host. To establish strategies to control parasite growth and restrict the spread of field dodder in crop fields, it is important to learn more about this pest, studying its life cycle, development, and parasitic-host interactions.

2 Biology and Ecology Characters of Field Dodder

Autotrophic flowering plants constitute the predominant group among weed species, but weeds also include some semiparasitic and parasitic flowering plants. The parasitic plants are represented by approximately 4200 species classified in 274 genera, which makes a little more than 1% of all flowering plants. Only some 11% of all genera include species that may be considered as parasites of cultivated plants. The worst economic damage in important host crops is caused by species from only four genera: *Cuscuta*, *Arceuthobium*, *Orobanche*, and *Striga* [18]. The genus *Cuscuta* L. (dodders) is one the most diverse and challenging groups of parasitic plants with more than 200 species and over 70 varieties [19–21]. The stem of a field dodder plant is threadlike and twining, and it is either leafless or the leaves are reduced to hardly visible scales. Fully matured field dodder seeds fall off and accumulate on the ground. They may then either germinate during the following season if a suitable host plant is growing in the vicinity or may stay dormant until such conditions have occurred [22]. These stem parasites attach to the host by haustoria and depend entirely (or nearly so) on their hosts for the necessary water and nutrient supplies [2, 23]. At an appropriate moment of maturation, a field dodder plant forms inflorescences with abounding hermaphrodite and actinomorphic flowers. The flowers are hermaphroditic, tiny, mostly white, reddish, or yellow. Petals are either individual or coalescent. The corona is bell-shaped or round, mostly with four or five petals (Picture 1a, b). The flower has five stamens. The fruit is a pod containing one to four seeds. The seed is tiny,

Picture 1 Flowers of field dodder (*C. campestris* Yunck.) (Saric-Krsmanovic 2013 – org. foto)



Picture 2 Seed of field dodder (*C. campestris* Yunck.) (Saric-Krsmanovic 2013 – org. foto)

spherical, rough, and light brown (Picture 2a, b). The seed of this parasitic flowering plant germinates on soil surface from May throughout June. Field dodder is a thermophilic species, and its optimal temperature for germination is 30 °C [24]. Dodder seeds retain vitality in soil over more than 10 years. A single plant is able to form up to 15,000 seeds, and their abundance constitutes the main mode of survival of that parasite in the environment [25]. Its reproduction may also be vegetative through segmentation of its threadlike stem. Such reproduction mode is frequent in alfalfa and clover crops after harvest and haying, which enables its transfer from infested plots to noninfested fields [26].

3 *Cuscuta* Life Cycle

The steps in the life cycle of parasite plants include (1) seed germination; (2) early development of the seedling; (3) search for a host plant, haustorium induction and invasion of the host, and haustorium maturation; and (4) interaction with the host plant [27, 28].

3.1 Seed Germination and Searching for a Host Plant

The life cycle of *Cuscuta,* as in other angiosperms, begins with seed germination. Germinating *Cuscuta* seedlings depends on limited seed reserves; they are unable to survive alone for a long time and must find an appropriate host plant stem within a few days [29]. *Cuscuta* seedlings normally live less than 3 weeks before becoming parasitic.

Seed dormancy is an important feature of *C. campestris* that ensures its survival as a parasite of crops [30]. There are three different types of seed dormancy (morphological, physical, and physiological), at least two of which have evolved on several separate occasions [31]. Dormancy of *C. campestris* occurs owing to its hard seed coat [32]. The percentage of hard seeds at dispersal varies among *C. campestris* [33] and *C. chinensis* plants [34]. Dormancy can be broken by the activity of soil microorganisms or by tillage, causing scarification of seed coat [35], etc. The dynamics of germination of *C. campestris* depends on a double mechanism of dormancy. After a period of primary dormancy (additional maturation caused by coat impermeability), the seed goes into an annual cycle of secondary dormancy. In *C. campestris*, secondary dormancy occurs at the end of summer, and it prevents germination during the following autumn and winter in order to avoid the season in which potential hosts of the temperate region would be scarce due to low temperatures. Secondary dormancy ends at the end of winter when temperature begins to grow and overall conditions for germination and growth of host plants improve [25]. Physical dormancy has been reported for seeds of several *Cuscuta* species: *C. campestris* [25, 30], *C. trifolii* [36], *C. monogyna* and *C. planiflora* [37], *C. chinensis* [34], *C. gronovii*, *C. umbrosa*, *C. epithymum*, and *C. epilinum* [38]. However, it is not common for *Cuscuta pedicellata* [39] because seeds of that species are readily water permeable due to a specific structure of their epidermis and endosperm.

To find and catch potential hosts, *Cuscuta* plants recognize plant volatiles as chemoattractants which guide seedling growth and increase the chances of successful establishment of a connection [29]. However, expert options vary as what is the necessary impulse for germination of field dodder seeds. Some researchers [40, 41] believe that *Cuscuta* spp. do not require host-root exudates to stimulate germination, similar to some important holoparasitic weeds of the genus *Orobanche* and some hemiparasitic weeds in the genus *Striga*. Field dodder as a stem parasite is strongly impacted by light signals, which stimulate germination of its seeds [42–44]. Field dodder seedlings tend to grow in the direction of light source, primarily red/far-red light, which help them find hosts, while far-red and blue light have a significant role in prehaustorium formation. Recognition of a host occurs through phototropic mechanisms, and some authors claim that chemotropism (movement induced by chemical stimulus) and thigmotropism (movement induced by mechanical stimulus, i.e., by touch) have equally important roles in host recognition process [45]. Mechanical stimulus, following initial contact with the host plant, induces cell differentiation and haustorium formation, and its subsequent penetration into the host stem. This is facilitated by the recruitment of stress-responsive and defense genes for host recognition and activity of cell wall-modifying enzymes [46–48]. Runyon et al. [29] found that volatile chemical substances were also important for movement of *Cuscuta*

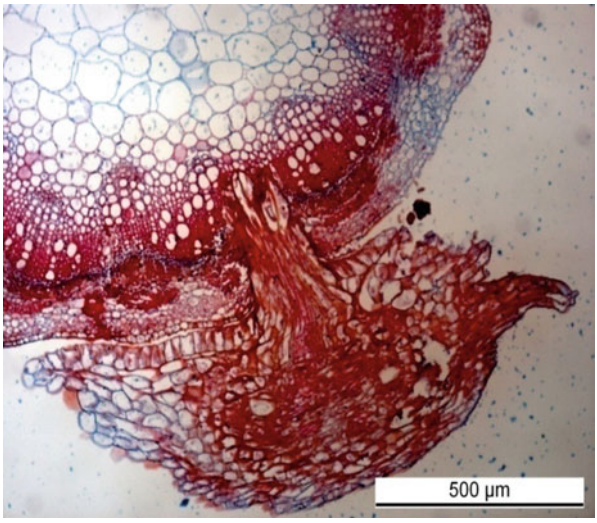
campestris seedlings in the dark. Saric-Krsmanovic et al. [49] examined the effect of host seeds on germination and initial growth of seedlings of field dodder plants in the dark, and under white light, the seeds of four host plants were used (watermelon, red clover, alfalfa, and sugar beet). The data of host seeds showed that light was a significant initial factor (83–95%, control 95%) for stimulating seed germination of field dodder plants, apart from host presence (73–79%, control 80%). *Cuscuta* can also change from one host to another and back. If the plant needs special volatile chemicals to search for a host, it is difficult to explain why it can parasitize so many different plants except there is a strong overlap between the volatile compositions of the various plants.

3.2 Attachment and Haustorium Development

The ability to form specialized organs for absorption, i.e., haustoria (Picture 3), is the chief adaptive character of all higher parasitic plants [50]. In field dodder plants, such structures are created from the stem meristem tissue of a parasitic plant, and they are considered as modified adventive roots [22]. Haustoria may develop even when no potential host is around [43, 51, 52]. The main stimulus for developing haustorial tissue may be simply the contact with another surface, such as glass [43, 53], filter paper [54], or plastic [55].

The development of haustoria may be roughly differentiated into three stages [56]: (1) attachment (i.e., establishing of a connection with the host tissue), (2) penetration (insertion into the host tissue), and (3) conductive stage (transmission of nutrients).

Sharp pointed haustoria develop from appressoria that enable the parasite to draw organic and mineral substances from its host. Obligate parasites are unable to

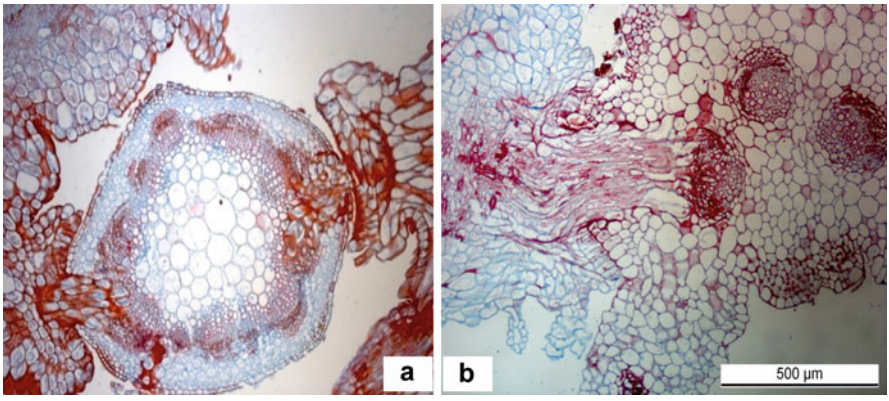


Picture 3 Haustorium of *Cuscuta campestris* Sarić-Krsmanović, M. (2013). Biology of field dodder (*Cuscuta campestris* Yunk.) and options for its control. Doctoral thesis, University of Belgrade, Faculty of Agriculture. (In Serbian)

develop without assimilates drawn from their host plants because they are unable to perform photosynthesis [23, 57] or their photosynthetic capacity is very weak [50]. Even though dodder plants possess a functional photosynthetic apparatus within a ring of cells surrounding vascular tissue [50], the amount of organic matter produced there is too small to provide for the plant sufficiently, so that 99% of the required carbon is still drawn from the host [58].

After finding an appropriate host plant, the first physical contact initiates the attachment phase, in which the parasitic epidermal and parenchymal cells begin to differentiate into a secondary meristem and develop prehaustoria, also known as adhesive disk [59, 60]. Important signals initiating and controlling this prehaustorium formation include mechanical pressure, osmotic potential, and phytohormones such as cytokinins and auxin [1, 61]. The prehaustorial cells start to produce and secrete adhesive substances, such as pectins and other polysaccharides, reinforcing the adhesion [47]. During the attachment phase, host cells in the proximity of *Cuscuta* haustoria respond with an increase in cytosolic calcium, detectable in host plants expressing aequorin as calcium reporter. Within the initial several hours of contact, *Cuscuta* also induces the host plant to produce its own sticky substances, such as arabinogalactan proteins, to promote adhesion [62]. These glycoproteins are secreted by the host plant and localized to the cell wall where they can force the adhesion together with other sticky components such as pectins.

The attachment phase is followed by penetration phase as prehaustoria develop into parasitic haustoria that penetrate the host stem through a fissure. This breach is effected by mechanical pressure [1] and is supported by biochemical degradation of host cell walls caused by secreted hydrolytic enzymes such as methylesterases [46] or complexes of lytic enzymes consisting of pectinases and cellulases [48]. Cells at the tip of the invading haustoria form "searching hyphae" which try to reach phloem or xylem cells of the host plant's vascular bundles (Picture 4). A day or two later, epidermal cells of "interior haustoria" begin to elongate and form unicellular



Picture 4 The haustorium searching hyphae of field dodder establishing a connection with both phloem and xylem tissues of alfalfa stem (**a**) and sugar beet petiole (**b**) (Sarić-Krsmanović 2013)

structures known as hyphae. In a compatible host, the hyphae searching for vascular tissue are able to expand from 800 to 2000 μm [1, 48], and their inter- and intracellular expansion into the host tissue depends on the mechanical as well as enzymatic processes [1]. These parasitic cells have been described as having ambivalent characters, functioning as both sieve elements and transfer cells [59, 63]. Interestingly, during this process, chimeric cell walls of host and parasite constituents are formed, and interspecific plasmodesmata build up a cytoplasmic syncytium between *Cuscuta* and its host plant [48, 64, 65]. To form a connection to the xylem, parasitic and host cells of the xylem parenchyma commence a synchronized development, fusing to build a continuous xylem tube from the host to the parasite [66]. With functional connections to the xylem and phloem of its host, the parasitic plant is supplied with water, nutrients, and carbohydrates [50, 58, 67].

4 Consequences of Field Dodder and Host Interaction

4.1 Impact on Host-Parasite Metabolites

After the establishment of a connection between host and parasite, the development of the parasite is based on the exchange of nutrients. In the process of establishing parasitic connections to its host, dodder uses a battery of hydrolytic enzymes, primarily cell wall-modifying glycosyl hydrolases [68], which have been observed directly through their activities [69] or indirectly through their structural consequences during host-tissue invasion [48]. Further, dodder appears to induce hydrolytic activities within its host [69, 70].

Transfer of fluids from the host to the parasitic plant occurs across a bridge created between the two organisms utilizing the difference in water potential of cell sap between the two plants. Parasitic flowering plants have a higher negative osmotic potential of cell sap that allows them to uptake organic nutrients from the host plant or, in other words, the phloems within vascular bundles of the parasite and the host become connected, creating a "physiological bridge" between the two plants' vascular tissues [50]. As *Cuscuta* has no roots and no effective photosynthesis system, most of the nutrients apparently come from the host phloem, but their haustoria reach into the xylem too for nutrients such as calcium. This makes *Cuscuta* a phloem feeder, and Haupt et al. [64] used fluorescent proteins to show a symplasmic connection with companion cells of phloem. A lower phloem flux here causes a reciprocal interaction between the host and the parasite. In certain cases, *Cuscuta* can be a mediator of virus infection for the host plant. Apoplasmic and symplasmic connections are found case by case. The presence of a plasmodesmata connection between *Cuscuta* and host plant was shown by Birschwilks et al. [65].

The connection between host and dodder vascular systems is continuous [65] and facilitates transport of not only water and minerals but also viruses, proteins [64], and mRNAs [71] from host to the parasite. Because plants possess hundreds of different phloem-mobile proteins and RNAs that play important roles in regulating plant development and stress responses [72], it is expected that the development and

stress tolerance of dodder could also be influenced by these host-derived mobile substances that are capable of interspecies trafficking.

The holostemparasitic plant *Cuscuta* can serve as an important system for studies on plant-plant interactions. Different responses from host plants to *Cuscuta* might be able to partially clarify some potential tendencies of plant stress response between different plant taxa and may also suggest unknown stress response mechanisms in host plants. Furuhashi et al. [73] used a unique experimental system to analyze *Cuscuta japonica* seedlings under FR light and/or with a contact signal attached to different host plants. *Cuscuta* attached to *Pueraria thunbergiana* showed a higher (>20%) mol percentage of pinitol both in the apical and middle regions (haustorium part). *Cuscuta japonica* attached to *Buxus microphylla* and *Conyza sumatrensis* contained less pinitol, and values were even lower than in *C. japonica* seedlings before parasitization. Although *C. japonica* attached to *Pueraria* did not contain large amounts of glucose and sucrose, *C. japonica* attached to *Buxus* and *Conyza* did especially in the haustorium-induced parts. Host plants without *C. japonica* parasitization clearly showed different metabolite profilings from *C. japonica* seedlings. Pinitol was dominant in *Pueraria*, and quinic acid was dominant in *Conyza* and *Buxus*. Also, glucose, myoinositol, and oxalic acid were bigger in both *Conyza* and *Buxus*, but not in *Pueraria*.

Parasite plants are clearly plants and have the same plant hormonal system and physiological response. This implies that host plants would not always be able to use the same defense strategy against parasite plants. This consideration gave rise to discussions about comparing parasite plants with herbivores [74]. Although parasite plants have been recognized as weeds that cause agricultural problems, triggering some interest [75, 76], parasitization does not always negatively influence the host plant. For example, tomatoes parasitized by *Cuscuta* altered certain plant hormones (e.g., salicylic acid) and can influence their defense system against insect herbivores [13]. Also, Runyon et al. [61] used a metabolomic profiling approach involving vapor phase extraction to measure changes in phytohormones occurring within tomato plants during parasitism by *C. pentagona*. Theirs results indicated that parasite seedlings elicit a relative paucity of host reactions when first attaching to 10-day-old tomato seedlings, whereas a second attachment by the growing parasite vine 10 days later induced large increases in several plant hormones and a strong HLR (hypersensitive-like response). Also, Runyon et al. [61] assessed the effectiveness of SA (salicylic acid)- and JA (jasmonic acid)-mediated host changes using transgenic and mutant plants. These methods give the first picture of the composition and timing of hormonal signalling induced in response to a parasitic plant. They conclude that as with herbivore and pathogen attack, plants are able to perceive invasion by parasitic plant haustoria and respond by activating induced defense pathways. Seedlings of *C. pentagona* elicited relatively few changes in the host upon first attachment to young tomato seedlings, possibly because of ontogenetic constraints in host defense or because the parasite is better able to manipulate young hosts. Older tomato plants responded to a second attachment by activating the JA and SA signalling pathways, both of which appear to mediate defenses that effectively reduce parasite growth. Parasitism also induced increases in ABA (abscisic

acid) and free fatty acids, but the roles of these compounds in defense remain uncertain. Although plant hormones play important roles for many plant interactions, including pathogenic responses, only little plant hormone research has been conducted on *Cuscuta*. Also, little is known about the influence of hormonal changes to *Cuscuta*, such as effect to haustorium induction and reciprocal interaction with host plant. Furuhashi et al. [84] firstly tested several host plant species for *Cuscuta* parasitization and also observed *Cuscuta* plant interaction in the field, in order to find interesting interactive relationship. They reported the new, unique phenomenon that a parasitic plant induced hypertrophy together with vascular tissue differentiation in the host plant stem. Plant hormone analysis clarified that cytokinin played a major role in this process. *Momordica charantia* hypertrophy response might be derived from resistance, while *Cuscuta* grow rapidly under the presence of hypertrophy response.

4.2 Impact on Host Pigment Content

Obligate parasites are not able to develop without assimilate supplies from their hosts because of their inability to perform any photosynthetic activity on their own or such photosynthetic capacity is very low [6, 50]. Their dependence on the host plant is therefore stronger, as well as their negative impact in terms of reducing chlorophyll and accessory pigments in the host plant [77]. Saric-Krsmanovic et al. [78, 79] showed a significant reduction in chlorophyll *a*, chlorophyll *b*, and carotenoids in infested alfalfa and sugar beet plants, compared to noninfested plants. Such reductions in chlorophyll *a*, chlorophyll *b*, and carotenoids were higher in infested alfalfa than infested sugar beet plants. Similarly, Fathoulla and Duhoky [80] found that different *Cuscuta* species caused not only morphological and anatomical changes in their hosts but also reduced their chlorophyll contents. Specifically, *C. campestris* and *C. chinensis* caused significant decrease in total chlorophyll contents in three tested hosts *Capsicum annuum*, *Coleus* spp., and *Helianthus annuus*, while the smallest reduction was caused by *C. monogyna*. Furthermore, these authors also revealed a significant variation in the chlorophyll content in the leaves of the same plant parasitized by different *Cuscuta* species. The differences in the infection between the different hosts by the same *Cuscuta* sp. may be related to the differences in nutrient status or sizes of the host (metabolic activities) [81].

4.3 Impact on Host Chlorophyll Fluorescence

Methods based on chlorophyll fluorescence have been used in many studies to monitor the effects of various stress factors on plants, such as water deficit, nitrogen deficit, extreme temperatures, and high salt concentrations, or to study changes in photosynthetic processes caused by herbicides or pathogen infection [82–85]. Saric-Krsmanovic et al. [78] have discovered possibilities that used chlorophyll fluorescence as an indicator of stress in host plants parasitized by field dodder. Most of the

measured parameters were affected by field dodder parasitism from the 1st day after infestation. An exception is the parameter F_v, whose lower value in infested plants was recorded on the 5th day after infestation (Table 1). The stressful influence of field dodder on alfalfa and sugar beet plants caused reductions in the parameters such as F_v, F_v/F_m, Φ_{PSII}, and IF. These findings are consistent with report from Vrbnicanin et al. [86] confirming lower values of these parameters in plants exposed to stress caused by various factors. They reported that several chlorophyll fluorescence parameters (F*v*, F_v/F_m, and Φ_{PSII}) of the host *Ambrosia trifida* were influenced by the parasitism of *C. campestris*. One of the possible reasons could be that, in host plant, field dodder suppressed photosynthesis by limiting gas diffusion over stomatal and photosynthetic metabolic processes. Furuhashi et al. [87] found that photosynthetic activity in *Momordica charantia* stems parasitized by *Cuscuta* fell with time, although values in leaves were not influenced by parasitization. As F_v/F_m- and F_v'/F_m'- values decreased, the PSII is probably mainly affected by parasitization. It is necessary to consider the impacts of *Cuscuta* infection on host plant's photosynthesis in the context of environmental factors. Also, many studies [88, 89] have shown

Table 1 Chlorophyll fluorescence in noninfested (N) and infested (I) sugar beet and alfalfa plants

Days after infestation in the plant sugar beet						
Parameters		1	5	10	15	20
Fv/Fm	N	0.7752	0.7621	0.7602	0.791	0.7963
	I	0.7385	0.685	0.6505	0.753	0.7093
Φ_{PSII}	N	0.7914	0.7926	0.7892	0.7923	0.7933
	I	0.748	0.6322	0.7313	0.7777	0.7013
Fo	N	0.5446	0.5379	0.5459	0.5582	0.559
	I	0.575	0.5555	0.6769	0.5847	0.5954
Fv	N	2.0446	1.9655	2.0033	2.0297	2.0317
	I	1.4341	1.2971	1.3786	1.6165	1.4712
IF	N	1.1185	1.1009	1.1477	1.0771	1.1213
	I	0.8693	1.3835	0.9083	0.9280	1.3331
Days after infestation in the plant alfalfa						
Parameters		1	5	10	15	20
Fv/Fm	N	0.8	0.8	0.7972	0.8104	0.813
	I	0.7542	0.7322	0.6482	0.7584	0.7842
Φ_{PSII}	N	0.782	0.8098	0.7862	0.775	0.782
	I	0.7568	0.6922	0.7376	0.8002	0.7568
Fo	N	0.4908	0.4908	0.504	0.4738	0.4738
	I	0.5638	0.5832	0.5508	0.5508	0.571
Fv	N	2.0072	1.9266	2.044	2.0842	2.0378
	I	1.9942	1.8342	1.8686	1.182	1.6182
IF	N	1.1783	1.1198	1.2124	1.1697	1.0600
	I	1.1039	1.2726	0.9487	1.1209	0.9040

Fm maximal fluorescence, *Fo* minimum fluorescence, *Fv* variable fluorescence, *Fv/Fm* maximum quantum efficiency of photosystem II, *IF* intensity of fluorescence, Φ_{PSII} effective fluorescence yield of photosystem II

that chlorophyll fluorescence parameters reacted to stress at different speeds, depending on a number of factors.

4.4 Impact on Host Mineral Nutrient Content

Parasitic plants restrain the growth and reproduction of their hosts by capturing nutrients and disturbing resource balance [2]. The presence of the parasite strongly reduces the biomass by acting as a competing sink for assimilate, but more importantly, by compromising the efficiency of mineral and organic nutrient assimilation. The holoparasitic *Cuscuta* is known to constitute an overwhelming competitive sink by diverting the major portion of the current photoassimilates of the host into its own tissues [1, 3, 90]. Hibberd and Jeschke [50] observed that nitrogen uptake by a parasite depends primarily on its availability and translocation through the conducting tissue of its host plant. Also, Press et al. [91] showed that the extent of parasites competing with hosts for carbon and other nutrients depends on their relative sink strength and the degree of autotrophy of the parasite. Increasing of nitrogen and potassium contents in *Mikania micrantha* was reported by Yu et al. [92], while no impact on phosphorus content was detected in the early stages after *C. campestris* infestation. Saric-Krsmanovic et al. [79] revealed increase of some nutrient content in the infested, compared to noninfested plants. Twenty days after infestation, K_2O and organic nutrient contents in infested alfalfa plants and N and organic nutrient contents in sugar beet were higher than in noninfested plants. Final assessment (40 DAI) revealed that field dodder increased the contents of N, P_2O_5, K_2O, and organic nutrients in the infested alfalfa plants, while the infested sugar beet plants had higher contents of N and organic nutrients, compared to noninfested plants (Table 2). Different responses from host plants to *Cuscuta* might be able to partially clarify some potential tendencies of plant stress response between different plant taxa and may also suggest unknown stress response mechanisms in host plants [73]. Also, the changeable contents of nitrogen, phosphorus, potassium, and organic

Table 2 Contents (%) of nitrogen, phosphorus, potassium, and organic and mineral nutrients in alfalfa and sugar beet plants

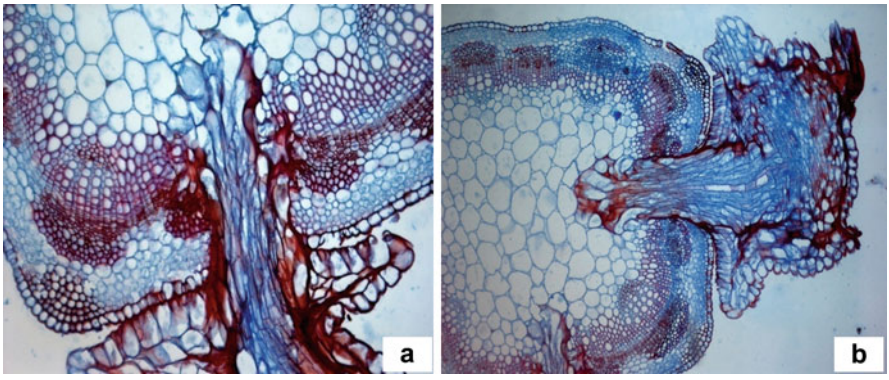
Parameters						
Assess	Treat	N%	P_2O_5%	K_2O%	Organic nutrients %	Mineral nutrients %
Alfalfa						
40 DAI	N	2.18 ± 0.11	0.36 ± 0.03	1.40 ± 0.05	91.49 ± 0.30	8.51 ± 0.30
	I	2.33 ± 0.10	0.42 ± 0.05	1.55 ± 0.22	92.24 ± 0.62	7.76 ± 0.62
Sugar beet						
40 DAI	N	1.12 ± 0.17	0.76 ± 0.06	3.53 ± 0.21	83.09 ± 2.32	16.92 ± 2.32
	I	2.03 ± 0.16	0.48 ± 0.18	2.84 ± 0.22	85.28 ± 1.56	14.72 ± 1.56

N noninfested alfalfa and sugar beet plants, *I* infested alfalfa and sugar beet plants, *DAI* days after infestation

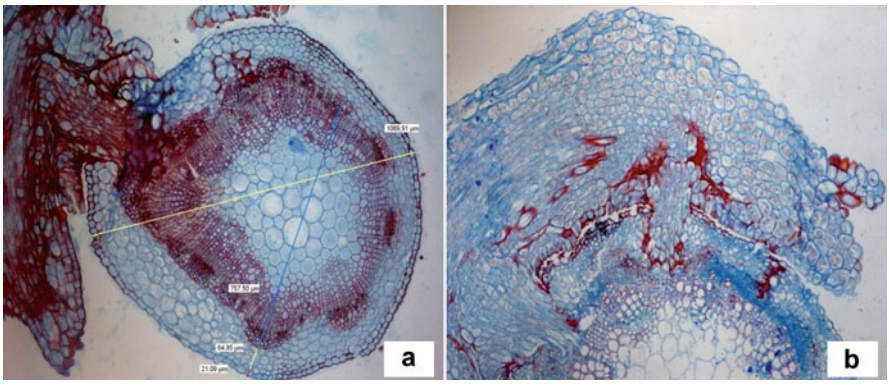
and mineral nutrients in noninfested and infested alfalfa and sugar beet plants may be considered as a response reaction of the host to parasitism, which mostly leads to accumulate nutrients because intensified metabolism creates a defense mechanism in the host. The changes in nutrient contents and fresh biomass have a crucial effect on the composition of plant communities and determine their invasiveness [93].

4.5 Impact on Host Anatomical Parameters

The effect of field dodder on the anatomy of cultivated host plants is still mostly an uninvestigated area. Field dodders cause changes in stalk anatomy and leaves of host plants (alfalfa and sugar beet) [79, 94, 95]. Regarding nearly all analyzed parameters of alfalfa stem (epidermis, cortex, pith, diameter), significantly lower values were recorded in infested than in noninfested plants 42 DAI (days after infestation) (Pictures 5 and 6). At the same time, our results showed that field dodder had a significant effect on most of the measured parameters (upper epidermis, palisade tissue, spongy tissue, leaf mesophyll, underside epidermis, vascular bundle cells) of alfalfa and sugar beet leaves. Furuhashi et al. [87] discovered hypertrophy and increasing number of vascular bundles in *Momordica* stems clearly induced by *Cuscuta hyphae*. This influence of the parasitic plant on its host resulted in decreasing of total photosynthetically active surface, as well as total photoassimilating tissue, which may lead to lower competitiveness of the infested plant and its weakened ability to set fruit and seed due to a major loss of nutrients assimilated by the parasite [50]. In early stages of field dodder infestation, the host plant reacts with a specific gene expression for calcium release, cell elongation, and changes in the cell wall [70, 96]. At a later stage, after hyphae have been formed, they are mostly connected to the xylem or phloem of the host, even though some of them may end up in the parenchyma. Possessing their ring-like structure, hyphae are able to connect to several sieve tubes of the host simultaneously, which increase their



Picture 5 The haustorium searching hyphae of field dodder connecting to the central cylinder (pith) tissue of alfalfa stem (**a**, **b**) (Sarić-Krsmanović 2013)



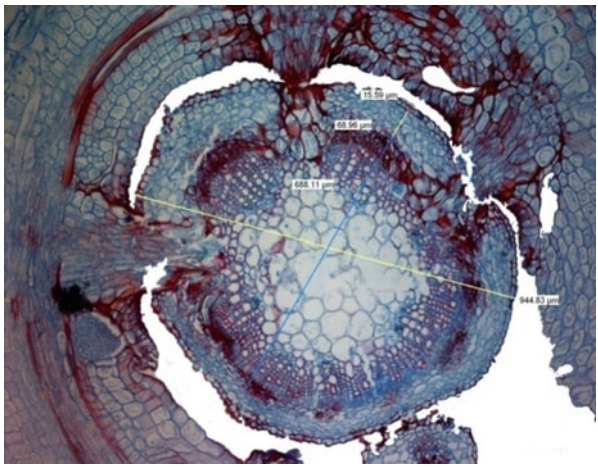
Picture 6 The haustorium searching hyphae of field dodder connecting to cortical parenchyma cells (**a**) and phloem tissue (**b**) of alfalfa stem (Sarić-Krsmanović 2013)

absorption strength, as well as their impact on the conducting tissue of the host [64]. Saric-Krsmanovic et al. [79, 95] examined the effect of field dodder on the petiole of sugar beet, and the data for the measured parameters (tracheid diameter, petiole hydraulic conductance, xylem surface, phloem cell diameter, and phloem area) indicated that this parasitic flowering plant has a significant influence on all measured parameters. In the infested sugar beet, field dodder significantly reduced the area of conducting tissues, as well as the hydraulic conductance of the petiole, compared to noninfested plants. Even though, the parasite is connected both with the host xylem and phloem, *Cuscuta* spp. mostly assimilates through the phloem [50]. In addition to the basic metabolic compounds, also some secondary products (such as alkaloids, etc.) and xenobiotics are adopted by dodder plants mostly from the phloem of the host [65]. But essential nutrients, which are deficient in the phloem, are assimilated from the host xylem [50].

In general, field dodder exhausts the host plant, so that it becomes weak, its lushness of growth declines, and fruit and seed maturation become significantly reduced [90]. Also, host plants change their habit as their axillary buds sometimes become suppressed [97], and the harm may result in total plant destruction (Picture 7).

5 Conclusions

Cuscuta, as a generalist type of holostemparasitic plants, interacts with various hosts, causing different morphological, anatomical, and physiological changes. Hosts are attacked non-specifically and sometimes even simultaneously, and one crop species may serve as a host for several dodder species. Depending on the infected plant species, *Cuscuta* infestation has more or less severe effects on the growth and reproduction of its host. Rather than causing host death, *Cuscuta* infestation seems to weaken host plants and to render them more susceptible to secondary diseases such as infection by microbes or insect and nematode infestation.



Picture 7 Field dodder haustoria (an example of hypersensitive reaction)

The parasitic process in *Cuscuta* begins in finding and attaching to a host plant and then developing a haustorium. The process does not always require any chemical signal but does require a light signal. A contact signal is also necessary for haustorium induction. The direct connection between *Cuscuta* and its host involves both the xylem and phloem, and mRNA and proteins can translocate. Several features indicate that *Cuscuta* is a useful model plant for parasite plant research as well as plant-plant interaction research. These include the simple anatomical structure and seedling development, no chemical requirement for haustorium induction, and the wide range of host plants. Their continuous growth and ability to successively change hosts make the occurrence of coevolution between *Cuscuta* and specific hosts unlikely. Different responses from host plants to *Cuscuta* might be able to partially clarify some potential tendencies of plant stress response between different plant taxa and may also suggest unknown stress response mechanisms in host plants. More extensive research is required in order to develop new means for parasitic weed control. It is important to learn more about this pest, studying its life cycle, development, and parasitic-host interactions.

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Molecular Interactions as Drivers of Changes in Marine Ecosystems

6

Fanny Defranoux and Ernesto Mollo

Contents

Abstract

Among the factors affecting community dynamics, bioactive natural products act as mediators of key biological processes, including competition, predation, defense, and reproduction. Their chemical diversity thus critically contributes to the stability of ecological systems. Accordingly, research in chemical ecology provides useful information for a better understanding of ecosystem functioning and biodiversity. On the other hand, the potential of bioactive molecules produced by invasive species to become disruptive to native communities has been recently emphasized in the literature, raising novel and urgent questions about the interactions of invasive metabolites with macromolecular counterparts of ecological and ecotoxicological interest. Relevant issues strongly emerged in the Mediterranean Sea where the green alga *Caulerpa cylindracea* and the seagrass *Halophila stipulacea*, both exotic macrophytes containing peculiar bioactive compounds, have become invasive. In particular, the study of these two species has led to the production of a recent literature focusing on "alien biomolecules" and their potential impact on the native community. This chapter summarizes the obtained results by giving special emphasis to the urgent need for individuating molecular interactions that are likely to exert cascade effects at all levels of biological organization, from molecules to ecosystems.

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Biological invasions · Alien biomolecules · *Caulerpa cylindracea* · *Halophila stipulacea* · Mediterranean Sea

1 Introduction

The nature of the interactions among species is crucial for structuring and stabilizing ecological systems. In spite of this, few studies assess the role of species interactions in driving ecosystem functioning [1]. This also applies to molecularly mediated biological interactions [2], the role of which at higher levels of biological organization largely remains unexplored [3]. Although chemical ecology has opened our "eyes" to the vast communicative interplay in which organisms use chemical signals to find their food and mates, to deter predators, or to prevent pathogen invasion [4], critical gaps remain in the study of natural products as mediators of ecological interactions at the level of populations, communities, and ecosystems. Indeed, chemoecological aspects have been only occasionally considered to draw conclusions about the impacts of bioactive natural products on biodiversity.

Terpenes, for example, represent the most abundant group of biogenic volatile organic compounds in the atmosphere [5] and a kind of complex chemical language mediating crucial ecological interactions [6]. Therefore, if we consider the critical roles played by terpenes in chemical communication between plants and animals in terrestrial environments, it becomes evident that they can affect biodiversity at all its levels of organization. This is also true for marine environments, where terpenes are also widespread and regulate interactions between benthic invertebrates [7], but also applies to other groups of compounds, such as alkaloids or flavonoids, affecting ecosystem stability as the long-term result of selective pressures and diffuse coevolutionary processes.

On the other hand, interactions between invasive and native species represent a serious threat to biodiversity, with a potential for dramatic ecosystem destabilization and for generating evolutionary changes occurring within faster time scales. Several studies have already shown that the evolution and spread of invasive species can be rapid and dramatic, rising some of the most important current environmental problems, but also offering interesting research opportunities to evolutionary biologists [8, 9]. In parallel, a chemoecological approach to biological invasions, especially in marine environments, has made it clear that the impact of marine invasive species also depends on their chemical ecology and, consequently, on the functional role of their bioactive natural products [9, 10]. Accordingly, a better understanding of the chemoecological factors that affect marine biological invasions has currently become an urgent research priority especially considering the exotic macrophytes that entered the Mediterranean Sea and are able of replacing keystone native species, causing environmental and economic damages [11]. By reviewing some of the available literature on this topic, this article places emphasis on the critical roles that biomolecules from invasive species can play in destabilizing marine

ecosystems. The available information on the pharmaceutically relevant activities of the alien metabolites will also be summarized paving the way for future researches that could clarify whether the properties that make those compounds important as drug candidates may be closely related to their impact on marine ecosystems.

2 Bioactive Metabolites from the Invasive Green Alga *Caulerpa cylindracea*

Marine green algae belonging to the genus *Caulerpa* are especially known to contain bioactive terpenes and alkaloids [12], among which the sesquiterpenoid caulerpenyne showing a diacetoxybutadiene moiety (**1**) and the bisindolic alkaloid caulerpin (**2**) have been especially investigated for their possible impact on the native Mediterranean community. The compounds have been isolated, in fact, from two *Caulerpa* species that have become invasive in the Mediterranean Sea: *Caulerpa taxifolia*, a feather-like species that has been included in the in the IUCN list of the 100 world's worst invasive species [9], and *Caulerpa cylindracea*, previously known as *Caulerpa racemosa* var. *cylindracea* (Fig. 1), which is now widespread in the whole Mediterranean.

A study by Raniello et al. [13] focused on the possible allelopathic role of secondary metabolites from *C. cylindracea* in interspecific competition with native Mediterranean seagrasses. Compounds **1** and **2**, along with the mixture of hydroxy amides caulerpicin (**3**) (Fig. 2), were evaluated for their possible toxic effect on the photosynthetic apparatus of the native seagrass *Cymodocea nodosa.* Leaf portions of the seagrass were exposed to purified compounds under controlled laboratory conditions to assess changes in the photosynthetic performance of the seagrass by monitoring its optimal quantum yield with a pulse amplitude modulated (PAM) fluorometer. Only caulerpenyne (**1**), however, turned out to be phytotoxic, while the other purified metabolites did not show any significant toxicity at the assayed concentrations. As a result, it was hypothesized a possible allelopathic effect of **1** toward native competitors, which may play a critical role in the successful competition of *C. cylindracea* with native seagrasses. This could explain the high efficiency

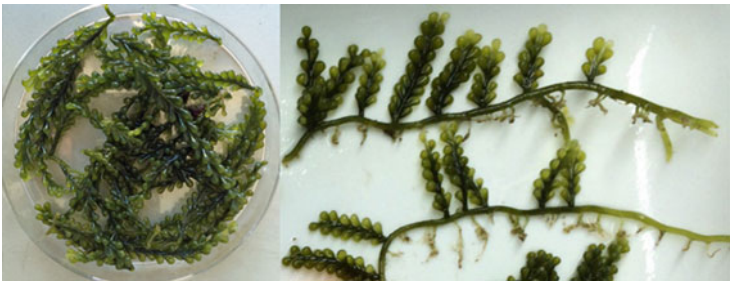

Fig. 1 *Caulerpa cylindracea*

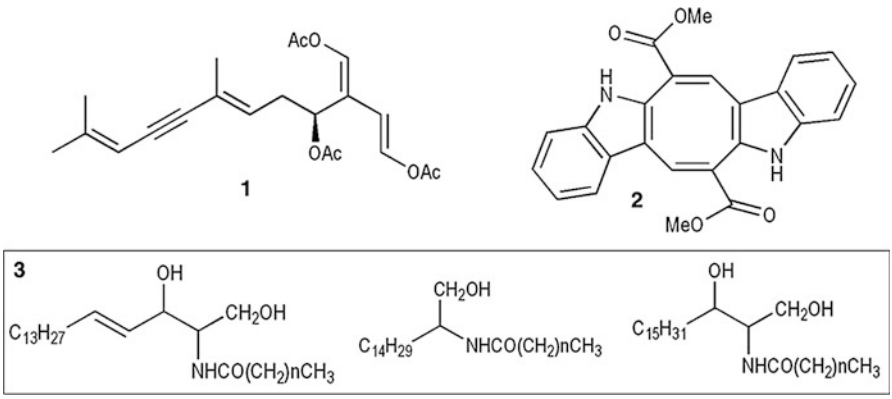

Fig. 2 Metabolites **1**–**3** from *Caulerpa cylindracea*

of this alga in colonizing seagrass meadows. If confirmed, the allelopathic effects of **1** would become a major conservation issue, especially toward seagrass communities that are considered the most productive and complex marine ecosystems in the Mediterranean [14]. However, in addition to *C. cylindracea*, compound **1** has been isolated from other *Caulerpa* species, including *Caulerpa prolifera* that is endemic of the Mediterranean [15], and it has been shown to be the most abundant metabolite of the highly invasive *C. taxifolia* [16]. However, differently from *C. taxifolia* and *C. prolifera*, *C. cylindracea* features a drastic decrease of its biomass in winter that could led to the caulerpenyne accumulation in the substrate leading to suppression of possible competitors [13]. Caulerpenyne (**1**) also showed a panel of biological activities of interest in pharmacology and biotechnology. It was found to be neurotoxic [17], to act as tubulin assembly inhibitor [18], antiproliferative and pro-apoptotic agent [19, 20], and as inhibitor of various enzymes, including alpha-amilase [21], lipoxygenase [22, 23], and xanthine oxidase [24]. Among the actions of potential ecotoxicological interest, it has been described that **1** acts as an inhibitor of cytochrome P450 dependent activities [25], which are important for the clearance of xenobiotics, and a potent noncompetitive inhibitor of zebrafish Oatp1d1 [26], a protein with a crucial role in absorption, distribution, metabolism, and elimination processes [27].

The red pigment caulerpin (**2**) has also attracted considerable attention both in the study of marine biological invasions and for possible biotechnological applications. The compound was originally isolated from three *Caulerpa* species: *Caulerpa racemosa*, *Caulerpa serrulata*, and *Caulerpa sertularioides* [28], but not from *C. prolifera* as erroneously reported [12]. However, the structure of **2** was subsequently revised as a pentacyclic bisindole alkaloid [29]. Compound **2** showed a panel of activities including antispasmodic [30], anticorrosive [31], antiviral [32], anti-nociceptive and anti-inflammatory [33], and mosquitocidal [34] activities. It was also found to act as plant growth regulator [35] and as an inhibitor of mitochondrial respiration [36, 37], protein tyrosine phosphatase 1B [38], and indoleamine 2,3-dioxygenase [39]. It also inhibited the multixenobiotic resistance (MXR) pump in

the gills of the mussel *Dreissena polymorpha* [40]. Compound **2** came to the fore of invasion biology especially when the commercial fish *Diplodus sargus* was noticed to have changed its alimentary habits in the Mediterranean Sea, consuming large amounts of the alga *C. cylindracea*. *D. sargus* is a native fish of high commercial relevance, also playing a major ecological role in controlling the abundance of keystone benthic herbivores in the Mediterranean [41, 42]. The novel "exotic diet" of the fish attracted the attention of researchers especially because the red pigment **2** was found to enter the food chain accumulating in the fish tissues. The levels of **2** in the fish were thus correlated with general biological condition markers associated with fish health and reproductive development, suggesting a possible detrimental effect of the dietary exposure to *C. cylindracea* on *D. sargus* [43]. The level of **2** in the fish tissues was also used as an indicator of the trophic exposure to the invasive pest and related to observed cellular and physiological alterations, including the activation of some enzymatic pathways (catalase, glutathione peroxidases, glutathione S-transferases, total glutathione and the total oxyradical scavenging capacity, and 7-ethoxy resorufin O-deethylase), and the inhibition of others (acetylcholinesterase and acylCoA oxidase), along with an increase of hepatosomatic index and decrease of gonadosomatic index [44]. The observed alterations supported a detrimental health status and altered behaviors in *D. sargus*, potentially preventing the reproductive success of fish populations, because of the *Caulerpa*-based diet.

Subsequent biomarkers analyses of fish exposed to *C. cylindracea* revealed limited alterations of the main antioxidant defenses, increased activities of cytochrome P450, glutathione S-transferases, and acyl CoA oxidase, as well as enhanced gene transcription for peroxisome proliferator-activated receptor alpha, cytochrome P4501A, and vitellogenin 1 [45].

In further study, the trophic exposure of *D. sargus* to *C. cylindracea* was related with a significant reduction in the amounts of essential fatty acids, suggesting that the novel diet affects the nutritional value of the fish flesh [46]. All above findings suggested that bioactive metabolites from *C. cylindracea* could modulate lipid metabolism in *D. sargus*. This hypothesis was then confirmed by feeding experiment followed by spectroscopic and multivariate analysis that provided the evidence of a direct effect of **2** on fish flesh lipid metabolic profile, with a significant loss of polyunsaturated fatty acids in fish fed with a caulerpin-enriched diet [47].

However, the macromolecular targets responsible for the observed effects remained unclear. The problem assumed wider proportions when it was discovered that other native Mediterranean sparid species feed on *C. cylindracea*, among which *Spondyliosoma cantharus* and *Sarpa salpa* accumulate caulerpin (**2**) in their tissues [48].

A first evidence of behavioral changes induced in *D. sargus* as an effect of **2** has been provided by experiments both on groups and on single fish under different doses of dietary **2**, showing that the aggressiveness of the fish decreases with the administration of **2**. It was thus demonstrated that not only a *Caulerpa*-based diet but also the purified metabolite **2** alone is able to alter the behavior on native species, with possible consequences on fish growth and population dynamics [49]. More light on neural mechanisms behind the altered behavior of *D. sargus* has been shed

by the finding of an increase of neuropeptide Y (NPY) transcriptional expression in the central nervous system of *D. sargus* fed with CAU enriched food [50].

In order to characterize the molecular interactions between bioactive metabolites from *C. cylindracea* that are responsible for the most relevant observed metabolic and behavioral effects, an interdisciplinary study has been carried out starting from *in silico* studies that led to predict the interaction of **2** with peroxisome proliferator activated receptors (PPARs) [51]. These nuclear receptors play essential roles in the regulation of metabolism and social behavior in vertebrates, mediating the effects of several nutrients and drugs through transcriptional regulation of their target genes. The prediction has been then validated by in vitro assays, coupled with *in vivo*, *ex vivo*, and *in vitro* transcriptional analysis of PPARα downstream genes related to fatty acid hepatic β-oxidation. Overall, the obtained results disclosed the unprecedented molecular interaction of **2** with PPARs, likely to exert cascade effects at all levels of biological organization, down to sea-based economy, with implications of interest for the development of functional foods for human nutrition and/or drugs for treating human chronic diseases [51].

3 Bioactive Metabolites from the Invasive Seagrass *Halophila stipulacea*

Halophila stipulacea (Fig. 3, left) is a marine seagrass that entered the Mediterranean after the opening of the Suez Canal as a "Lessepsian invader." Fragments of the plant were found in the stomach of the mollusk *Syphonota geographica* (Fig. 3, right) [52], a circumtropical sea hare collected along Greek coasts, during December 2002.

A first study of the chemical composition of the skin of the mollusk led to the isolation of two unprecedented degraded sterols, aplykurodinone-1 and -2, for which a biosynthetic origin from a sterol precursor has been hypothesized. Since both compounds were found to be selectively localized in the skin of the animal, the more exposed parts of the body, their involvement in the defensive mechanisms of the mollusk were also hypothesized. Instead, the study of the extracts from the internal

Fig. 3 *Halophila stipulacea* (right) and *Syphonota geographica* (left)

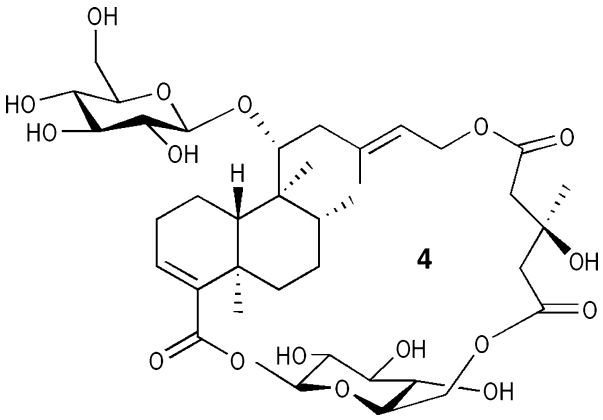

Fig. 4 Structure of syphonoside (**4**)

organs of *S. geographica* led to the isolation of a peculiar compound possessing a novel macrocyclic glycoterpenoid skeleton, which was called syphonoside (**4**) (Fig. 4). The compound was also isolated in a sample of *H. stipulacea* collected from the same site as the mollusk, confirming the trophic relationship between *S. geographica* and the sea-grass. Compound **4** was able to inhibit high-density induced apoptosis in a number of human and murine carcinoma cell lines. This led to hypothesize that **4** may play an important role in regulating cell survival and cell death. Additional chemical investigations on both the mollusk and the seagrass led to isolate three novel macrocyclic glycoterpenoids, structurally related to **4**, one of which was isolated only from *H. stipulacea*, whereas the remaining two compounds were found only in *S. geographica*. This suggested that the mollusk is able to biotransform the dietary metabolite syphonoside (**4**). Furthermore, it was observed that the relative amount of **4** was much higher in the mollusk that in the seagrass, supporting a phenomenon of dietary bioaccumulation [53].

Along with **4**, the known bioactive flavonoids apigenin (**5**), genkwanin (**6**), and chrisoeriol (**7**) (Fig. 5) were subsequently isolated both from *H. stipulacea* and in the viscera of each studied individual of *S. geographica* [10]. This finding strongly supported a dietary dependency, suggesting that the establishment of *H. stipulacea* in the Mediterranean Sea could have enabled the subsequent migration of its specialist grazer, facilitating its invasion in terms of alimentary resources [10]. However, it remains to be clarified whether the compounds present in *H. stipulacea* can act as kairomones, indicating to the herbivore specialist *S. geographica* the presence of its favorite food source.

Further investigations of the chemical constituents of *H. stipulacea* resulted in the isolation of a new malonylated glucopyranosyl flavone, along with five related flavones and the malonylated glucopyranosylapigenin [54]. It was the first finding of malonylated flavone glycosides in the marine environment, while malonyl flavone glucosides derivatives have been reported from many terrestrial sources. This confirmed that seagrasses share most features of their secondary metabolism with land plants from which they derive, having secondarily returned to the sea [55].

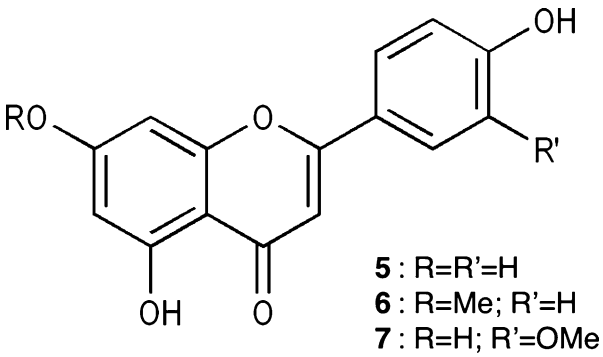

Fig. 5 Structures of apigenin (**5**), genkwanin (**6**), and chrisoeriol (**7**)

It is worth to mention here that flavonoids showed important health functionality of interest for humans. In particular, the flavone apigenin (**5**) isolated from *H. stipulacea* is widely distributed in terrestrial plants, fruits, herbs, and plant-based beverages with health-promoting effects and interesting therapeutic functions [56, 57], including antioxidant, anti-inflammatory, antimutagenic, antitumorigenic, and neuroprotective properties [58]. Compound **5** is also known to inhibit the activity of 5′-nucleotidase [59], aromatase (CYP19) [60], phospholipase A2 [61], platelet-derived growth factor (PDGF) [62], and angiotensin converting enzyme (ACE) [63], and to inhibit the isoform CYP2C9 of the cytochrome P450, which is among the most important drug-metabolizing enzymes in humans [64]. This latter finding suggests that **5** could inhibit detoxification metabolism, increasing the toxicity of other compounds when taken simultaneously, with potential high ecotoxicological impact, since the level of toxicity of chemical agents is dependent on detoxification processes. On the other hand, flavonoids have a number of important functions in plants, acting as regulators of symbiotic interactions with microorganisms, maintaining a redox state in cells, and participating in protective strategies against herbivores and pathogens, as well as against abiotic stresses, such as UV radiation and heat [65]. As a result, they could confer to *H. stipulacea* a competitive advantage over native species, sensibly contributing to its success as invader in the Mediterranean Sea. It would therefore be appropriate to carry out further studies to confirm this hypothesis.

4 Conclusions

Marine invasive species are having a tremendous impact on the Mediterranean biota, which is losing its biological distinctiveness under the continuous pressure of biological invasions [10]. In particular, since their eradication is considered unrealistic, a major challenge for environmental management institutions is how to deal with the invasive macrophytes that are dramatically taking the place of keystone species, altering the food web, threatening native species of commercial interest, and negatively affecting both tourism and fisheries. It gives urgency to

a better understanding of the factors that affect marine biological invasions, and to the development of effective and knowledge-based strategies to face these relevant threats to local biodiversity.

In this article, we focused our attention on the bioactive molecules that two invasive macrophytes, *C. cylindracea* and *H. stipulacea*, brought with them when they invaded the Mediterranean Sea. Although little is known about the natural function of those compounds, we have shown that a great deal of information on their biological activities of interest in biotechnology and pharmacology is already available in the literature. Nevertheless, we believe that it is crucially important to understand how such actions relate to the roles played by the compounds in nature. Natural substances have evolved as important adaptations for the organisms producing them in their native environments, and these activities may well be closely related to the properties that make them so interesting for possible applications in human health and biotechnology. They evolved to give protection to their producers from predators, parasites, and pathogens and to act as weapons against competitors, as the result of diffuse coevolutionary processes. However, when those molecules invade a new environment, they will start to interact with native species who had never encountered them before. What should we expect from this? Can they contribute to the success of the invaders? Do they represent threats to native species and drivers of community change?

Our overview of the variety of biological activities that characterize some of the compounds isolated from *C. cylindracea* and *H. stipulacea* suggests that these molecules can actually act as drivers of changes in the Mediterranean by especially providing the invaders with tools for outcompeting and replacing native species, within rapid coevolutionary processes. Indeed, there is reason to hope for a greater synergy between chemical ecology and invasion biology when approaching the complex issues raised by invasive species.

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7 Co-evolution of the Shrimp *Hippolyte inermis* and the Diatoms *Cocconeis* spp. in *Posidonia oceanica*: Sexual Adaptations Explained by Ecological Fitting

Valerio Zupo

Contents

Abstract

Microalgae influence the life of grazers in such stable ecosystems as *Posidonia oceanica* meadows. Competition and co-existence require adaptations for both organisms: algae produce metabolites able to reduce the grazing activity, and invertebrate react to the chemical weapons of algae, for feeding on their thalli. Several diatoms produce wound-activated compounds and some of them have been demonstrated to trigger apoptosis and teratogenic effects in planktonic copepods. The case of *Hippolyte inermis* and its diatom food is different and peculiar because the shrimp transformed the effects of apoptogenic compounds produced by *Cocconeis* into a spring signal to obtain a higher abundance of females, so stabilizing its natural populations. As in crustacean decapods, the sex is determined by the presence/absence of a single gland (the Androgenic Gland; A.G.), in *H. inermis* the apoptogenic effect of secondary metabolites is limited to the destruction of the A.G. in spring, when various species of *Cocconeis* dominate the epiphytic layer of *Posidonia* leaves. This relationship, evidently co-evolved through a competitive relationship, allows the shrimp to produce a

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secondary reproduction burst in fall, when *Cocconeis* spp. are less abundant on the leaves of the plant. Co-evolutionary relationships are often viewed in light of mutual cooperation between two species. However, the peculiar case of *H. inermis* indicates the need to widen the concept, integrating various adaptations that may lead to different degrees of advantages for two co-evolving organisms. Shrimp's populations are stabilized in *P. oceanica* meadows thanks to this very specific relationship, and they can survive a high predation pressure by fish and other invertebrates because the secondary reproductive burst in fall produces sufficient specimens for the next spring.

Keywords
Chemical ecology · Adaptation · Co-existence · Feeding · Food webs · Apoptosis

1 Introduction

Co-evolutionary processes often lead to mutualistic associations where two partners gain reciprocal advantages from the interactions in the same environment [1], by following a process of joint adaptations between species [2]. The concept was initially developed to explain the evolutionary forces driving the selection of two species having close physiologic or ecologic relationships, to obtain mutual advantages. In contrast, the example of the shrimp Hippolyte inermis and the diatoms of the genus Cocconeis indicates the evolution of a struggle for survival, leading to organic diversification of both species, aimed at surviving in a complex but relatively stable environment as the one represented by Posidonia oceanica meadows. Since the infancy of coevolution studies, "the examination of patterns of interaction between two major groups of organisms with a close and evident ecological relationship, such as plants and herbivores" is considered a fundamental topic to be investigated [3]. Thus, it is worth considering the case of the shrimp H. inermis, that is a grazer of benthic diatoms of the genus Cocconeis, to understand if, in the absence of fossil records, the ecological and physiological patterns discovered up to date aid in separating the rate and time components of evolutionary changes in either or both organisms. Evidently a selective pressure is mutually exerted, but in this case, a skewed pattern of advantages is observed in the two species, because the shrimp evolved the ability to use teratogenic compounds, normally produced as anti-grazer agents, to improve its sexual maturation and the fitness of natural populations. As in the case of other long-term biological interactions (e.g., symbiosis vs. commensalism, parasitism, etc.) we propose here to widen the concept distinguishing between the "evolution of cooperation" and the "evolution of competition", thus introducing the theoretical notion of "competitive co-evolution" [4], according to the evolutionary game theory [5], as an agonist alternative to the "cooperation co-evolution". In both cases, the establishment of either "cooperator–cooperator" or "defector–defector" links facilitate the formation of a hierarchical interaction structure leading to a favorable environment for two or more species [6].

2 Food Choice and Evolutionary Constraints

The choice, by any animal grazer, of plant foods available in the same geographical and ecological range [7], is of paramount importance to determine its fitness and represents a selective advantage. Mechanical, physiological and chemical factors influence these choices [8]. For example, the selection of macroalgal food by various marine invertebrates is hardly influenced by the toughness and the mechanical properties of thalli [9], but also by the chemical defenses (both constitutive and activated) produced by various algae. Mechanical properties represent a first level of defense, but plants are mainly efficient for the production of a large variety of chemical weapons [10], including oxylipins, terpenoids, alkaloids, quinones, terpenoids, flavonoids, glycosides, organic acids and other compounds (comprising cyanogenic and carcinogenic compounds) able to make them unpalatable or even toxic for various potential consumers [11]. Diatoms, in this view, are among the most productive organisms, since they are mechanically protected by silica frustules and chemically protected by interesting and quite innovative compounds [12]. For example, the hatching success of herbivore copepods is impaired by the feeding on diatom-dominated blooms, because these microalgae produce wound-activated oxylipins and aldehydes [13] triggering mortality or teratogenic modifications in copepod larvae. The production of wound-activated chemical defenses is widespread within marine microorganisms [14]. The evolution of chemical weapons, according to the evolution of invertebrates able to detoxify [15], incorporate [16] or use them for various purposes [17] represent spectacular examples of invertebrate-microorganism co-evolution [18]. Thus, various substances produced by algae and diatoms as repellents may acquire important physiological roles and become decisive in patterns of food plant selection, as also observed since the last century in terrestrial organisms [19].

3 The Diversity of Marine Diatoms

Diatoms are a major group of microalgae colonizing any marine and freshwater environment, including humid zones, and they are quite abundant both as part of the phytoplankton and in the benthos [20]. The large variability of environmental factors influencing their ecology and morphology produced an extraordinary diversity of forms and adaptations [21]. The impact of diatoms on the primary production of oceans is quite high and their global scale contribution is estimated to be 30–40% of the world's primary production. Apparently, the diversity of diatom genomes (and, consequently, their physiological plasticity) is so high to justify their success and dominance in any aquatic environment, thanks to their ability to react to environmental changes, also producing spores and defense compounds [22]. In addition, environmental factors drive their morphological variations and their frustules, appearing as static structures, may adapt to both physicochemical conditions and ecological constraints, leading to morphological variations of valves towards the best solutions to face adverse or favorable conditions [23], according to their genetic diversity. Most planktonic [24] and benthic diatoms have been demonstrated to

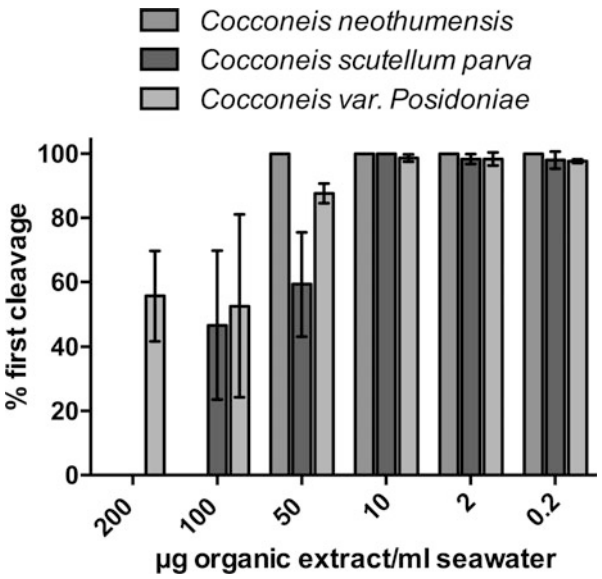

Fig. 1 The toxicity of three species of *Cocconeis* diatoms tested by observing the percentage of first cleavage of sea urchin embryos. The different toxicity of three species is compared, taking into account that these species are all commonly ingested by *Hippolyte inermis* and they do not produce any toxic effect, but the sex reversal, when consumed in the first days of post-larval development

produce a variety of secondary metabolites [25] and several of them are wound-activated [26, 27]. Some wound activated compounds produced by benthic diatoms after the ingestion by various grazers have the role of infochemicals [28] and they influence the behavior of invertebrates [29], because they evolved chemotactic reactions using the volatile organic compounds of diatoms to detect the possible presence of predators or the locations of specific food items [30]. Other compounds are toxic for various invertebrates but still, evolutionary relationships may tune their activity [31]. For example, it has been demonstrated [29] that the toxicity of diatoms [32, 33] is inversely correlated to the ability of animals to recognize their odors and this fits the scope of their insidious defense compounds [13], but benthic invertebrates tend to develop "good noses" for those species that are stable components of their environments, probably due to co-evolutionary processes [34]. Thus, the production of toxic compounds (Fig. 1) and their recognition by invertebrates is a factor able to stabilize local associations, because only alien species, unable to recognize (or detoxify) the most noxious species, feed on them and are exposed to the toxigenic effects (Fig. 2). All this demonstrates how the morphological diversity of diatoms is encompassed only by the diversity of their chemical defenses, and how co-evolutionary processes may modulate their ecological relationships with associated animal communities.

4 Diatoms as Food for Invertebrates

Diatoms are a quite interesting prey for several invertebrates, since they appear as a portion of proteins, starch and fatty acids, well packed into a glass frustule [35]. For this reason they are elective food for copepods, in the planktonic environment [36],

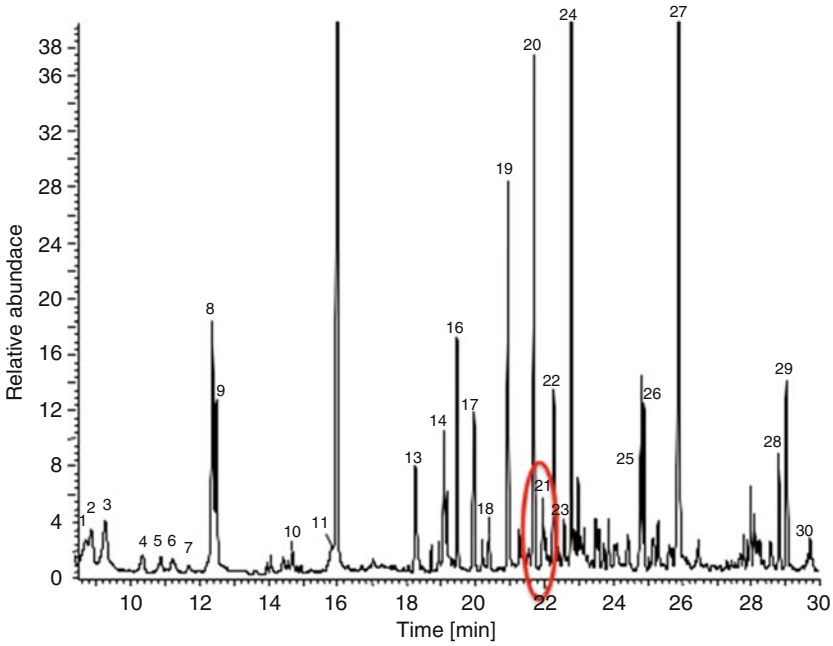

Fig. 2 An HTML profile of *Cocconeis scutellum parva* lipophilic fraction. The active compound responsible for the apoptotic destruction of the A.G. of *Hippolyte inermis* is considered to be in the area indicated by a red ellipse

but also a very important food source for benthic organisms [37, 38], due to large seasonal abundances, the above-mentioned diversity and the relationships they evolved with given species [39]. For example, the Posidonia oceanica environment contains several species of diatoms [40] and some of them are selectively present on the plant leaves, if not exclusive, due to a long evolutionary history [41]. The genus Cocconeis (Fig. 3), in particular, contains some species (e.g., Cocconeis scutellum var. posidoniae and C. scutellum var. neothumensis) that were identified in very specific areas and in presence of the plant substratum [40]. Cocconeis spp. diatoms are well adapted to the life on P. oceanica leaves because they are generally sciaphylic [42], very adhesive and slow growing, able to seasonally cover almost the entire surface of plant leaves. They represent an excellent trophic resource for various micro-grazers [43], due to a complete spectrum of fatty acids specifically produced, and low generic toxicity [29].

5 The Life Cycle of *Hippolyte inermis*

The peculiar case of Hippolyte inermis Leach, 1815 is emblematic in this view [44]. H. inermis lives in shallow waters of the Mediterranean and along the Atlantic coast of Spain [45]. It forms stable populations in seagrass meadows [46], mainly in Posidonia oceanica and Cymodocea nodosa [47]. Most individuals exhibit mimic

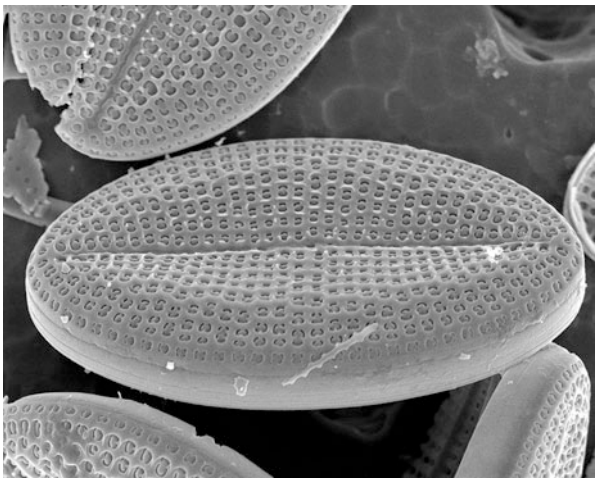

Fig. 3 SEM microphoto of *Cocconeis neothumensis*, one of the species characterizing the leaf stratum of *Posidonia oceanica* and seasonally reaching extremely high densities

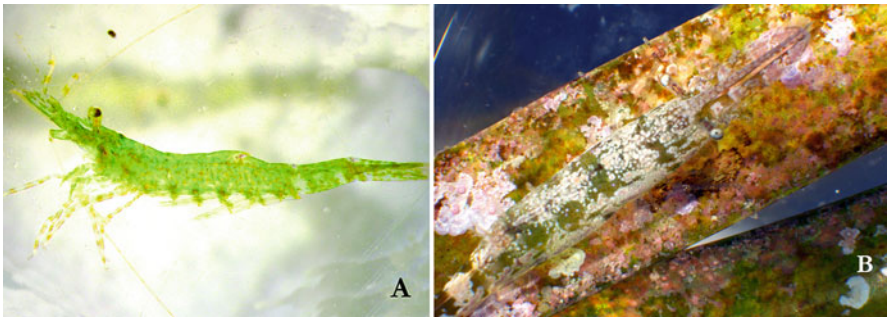

Fig. 4 (**a**) *Hippolyte inermis*, male of 10 mm total length. The vivid green color resembles the surface of seagrass leaves. (**b**) A female of 14 mm total length. The patterns of color resemble those of coralline algae epiphytizing the *Posidonia oceanica* leaf, on which the shrimp is located

colors [48] resembling the leaves of the plant and their epiphytes, as a vivid demonstration of the close ecological association with the seagrass (Fig. 4).

On the other end, the influence of diatoms on the reproductive ecology and the life cycle of other crustaceans [13, 49], mainly copepods [50], was previously demonstrated. The production of diatom compounds, detrimental to the development and survival of grazers, has major impacts on secondary production [51, 13]. Opposite effects were hypothesized, however, in H. inermis [52, 53], according to co-evolutionary processes [54]: it is largely adapted to the life in P. oceanica [55] and the toxic effect of diatoms is translated into a spring signal (Fig. 5) for the development of beta females [56], whose presence is a crucial factor for maintaining a constant sex ratio [53, 57].

The sex determination in crustaceans is due [58] to the presence of a single Androgenic Gland (A.G.) producing an insulin-like hormone [59]. Thus, protandric

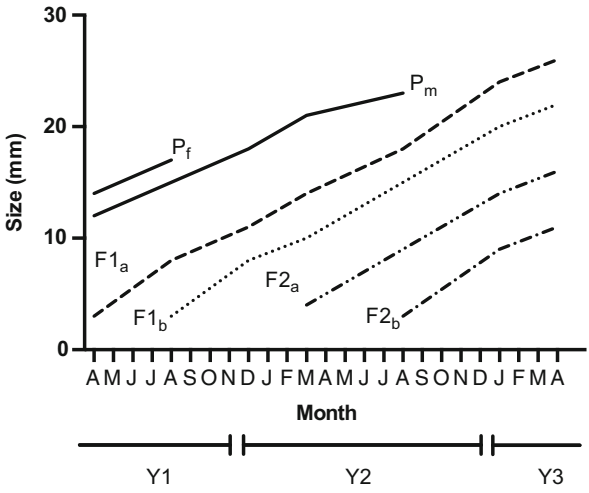

Fig. 5 Population dynamics of *Hippolyte inermis* (From Zupo 1994, modified). Three cohorts are represented: P (parental), F1 (first generation) and F2 (second generation). Each cohort produces two reproductive bursts, the first in spring (April) and the second in fall (September)

shrimps normally develop an A.G. in the first phases of their post-larval life and loose it during their life. While the gland regresses, testis tissues are transformed into an ovary. During the process a new intermediate gonadic stage named "ovotestis" is produced and this phase is stabilized in some species, producing contemporary hermaphroditism [60]. In *H. inermis* the destruction of the A.G. is a very rapid process, often completed during a single molting cycle, and it leads to the rapid sex shift to female sex [61]. The sex reversal was initially supposed to be completed after about 1 year [62], as observed in other hippolytid shrimps. In contrast, it has been demonstrated [53] that the shrimp is characterized by two reproduction bursts (in spring and in fall) exhibiting very different properties. Individuals born in spring feed on diatoms of the genus *Cocconeis* [63] and they develop both as males and females, appearing as a gonochoristic species [64]. Individuals born in fall are out of the *Cocconeis* blooms on *Posidonia* leaves and their guts do not contain these diatoms [63]. They produce young males (Fig. 6), that shift their sex to females after about 1 year [53], according to a proterandric strategy.

It has been demonstrated [54] that the process observed in spring is due to the apoptosis of the A.G., triggered by compounds present in the ingested diatoms, able to destroy selectively the gland tissues (Fig. 7). The process is rather rapid (it takes place from the 5th to the 12th day of post-larval growth [44] and it is immediately followed by the destruction of the shrimp testes, along with the vas deferens (Fig. 8).

This leads to the shift to the female sex and this peculiar life strategy (double period of reproduction with different sexual strategy applied by young individuals and a variable proportion of resources invested in large and small females, respectively) may be viewed as a smart stratagem to increase mating opportunities and adjust sex allocations [65, 66], seasonally, in order to improve its fitness to a high predation pressure [53]. The process is in perfect coherence with the abundance of epiphytes covering the leaf stratum, with which the life cycle of the shrimp is synchronized (Fig. 9).

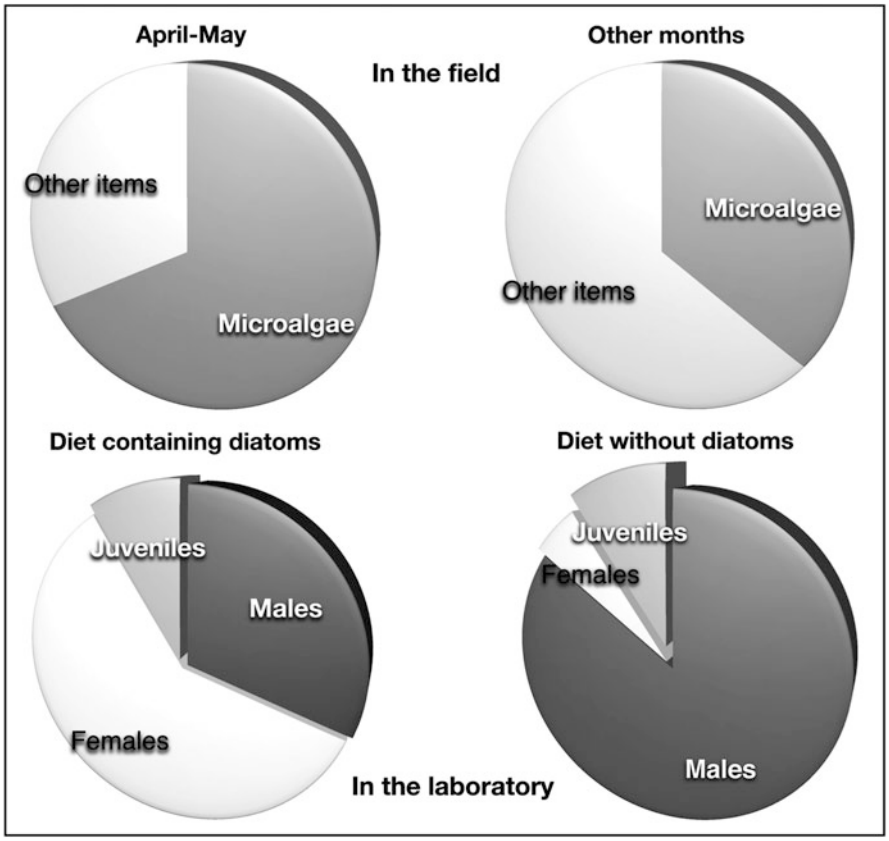

Fig. 6 Top plots. Analysis of sex in individuals of *H. inermis* collected in the field in April–May (when young females are produced) and in other months (when shrimps show a typical proterandric development). Microalgae containing large amounts of *Cocconeis* dominate their gut contents. Lower plots. Analysis of sex of individuals of *H. inermis* cultured in the laboratory and fed diets containing *Cocconeis* (left) and not containing diatoms (right). Evidently the diet containing diatoms triggers the production of larger amounts of females, resembling those collected in spring in the field

6 Conclusions

Several diatoms produce wound-activated compounds able to trigger apoptosis processes in crustaceans [49]. These secondary metabolites, synthesized within the oxylipin pathway, are generally meant as induced defenses against grazer consumers. The shrimp here considered translated this effect into a spring signal to produce young females [53], to increase the reproductive burst in fall. Although the example here discussed of a close interaction between a shrimp and a diatom appears, at the best of our knowledge, as a unique case for chemical ecology and

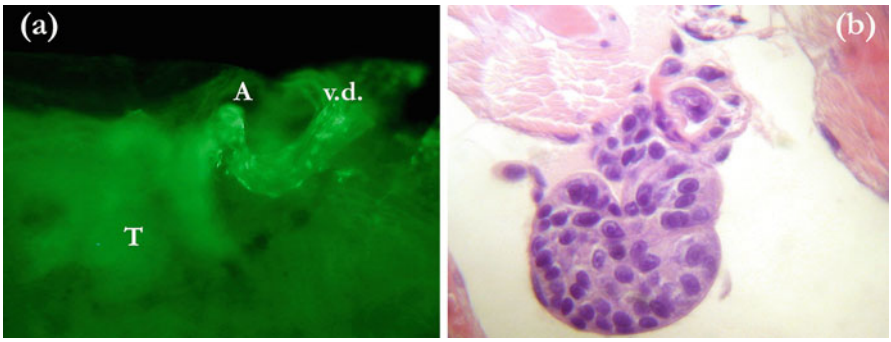

Fig. 7 (**a**) Analysis under the complanar microscope of a male shrimp fed on *Cocconeis* diatoms and treated with the TUNEL technique for the detection of apoptosis. The fluorescent areas indicate apoptotic processes. *A* Androgenic gland, *T* testis, *v.d.* vas deferens of the male. (**b**) A normal Androgenic Gland in a young male observed under the light microscopy

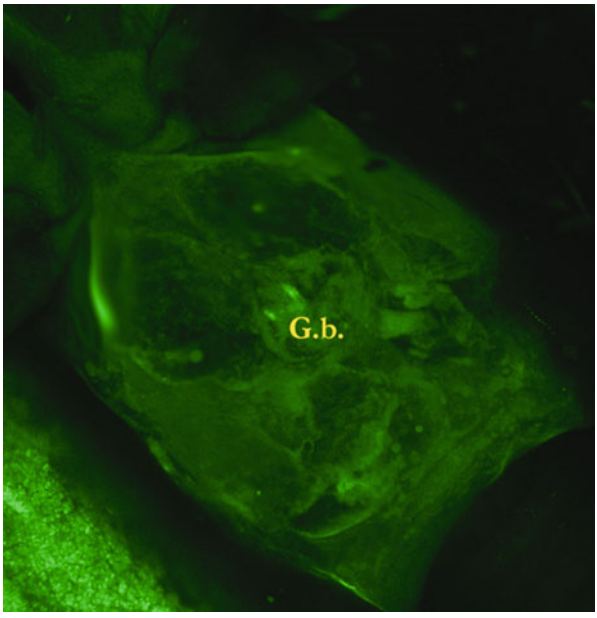

Fig. 8 Analysis under the complanar microscope of a 7 day old male fed on *Cocconeis* diatoms and treated with the TUNEL technique for the detection of apoptosis. The fluorescent areas indicate apoptotic processes. The gonadic buds (G.b.) under development are clearly being destroyed by apoptotic processes

co-evolution studies, some generalities can be drawn from the observed patterns, if we wish to understand the arrangements of use of different plant groups by invertebrates, to understand general forces ruling their co-evolutionary adaptations. In particular, the case here reported indicates that in the frame of "competitive co-evolution", the chemical weapons produced by a species may be used for different purposes by their competitors/consumers, even without incorporating or modifying their structure [16, 17], but simply evolving physiological adaptations that lead to

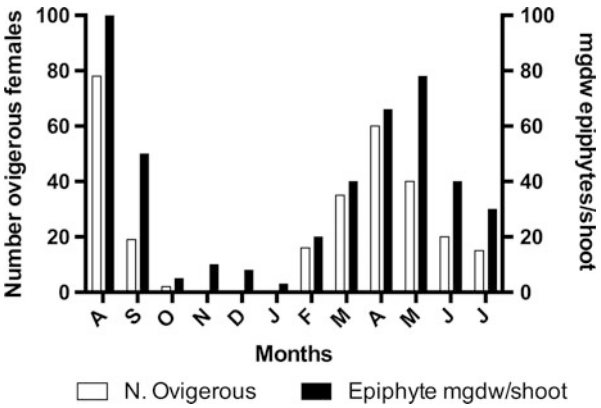

Fig. 9 Number of Ovigerous females per square meter collected in the *P. oceanica* meadow in Lacco Ameno d'Ischia (Bay of Napoli, Italy) and abundance of epiphytes covering the leaves of the seagrass. The two measures are largely in accordance during the year

improvement of their fitness to a common environment (Posidonia oceanica leaves), as in the case of the rapid sex shift exhibited by the model shrimp. The described process is due to a very specific apoptogenic effect, physiologically restricted by the shrimp to the tissues of its A.G., as an adaptation to the generic apoptogenic activity [67] triggered by diatoms for anti-grazing purposes [68]. These observations indicate the need to distinguish between the concept of "competitive co-evolution" [4], as an agonist alternative to the "cooperation co-evolution" taking into account that a continuous range of intermediate adaptations may be documented in various organisms.

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Part II

Evolution of Chemical Ecology

8 Evolution of the Angiosperms and Co-evolution of Secondary Metabolites, Especially of Alkaloids

Michael Wink

Contents

Abstract

Plants produce a wide variety of secondary metabolites (PSM) for protection against herbivores, microorganisms, and competing plants. PSM also function as signal compounds to attract pollinating and fruit-dispersing animals. PSM occur in complex mixtures, which vary between organs and developmental stages of a plant. PSM have been structurally optimized during evolution to affect molecular targets in animals, other plants, and microbes. Many insect herbivores have adapted to the defense chemistry of their host plants and are mono- and oligophagous. The largest class of PSM are alkaloids, which often function as strong neurotoxins against insects and vertebrates. Whereas the production of alkaloids is very limited in spore bearing plants and gymnosperms, they

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are dominant in angiosperms, which comprise more than 90% of all living plants. Angiosperms develop showy flowers to attract pollinators. However, these pollinators should only feed on nectar but not on the aerial parts or flowers of a plant. It is argued that the diversification of angiosperms was a driving force for the radiation and diversification of insects, which comprise the majority of animals with more than 1.4 million species. As a sort of co-evolution, angiosperms, which rely on animal pollination, started to produce a wide diversity of neurotoxic and fast-acting alkaloids to keep their animal visitors under control.

Keywords
Plant phylogeny · Secondary metabolites · Alkaloids · Co-evolution · Pollination · Herbivory · Chemical defense

Abbreviations

AMP	Antimicrobial peptide
PSM	Plant secondary metabolite

1 Introduction: Evolution and Functions of Secondary Metabolites

1.1 Plant Secondary Metabolism

A characteristic feature of plants is the production of low-molecular weight compounds, termed secondary metabolites (PSM) or natural products (Fig. 1) [1–10]. PSM are produced in specific biosynthetic pathways with the aid of substrate specific enzymes. The precursors of PSM derive from primary metabolites of plant metabolism, such as amino acids, sugars, fatty acids, or acetyl-CoA. Thus, primary and secondary metabolism are tightly connected [2, 11].

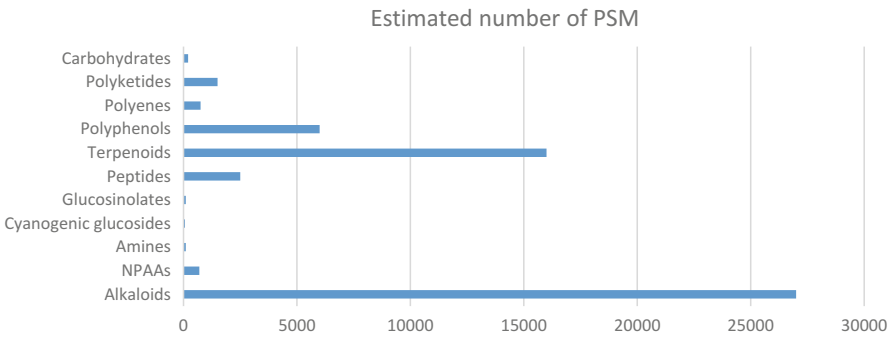

Fig. 1 Estimated number of PSM

Most PSM are stored in plants in relatively high concentrations, which can exceed more than 10% of total dry weight. Water-soluble PSM are usually stored in the vacuole of plant cells, often the epidermal cells. Lipophilic compounds are not sequestered in the vacuole but excreted into raisin ducts, lacticifers, oil cells, trichomes, dead cells, or the cuticle [5, 11, 12].

PSM can be synthesized in almost any plant organ, but this differs from plant species to another or types of PSM. Some plants produce a PSM in the root, but store it in the aerial parts (e.g., tropane alkaloids or *Nicotiana* alkaloids). In order to reach the aerial parts, PSM undergo long-distance transport via the xylem. Other plants produce PSM in the leaves, sometimes even in chloroplasts. If these PSM are accumulated in roots, stems, flowers, or seeds, they are usually transported there via the phloem (e.g., quinolizidine alkaloids) [11].

Plants usually do not produce a single PSM but a couple of main PSM and several derivatives, differing by additional hydroxyl, methoxy, epoxy, aldehyde, or ester moieties or the degree of oxidation. These additional functional groups can influence the biological activity of a PSM [13, 14]. In many instances, plants not only produce a mixture of PSM from the same class of PSM but from several classes. The composition of these mixtures differs between plant organs, i.e., PSM of roots differ from those of leaves or seeds. Furthermore, PSM profiles differ between developmental stages; i.e., profiles from seedlings differ from those of mature flowering or senescing plants [11]. This feature is important for our discussion of co-evolution of angiosperms and herbivores. Within a population, a substantial variation in content and composition of PSM can be expected. This variation can be due to genetic or environmental factors. Plants growing at a sunny site have a different PSM profile from those living in the shade, or mountain populations differ from low-land populations.

Most PSM are not end products but can undergo metabolism. Nitrogen--containing PSM are often used as nitrogen-storage compounds by some plants. Legumes (family Fabaceae), which store quinolizidine alkaloids, lectins, protease inhibitors, alkaloids, or nonprotein amino acids in their seeds, mobilize these PSM after germination and use their nitrogen for the developing young plant (Fig. 2). This feature is important, because nitrogen is a limiting factor for most plants and thus its use must be economic [2, 5, 11].

Plant secondary metabolism is not static. When plants are infected by a pathogen or wounded by an herbivore (an animals which feeds on plants), the secondary metabolism is often activated. Preformed PSMs are activated after cleavage of a sugar moiety by beta glucosidase or an esterase; examples are cyanogenic glucosides, glucosinolates, saponins, or flavonoid glycosides [15–17]. In other cases, the formation of existing PSMs is stimulated or in some plants completely new PSM are synthesized, mostly oriented against microorganisms. Plant pathologist have termed the new compounds “phytoalexins.” Plant hormones, such as gibberellic acid, jasmonic acid, salicylic acid, play important roles in the corresponding signal pathways (involving calcium signaling) and differential gene expression, which regulate plant responses to the environment [11, 15, 18].

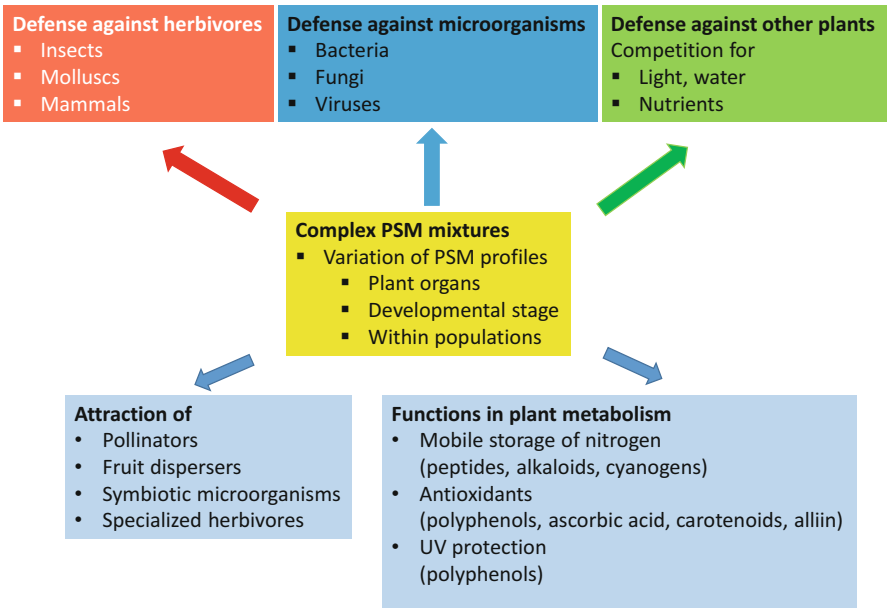

Fig. 2 Functions of PSM as defense, signal, or otherwise useful compounds

1.2 Functions of Plant Secondary Metabolites as Defense Compounds

During the last decades, it became more and more evident that PSM are not worthless waste products, but important for the survival and ecological fitness of a plant producing them [2, 5, 6, 11, 12, 15]. It is a trivial observation that plants cannot run away when they are attacked by an herbivore nor do they have an adaptive immune system (as animals have) to ward off microbial pathogens or parasites. The evolutionary solution of plants and often of other immobile organisms was the synthesis of defense compounds, which could interfere with the physiology, metabolism, or reproduction of potential enemies (Fig. 2). In common language, we would call such compounds toxins or antimicrobials. Thus, PSM, which we see today, have undergone several cycles of evolution screening by natural selection to make them biologically active. For this reason, many PSM or the herbs producing them can be used in pharmacy or medicine to treat infections and some health conditions [4, 16, 17]. I have thus termed the result of this process "evolutionary pharmacology" [14, 16, 17]. In conclusion, the main function of PSM mixtures is defense against herbivores (grazing mammals, insects, slugs, and mollusks). Plants also use mechanical features against herbivores, such as spines, thorns, stinging hairs, or a thick strongly textured bark. In addition, most plants have open growth and can replace plants parts, which have been damaged.

Sometimes, the same or other PSM are more directed against microorganisms and help to ward off bacteria, fungi and viruses, which are abundant in the environment. Antimicrobial PSM interfere with biomembranes of microbes (saponins, mono- and sesquiterpenes), of proteins (polyphenols), protein biosynthesis, and DNA replication and transcription (many alkaloids). In addition to antimicrobial PSM and antimicrobial peptides (AMPs), plants can block vessels by storing callose or can inhibit microbial infection by secreting chitinase, glucanase, and peroxidase [5, 11, 13–15].

Plants compete with other plants for light, water, and nutrients. In some instances, for example, in desert plants, a strong competition between plants of the same or different species can be observed, in a way that individual plants are surrounded by an empty space. It could be demonstrated that plants excrete PSM from their rhizosphere or from leaves, which can inhibit the germination or development of other competing plants. This phenomenon has been termed "allelopathy" (Fig. 2) [2, 5, 7, 19].

1.3 Functions of Plant Secondary Metabolites as Signal Compounds

It appears to be contradictory that plants also use PSM as signal compounds to attract pollinating insects, fruit-dispersing animals, or symbiotic root bacteria (Fig. 2). Mostly, we see the same PSM, which are employed in defense against herbivores. How to explain this contradiction? Flowering plants (angiosperms) produce conspicuous flowers to attract pollinators. Attraction is achieved via colors (mostly anthocyanins, some flavonoids, and carotenoids) which insect can perceive in normal but also UV light. In addition, many plants employ aromatic PSM (mostly terpenoids) as an additional olfactory attractants. Some plants produce foully smelling PSM, such as amines to attract flies and beetles [2, 5, 6, 19–21]. These strategies help to attract pollinators to the vicinity of a flower. But the pollinator should not feed on the flower itself, but instead is rewarded by nectar. Nectar is usually rich in sucrose or glucose and may contain some lipids and amino acids. In some plants, the nectar also sequesters PSM [22–24]. Thus, the PSM in flowers function as deterrent at low distance. In addition to insects (honey bees, solitary bees, bumblebees, pollen wasps, ants, flies, bee flies, hover flies, mosquitos, butterflies, moths, flower beetles), some plants (often with red flowers) employ birds (sugar birds, sun birds, humming birds, bats) as pollinators [2].

In contrast to plants which use animals (entomophilous and zoophilous species), the majority of gymnosperms and many angiosperms (taxa within Poales, such as grasses, sedges, and rushes; Fagaceae, Betalaceae, Junglandaceae, Vitaceae) use wind-pollination (anemophily). Anemophilous plants do not produce showy flowers, lack nectar but produce large amounts of pollen grains. As discussed later, wind-pollinated plants are often without strong poisons, such as alkaloids.

Some plants, which produce fruits, are interested that their seeds are dispersed away from the producing plants [3–5]. To achieve this purpose, plants produce fruits,

which are attractive to fruit eating animals (frugivores), such as certain birds, primates, or bats. In this case, the seeds can withstand the digestive process and are discarded in the feces, usually at a different site. To be attractive, mature fruits are often rich in nutritive sugars and have an aromatic smell. As only mature fruits should be consumed, unripe fruits are often rich in bitter or acidic PSMs and do not advertise themselves by conspicuous colors, i.e., they are mostly green [11, 15].

Plants use some of their PSM in addition as mobile nitrogen storage compounds, as antioxidants or for UV defense (Fig. 2) [11, 15]. Thus, PSMs can have multiple purposes for the plants producing and storing them.

1.4 Adaptation of Herbivores

Whereas chemical defense works in most instances against most polyphagous herbivores, some monophagous herbivores have evolved a tolerance towards a particular toxin of its host plants. Some insects can quickly detoxify and eliminate dietary PSMs, a few other sequester these PSM and use them for their own defense against predators. These specialists can thus exploit a specific host plant without being poisoned. But they cannot feed on other unrelated plants [2, 20, 21, 25–30].

Special adaptations are required for this process, often involving several mutational steps. For example, larvae of the Monarch butterfly can feed on *Asclepias* plants, which produce cardiac glycosides which are poisonous for most animals. In the Monarch, a mutation of Na^+, K^+ -ATPase in the binding site for cardiac glycosides confers resistance. Larvae and adult Monarch butterflies become toxic themselves and are avoided by insectivorous birds [31–35]. Similar adaptations have been observed in insects, mostly in moths, butterflies, beetles, and bugs, and comprise several neurotoxic PSM, such as cardiac glycosides, pyrrolizidine alkaloids, quinolizidine alkaloids, cyanogenic glucosides, but also iridoid glucosides, glucosinolates, and polyphenols [2, 13, 21, 36–43]. In most instances, the exact molecular adaptations have not been discovered.

As a consequence of these adaptations, many insects are mono- and oligophagous.

2 Evolution and Function of Alkaloids

As mentioned above, plants produce a wide diversity of PSMs, which can be classified by their ring structures or biochemical pathways from which they derived (Fig. 1). Formally, we can distinguish between nitrogen-containing PSM and nitrogen-free PSM, such as terpenoids, phenolics and tannins, anthraquinones and polyenes. The class of PSM with nitrogen in their molecule is dominated by alkaloids, of which more than 27,000 structures have been described. In this class, we also find nonprotein amino acids (NPAA; more than 700 structures), glucosinolates (ca. 100 structures), cyanogenic glucosides (80 structures), and

amines and several peptides [lectins, protease inhibitors, and antimicrobial peptides (AMPs)] [1, 3–5, 7, 8, 11, 16, 17, 44].

Whereas alkaloids are often powerful neurotoxins, NPAAs are mimics of the 20 proteinogenic amino acids and can be incorporated into proteins. These proteins show a wrong secondary and tertiary structure and thus are often functionless. Thus, NPAAs function as metabolic disruptors [14, 16, 17, 21, 44]. When cyanogenic glucosides are hydrolyzed, they release HCN, which is a powerful inhibitor of the respiratory chain in mitochondria and thus inhibits the formation of ATP. They are thus powerful metabolic poisons [2, 4, 21]. When glucosinolates are cleaved, lipophilic mustard oils or isothiocyanates are liberated. They disturb membrane fluidity, bind to proteins and DNA bases, and cause inflammation, a pungent taste, and pain [14, 16, 17]. Lectins often interfere with ribosomal protein biosynthesis, protease inhibitors inhibit proteases of the digestion process, and AMPs influence membrane fluidity and stability in microbial and eukaryotic cells.

Alkaloids contain one or several nitrogen atoms, mostly in their ring structures. They form a free base under alkaline conditions and are charged molecules below pH 7 (i.e., in most plants and in target organisms, alkaloids are mostly present in a protonated form). However, when we draw them, we usually show the free base.

Depending on the biosynthetic pathways and ring structures, alkaloids are divided into the following groups: pyridine, pyrimidine, pyrrol, piperidine, pyrrolizidine, quinolizidine, indolizidine, isoquinoline, quinoline, indol, monoterpene indole, terpenoid, and steroidal alkaloids. The main amino acids, which serve as precursors, are phenylalanine/tyrosine (isoquinoline alkaloids, including protoberberine and morphinane alkaloids), tryptophan (indol alkaloids, monoterpene indole alkaloids, and quinoline alkaloids), lysine (piperidine and quinolizidine alkaloids), ornithine/arginine (tropane and pyrrolizidine alkaloids) [45–51].

As mentioned above, alkaloids often function as neurotoxins, others are cytotoxic, as they interfere with biomembranes, microtubules, actin filaments, enzymes, and DNA/RNA and corresponding enzymes [5, 6, 15, 44, 45, 47, 51–54]. In case of neurotoxic alkaloids, they often mimic the structure of neurotransmitters, such as acetylcholine, noradrenaline, adrenaline, serotonin, dopamine, or endorphins (Table 1). They can either block the neurotransmitter receptor as antagonist or stimulate it as agonist. Some alkaloids inhibit the activity of enzymes, which degrade neurotransmitters, such as acetylcholine esterase or monoamine oxidase (MAO). Also, the uptake protein for neurotransmitters into the presynapse or the neurovesicles can be inhibited. Several toxic alkaloids inhibit or activate ion channels (Na^+, K^+, Ca^{++}) or Na^+, K^+-ATPase [14, 16, 17, 43, 44, 48, 53–55]. Some alkaloids have a single target, many others and the majority of PSM are multitarget compounds, which are directed against several targets in animals and/or microorganisms. In conclusion, most alkaloids are known for their pronounced toxicological properties and some of them are lethal poisons others are used in medicine to treat health conditions [14, 16, 17]. From a plants point of view, alkaloids are mostly employed as defense compounds against herbivores and many of the alkaloid-accumulating plants are avoided by herbivores.

Table 1 Interaction of some alkaloids with neuroreceptors and other elements of neuronal signaling. (Source: Refs. [51, 53])

Target	Natural ligand/ substrate	Alkaloid	Occurrence
Acetylcholine receptor			
Nicotinic receptor	Acetylcholine	Nicotine	*Nicotiana, Duboisia*
		C-toxiferine	*Strychnos*
		Tubocurarine	*Chondrodendron*
		Coniine	*Conium*
		Cytisine and other QAs	Several Fabaceae
		Lobeline	*Lobelia*
		Anabasine	*Anabasis, Nicotiana*
Muscarinic receptor	Acetylcholine	Hyoscyamine (atropine)	*Atropa, Hyoscyamus, Datura, Mandragora*
		Scopolamine	Several Solanaceae
		Arecoline	*Areca*
		Pilocarpine	*Pilocarpus*
		Muscarine	*Amanita, Clitocybe,* other fungi
		Sparteine and other QAs	Several Fabaceae
Adrenergic receptors	Noradrenaline/ adrenaline	Ergot alkaloids	*Claviceps*
		Yohimbine	*Pausinystalia, Aspidosperma*
		Rauwolscine	*Rauvolfia*
		Corynanthine	*Rauvolfia*
		Norlaudanosollne	Papaveraceae
		Ephedrine, norephedrine	*Ephedra, Catha*
Dopamine receptor	Dopamine	Ergot alkaloids	*Claviceps*
		Bulbocapnine	*Corydalis*
Serotonin receptor	Serotonin	Ergot alkaloids	*Claviceps*
		Psilocin, psilocybin	*Psilocybe,* other fungi
		N,N-dimethyltryptamine	Several plants and toads
		Bufotenine	*Virola, Anadenanthera*
		Beta-carboline alkaloids	*Banisteriopsis, Peganum*
		Mescaline	*Lophophora, other cacti*

(*continued*)

Table 1 (continued)

Target	Natural ligand/ substrate	Alkaloid	Occurrence
GABA receptor	Gamma aminobutyric acid (GABA)	Bicuculline	*Dicentra cucullaria, Corydalis,*
		Muscimol	*Amanita*
		Beta-carboline alkaloids	*Peganum, Banisteriopsis*
Adenosine receptor	Adenosine	Caffeine	*Coffea, Camellia, / lex, Paullinia*
		Theophylline, Theobromine	*Theobroma*
Glycine receptor	Glycine	Brucine	*Strychnos*
		Strychnine	*Strychnos*
Opioid receptor	Endorphins	Morphine	*Papaver somniferum*
Acetylcholine esterase	Acetylcholine	Physostigmine (eserine)	*Physostigma venenosum*
		Berberine	Several Papaveraceae
		Coptisine	Several Papaveraceae
		Galantamine	Several Amaryllidaceae
		Solanine and other steroid alkaloids	*Solanum*
		Huperzine A	*Huperzia serrata*
Monoamine oxidase (MAO)	Noradrenaline, dopamine, serotonin, histamine	Harmaline, harmine	*Peganum*
		Salsolinol	Chenopodiaceae
Catechol-*O*-methyltransferase	Noradrenaline, adrenaline, dopamine	Tetrahydroisoquinoline	Papaveraceae
Na^+/K^+ channels	Na^+, K^+	Aconitine	*Aconitum*
		Quinidine	*Cinchona*
		Sparteine, lupanine	*Lupinus, Cytisus*
		Protoveratrine A	*Veratrum*

2.1 Evolution of Alkaloids

When were PSM invented during evolution? For our discussion, Fig. 3 illustrates a molecular phylogeny of plants, which is based on DNA sequences and transcriptome analyses [56, 57]. From a global perspective, we can state that already the early land plants needed chemical defense against herbivores and microbes as these organisms already existed when land plants evolved in the Devonian. Widely present in extant spore-bearing plants, gymnosperms and angiosperms are terpenoids and polyphenols. It can be speculated that these PSMs already evolved more than 500 million years ago.

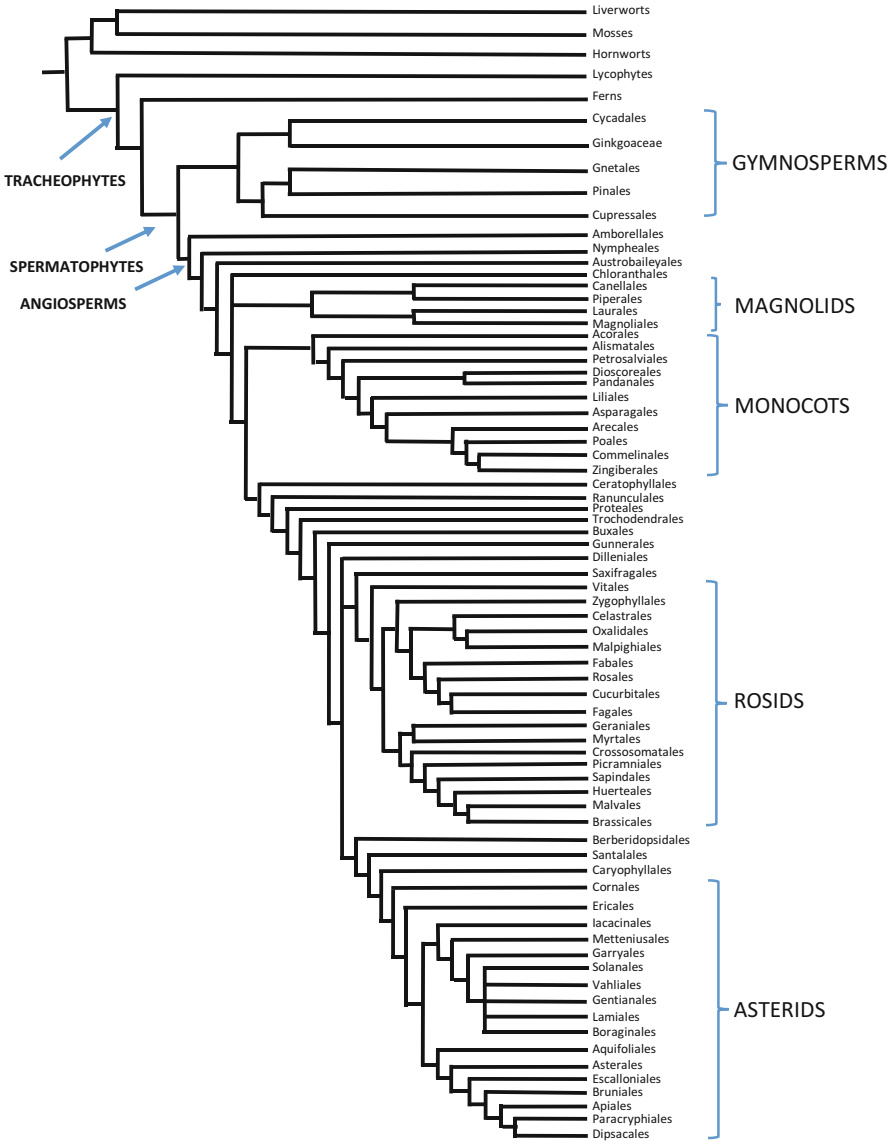

Fig. 3 Phylogeny reconstruction of plants according to APGIII and One Thousand Plant Transcriptomes Initiative [57]

Genes of corresponding biosynthetic pathways exist in all plants and algae and even in many microorganisms, suggesting that these pathways are very old [5, 7, 8, 58].

What about alkaloids? Alkaloids are common among angiosperms, but much more restricted in gymnosperms and spore bearing plants (Table 2; Fig. 4). Among spore bearing plants (mosses, lycopods, ferns, horsetails), only lycopods commonly

Table 2 Occurrences of selected alkaloids among plants. (Source: Refs: [3, 9, 60])

Alkaloid type	Example	Order	Family	Genus (selection)
Amaryllidaceae alkaloids	Ambelline, brunsvigine, galanthamine, haemanthamine, lycorine	Asparagales	Amaryllidaceae	*Amaryllis*, *Ammocharis*, *Boophane*, *Brunsvigia*, *Clivia*, *Crinum*, *Galanthus*, *Haemanthus*, *Hippeastrum*, *Hymenocallis*, *Leucojum*, *Lycoris*, *Narcissus*, *Nerine*, *Pancratium*, *Sternbergia*, *Ungermia*, *Zephyranthes*
Betalain alkaloids	Amaranthin, betanin, miraxanthin, vulgaxanthin-I	Caryophyllales	Aizoaceae, Amaranthaceae/ Chenopodiaceae, Cactaceae, Nyctaginaceae, Phytolaccaceae, Portulacaceae	*Amaranthus*, *Atriplex*, *Beta*, *Bougainvillea*, *Celosia*, *Chenopodium*, *Gomphrena*, *Mesembryanthemum*, *Mirabilis*, *Opuntia*, *Portulaca*, *Phytolacca*
Diterpenoid alkaloids	Aconitine, delphinine, delsonine, lycaconithine, lycoctonine, spiradine A	Ranunculales	Ranunculaceae	*Aconitum*, *Atragene*, *Consolida*, *Delphinium*, *Thalictrum*
	Anopterine	Saxifragales	Saxifragaceae	*Anopterus*
	Cassaine	Fabales	Fabaceae	*Erythrophleum*
	Cuauchichine, garryine, lindheimerine,	Garryales	Garryaceae	*Garrya*
	Icaceine	Icacinales	Iacacinaceae	*Icacina*
	Ryanodine	Malpighiales	Salicaceae	*Ryania*
	Spiradine A	Rosales	Rosaceae	*Spirea*
Indole alkaloids	Ajmalicine, ajmaline, akuammidine, alstonine, brucine, calebassine, catharanthine, C-curarine, eburnamine, ellipticine, gelsemine, ibogaine, mitragynine, reserpine, rutaecarpine, sempervirine, serpentine, strychnine, tabernamine,	Gentianales	Apocynaceae, Loganiaceae, Rubiaceae	*Adina*, *Alstonia*, *Amsonia*, *Aspidosperma*, *Borreria*, *Catharanthus*, *Cephaelis*, *Cinchona*, *Corynanthe*, *Evodia*, *Geissospermum*, *Gelsemium*, *Flindersia*, *Hortia*, *Hunteria*, *Melodinus*, *Mitragyna*, *Murraya*,

(*continued*)

Table 2 (continued)

Alkaloid type	Example	Order	Family	Genus (selection)
	tabersonine, toxiferine-I, vinblastine, vincamine, yohimbine			*Ochrosia*, *Pausinystalia*, *Picralima*, *Rauwolfia*, *Rhazya*, *Strychnos*, *Tabernaemontana*, *Uncaria*, *Vallesia*, *Vinca*, *Voacanga*, *Zanthoxylum*
	Agroclavine, chanoclavine-I, ergine	Solanales	Convolvulaceae	*Ipomoea*, *Rivea*
	Canthinone, 11-hydroxycanthin-6-one	Sapindales	Simaroubaceae	*Amaroria*, *Picrasma*, *Quassia*
	Eseramine, physostigmine	Fabales	Fabaceae	*Physostigma*
	Harmaline, harman	Zygophyllales	Zygophyllaceae	*Peganum*, *Zygophyllum*
	Harmaline	Malpighiales	Malpighiaceae	*Banisteriopsis*
	Mesembrine	Caryophyllales	Aizoaceae	*Sceletium*
Isoquinoline alkaloids	Actinodaphnine, atherospermoline, bebeerine, berbamine, boldine, glaucine, laudanidine, obaberine, reticuline	Laurales	Atherospermaceae, Hernandiaceae, Lauraceae, Monimiaceae	*Actinodaphne*, *Albertisia*, *Atherosperma*, *Beilschmidia*, *Boldea*, *Cassytha*, *Cryptocarpa*, *Daphnandra*, *Doryphora*, *Gyrocarpus*, *Hernandia*, *Larus*, *Laurelia*, *Litsea*, *Machilus*, *Monimia*, *Nectandra*, *Ocotea*, *Peumus*, *Phoebe*, *Sassafras*
	Adlumine, aknadicine, alpinine, argemonine, bebeerine, berberine, bicuculline, bulbocapnine, canadine, codeine, glaucine, hernandezine, jatrorrhizine, macarpine, magnoflorine, morphine, narceine, noscapine, palmatine, papaverine, reticuline, sanguinarine, tetrandrine, thalicarpine, thebaine, tubocurarine	Ranunculales	Berberidaceae, Menispermaceae, Papaveraceae, Ranunculaceae	*Abuta*, *Aconitum*, *Adlumia*, *Anisocycla*, *Argemone*, *Berberis*, *Bocconia*, *Chelidonium*, *Chondodendron*, *Cissampelos*, *Cocculus*, *Coptis*, *Corydalis*, *Dicentra*, *Dicranostigma*, *Eschscholzia*, *Glaucium*, *Hydrastis*, *Jateorrhiza*, *Leontice*, *Macleaya*, *Mahonia*, *Meconopsis*, *Menispermum*, *Nandina*, *Papaver*,

				Pycnarrhenia, *Romneya*, *Sanguinaria*, *Sinomenium*, *Stephania*, *Stylophorum*, *Thalictrum*, *Tiliacora*, *Zanthorrhiza*
	Alamarine, alangicine, ankorine, psychotrine	Cornales	Alangiaceae	*Alangia*
	Ancistrocladine, carnegine, gigantine, pellotine, pilocereine, salsoline	Caryophyllales	Ancistrocladaceae, Cactaceae, Chenopodiaceae	*Ancistrocladus*, *Carnegia*, *Cephalocereus*, *Cereus*, *Lophocereus*, *Lophophora*, *Pachycereus*, *Salsola*
	Angoline, berberine, canadine, dihydrosanguinarine, fagaridine, magnoflorine, sanguinarine	Sapindales	Rutaceae, Sapindaceae	*Evodia*, *Fagara*, *Pteridophyllum* *Toddalia*, *Zanthoxylum*
	Annolobine, berberine, boldine, jatrorrhizine, laudanidine, liriodenine, magnoflorine	Magnoliales	Annonaceae, Magnoliaceae	*Annona*, *Arbabotrys*, *Asimia*, *Coelocline*, *Desmos*, *Isolona*, *Liriodendron*, *Magnolia*, *Michelia*, *Phaeanthus*, *Xylopia*
	Annonaine	Nympheales	Nymphaceae	*Nelumbo*
	Bebeerine	Buxales	Buxaceae	*Buxus*
	Boldine	Rosales	Rhamnaceae	*Retanilla,*
	Caaverine	Ericales	Symplocaceae	*Symplocos*
	Cephaeline, emetine, papaverine, psychotrine	Gentianales	Apocynaceae, Rubiaceae	*Cephaelis*, *Rauwolfia*
	Cephaeline	Cornales	Cornaceae	*Alangium*
	Erythratidine, erythroidine	Fabales	Fabaceae	*Erythrina*
	Aristolochic acid, magnoflorine	Piperales	Aristolochiaceae	*Arstolochia*
	Pronuciferine	Proteales	Nelumbonaceae	*Nelumbo*
	Salutaridine	Malpighiales	Euphorbiaceae	*Croton*
	Colchicine	Liliales	Colchicaceae	*Colchicum*

(*continued*)

Table 2 (continued)

Alkaloid type	Example	Order	Family	Genus (selection)
Lycopodium alkaloids	Annotidine, cernuine, lycopodine, obscurine, selagine	Lycopodiales	Lycopodiaceae	*Lycopodium*
Pyrrolidine and piperidine alkaloids	Adenocarpine, ammodendrine, australine, cassine, castanospermine, juliflorine, pelletierine, santiaguine, swainsonine	Fabales	Fabaceae	*Adenocarpus*, *Ammodendron*, *Cassia*, *Castanospermum*, *Lupinus*, *Prosopis*, *Sophora*, *Swainsonia*
	Anabasine, anaferine, hygrine, myosmine, nicotine, nornicotine, pelletierine	Solanales	Convolvulaceae, Solanaceae	*Atropa*, *Convolvulus*, *Duboisia*, *Nicotiana*, *Withania*
	Anabasine	Caryophyllales	Chenopodiaceae	*Anabasis*
	Anabasine, lobeline	Asterales	Asteraceae, Campanulaceae	*Campanula*, *Lobelia*, *Zollikoferia*
	Anabasine	Cornales	Cornaceae	*Alangium*
	Arecaidine, arecoline	Arecales	Arecaceae	*Areca*
	Astrocasine, astrophylline, ricinine	Malpighiales	Euphorbiaceae	*Astrocasia*, *Ricinus*
	Carpaine, hygrine	Brassicales	Brassicaceae, Carcicaceae	*Carica*, *Cochlearia*, *Vasconcellea*
	Coniine, coniceine	Apiales	Apiaceae	*Conium*
	Hygrine	Asparagales	Orchidaceae	*Dendrobium*
	Pelletierine	Myrtales	Lythraceae	*Punica*
	Pelletierine, sedamine	Saxifragales	Crassulaceae	*Sedum*
	Pinidine	Pinales	Pinaceae	*Pinus*
	Piperine	Piperales	Piperaceae	*Piper*
Pyrrolizidine alkaloids	Anacrotine, fulvine, monocrotaline, platyphylline, retronecine, retrorsine, riddeline, seneciphylline, senkirkine	Fabales	Fabaceae	*Adenostyles*, *Crotalaria*, *Lotononis*
	Amabiline, clivorine, echimidine, europine, heliosupine, heliotrine, indicine, integerrimine, intermedine,	Boraginales	Boraginaceae	*Alkanna*, *Amsinckia*, *Anchusa*, *Borago*, *Cynoglossum*, *Echium*, *Heliotropium*, *Lappula*, *Lindelofia*,

	lasiocarpine, lycopsamine, monocrotaline, retronecine, rinderine, supinine, symphytine			*Messerschmidia*, *Myosotis*, *Rindera*, *Solenanthus*, *Symphytum*, *Tournefortia*, *Trichodesma*
	Auriculine	Asparagales	Orchidaceae	*Liparis*
	Angularine, clivorine, doronine, integerrimine, intermedine, jacobine, lycopsamine, petasitenine, platyphylline, retronecine, retrorsine, riddeline, rinderine, senecionine, seneciphylline, senkirkine, supinine	Asterales	Asteraceae	*Brachyglottis*, *Cacalia*, *Conoclinum*, *Doronicum*, *Emilia*, *Eupatorium*, *Farfugium*, *Ligularia*, *Petasites*, *Senecio, Tussilago*
	Lycopsamine	Gentianales	Apocynaceae	*Parsonsia*
Quinoline alkaloids	Acronidine, acronycine, arborine, bucharaine, dictamnine, evoxine, fagarine, graveoline, kokusaginine, maculine, melicopine, pteleatine, rutacridone, skimmianine	Sapindales	Rutaceae	*Acronychia*, *Adiscanthus*, *Aegle*, *Afraele*, *Atalantia*, *Balfourodendron*, *Bauerella*, *Cusparia*, *Dictamnus*, *Esenbeckia*, *Euodia*, *Fagra*, *Flindersia*, *Galipea*, *Glycosmis*, *Haplophyllum*, *Helietta*, *Lunasia*, *Melicope*, *Monnieria*, *Murraya*, *Orixa*, *Ptelea*, *Ruta*, *Sarcomelicope*, *Skimmia*, *Teclea*, *Zanthoxylum*
	Aniflorine, anisotine, peganine, vasicinol, vasicinone	Lamiales	Acanthaceae	*Adhatoda*, *Anisotes*
	Camptothecin, febrifugine	Cornales	Nyssaceae, Hydrangeaceae	*Camptotheca*, *Dichroa*, *Hydrangea*
	Camptothecin,	Icacinales	Icacinaceae	*Mappia*
	Camptothecin, Cinchonidine, quinine	Gentianales	Rubiaceae	*Cinchona*, *Ophiorrhiza*, *Remijia*
	Echinopsine	Asterales	Asteraceae	*Echinops*
	Peganine, vasicinone	Zygophyllales	Zygophyllaceae	*Nitraria*, *Peganum*
Quinolizidine alkaloids	Anagyrine, aloperine, ammonthamnine, angustifoline, aphylline, baptifoline, methylcytisine, cytisine, 13-	Fabales	Fabaceae	*Ammodendron*, *Ammothamnus*, *Anagyris*, *Argyrolobium, Baptisia*, *Cadia*, *Alpurnia*, *Cytisus*, *Euchresta*, *Genista*, *Goebelia*, *Hovea*,

(*continued*)

Table 2 (continued)

Alkaloid type	Example	Order	Family	Genus (selection)
	hydroxylupanine, lupanine, lupinine, matrine, multiflorine, ormosanine, piptanthine, retamine, sparteine, thermopsine			*Laburnum*, *Lamprolobium*, *Lupinus*, *Ormosia*, *Piptanthus*, *Retama*, *Podopetalum*, *Sophora*, *Spartium*, *Templetonia*, *Thermopsis*, *Ulex*, *Vexibia*
	Aphylline, lupinine	Caryophyllales	Chenopodiaceae	*Anabasis*
	Baptifoline, leontiformine	Ranunculales	Berberidaceae	*Caulophyllum*, *Leontice*
Steroidal alkaloids	Buxamine, cyclobuxine, terminaline	Buxales	Buxaceae	*Buxus*, *Pachysandra*
	Chaconine, demissine, solamargine, solanidine, solanine, solasonine, tomatidine	Solanales	Solanaceae	*Solanum*
	Chaconine, cyclopamine, germine, jervine, protoveratrine A and B, veracevine, verticine, zygadenine	Liliales	Liliaceae, Melianthaceae	*Fritillaria*, *Notholiron*, *Schoenocaulon*, *Veratrum*, *Zigadenus*
	Conessine, funtumine, paravallarine	Gentianales	Apocynaceae	*Funtumia, Holarrhena*, *Kibatalia*, *Paravallaris*
Tropane alkaloids	Atropine, convolamine, convolvine, hyoscyamine, littorrine, meteloidine, scopolamine, tigloidine	Solanales	Convolvulaceae, Solanaceae	*Anthocercis*, *Atropa*, *Datura*, *Duboisia*, *Hyoscyamus*, *Scopolia*, *Solandra*
	Benzylecgonine, cinnamoylcocaine, cocaine, ecgonine, meteloidine	Malpighiales	Erythroxylaceae	*Erythroxylum*
Cephalotaxus alkaloids	Cephalotaxine, harringtonine	Pinales	Cephalotaxaceae	*Cephalotaxus*
Equietum alkaloids	Palustrine	Equisetales	Equisetaceae	*Equisetum*
Taxus alkaloids	Paclitaxel (taxol), taxine A	Pinales	Taxaceae	*Taxus*

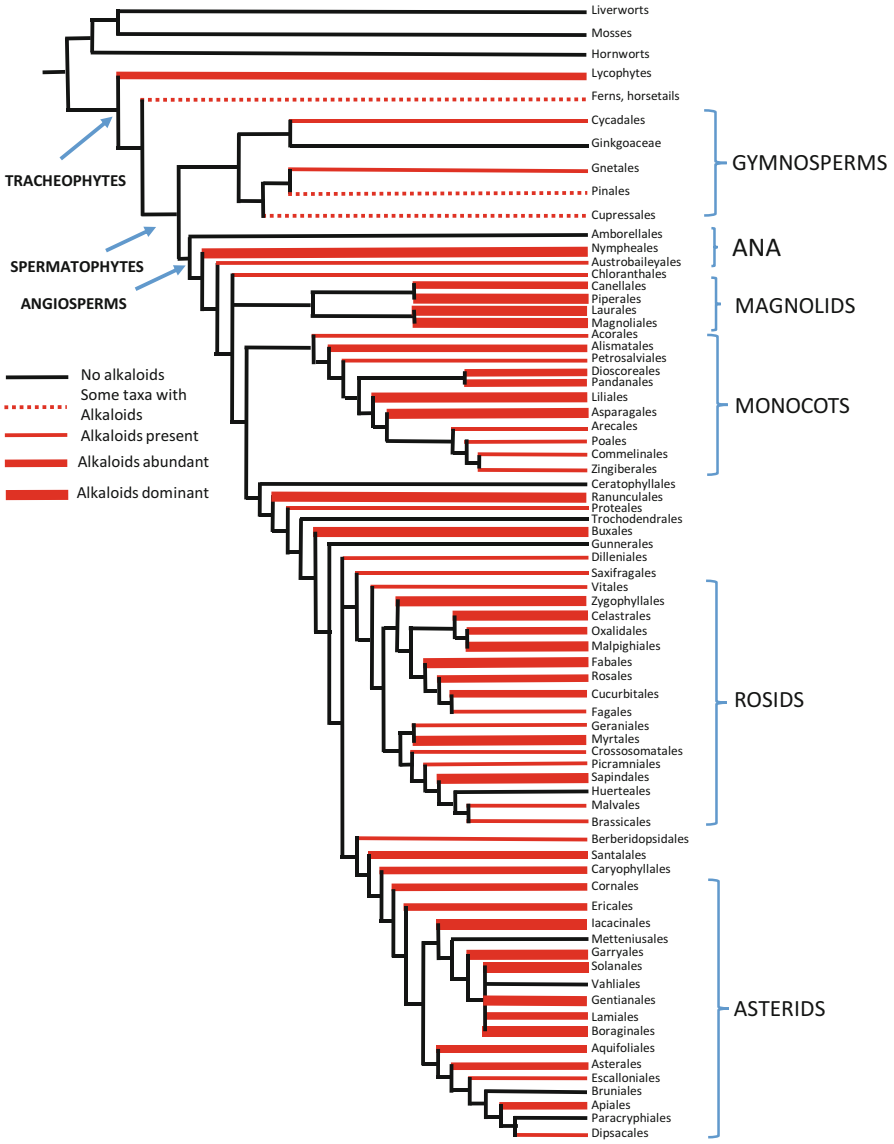

Fig. 4 Distribution of alkaloids in the plant kingdom

produce alkaloids of the quinolizidine type, where alkaloids are rare or absent in ferns, horsetails (except the alkaloid palustrine), and mosses [3–5, 59].

Among gymnosperms, cycads produce cycasin as an alkaloid, and the Ephedraceae the simple alkaloid ephedrine (and derivatives). Among plants of the orders Pinales and Cupressales, alkaloids are rarely produced in a few genera (Table 2) [3–5, 59].

Among angiosperms, some orders have more families producing alkaloids, than others (Fig. 4): Alkaloid-rich are Nympheales, Piperales, Magnoliales, Laurales, Liliales, Ranunculales, Buxales, Zygophyllales, Malpighiales, Fabales, Sapindales, Solanales, Gentianales, and Boraginales [3–5, 46, 47, 59].

Some types of alkaloids occur in closely related taxa (Table 2), which was the base for chemotaxonomy some time ago [59–63]. But a closer look shows that this feature is not consistent. For example, quinolizidine alkaloids occur predominantly in the tribe Genisteae of Fabaceae. But alkaloids with identical structures have also been detected in unrelated orders/families, such as Chenopodiaceae or Ranunculaceae. Or pyrrolizidine alkaloids occur in Boraginaceae, the tribe Crotalarieae of Fabaceae, and in the tribe Senecioneae of Asteraceae, which are unrelated families. Similar examples can be found for tropane, indole, and isoquinoline alkaloids (Table 2) [6–9, 15]. How to explain the irregular occurrences? It could be argued that the occurrence of a PSM in unrelated families is due to convergent evolution. Although convergence cannot be ruled out in all instances, genetic data favor a different hypothesis. When genomes of different plants and microorganisms were analyzed for the presence/absence of key enzymes of PSM biosynthesis, it turned out that the key enzymes are widely distributed in the plant kingdom, irrespective of whether a particular species produces a particular PSM or not. We have therefore postulated that the key genes of PSM biosynthesis are present in most if not in all plants and that the PSM profile observed in a particular plant is a consequence of differential gene expression. Furthermore, the origin of the key genes may be found in microorganisms, which also are active producers of secondary metabolites. These microbial genes probably entered plant genomes by horizontal gene transfer. According to the endosymbiont theory, plants have mitochondria, which derived from alpha Protobacteria and chloroplasts, which came from Cyanobacteria. The originally microbial genomes were incorporated into plant genomes, which thus obtained many genes which could be used later for the biosynthesis of PSM [7–9, 59, 64].

3 Evolution of Angiosperms, Pollinating Insects, and Defense via Alkaloids

First land plants occurred around 420 million years ago in the Silurian and became more common in the Devonian 416–359 million years ago. Fossils from this period mainly show plants which produced spores and resembled ferns (Psilophytatae). Later in the Devonian, tree-like lycopods, ferns, and horsetails followed which formed dense forests in the Devonian and Carbon (a source of our coal today). Starting in the Carboniferous, first gymnosperms occurred with cycads and gingko. Gymnosperms were abundant in Perm and the following Mesozoicum. About 140 million years ago in the Cretaceous, the largest group of plants, the angiosperms evolved and became the dominant flora, especially in the Palaeogene. Roots of most angiosperm orders go back to 100–120 million years ago. However, radiation and

diversification within extant plant families occurred during the last 50 million years ago (Fig. 3) [58, 65].

The numbers of known plants is increasing over the years as new species are constantly being discovered: For our discussion, we only need to consider the actual numbers (not speculating on the unknown numbers of undescribed taxa): Species numbers: Bryophyta: 20,000; Pteridophyta: 13,000; Gymnosperms: 1100 and Angiosperms: 369,000 (Fig. 5a) [58, 65, 66].

Spore-bearing plants reproduce without the aid of animals. Also, most gymnosperms are wind-pollinated. Only cycads and Ephedra are visited by insects, which help to pollinate. In cycads, insects are mostly beetles and not flies and hymenopterans as in angiosperms.

The key innovation of angiosperms was the formation conspicuous flowers, with animals playing a role in pollination, whereas wind-pollinated angiosperms have inconspicuous flowers, such as in grasses. Flowers evolved in a way to attract animals for pollination. As already discussed above, flowers accumulate aromatic and colored PSM, such as anthocyanins, flavonoids, and carotenoids, which can be detected by insects and birds (nectar specialists, such as humming birds, sun birds). In some flowers, coloration (sometimes only visible in UV light) leads to the nectar source, which is the reward for the pollinating organism. The nectar of some plants contains bitter-tasting PSM, such as alkaloids [22, 23]. There is evidence that these PSMs provide a niche for some pollinators, which are adapted to the bitter-tasting PSM. An example is Anna's Hummingbird [24]. In this case, the nectar of a particular species is exclusive for a few pollinators, which may be of benefit for the plants, as this improved the likelihood that pollen of the same species is transferred by a pollinator.

In addition, many flowers produce aromatic and foully smelling fragrances, which are attractive for many insects (flies, bees, bumble bees, syrphid flies, butterflies). In some plants, these fragrances are mainly produced at night when certain kind of moth roam around for pollination [2, 21].

4 Co-evolution of Angiosperms, Animals, and Alkaloids

Among animals, chordates (which include mammals, birds, reptiles) consist of about 65,000 species in contrast to more than 1,360,000 species of invertebrates (Fig. 5b). The largest group within the invertebrates are insects with over one million species. Species numbers are uneven within insects: The major groups are Coleoptera (beetles) with 360,000–400,000 species, followed by Lepidoptera (moths and butterflies) with 175,000 species, Diptera (flies) with 153,000 species, Hymenoptera (bees, wasps, ants) with 115,000 species, and Hemiptera (aphids, bugs) with 88,000 species [58, 66]. A common theme for the species-rich insect groups is that many are pollinators and feed on plants at least in one developmental stage (e.g., as larvae many butterflies are herbivores and nectar feeders as adults).

It has been argued that the large number of insects is a consequence of the diversification of angiosperms, which constitute more than 94% of all vascular

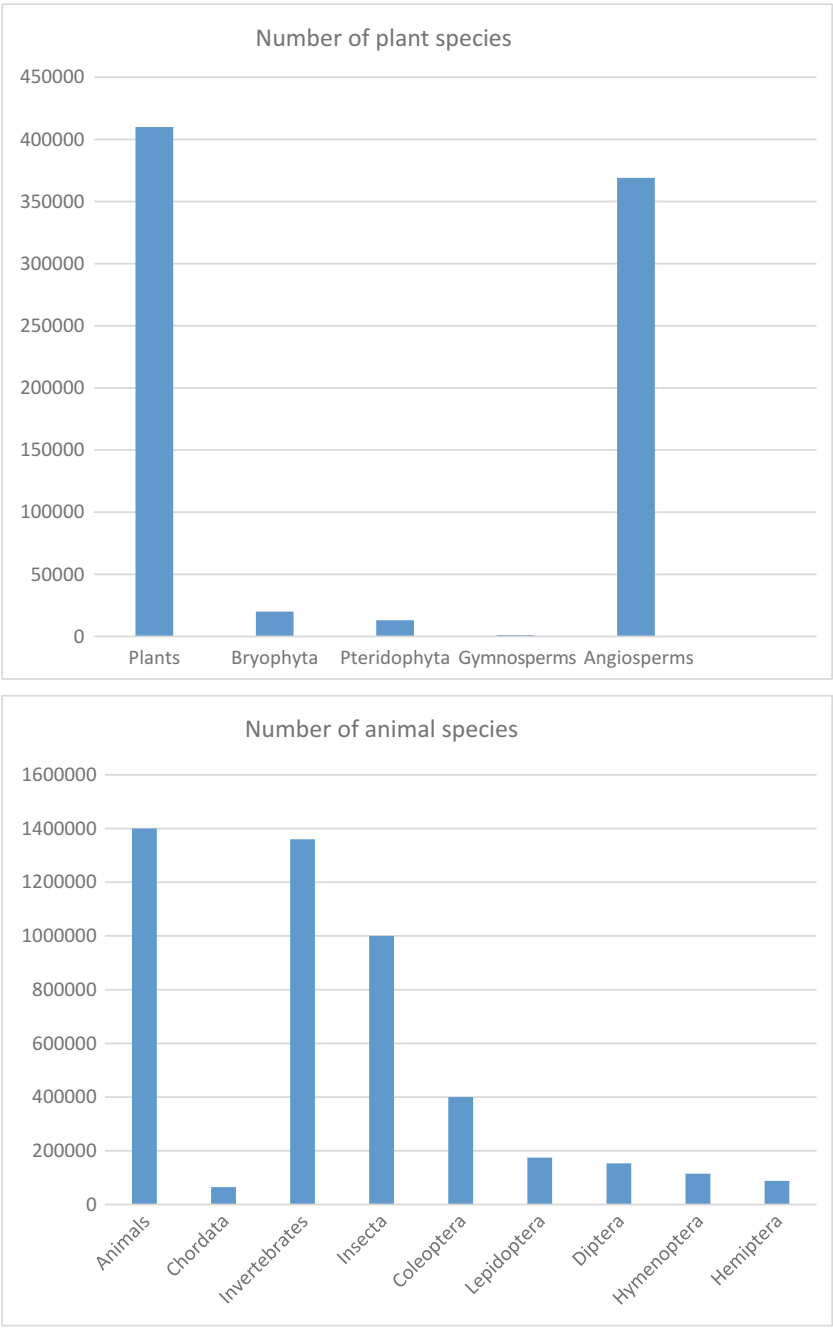

Fig. 5 Number of known plant (**a**) and animal (**b**) species

plants (Pteridophytes, Gymnosperms, Angiosperms) (Fig. 5a). A common theme for the largest groups of invertebrates is that they either feed on plants and/or that they are pollinators [19, 21, 67, 68]. Many insects are monophagous or oligophagous, meaning that they have specialized on one or a few host plants [21]. There is an estimate that a single plant species can be a host for more than 10 insect species. (In the biodiversity crisis, which we see today, a dramatic loss of insects has been recorded. This appears to be due to the increased application of insecticides in agriculture. In addition, the loss of plant diversity due to herbicides and over fertilization needs to considered. If we destroy the diversity of plants, we also destroy a food source for all the adapted herbivores.)

Why are there so many mono- and oligophagous insects? As discussed before, plants produce a wide diversity of PSM with deterrent and toxic properties, whose composition differs between plant parts and developmental stages (Fig. 2). Many insects nevertheless evolved strategies to feed on chemically defended plants or more precise a particular organ, by acquiring tolerance against the respective PSMs. If a PSM is directed to a specific target in insects, such as a receptor, the binding site might be changed by mutations so that the PSM can no longer bind to it (examples are Na^+, K^+ ATPase, or amino acyl tRNA synthetase). Other strategies involve a sequestration of a toxic PSM into a compartment where it does not affect the metabolism. Or the degradation of PSM with cytochrome p450 enzymes or their export via ABC transporters can be additional strategies. These adaptive mechanisms are not general but relevant for a single group of compounds. Thus, there are several beetles which can cope with the defense chemistry of seeds of a particular plant species but not with those of its leaves or roots [2, 8, 20, 21, 25, 26, 36, 37, 41].

On top of this, we have the specialization for nectar feeding in insects, several of which are also specific for a restricted selection of plant species. Thus, the evolution of a large number of angiosperm species probably offered many ecological niches for speciation of specialized insects.

As discussed before, the insects visiting a flower should not feed on the carpels or other parts of the flower, but only transfer the pollen, which they carry on their integument, to the stigma of the pistil. We can speculate that plants should protect their reproductive organ against herbivores. This is indeed the situation, and flowers usually harbor substantial amounts of PSM, which could function as repellents or deterrents. As mentioned before, many of the insect-pollinated angiosperms produce neurotoxic alkaloids, which also accumulate in all parts of the flower (only rarely in the nectar). Since neurotoxicity occurs immediately, such toxins should be well suited to ward off herbivores and to prevent a pollinating insect to feed on parts of the flower.

5 Conclusions

The abundance of alkaloid-producing plants among animal-pollinated angiosperms (Fig. 4) suggests that alkaloid evolution was enhanced during the last 150 million years when angiosperms started to radiate and to produce showy flowers that needed

chemical protection. It is thus tempting to speculate that the evolution of alkaloids, but also of glucosinolates or cyanogenic glucosides in angiosperms, is the consequence of the co-evolution of angiosperms and pollinating insects or herbivores.

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Lichen Metabolites: An Overview of Some Secondary Metabolites and Their Biological Potential

9

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Abstract

Lichens present a symbiotic association between two or more organisms. These unique organisms produce many chemical compounds, known as secondary metabolites or lichen acids. Most of them are localized in the cortex and form specific crystals on the surface of the fungal hyphae. Approximately 1000 secondary metabolites were discovered so far and most of them are specific for lichens. Lichen secondary metabolites showed many pharmaceutical activities, including antimicrobial, antiproliferative, antioxidant, antiviral, anti-inflammatory, and further allelopathic, antiherbivore, photoprotective activities. Lichens are important source of bioactive compounds, and despite a lot of studies dealing with activity of lichen secondary metabolites, their production in lichens and their role is still very enigmatic. In this chapter, we demonstrated all three main pathways of how secondary compounds originate and chose most characteristic acids with their proposed biological and ecological activities. This chapter gives a basic overview of lichens, secondary metabolites, and their properties.

Keywords

Symbiosis · Lichens · Biosynthetic pathways · Secondary metabolites · Pharmaceutical activities

1 Introduction

Colonization of land by phototrophic organisms started in Silurian era around 450 million years ago [1]. The environment was not friendly for these organisms because they needed to counter a low content of mineral nutrition, harmful UV-radiation from the sun, high oscillation of temperature, as well as a lower content of water or even its absence. All of these abiotic factors played important role in adaptations to the terrestrial environment. Living forms, which would like to stand in these adaptations, needed phosphorus to create nucleic acids and ATP (adenosine triphosphate). One example of how to solve problem with phosphorus uptake was that first colonizing organisms formed associations with mycorrhizal fungi [2, 3]. It needs to be mentioned that early organisms were forced to establish a form of mutualism which means the interaction between at least two different species of the individuals. Mutualism provided various adaptations for terrestrial plants and played a crucial part for settling on soil as well as in the evolution of land phototrophs [4].

Lichens (lichen-forming fungi) represent nearly one-fifth of all known fungal species so far [5]. They are typical examples of mutualistic symbiosis, where both partners need each other to benefit. The total number of lichens is still not known, but around 18,500 species were already described around the world [6]. Lichens are the dominant vegetation of approximately 8% of terrestrial ecosystems [7] and are typically found in environments subjected to extremes such as temperature, desiccation, and nutrient status.

This symbiotic partnership consists of fungal partner (called also mycobiont) and one or more photoautotrophic partners (called photobiont or phycobiont) [8]. For almost 150 years, lichens had been the model organisms of symbiosis on the lands until the researchers uncovered an unexpected third partner in the lichen cortex – yeast [9].

Lichens as fossils are scarce. Fossil evidence for the interactions of fungi with other organisms, including phototrophs, has been found originating from an era approximately 400 million years ago, in the area of Rhynie chert in Scotland. However, the discovery of lichen-like fossils preserved in marine phosphorite of the Doushantuo Formation (approximately 600 million years old) at Weng'an in southern China indicates that lichenization could have arisen even before the evolution of vascular plants [10]. In addition, recent molecular data suggest that lichen symbioses arose repeatedly during the evolution of fungi [11] and had a very important role in the evolution of Ascomycota [12].

2 Lichen Symbiotic Partners

Based on the most definitions, the lichen is the organism that represents the symbiotic association between the fungus (mycobiont) and the photosynthetic partner (photobiont). Photobiont coexistence with the lichen mycobiont brings many benefits that none of the organisms itself cannot achieve [13]. Although the dual nature of most lichens is now widely established, it is less commonly known that some lichens are symbioses involving three or more partners [8]. It has been suggested that they are mainly bacteria involved in the formation of complete lichen thalli [14].

2.1 Mycobiont

Most of the fungal partners belong to Ascomycota [15, 16], but we can also find species belonging to the Basidiomycota and anamorphic fungi. Mycobiont (Fig. 1a) is the dominant component of the lichen thallus. Separated "biont" cells in most cases are in direct contact, where fungal hyphae try to penetrate to cells of photobiont. There are known some cases where mycobiont is in contact not only with one type of photobiont but with two or even more. This leads to the creation of specific structure, cephalodium (e.g., *Peltigera aphthosa*). Because mycobiont is unable to produce the organic substances necessary for its growth, hence it must acquire them from a symbiosis. Heterotrophic mycobiont acquires fixed carbon in symbiosis from an autotrophic green algae or cyanobacteria. These are photosynthesis products (ribitol, sorbitol, glucose). In the lichen symbiosis, mycobiont ensures the intake of water and minerals for lichen thallus. It creates morphology and structures that are involved in both sexual and nonsexual reproduction. One of the most important roles

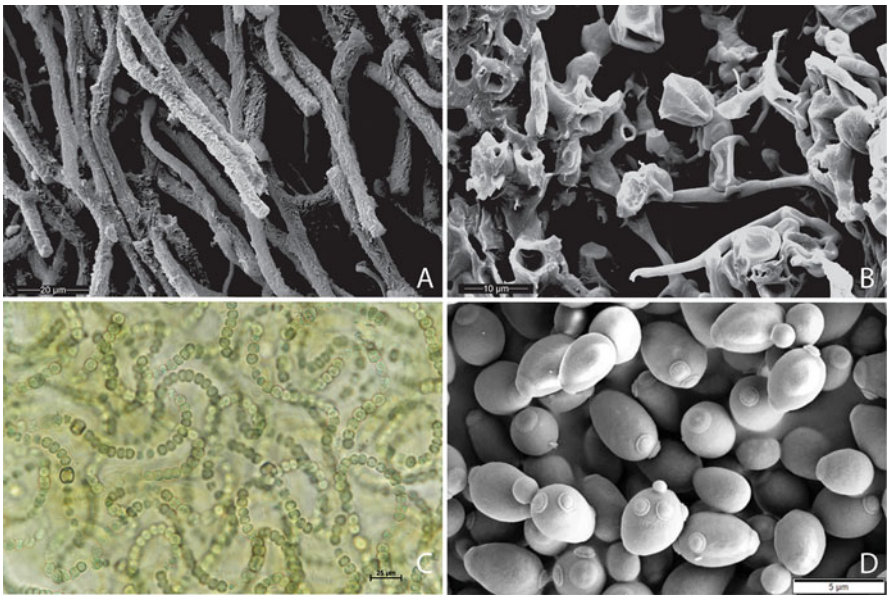


Fig. 1 (**a**) Mycobionts cells consisting of hyphae, (**b**) photobiont cells consisting of green algae, (**c**) photobiont cells consisting of cyanobacteria, (**d**) third symbiotic partner cells consisting of yeasts

of mycobiont is protection of photobiont from exposure to intense sunlight and desiccation by production of secondary metabolites. There is a predominant view that mycobiont has a higher tolerance to various environmental factors [17].

2.2 Photobiont

The role of photobionts in the lichen thallus can play nearly 40 genera of algae and cyanobacteria [18, 19]. The vast majority are eukaryotic photobionts, which belong to green algae (*Chlorophyta*) (Fig. 1b). They have a large number of common cytological features and their pigmentation, such as the presence of chlorophylls *a* and *b*, whose presence is common with higher plants [20, 21]. In only a small percentage of lichens, the photobionts are represented by prokaryotic cyanobacteria (Fig. 1c), sometimes called "cyanobionts." There are also known examples in which both groups of obligatory photobionts were observed simultaneously in the lichen thalli. The most frequent photobionts are represented by the genera *Trebouxia*, *Trentepohlia*, and *Nostoc*. Unlike green algae, cyanobacteria are diazotrophic because they can fix atmospheric nitrogen. The diversity of these photosynthetic partners is related to the variety of substrates that individual species are able to colonize within the genus. Main role of autotrophic photobiont is to synthesize organic compounds from carbon dioxide. Transfer of metabolites from photobiont to the mycobiont depends on the type of autotrophic photobiont involved [8].

2.3 Third Symbiont "Yeast"

In the study of Spribille et al. [9] is stated that many common lichens consist of a known ascomycete, the autotrophic photosynthesizing partner, and unexpectedly specific basidiomycete yeast (Fig. 1d). These yeasts are anchored in the cortex of lichen thallus and their abundance correlates with previously unexplained variations of the phenotype.

3 Anatomy and Morphology

Lichen morphology and anatomy is highly adapted to environmental restrictions; the mycobiont forms the exhabitant and the photobiont is the inhabitant [22]. The lichen "body" is called thallus. In the cross section (Fig. 2c), the lichen thallus usually consists of the upper cortex, a photosynthetic layer, the medulla, and the lower cortex. Some species also developed a central cord which has a support function (Fig. 2a, b). The thickness of the layers can vary in different species which is a response to the different environmental conditions.

Symbiosis is a source of dynamic evolution which is reflected by the different growth forms of thalli [23]. Many different thalli structures are known [24, 25], but they can be divided into three morphological types: (Fig. 3a) fruticose, (Fig. 3b) crustose, and (Fig. 3c) foliose. Other types can be included into these main three types. For example, *Cladonia macilenta* is lichen with squamulose bases and fruticose fruiting structures which are called podetia (Fig. 3d). *Lepraria* species have leprose, crustose lichen thalli with a powdery or granular surface. Genus *Collema*, *Leptogium*, or *Lathagrium* are characteristic of their gelatinous foliose thallus (Fig. 3e).

Crustose (Fig. 3a) lichens are tightly attached to the substrate by whole thalli, and it is very hard to remove them without any damage. These lichens usually grow on rocks or barks and colonize extreme habitats, including metal rich substrates. Unfortunately, the physiological studies of these lichens are very poor due to the complicated removal from substrate and low biomass production for routine analysis [26]. Extreme examples are endolithic species which penetrate a rock surface and only fruiting bodies are exposed (Fig. 3f).

Foliose lichens (Fig. 3b) are known as leafy-like. They are partially attached to the substrate or in one single point. The thallus is usually divided into lobes (*Parmelia sp.*) with various degrees of branching, but in some species (*Umbilicaria sp.*) the thallus is from one single unbranched lobe or a "multilobe" with limited branching [8]. According to their biomass and easy collection, they are used in biochemical and ecophysiological studies [26].

Fruticose lichens (Fig. 3c, d) are known as hair-like or strap-shaped. The lobes are usually flat or cylindrical. The thallus can grow horizontally or vertically (*Cladonia sp.*) or even hanging (*Usnea sp.*). The branching of lobes may be different within the systematic groups or even a single genus. Fruticose lichens are growing usually on tree barks but also on the ground. As with the foliose types, lichenologists prefer for experiments fruticose growth forms of thalli due to the easy removal from surface.

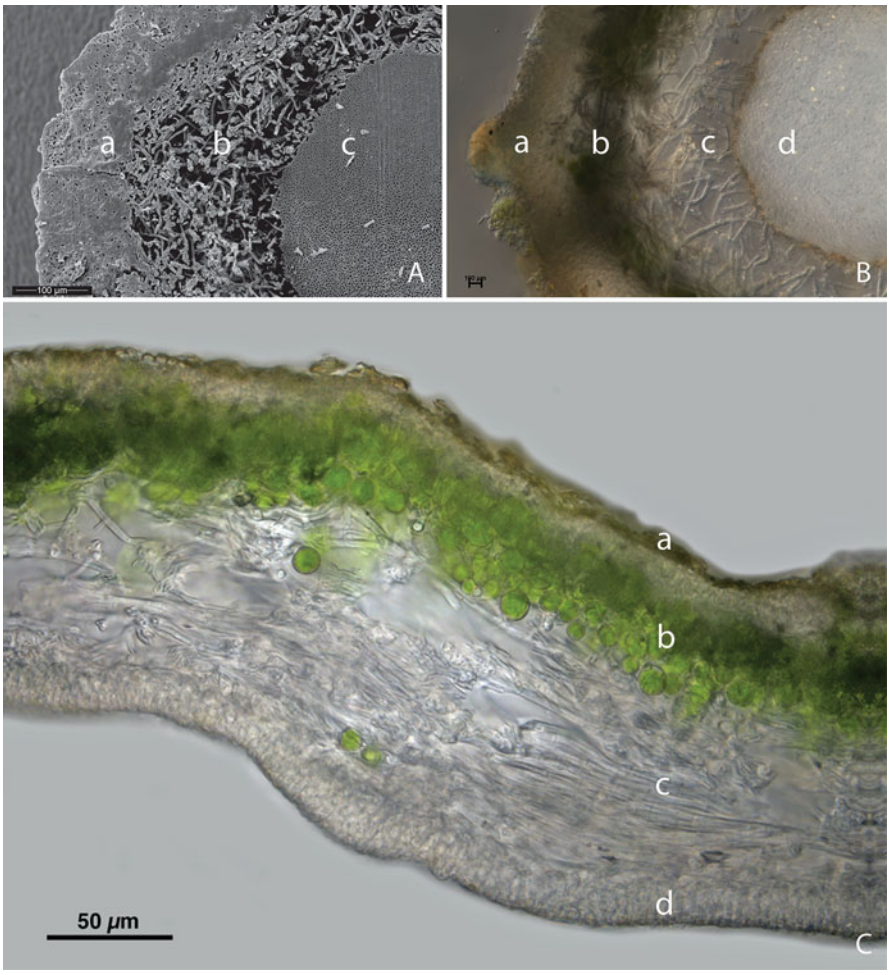


Fig. 2 (**a**) SEM photo of lichen *Usnea* sp. (a) upper cortex, (b) medulla, (c) central cord, (**b**) light microscopy (LM) photo of lichen *Usnea* sp. (a) upper cortex, (b) photosynthetic layer consisting of green algae cortical metabolites, (c) medulla, (d) central cord, (**c**) cross section of lichen thallus (*Xanthoria parietina*) in LM consisting of (a) upper cortex, (b) photosynthetic layer, (c) medulla, (d) lower cortex

4 Lichen Secondary Metabolites

Lichens present pioneer organisms, which can live in extreme habitats. These symbiotic organisms can deal with very specific conditions of environment because they produce secondary metabolites, which provide them with a good protection against various negative physical and biological influences [27]. As Lawrey [28] described, lichens produce two main groups of metabolites: primary (intracellular)

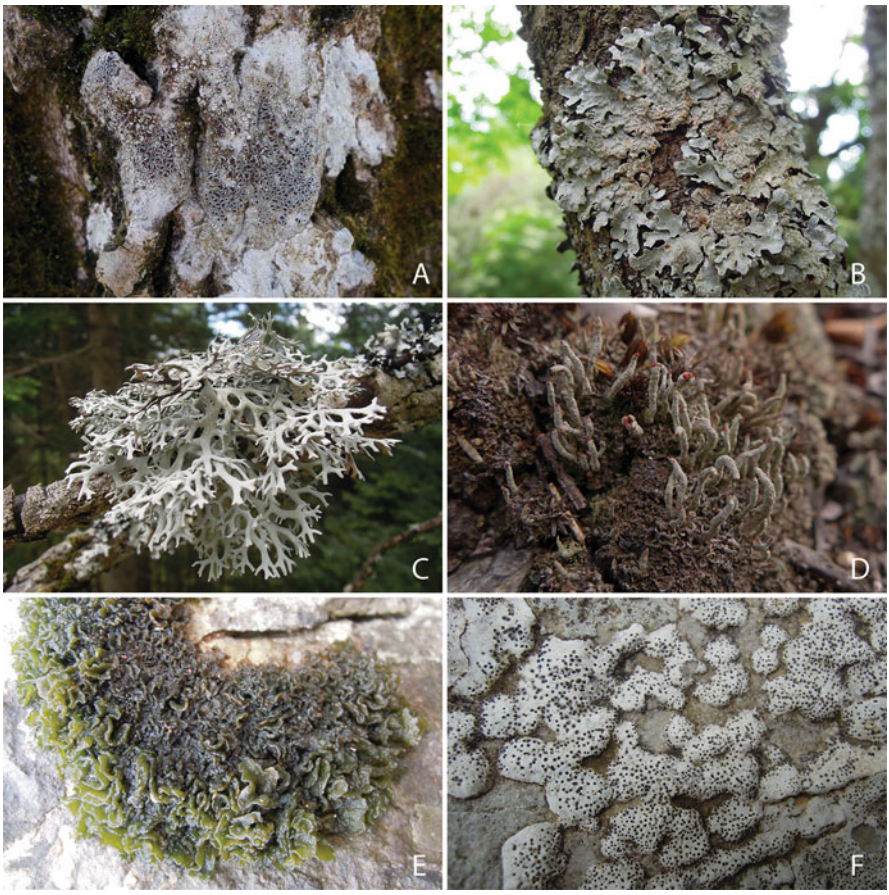


Fig. 3 Morphology of lichen thallus: (**a**) crustose thallus (*Lecanora argentata*), (**b**) foliose thallus (*Parmelia sulcata*), (**c**) fruticose thallus (*Pseudevernia furfuracea*), (**d**) bipartite thallus (*Cladonia macilenta*), (**e**) gelatinous thallus (*Lathagrium* sp.), (**f**) endolithic thallus (*Bagliettoa* sp.)

and secondary (extracellular). More than 1000 lichen substances are already known. The isolation, identification, and structures are described in the handbook of Huneck and Yoshimura [29].

Into the products of primary metabolism, we can include amino acids, amines, peptides, proteins, polyols, saccharides (mono-, oligo-, poly-) carotenoids, and vitamins, which are bound in the cell walls and the protoplasts. Most of them are soluble in water and can be extracted with boiling water [30]. Some of the primary metabolites are produced by fungal and some of them by photosynthetic partner. Many of these primary metabolites are not specific only for lichens and can be easily found in free-living fungi, algae, as well as higher plants [31]. Lichens dispose a similar amount of free amino acids as do the other plants. Lichen thallus present from 1.6% to 11.4% dry weight of nitrogen compounds [31], 1.5 to 24 mg/g dry weight of carotenoids, and 3–5% dry weight of polysaccharides [32].

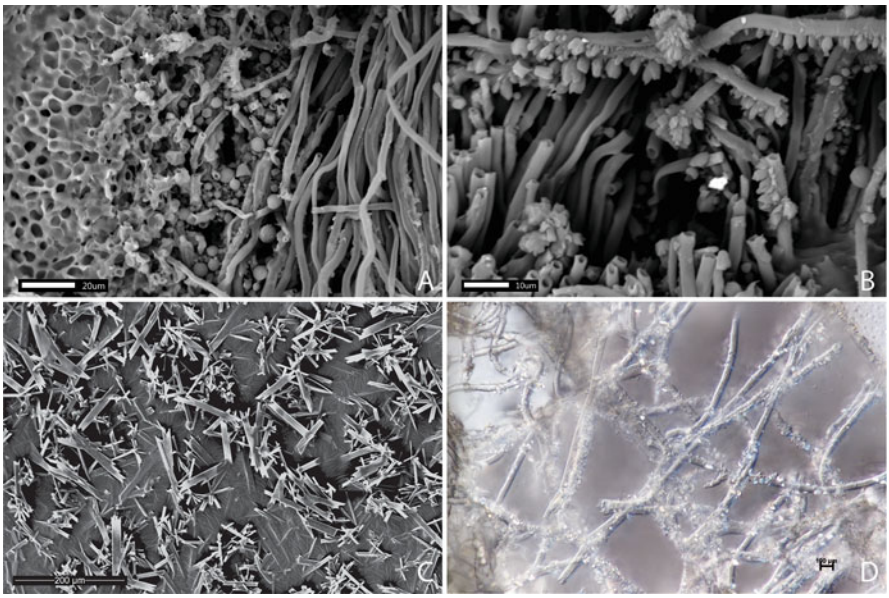


Fig. 4 (**a**) Various shape of lichen crystals, (**b**) secondary metabolites as crystals on hyphae, (**c**) crystals of usnic acid after recrystallization (SEM), (**d**) lichen crystals attached on mycobiont hyphae (LM)

The major group of these organic compounds which are found in lichens are products of secondary metabolism. The amount of secondary metabolites varies usually between 0.1% to 10% of dry weight of thallus but sometimes up to 30% [33–35]. All of the secondary metabolites (Fig. 4) of lichens are of fungal origin [6]. These lichen substances can be found in crystal form deposited on the surface of the hyphae of mycobiont. The solubility in water is very poor and mostly organic solvents can be used for their isolation.

Crystals of secondary metabolites are very stable, once they are formed, which was confirmed in several studies. Herbarium specimens of the lichens showed no significant decrease in concentrations of secondary metabolites [36].

Production of secondary metabolites in lichens is influenced by environmental factors including a light, UV-exposure, elevation, temperature fluctuations, and seasonality [6]. The age of lichens also plays significant role in production of lichen compounds and their location in lichen thallus as well.

4.1 Lichen Biosynthetic Pathways

Lichen secondary metabolites are classified by Culberson and Elix [37] according to their biosynthetic origins and chemical structures [38]. Three chemical pathways are

known in lichens: acetyl-malonate pathway, shikimate pathway, and mevalonate pathway.

4.1.1 Acetyl-Malonate Pathway

The formation of the polyketide chain could be envisaged as a series of Claisen reactions between the starting acetyl CoA and various number of malonyl CoA since every step ends by decarboxylation reaction. Orsellinic acid, the main intermediate in the biosynthesis of depsides and depsidones, is formed by intramolecular aldol reaction of the polyketide containing four keto groups and subsequent enolization and hydrolysis. Esterification of two orsellinic acid molecules affords lecanoric acid as member of depsides class. The most known orcinol-type depsidones have an α- or a β-keto group in the side chain of the first ring. It is well known that this functional group has a strong effect upon the ester linkage between the two rings since enol lactones form readily. Oxidative cyclization of depsides to depsidones usually joins the 2-hydroxyl of ring A and the 5-position of ring B.

C-methylation, Claisen reaction, and subsequent aromatization of the same polyketide leads to methylphloracetophenone. Radical coupling of two radicals derived from this intermediate affords bis dienone from which usnic acid is formed.

By Claisen reaction, aromatization and subsequent cyclization reactions of the polyketide containing five keto groups 5,7-dihydroxy-2-methylchromone are formed as key intermediate for synthesis of chromones and xanthones.

Polyketide containing eight keto groups undergoes several aldol reactions followed by reactions such as enolization, oxidation, decarboxylation, and selective methylation to give parietin, member of the anthraquinone group. Classes of lichen substances, which are derived by acetyl-malonate pathway, are depsides, depsidones, dibenzofurans, anthraquinones, chromones, and xanthones (Fig. 5).

Depsides

Polyphenolic compounds consisting of two or more monocyclic aromatic units linked by an ester bond are called depsides. The most common are products of intermolecular esterification of similar or identical units. Second esterification leads to tridepsides.

Evernic Acid

Evernic acid showed strong antioxidant, antimicrobial, and anticancer activities (Fig. 6). Antiherbicidal activity was also reported. Kosanić et al. [39] found varying antioxidant activity of evernic acid in free radical scavenging, superoxide anion radical scavenging. Strong antibacterial activity was reported against Gram-positive bacteria (*Staphylococcus aureus*, *Bacillus subtilis*, and *Bacillus megaterium*) [28]. Antitumor activity of evernic acid against HeLa cancer cell lines was also reported [40]. Evernic acid acts also as photosystem II inhibitor [41].

Lecanoric Acid

The antitumor, antioxidant, antibacterial, and antifungal activities of lichen compound lecanoric acid were confirmed (Fig. 7). Bogo et al. [42] tested cytotoxicity of

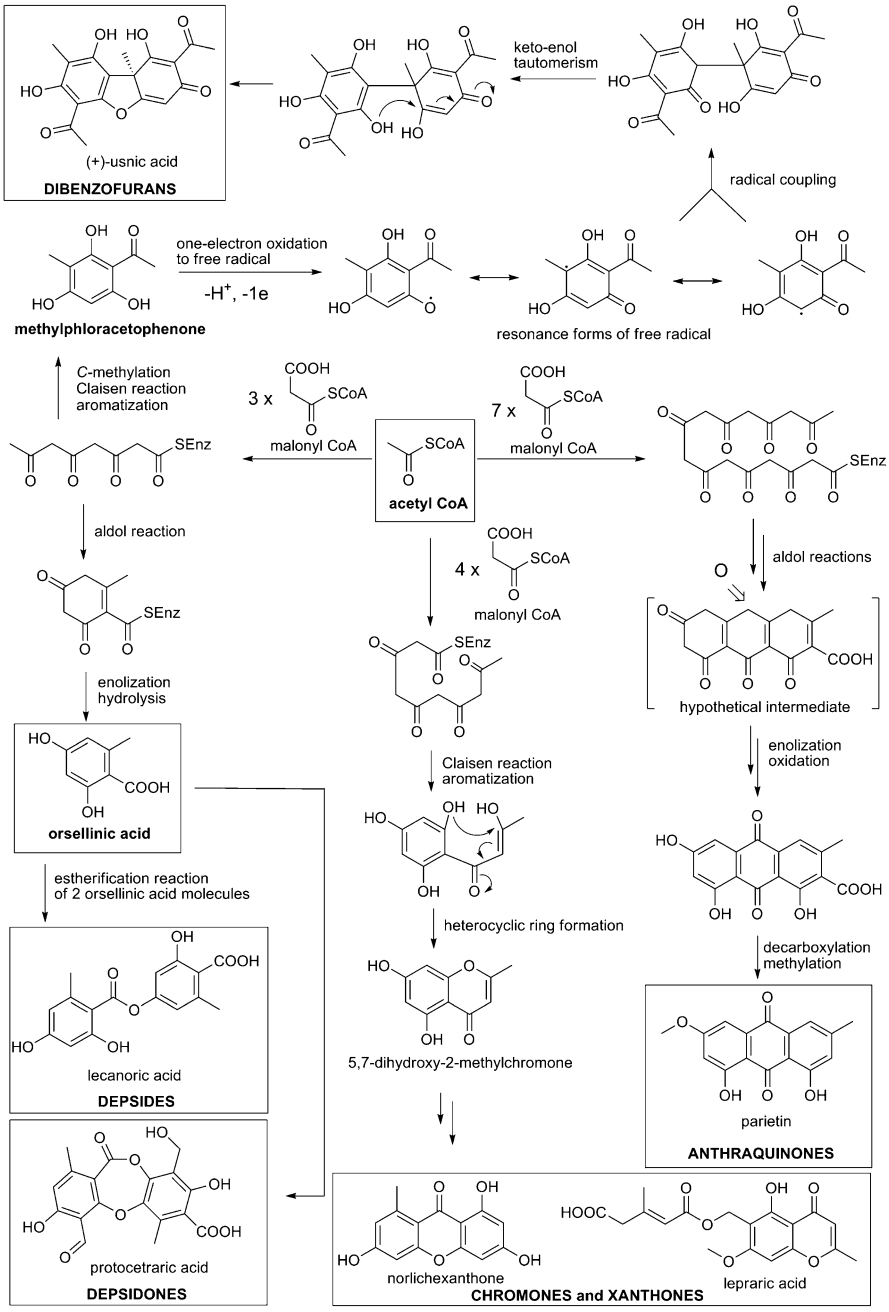


Fig. 5 Classes of lichen substances which are derived by acetyl-malonate pathway

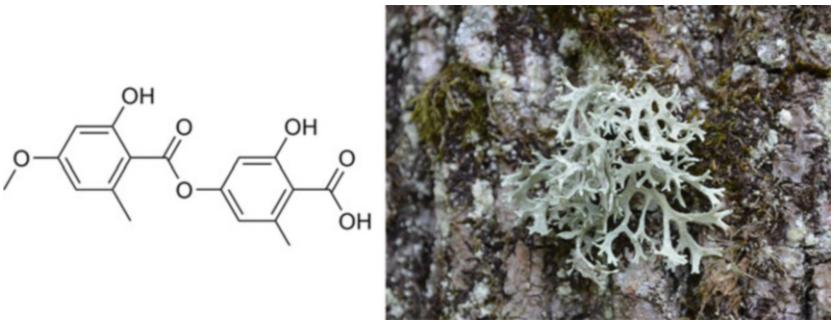


Fig. 6 Evernic acid structure (*Evernia prunastri*)

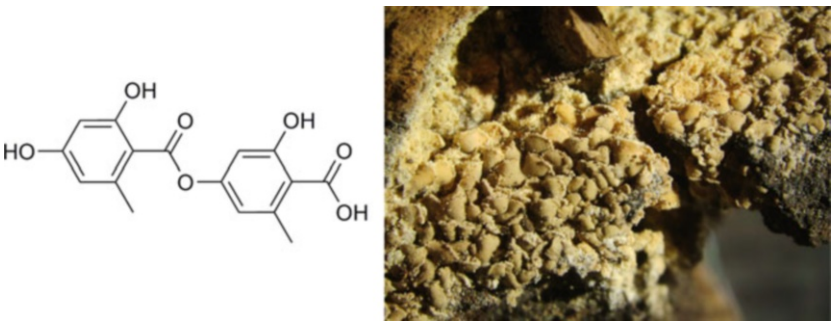


Fig. 7 Lecanoric acid structure (*Hypocenomyce scalaris*)

lecanoric acid and its orsellinate derivates against cancer cell lines (HEP-2, MCF-7, 786-0, and murine melanoma cell) and structural modifications increased activity. Promising antioxidant activity of lecanoric acid in SOR (superoxide radical) was demonstrated [43]. This compound showed relatively strong antimicrobial effects against 6 bacteria and 10 fungi containing human, animal, and plant pathogens, mycotoxin producers, as well as food-spoilage organisms [44, 45]. Lecanoric acid was also reported as a potent fungitoxic compound, which was tested against fungus *Cladosporium sphaerospermum* [46].

Gyrophoric Acid

Gyrophoric acid demonstrated antioxidant, antibacterial, cytotoxic, and antitumor activities (Fig. 8). This lichen compound is a common metabolite in *Umbilicaria* lichen species. Antioxidant activity of lichen members in family *Umbilicariaceae* was demonstrated [47]. Antibacterial activity of gyrophoric acid was showed against some foodborne bacteria and fungi [48]. Gyrophoric acid was highly effective against cancer cell lines (HL-60, A2780, Jurkat), where cytotoxicity and pro-apoptosis activity were confirmed [49].

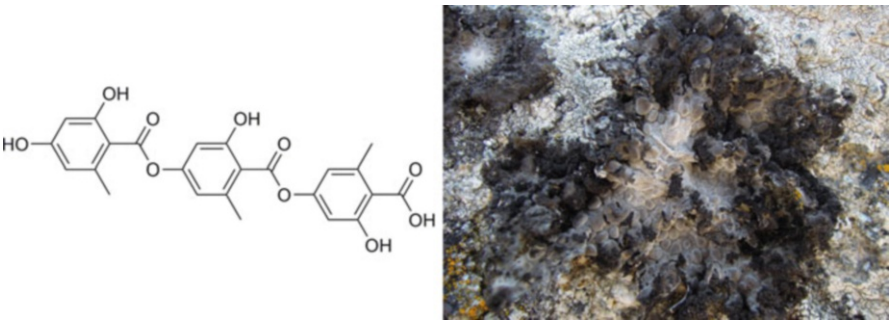


Fig. 8 Gyrophoric acid structure (*Lasallia pustulata*)

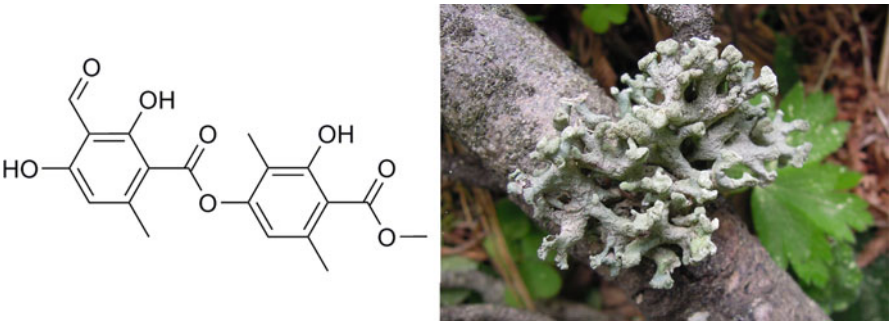


Fig. 9 Atranorin structure (*Hypogymnia tubulosa*)

Atranorin

Atranorin has strong antioxidant and antitumor properties (Fig. 9). This lichen compound has one of the largest free radical scavenging activities from lichen substances tested and the most effective reducing power and superoxide radical scavenging so far [50]. Another property of atranorin is anticancer activity against cancer cell lines (A2780 and HT-29) which was demonstrated by Bačkorová et al. [51]. This depside demonstrated strong pro-apoptic action and inhibition of cancer cell proliferation. Atranorin is counted also as a potential anticancer agent in hepatocytes from rat [52]. Antibacterial activities of this metabolite were also tested [44, 45].

Thamnolic Acid

In the study of Cankılıç et al. [53], thamnolic acid showed potential antibacterial, antituberculosis, and antifungal activities (Fig. 10). Strong effect of this compound was determined also against bacteria and yeasts. This compound can be used as potential antimicrobial agent in food industry and for the purpose of controlling different diseases.

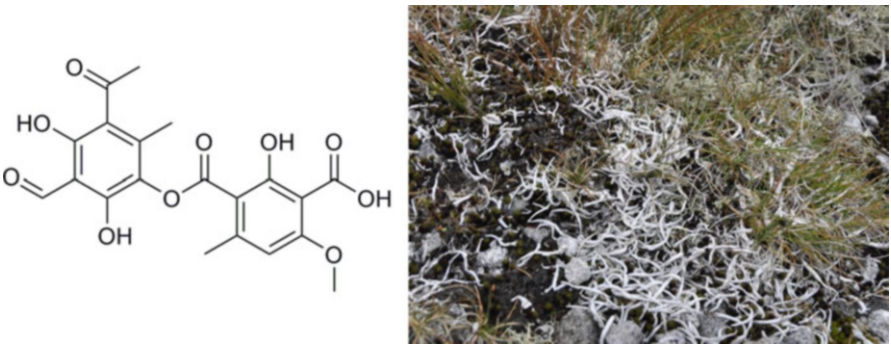


Fig. 10 Thamnolic acid structure (*Thamnolia vermicularis*)

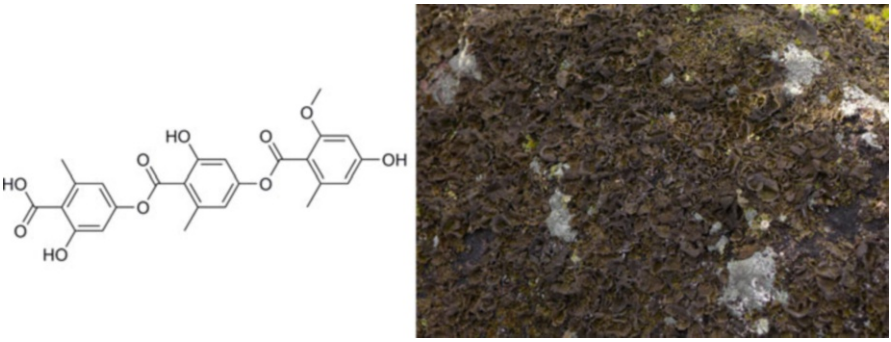


Fig. 11 Umbilicaric acid structure (*Umbilicaria polyphylla*)

Umbilicaric Acid

Umbilicaric acid is a common lichen substance in family *Umbilicariaceae* (Fig. 11). Antioxidant and antimicrobial activities of this metabolite were demonstrated. Umbilicaric acid was tested for potential antioxidant ability and showed the highest antioxidant activity with 68.14% inhibition among all tested metabolites [47]. Inhibitory effect on three Gram-positive bacteria and two yeasts, which are known as foodborne microorganisms and lead to infections in humans, was observed.

Depsidones

Orcinol-type depsidones have keto-group in the side chain of the first ring. It is well known that this functional group has a strong effect upon the ester linkage between the two rings, since enol lactones form readily. Oxidative cyclization of depsides to depsidones usually joins the 2-hydroxyl of ring A and the 5-position of ring B.

Protocetraric Acid

Antibacterial, antifungal, antioxidant and anticancer potential was found in protocetraric acid (Fig. 12). Antibacterial activity of protocetraric acid against

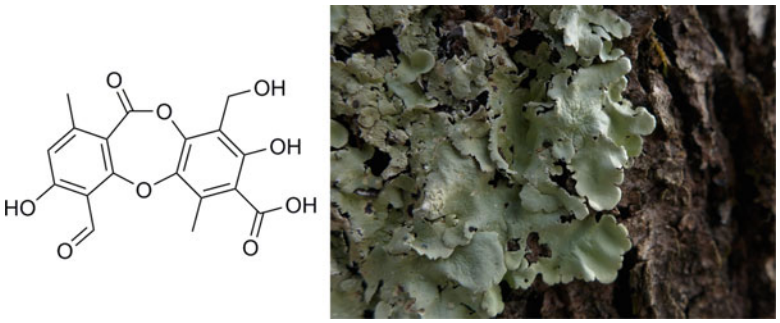


Fig. 12 Protocetraric acid structure (*Flavoparmelia caperata*)

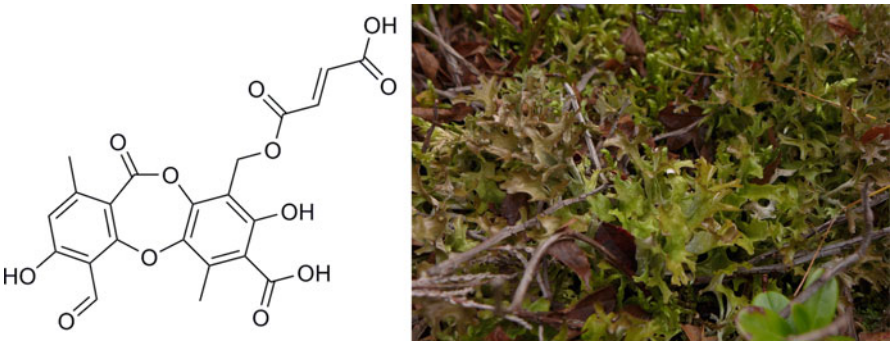


Fig. 13 Fumarprotocetraric acid structure (*Cetraria islandica*)

Salmonella typhi (0.5 μg/mL) and significant antifungal effect against *Tripthyton rubrum* (1 μg/1 mL) were reported. Protocetraric acid can be used as potential antimicrobial drug against human pathogenic microbes [54]. Antitubercular activity of several lichen substances was also tested. Protocetraric acid (MIC value 125 μg/mL, 334 μM) showed moderate inhibitory activity [55]. Antiproliferative activity of protocetraric acid against FemX (human melanoma) and LS174 (human colon carcinoma) cell lines with IC_{50} values from 35.67 to 60.18 μg/mL was confirmed.

Fumarprotocetraric Acid

Fumarprotocetraric acid as one of the bioactive compounds of lichen (Fig. 13) was tested as expectorant and for its antioxidant activities (Fig. 13). Orally administered compound (25 and 50 mg/kg) showed significantly greater dose-dependent phenol red activity in the bronchoalveolar lavage and expectorant activity ($p < 0.05$). Lipid peroxidation was also reduced by 50% in the lung tissue [56]. The growth inhibition of bacteria (*Bacillus cereus, Bacillus subtilis, Listeria monocytogenes*) and yeasts (*Candida albicans, Candida glabrata*) was observed after use of fumarprotocetraric acid (MIC 4.6 μg/mL, 0.33 mM for bacteria, and 18.7 μg/mL and 1.32 mM for yeasts) [57].

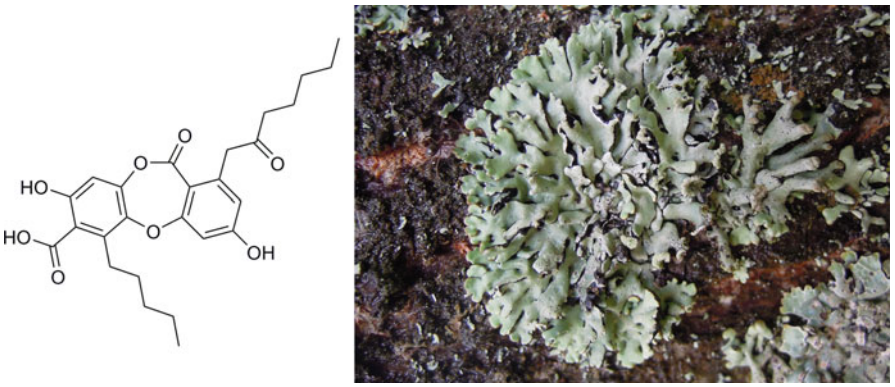


Fig. 14 Physodic acid structure (*Hypogymnia physodes*)

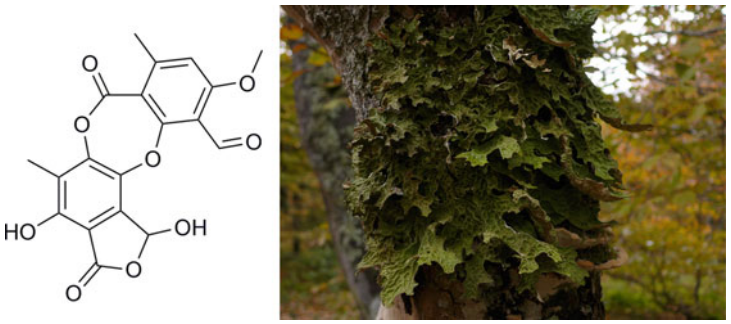


Fig. 15 Stictic acid structure (*Lobaria pulmonaria*)

Physodic Acid

Depsidone physodic acid was tested for anticancer activity (Fig. 14). This compound activated an apoptic process on A375 cells in the concentration of 6.25–50 μM. It probably involves the reduction of Hsp70 expression [58]. Another cytotoxic activity of physodic acid was tested on tumorigenic (MDA-MB-231, MCF-7, and T-47D) and nontumorigenic (MCF-10A) cell lines. Strong activity was observed against tumoric cell lines (IC_{50} = 46–94 μM) and inactivity of compound against nontumoric cell line (IC_{50} > 100 μM). In study of antimicrobial activity, physodic acid was active against the same bacteria or yeasts and inactive against all of the filamentous fungi, which were tested [59].

Stictic Acid

Stictic acid (Fig. 15) showed neuroprotection through the antioxidant activity against U373MG cell line (5 and 10 μg/mL) by decreasing productivity of ROS induced by hydrogen peroxide [60]. Antioxidant activity of concentration range 0.012–0.015 mg/mL was observed according to radical scavenging Co (II) EDTA-induced luminol plateau chemiluminescence assay [61]. Growth inhibition of cancer cell lines HT-29

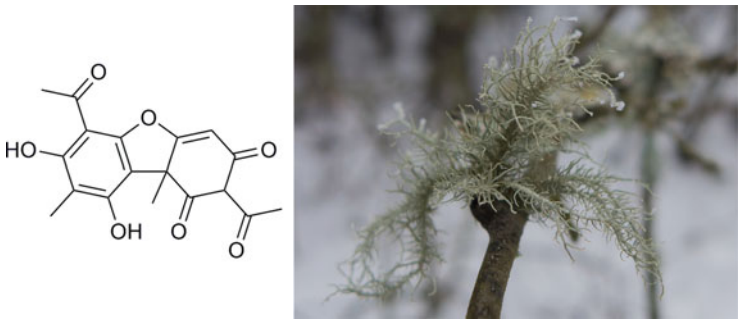


Fig. 16 Usnic acid structure (*Usnea* sp.)

and MCF-7 was tested as potential anticancer activity of stictic acid. Results showed strong potential of this compound as anticancer agent (IC_{50} = 29,29 μg/mL). For comparison was tested normal cell line MRC-5 with IC_{50} = 2478.40 μg/mL [62].

Dibenzofurans

Dibenzofurans are heterocyclic aromatic organic compounds with two benzene rings fused to a central furan ring. As secondary metabolites, the phenolic units are derived by the orsellinic acid-type cyclization. The dibenzofurans appear to form by carbon–carbon coupling and cyclodehydration of two such acetate–polymalonate-derived phenolic acid units.

Usnic Acid

One of the most studied secondary metabolite of lichen is usnic acid (Fig. 16). Based on the wide biological and ecological activities, it is used in cosmetics, deodorants, toothpastes, and medical creams. It also exhibits antimitotic, anti-inflammatory, analgesic, antiviral, antiprotozoal activities, as well as preserving properties, anti-growth, and antiherbivore activity [63]. Usnic acid serves as a repellent against insect feeding. Larvae of *Cleorodes lichenaria* were affected by retarded growth, increased mortality, and enhanced concentrations of usnic acid in the animal tissue [64]. Usnic acid is an effective UV-absorbing compound, which is also one of the known roles of secondary metabolites, and protects algal layer from intense light levels [65]. This compound also decreased the proliferation of human breast cancer cells and human lung cancer cells without any DNA damage [66]. Strong hepato-toxic activity was also observed against monogastric murine hepatocytes, with inhibition of the electron transport chain in the mitochondria and induction of oxidative stress in cells [67]. Usnic acid plays also important role as an allelopathic agent in competition between lichens and mosses. Growth inhibition of protonemata and reduced development of gametophores was observed. Usnic acid has a strong effect on cell division in protonemata [68]. The level of ploidy in mosses is also influenced by presence of usnic acid and can be counted as a physiological change after stress [69].

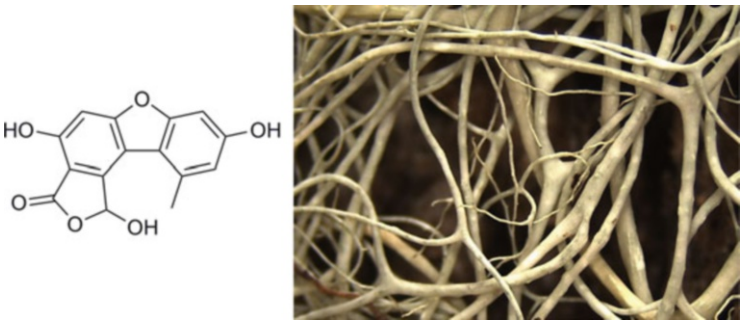


Fig. 17 Alectosarmentin structure (*Alectoria sarmentosa*)

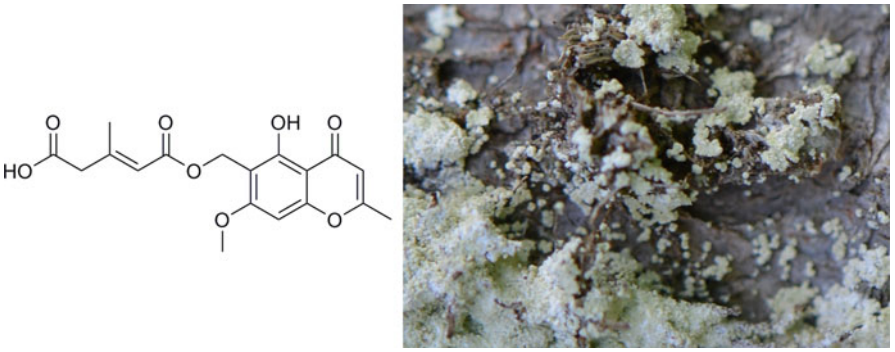


Fig. 18 Lepraric acid structure (*Lepraria* sp.)

Alectosarmentin

Alectosarmentin is a relatively newly discovered compound identified in lichen *Alectoria sarmentosa* (Fig. 17). This compound has antibacterial activity including microorganisms *Staphylococcus aureus* and *Mycobacterium smegmatis* [70].

Chromones

Chromone, parent compound of the chromones group, is derivative of benzopyran with substituted keto group on the pyran ring. Chromones are probably formed by internal cyclization of a single, folded polyketide chain and are often identical or analogous to products of nonlichen-forming fungi or higher plants.

Lepraric Acid

Lepraric acid (Fig. 18) can be used as chemotaxonomic marker in *Hypoxylon aeruginosum, Chlorostroma subcubisporum*, and *Chlorostroma cyaninum* [71].

Xanthones

Xanthones are known in free-living fungi and recent studies indicate that they are rather common in lichens too. Unlike the fungal xanthones, many lichen xanthones

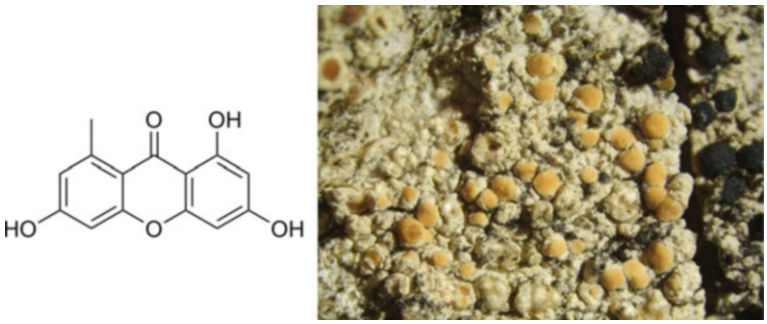


Fig. 19 Norlichexanthone structure (*Lecanora symmicta*)

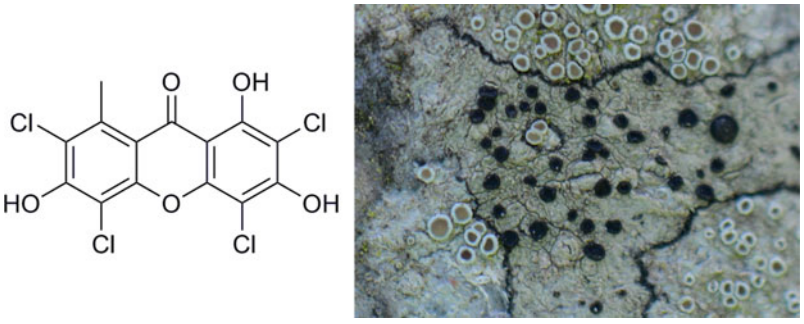


Fig. 20 Thiophanic acid structure (*Lecidella elaeochroma*)

have one or more nuclear chlorine substituents. The fundamental structure of the known lichen xanthones could be derived directly by linear condensation of seven acetate and malonate units with one orsellinic acid-type cyclization. The two rings are joined by a ketonic carbon and by an ether-oxygen arising from cyclodehydration.

Norlichexanthone

Norlichexanthone (Fig. 19) is lichen compound that fully inhibits $p56^{lck}$ tyrosine kinase at 200 μg/mL [72] and inhibits the activity of the protein kinases aurora-B, PIM1, and VEGF-R2, where IC_{50} values from 0.3 to 12 μM [73].

Thiophanic Acid

Allelopathic effect of thiophanic acid (Fig. 20) on wide number of higher plants was demonstrated [74]. Fungicidal activity of thiophanic acid and thiophaninic acid was recorded as well [75].

Anthraquinones

Anthraquinones is a class of phenolic compounds based on the 9,10-anthraquinone skeleton and is probably formed by internal cyclization of a single polyacetyl chain.

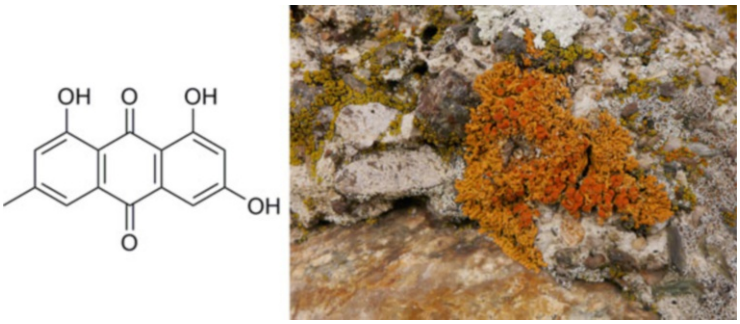


Fig. 21 Emodin structure (*Xanthoria elegans*)

Fig. 22 Structure of parietin (*Xanthoria parietina*)

These substances are typical for members of family *Teloschistaceae* within genera *Caloplaca*, *Teloschistes* and *Xanthoria* [76]. Anthraquinones are produced by lichens, as well as by nonlichenized fungi [77]. Biological activities of various anthraquinones were confirmed in several studies such as antitumoral, anti-inflammatory, and bactericide effects [77–79]. They are all pigmented compounds which also acting as a light filters.

Emodin

Generally, anthraquinones are potential antiviral agents against HIV virus [80]. Emodin (Fig. 21), 7-chloroemodin, and 7-chloro-1-O-methylemodin showed partial inactivation of the herpes simplex virus type 1. With an increasing substitution of chlorine in the anthraquinone nucleus, an antiviral activity increases [81]. Derivatives of emodin revealed anticancer activity against leukemia cells [82].

Parietin

Parietin (Fig. 22) is an orange anthraquinone pigment and it is widespread in lichens, which are characteristic for sun-exposed habitats. Mainly it is localized in the upper cortex of lichen genera *Xanthoria, Teloschistes*, *and Caloplaca*. According to Hill and Woolhouse [83], the content of parietin is positively correlated to intensity of

light in habitat. Since parietin absorbs light, it may help to protect the photosynthetic apparatus of the photobiont against damage by high light levels [36, 84]. Solhaug and Gauslaa [85] reported that UV-B radiation may trigger the resynthesis of this cortical pigment parietin (= physcion) in the lichen *Xanthoria parietina*. Despite the long-term study of parietin, we still cannot claim that this secondary metabolite serves as UV-B or PAR screening pigment [86]. It is possible that parietin also acts as an antioxidant [87].

4.1.2 Mevalonate Pathway

The terpenoids form a large and structurally diverse family of natural products derived from C_5 isoprene units joined in a head-to-tail fashion. Isoprene is produced naturally but is not involved in the formation of these compounds, and the biochemically active isoprene units were identified as the diphosphate esters – dimethylallyl pyrophosphate and isopenthenyl pyrophosphate.

Two molecules of acetyl-coenzyme A combine initially in a Claisen condensation to give acetoacetyl-CoA, and a third is incorporated via a stereospecific aldol addition giving the branched-chain ester. The thioester is then reduced to primary alcohol via hemithioacetal and aldehyde to give mevalonic acid. The six-carbon mevalonic acid is then transformed into the five-carbon phosphorylated isoprene units in a series of reactions, beginning with phosphorylation of the primary alcohol group. Two different ATP-dependent enzymes are involved, resulting in mevalonic acid diphosphate, and decarboxylation/dehydration then follows to give isopentenyl pyrophosphate. Combination of two isoprene units head-to-tail forms monoterpenes. Limonene is formed by cyclization reactions of geranyl pyrophosphate. Diterpenes consist of four isoprene units. Geranyl PP reacts with first isoprene unit to give farnesyl PP which reacts with second isoprene to give geranylgeranyl PP. Phytol is one of the best known diterpenes. Triterpenes are derived from squalene, six isoprene units containing compound. Steroids are then formed by cyclization of squalene. Another well-known class of terpenoids – carotenes – are derivatives of tetraterpene lycopene, which is formed by combination of two geranylgeranyl PP units. Classes of lichen substances that are derived by mevalonate pathway are terpenes, steroids, and carotenoids (Fig. 23).

Terpenes

Terpenes are large and diverse class of organic compounds. Terpenes are formed biosynthetically from units of isopentenyl pyrophosphate, which is the product of mevalonate pathway.

Limonene

In study of Kahriman et al. [88], the antimicrobial and antifungal activity of the essential oil obtained by hydrodistillation from *Evernia prunastri* (L.) Ach. and *Evernia divaricata* (L.) has been analyzed. The major substances in the essential oil of *Evernia prunastri* and *Evernia divaricata* were β-pinene (6.3 and 8.0%), α-pinene (6.6%, 7.2%), limonene (1.6%, 6.3%), α-phellandrene (3.3%, 4.4%), camphene (3.0%, 3.1%), and p-cymene (1.5%, 1.8%), respectively. The antimicrobial and

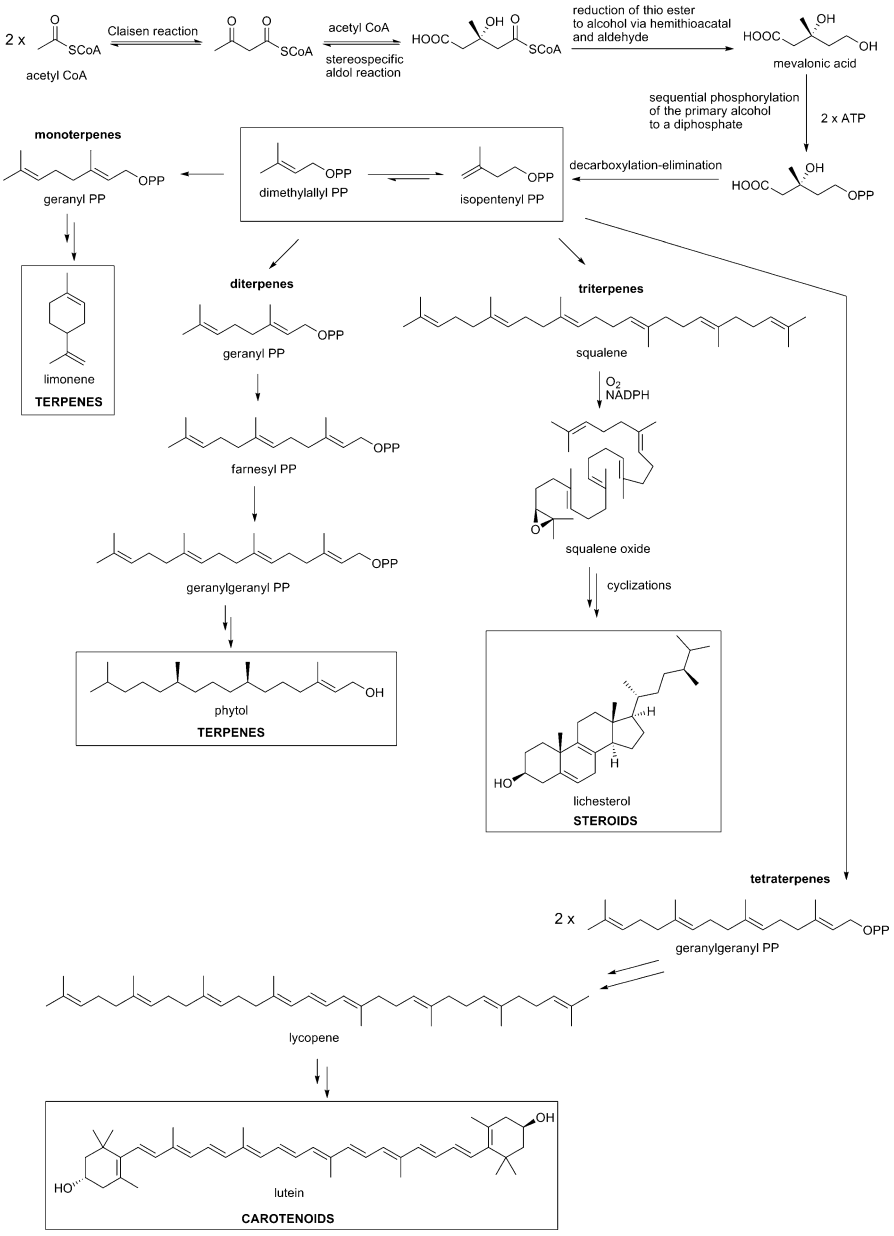


Fig. 23 Classes of lichen substances that are derived by mevalonate pathway

antifungal activities of the essential oil of *Evernia prunastri* and *Evernia divaricata* were tested in vitro against the bacteria *E. coli, Y. pseudotuberculosis, S. aureus, E. faecalis, B. cereus, C. albicans*. *Evernia divaricata* showed antimicrobial activity

Fig. 24 Limonene structure (*Evernia prunastri*)

and antifungal activity. Essential oil of *Evernia prunastri* exhibited only antifungal activity (Fig. 24).

Phytol

This secondary metabolite of terpenoid origin showed mainly antimycobacterial activity. Rajab et al. [89] tested (E)-phytol (Fig. 25) as the principal antimycobacterial constituent against *Mycobacterium tuberculosis* with a minimum inhibitory concentration (MIC) of 2 μg/ml. Inhibitory value was also observed for (3R,5,7R,11R)-phytanol, (Z)-phytol, and a commercially available 2: 1 mixture of (E)- and (Z)-phytol with lower antimycobacterial activity with MIC $>$ 128 μg/ml.

Zeorin

Zeorin (Fig. 26) (6α,22-dihydroxyhopane) is the main triterpene in various species of lichens [90]. In the study of Kosanić et al. [91] has been tested antibacterial and antifungal activity of the lichen *Lecanora frustulosa* and *Parmeliopsis hyperopta* and their zeorin constituents and divaricatic acid. Acetone, methanol, and aqueous extracts of these lichens have been tested in vitro against: *Bacillus mycoides, Bacillus subtilis, Staphylococcus aureus, Enterobacter cloaceae, Escherichia coli, Klebsiella pneumoniae, Aspergillus flavus, Aspergillus fumigatus, Botrytis cinerea, Candida albicans, Fusarium oxysporum, Mucor mucedo, Paecilomyces variotii, Penicillium purpurescens, Penicillium verrucosum*, and *Trichoderma harzianum.* According to this study, zeorin exhibited stronger antibacterial activity than divaricatic acid at a concentration of 0.39 mg/ml which inhibited 4 out of 6 tested bacteria.

Steroids

Steroids are products of the mevalonate pathway and are highly often present in lichens. Steroids are derived from cyclization of the triterpene squalene.

It has been reported that sterol compounds can play an important role in the medicine. They possess different types of pharmacological activities like anti-

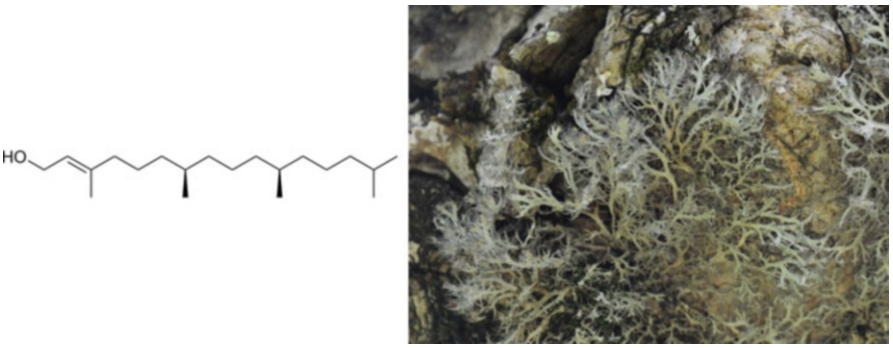


Fig. 25 Phytol structure (*Anaptychia ciliaris*)

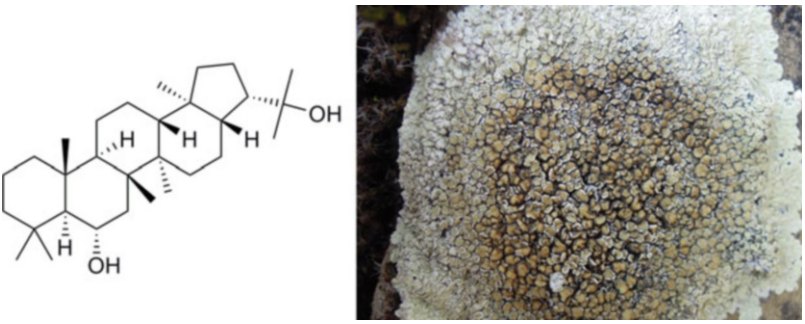


Fig. 26 Zeorin structure (*Protoparmeliopsis muralis*)

inflammatory, antiulcerogenic, antibacterial, antifungal, and antirheumatic activities [90].

Brassicasterol

Brassicasterol (Fig. 27) known in higher plant is also found in the various lichens [90]. In acetone extract of the lichen *Stereocaulon azoreum* were identified several substances by column chromatography. In addition to the main compounds, three sterols such as brassicasterol, ergosterol peroxide, and cerevisterol were obtained [92].

Ergosterol

It has been demonstrated in previous studies that sterols may play a role in the membrane permeability in the lichen thallus. The content of ergosterol (Fig. 28) (Ergosta-5,7,22-trien-3β-ol) in the lichens, which is the major sterol of the fungal plasma membrane, responds rapidly to the presence of xenobiotics in the environment, including the presence of heavy metals. Ergosterol can be considered as marker of the fungal metabolic activity [93].

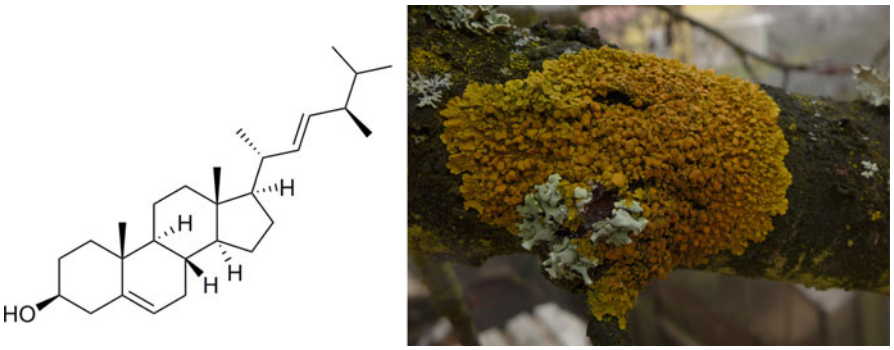


Fig. 27 Brassicasterol (*Xanthoria parietina*)

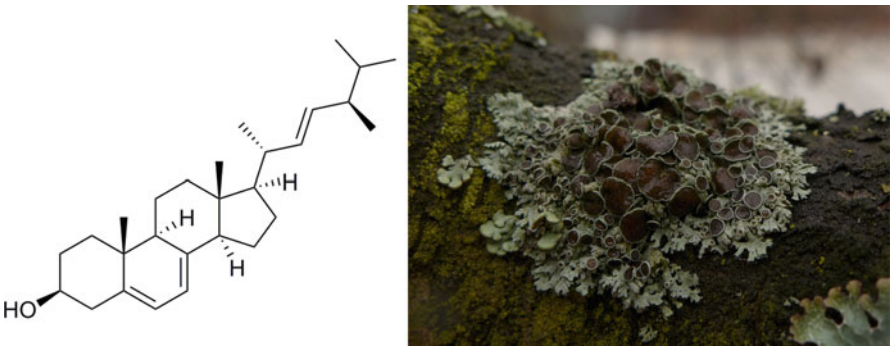


Fig. 28 Ergosterol structure (*Physcia stellaris*)

Lichesterol

Lichesterol (Fig. 29) or Ergosta-5,8,22-trien-3β-ol has been isolated and characterized in lichens *Usnea longissima*, *Lobaria pulmonaria*, *Lobaria scrobiculata* [94], and *Ramalina africana* [95].

Carotenoids

These linear molecules with multiple conjugated double bonds are found in all photosynthetic organisms. They are products of primary (intracellular) metabolism such as proteins, amino acids, polysaccharides, and vitamins. Carotenoids are products of both symbionts – fungi and algae.

In lichens with green algae photobionts following carotenoids are usually present β-carotene, lutein, violaxanthin, antheraxanthin, zeaxanthin, and neoxanthin [96, 97]. In lichens with cyanobacterial photobionts occur mainly β -carotene, zeaxanthin, canthaxanthin, and echinenone.

β-Carotene

β-Carotene (Fig. 30) is a pigment frequently found in lichen thalli, which has been analyzed using different methods. Czeczuga et al. [98] investigated in ten lichen

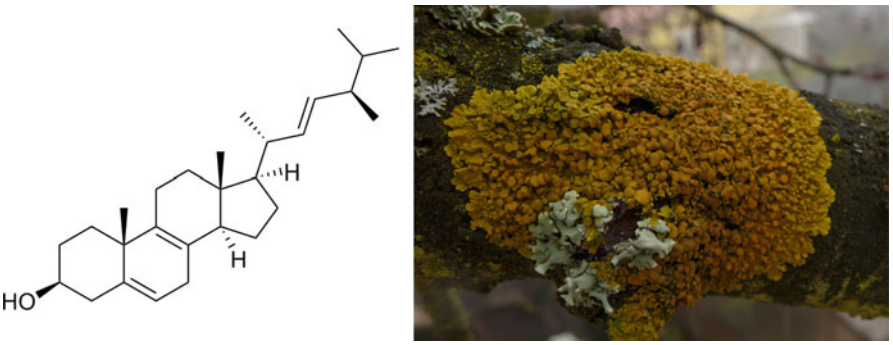


Fig. 29 Lichesterol structure (*Xanthoria parietina*)

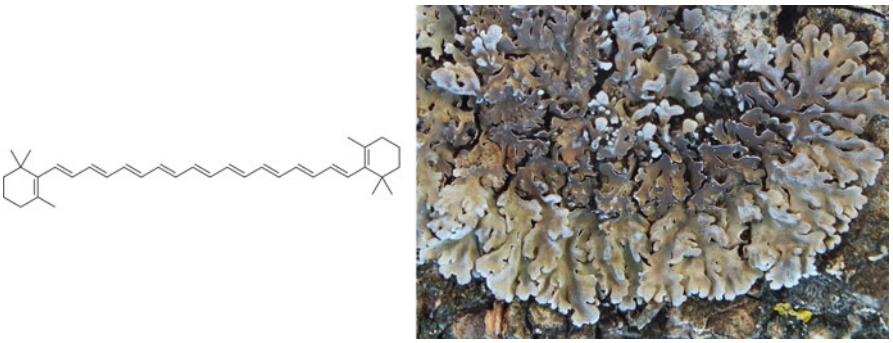


Fig. 30 β-Carotene structure (*Physconia distorta*)

species carotenoids by column and thin-layer chromatography. Lichen encrustations from *Diploschistes scruposus* showed characteristic vibrational spectra using Raman spectroscopy [99]. Nowadays it is a frequent practice to measure total carotenoids spectrophotometrically and using high-performance liquid chromatography (HPLC) techniques which facilitated the separation and identification of plastid pigments.

Lutein

Lutein (Fig. 31) mainly occurs in higher plants but was also found in lichens and algae growing near to shaded habitats, because at sunny sites it is replaced by lutein epoxide. The growth of lichens in poorly lit places is possible due to the mechanism called chromatic adaptation by an increasing of photosynthetically active pigments [100].

Zeaxanthin

The presence of this carotenoid (Fig. 32) together with violaxanthin in plants is influenced by intensity of light in xanthophyll cycle. In case that insolation is intensive, the accumulation of zeaxanthin occurs. When light intensity decreases,

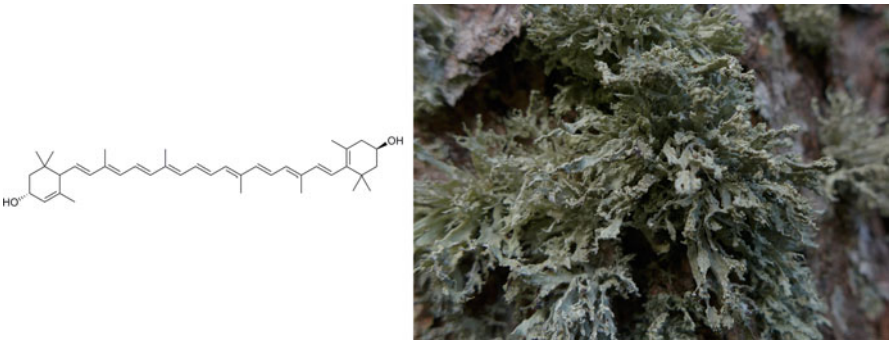


Fig. 31 Lutein structure (*Ramalina farinacea*)

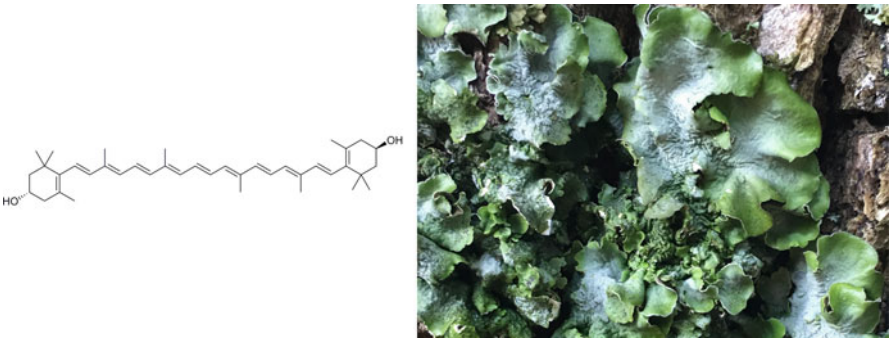


Fig. 32 Zeaxanthin structure (*Pleurosticta acetabulum*)

the zeaxanthin is converted through antheraxanthin into violaxanthin and vice versa [101]. It may be considered that this cycle occurs in the photobionts of lichens.

4.1.3 Shikimate Pathway

The shikimate pathway provides a route to aromatic compounds, particularly the aromatic amino acids and their derivatives. The pathway is employed by microorganisms and plants but not by animals. A central intermediate in the pathway is shikimic acid. The shikimate pathway begins with a coupling of phosphoenolpyruvate (from glycolysis) and D-erythrose 4-phosphate (from the pentose phosphate cycle) by aldol-type reaction. Then by elimination of phosphate and another aldol-type reaction, a cyclic product 3-dehydroquinic acid is formed. Next step involves dehydration and reduction of carbonyl function.

Phosphoenolpyruvate combines with shikimic acid 3-phosphate to an intermediate in which 1,2-elimination of phosphoric acid in side-chain and then 1,4-elimination of phosphoric acid leads to chorismic acid. The reaction transforming chorismic acid to prephenic acid is Claisen rearrangement which transfer the side-chain so that it becomes directly bonded to the carbocycle. Next reaction

steps leading to the C_6-C_3 building block (phenylpyruvic acid, L-phenylalanine) include decarboxylation, aromatization, and dehydroxylation. Generally two of the C_6-C_3 building blocks combine to form terphenylquinones and reaction pathway continues to pulvinic acid derivatives. Classes of lichen substances which are derived by shikimate pathway are therphenylquinones and pulvinic acid derivates (Fig. 33)

Terphenylquinones

Phenylquinones are well-documented examples of lichen secondary products derived by the shikimic acid pathway and are widespread especially among fungi. Terphenylquinones are formed by condensation of two (probably activated) phenylpyruvic acid derivatives. Only two terphenylquinones, polyporic acid and thelephoric acid, are known.

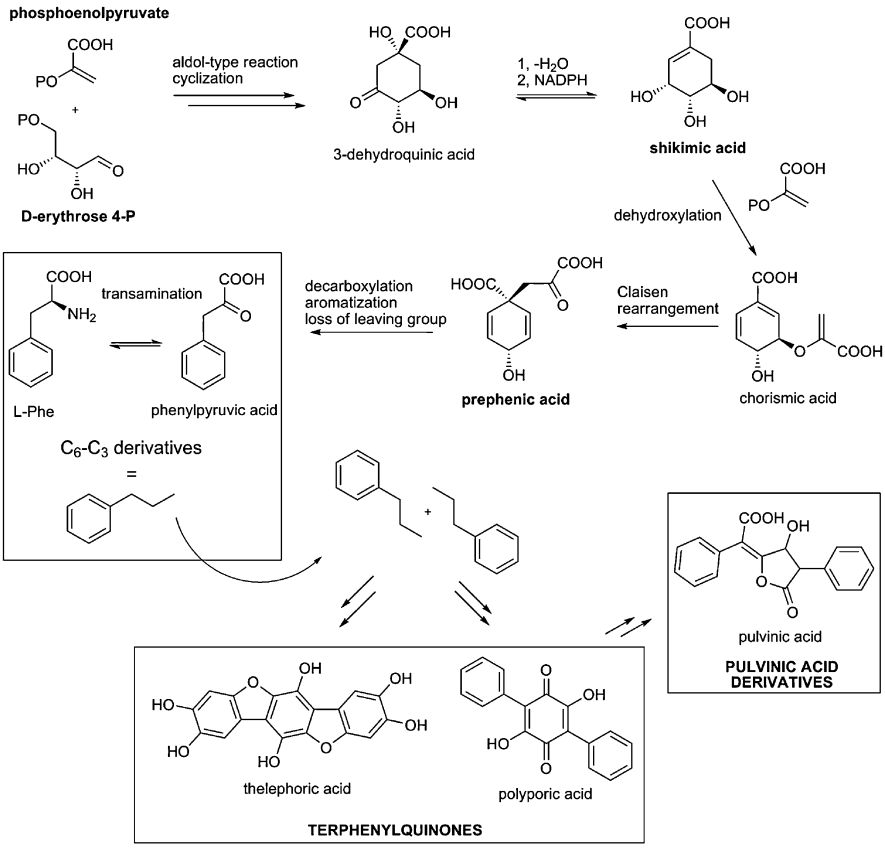


Fig. 33 Classes of lichen substances which are derived by shikimate pathway

Fig. 34 Polyporic acid structure

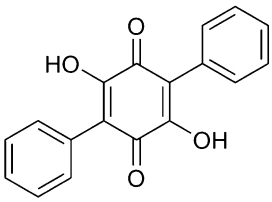
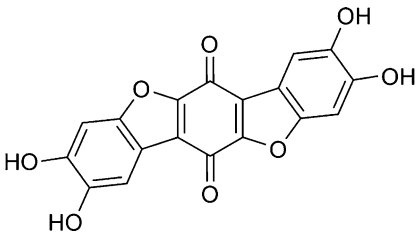


Fig. 35 Thelephoric acid structure



Polyporic Acid

Polyporic acid (Fig. 34) extracted from fungus *Hapalopilus rutilans* decreased activity of DHOdehase enzyme in rats by 20–30% due to its inhibitory effect. DHOdehase enzyme catalyzes reaction of pyrimidine de novo synthesis at the inner mitochondrial membrane. Activity of the human enzyme was not affected [102]. Another study conducted on rats showed strong inhibitory effect of polyporic acid; the rats exhibited reduced locomotor activity, hepatorenal failure, and metabolic acidosis [103]. Burton and Cain [104] showed antileukemic activity of polyporic acid isolated from lichen *Sticta coronata* on mice.

Thelephoric Acid

Thelephoric acid (Fig. 35) from fungus *Polyozellus multiplex* exhibited inhibitory effect against prolyl endopeptidase, in which increased level is involved in the development of Alzheimer's type senile dementia [105]. Antioxidative properties were investigated by Chung et al. [106] where results were conclusive for superoxide anion radical, hydroxyl radical, and DPPH radical. Rao et al. [107] found theleophoric acid in lichen *Lobaria insidiosa* from Western Himalayas. This acid is also found in *Thelephora* spp. and *Hydnum* spp.

Pulvinic Acid Derivatives

Pulvinic acid derivatives are lichen secondary products which are derived by the shikimic acid pathway. Pulvinic acid derivatives are not present in all lichen species that contain blue-green algae. Nitrogen fixing algae present in some lichens are no necessary in species, where pulvinic acid pigments were observed.

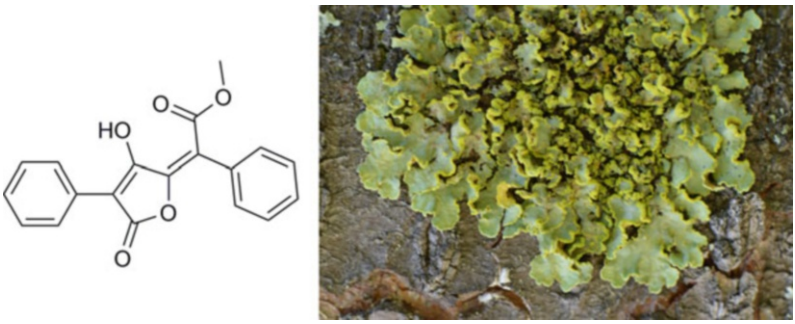


Fig. 36 Vulpinic acid structure (*Vulpicida pinastri*)

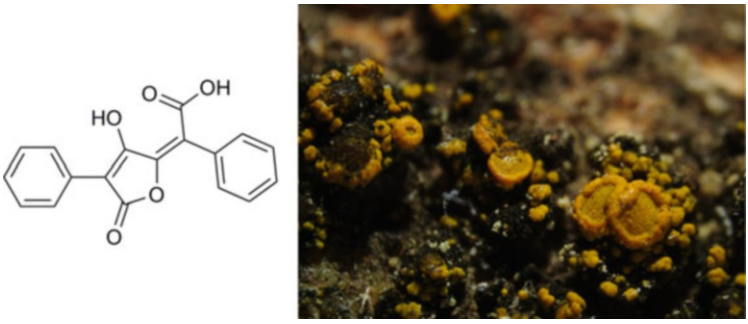


Fig. 37 Pulvinic acid structure (*Candelariella vitellina*)

Vulpinic Acid

Vulpinic acid (Fig. 36) isolated from *Letharia vulpina* induced uncoupling by acting on the inner mitochondrial membrane in mice liver in vitro [108]. Extract from *Vulpicida pinastri* (containing vulpinic acid, pinastric acid, usnic acid) acts as a UV-A and UV-B blocker agent due to its superoxide anion scavenging activity [109, 110]. Application of vulpinic acid strongly influenced growth of lichen photobiont *Trebouxia irregularis* [111]. When used on larvae of the polyphagous insect herbivore *Spodoptera littoralis*, vulpinic acid showed strong mortality and growth retardation in concentration lower than naturally occurring in lichens [112]. Antiproliferative effect of vulpinic acid was tested on HepG2 and NS20Y cancer cell lines, exhibited strong antiangiogenic potential, and showed no toxic effects on noncancerous cells [113].

Pulvinic Acid

Pulvinic acid (Fig. 37) derivate pulvinamide exhibited antioxidant properties [114]. Another set of derivates – atromentic acid, variegatic acid, and xerocomic acid – showed nonspecific inhibitory effects on four cytochrome P450 (CYP) – 1A2, 2C9, 2D6, and 3A4 – probably by reduction of ferryl heme to ferric heme [115].

5 Conclusion

Lichens are very typical symbiotic organisms, which can be found everywhere around the world and dominantly present in 8% of earth's land surface. Due to the fact that they belong to the slowest growing organisms, they are very important and interesting because of their secondary metabolites. One of the first descriptions of uses is from time of early Chinese and Egyptian civilizations.

Since the sixteenth century, lichens have been used in the perfume and cosmetic industries. They are attractive also for their typical color hence used as dyes. Lichen secondary compounds are studied for more than one hundred years for their pharmaceutical, biological, and ecological potential, which was described in many studies.

Based on the pathways how lichen secondary metabolites are produced, the acetate-polymalonate pathway is unique for lichens. Most of bioactive compounds are synthetized by this pathway, and their biological properties are very promising and still the aim of study around the world. In this chapter, we showed all three pathways, which can serve for better understanding of synthesis of lichen compounds. Main groups that belong to the pathways are also described as well as their typical secondary compounds with their pharmaceutical, biological, and ecological uses. It is evident that secondary compounds of lichens have wide area where they can be applied.

Approximately 1000 secondary metabolites of lichens were discovered and described. Most of them are solely present in lichens. Their antiproliferative, antibacterial, antiviral, allelopathic, antiherbivore, UV-protective, antioxidant, anti-inflammatory, analgesic, antipyretic potential is evident. Lichens are still source of many bioactive compounds, which application is still in process of research.

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Fusarium Secondary Metabolism Biosynthetic Pathways: So Close but So Far Away

10

Łukasz Stępień, Justyna Lalak-Kańczugowska, Natalia Witaszak, and Monika Urbaniak

Contents

Abstract

Fusarium species are casual filamentous fungi, including opportunistic pathogens infecting plants worldwide, but also able to grow as saprotrophs in a range of climatic zones. The genus is extremely variable in terms of genetics, biology, ecology, and, consequently, secondary metabolism, which directly relates to ecological conditions and niches occupied by individual species. Fungal secondary metabolites are the main "weapon" of the pathogenic species before, during, and after the infection process, allowing for the communication with the organism that is being attacked. Many of secondary metabolites are common for diverse fungal microorganisms, and their mode of action is similar for various plant-pathogen systems. *Fusaria* are able to produce a range of quite specific metabolites, some of which have yet unknown biological functions.

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Nevertheless, genetic and biochemical pathways responsible for their biosynthesis remain under strong selection pressure, which keeps their structures and functions relatively stable, regardless of the producing organism. Here, we summarize the data available in recent literature reports on genetic and biochemical diversity occurring in the studies of main secondary metabolites produced by *Fusarium* species differing in origin and ecology.

Keywords
Fumonisins · Fungal ecology · Metabolic pathways · Mycotoxins · Phylogeny · Trichothecenes · Zearalenone

List of Abbreviations

AcDON	Acetylated DON derivatives
BEA	Beauvericin
bik	Bikaverin biosynthetic gene cluster
c*ar*	Carotenoid biosynthetic gene cluster
DAS	Diacetoxyscirpenol
DMATS	Dimethylallyltryptophan synthase
DON	Deoxynivalenol
ENN	Enniatin
eqx	Equisetin biosynthetic gene cluster
FA	Fusaric acid
FB	Fumonisin B
FESC	*F. equiseti* species complex
FFSC	*F. fujikuroi* species complex
FGSC	*F. graminearum* species complex
FOSC	*F. oxysporum* species complex
FPP	Farnesyl pyrophosphate
Fsr	Fusarubin biosynthetic gene cluster
FSSC	*F. solani* species complex
FUB	Fusaric acid biosynthetic gene cluster
FUM	Fumonisin biosynthetic gene cluster
FUS	Fusarin C biosynthetic gene cluster
GA	Gibberellins
GGPP	Geranylgeranyl pyrophosphate
MAPK	Mitogen-activated protein kinase
MON	Moniliformin
NIV	Nivalenol
NRPS	Nonribosomal peptide synthetase
PKS	Polyketide synthase
PM	Primary metabolism
SM	Secondary metabolite
TC	Terpene cyclase
TF	Transcription factor
TRI	Trichothecene biosynthetic gene cluster
ZEA	Zearalenone

1 Introduction

Secondary metabolites (SMs) are universal messengers between plants and pathogens, of which the most widespread are filamentous fungi. SMs are responsible for pathogen recognition by the plant host and for pathogen actions during host infection. They belong to multiple classes concerning their chemical structures and influence diverse biochemical processes exhibiting signaling, toxic, eliciting, priming, growth-promoting, or defense response-inducing actions [1]. The ability to produce the SMs is often governed by the presence and activity of the specific gene clusters present in fungal genomes, which usually contain several enzyme-encoding genes devoted exclusively to biosynthesize specific group of compounds [2, 3]. The distribution of these gene clusters among fungal taxa generally resembles their phylogenetic relationships but sometimes may serve as the evidence of the past horizontal gene transfer events, since the same biosynthetic pathways may be found in species that not share close relationship [4, 5]. Moreover, it seems that SM biosynthetic gene clusters not only undergo common regulation and expression patterns but also share evolutionary fate, which often depends strongly on the genomic context and differs from main primary metabolism (PM) regions. Many SMs are universal for diverse fungal microorganisms, and their actions are similar for various plant-pathogen systems; nevertheless, significant level of specificity may be observed in comparative metabolomic analyses of pathogenic fungi.

Fusarium genus consists of a large number of diverse species of different lifestyles. Many of them are opportunistic pathogens infecting multiple plant species in a range of climatic zones (e.g., *F. graminearum* species complex (FGSC), *F. fujikuroi* species complex (FFSC), *F. equiseti* species complex (FESC), *F. avenaceum*, and *F. culmorum*), and some are more typical soil-borne pathogens and are more likely isolated from the rhizosphere of plants (mainly *F. oxysporum* species complex (FOSC) and *F. solani* species complex (FSSC)).

Fusaria are extremely variable in terms of genetics, biology, and ecology; thus, they produce also very diverse repertoire of SMs. This divergence relates partially to the ecological niches occupied by individual species but also seems to play some, yet unknown role in the organism ecological flexibility. On the other hand, closely related species may vary in biosynthetic potentials. Fumonisins may serve as an example. *F. verticillioides* and *F. proliferatum* are the main producers of fumonisins, both capable of infecting maize as the typical host. Yet, the sequence divergence of the *FUM* biosynthetic cluster responsible for fumonisin biosynthetic ability reaches 20% when those two species are compared [2, 6–8]; in *F. oxysporum*, *FUM* cluster has been found and characterized for just one strain O-1890 [9], and another maize pathogens from the FFSC – *F. subglutinans* and *F. temperatum* – are essentially fumonisin nonproducers [10, 11].

Genes inside the clusters responsible for the SMs' biosynthesis remain under strong selection pressure, exerted by ecological factors (environment, competitive organisms, host availability, and resistance) which keep the structures and functions of encoded enzymes relatively stable. Still, even intraspecific polymorphism can be observed for some of the pathways, like *FUM* cluster divergence in populations of *F. proliferatum* [12, 13]. Similar examples of discrepancies in phylogenetic

relationships between closely related taxa and their SM biosynthetic abilities are frequent in *Fusarium* genus and are presented and discussed in this chapter. Main *Fusarium*-produced mycotoxin pathways were reviewed in terms of genetic divergence and biochemical and chemotypic population shifts. We also summarized the data on genetic and biochemical diversity occurring in the studies of main secondary metabolites produced by *Fusaria* differing in origin and ecology.

2 *Fusarium*: Clades and Species

First description of *Fusarium* was reported in 1809 by Link and since than more than a thousand species have been identified, of which 70 is well-known. The first taxonomic classifications have been created based on morphological characters of species and test crosses [14]. Later, thanks to the genetic and bioinformatic tools, species became classified using phylogenetic analyses. Aoki et al. in 2014 divided *Fusarium* species into four complexes based on RNA polymerase II subunit gene sequences (*Fusarium fujikuroi* species complex (FFSC), *Fusarium graminearum* species complex (FGSC), *Fusarium oxysporum* species complex (FOSC), and *Fusarium solani* species complex (FSSC)), but some well-known species were not assigned to any of these [15]. In 2011, Watanabe et al. used maximum likelihood method for reconstruction of the phylogenetic relationships using the following genetic markers: rDNA cluster region, *β*-tubulin (*β-tub*), translation elongation factor 1*α* (*EF*-1α), and aminoadipate reductase (*lys2*). Based on the resulting phylogenetic tree, they proposed a new classification divided into seven clades (Table 1) [16].

Obviously, this classification contained some flaws, related to the limited number of strains used, but mainly followed earlier dividing *Fusarium* into "sections" which is no longer used. More detailed studies allowed to discriminate closely related species inside the clades, and currently, many reports describing new species or chemotypes are becoming available, particularly concerning trichothecene producers from the FGSC and fumonisin producers from the FFSC.

3 Ecological Niches: From Saprotrophs to Human Pathogens

As a worldwide occurring genus, *Fusaria* are adapted to survive and spread in a wide spectrum of environmental conditions. The genus is known at best as a plant pathogen that causes yearly huge economic losses in yields of almost all crops cultivated all over the world. Spores of *Fusarium* infect plants and then develop hyphae within plant organs (e.g., leaves, stems, seeds, flowers, roots) which cause changes in host cells' metabolism, tissue destruction, and, eventually, the development of numerous diseases.

Some *Fusarium* species complexes are still classified as specialized groups within the species, so-called *formae specialis* (f.sp.) based on specific host that they are able to infect. *F. oxysporum* is the species with the largest number of *formae*

Table 1 *Fusarium* clade classification based on the multilocus sequence analysis [according to Ref. 16]

Clade	*Fusarium* species
Clade I	*F. larvarum*
	F. merismoides
Clade II	*F. dimerium*
Clade III	*F. solani*
Clade IV	*F. decemcellulare*
Clade V	*F. oxysporum*
	F. proliferatum
	F. subglutinans
	F. verticillioides
Clade VI	*F. avenaceum*
	F. lateritium
	F. tricinctum
Clade VII	*F. acuminatum*
	F. culmorum
	F. graminearum
	F. kyushuense
	F. langsethiae
	F. poae
	F. sporotrichioides

specialis. For instance, *F. oxysporum* f.sp. *lycopersici* causes wilt in tomato, while *F. oxysporum* f.sp. *cubense* causes Panama disease on banana. Other specific examples of *Fusarium* plant diseases (also called fusiariosis) are ear rot of maize (*F. verticillioides*), *Fusarium* head blight of wheat and barley (*F. avenaceum*, *F. culmorum*, *F. graminearum*), and root rot of soybean (*F. solani*) [17].

Species belonging to *Fusarium* genus are generally saprotrophic, and necrotrophs, being potentially pathogenic, are not obligatory pathogens, like biotrophic *Puccinia* spp. causing rusts in small grain cereals (mainly wheat, barley, and triticale). After the harvest, fragments of infected tissues get into the soil, where the fungi develop feeding on decayed organic matter and can survive unfavorable environmental conditions. Some of the species can form fruiting bodies which can remain viable for a very long time and then germinate at the appearance of appropriate conditions to infect plant roots. This process is a part of the vegetative development of some *Fusaria*, for example, *F. culmorum*, *F. oxysporum*, and *F. graminearum* [18–21].

Animal and human fusariosis are not as common as plant fusariosis. Human fusariosis usually occur in people with tissue breakdown or patients with impaired immune system. The symptoms of these diseases are usually keratitis and onychomycosis which are caused by *F. verticillioides*, *F. oxysporum*, and *F. solani* [22]. Mycotoxicoses occur more often than fusariosis and are the effects of ingestion of toxic fungal secondary metabolites. Exposure to mycotoxins occurs

mainly through the consumption of contaminated food, but inhalation with the air is also possible. Many of these compounds do not degrade during technological processes even under high temperature and pressure conditions. Mycotoxins are biosynthesized by fungi and secreted into host tissues where they are accumulated and transferred into food and feedstuffs. Exposure to mycotoxins may also occur through the consumption of contaminated animal products such as meat, milk, or eggs because some compounds pass from plant-derived materials to animal tissues and may be excreted with milk (e.g., aflatoxins). Numerous reports on diseases caused by *Fusarium* mycotoxins are available. Fumonisins B_1 and B_2 causing equine encephalomalacia, deoxynivalenol causing vomiting as well as diarrhea, and zearalenone causing breast cancer are among the most frequent ones [1, 23, 24].

4 Phylogeny and New Species Discovery

Exact taxonomic positioning of the *Fusarium* genotype studied is one of the most basic questions faced by researchers interested in *Fusarium* research, because mistake at this stage may have serious consequences. During last decades, three kinds of species concepts were proposed to identify *Fusarium* species:

- Morphological species concept (defined by the morphological characters of pure fungal cultures on standardized laboratory media)
- Biological species concept (using sexual crosses, aggressiveness tests)
- Phylogenetical species concept (molecular analyses defining similarities between related strains)

Sometimes, the combination of two or three of these species' concepts can be found [25–27]. Nevertheless, the use of morphological species concept for identifying species requires a skilled and experienced researcher with wide knowledge of classical taxonomy, which is nowadays more and more difficult to find. Additional problem bears in overlapping morphological characters among closely related species and new species described practically each year.

The biological characteristics based on sexual compatibility show numerous problems, such as environmental factors suppressing sexual reproduction, unequal frequencies of mating-type alleles in different populations, or failure of compatible isolates to reproduce due to male or female dominance. Moreover, the environmental conditions and genetics of the host may play significant roles in aggressiveness tests on specific host plant [28]. According to Moretti (2009), it is a great challenge to determine the taxonomic status of *Fusarium* species on the basic of their phenotypic characteristics alone, including pathogenicity and toxigenicity [29].

After the year 2000, most scientists have utilized the molecular phylogenetic approaches to ascertain the taxonomy of *Fusarium* species and have proposed new taxonomic systems based on the phylogenetic species concept [3, 7, 10, 12, 30–35]. This was a requirement that arose from the fact that several of the "traditional"

species based on morphological identification are now considered to be species complexes composed of many species [33, 36, 37].

Phylogenetic analyses of *Fusarium* isolates are being performed based on numerous diagnostic marker sequences. Among them the most common are calmodulin (*cmd*) [38], histone 3 (*HIS3*), *Tri101* [39], mating-type (*MAT*) locus [36], internally transcribed spacer regions in the ribosomal repeat region (ITS1 and ITS2) [40, 41], the intergenic spacer region (IGS) [42], the nuclear ribosomal RNA large subunit (28S or LSU rDNA), and the mitochondrial small subunit (mtSSU rDNA) [27]. Protein-coding genes are also in use, such as RNA polymerase (*RPB2*), β-tubulin (*tub2*) [43], translation elongation factor (*EF*-1α) [43–45], and ATP citrate lyase (*ACL1*) [46]. Notably, not all of these sequences work equally well showing significant polymorphism for all *Fusarium* species. For instance, the ITS regions have shown its limited usefulness within many *Fusarium* species, such as *F. avenaceum*, *F. arthrosporioides*/*F. tricinctum*, *F. sporotrichioides*/*F. langsethiae*, and the lineages of *F. graminearum* species complex, due to the occurrence of non-orthologous copies [25, 38]. Correspondingly, *β*-tubulin gene is not discriminative for genotypes from the *Fusarium solani* species complex [47]. Nevertheless, *EF*-1α, *RPB1*, and/or *RPB2* gene fragments have gained the most of the researcher's interest for the following reasons: (i) highly informative at the species level, (ii) non-orthologous copies, (iii) amplified from all species of the genus using single pairs of universal primers, and (iv) sequences from these three genes are well represented in the reference database (i.e., FUSARIUM-ID, Fusarium MLST, and NCBI GenBank) [48–50].

Phylogenetical characterization based on genealogical concordance (GCPSR), a robust method for determined species boundaries [31], has shown the severe limitations of morphological and biological species identification in *Fusarium* and accelerated species discovery inside the genus. To date, approximately two-thirds of the 300 phylogenetically distinct species-level *Fusaria* were discovered using GCPSR-based studies [51]. Moreover, continuous research investments have provided tremendous insight into evolutionary relationships within the *Fusarium* genus inferred from partial *RPB1* and *RPB2* sequences. The study determined 20 monophyletic species complexes and 9 monotypic lineages, which were named informally to facilitate the communication of an isolate's clade membership and genetic diversity [24, 52]. Based on newly discovered species, two of these monotypic lineages are currently considered as species complexes [24, 53, 54].

5 Secondary Metabolism Biosynthetic Pathways

Recently, it appeared that genomic regions involved in secondary metabolism present similarly useful or sometimes better targets for designing phylogenetic markers and their analysis [3]. The weak side of such approach is that only some of *Fusarium* species may possess the gene cluster of interest but the resolution of the genotypes obtained with SM biosynthetic sequences may be higher than that

of PM ones. Therefore, each of the clusters should be carefully and individually checked for its usefulness in the species studied.

5.1 Mycotoxins

Mycotoxins are SMs produced by vast majority of filamentous fungi, mostly under favorable environmental conditions. *Fusarium* species have the genetic potential to produce hundreds of structurally diverse SMs, most of which have poorly understood or completely unknown ecological functions [24, 55–57]. These substances are usually produced in complex biochemical processes, including polyketide, terpenoid, and amino acid metabolic pathways, and can be accumulated in crop plants. Thereby, they pose a health risk to human and livestock [4, 58–60]. Many known mycotoxins are the virulence factors related to plant disease development [61], or they might play a role in improvement of the survival of the spores and, consequently, influence the development of the producing organism by enhancing the fitness of a given community/species [59].

Throughout the past two decades, numerous studies have been made to better understand the molecular mechanisms of mycotoxin biosynthesis and the direct and indirect regulatory agents and patterns controlling these processes. Mycotoxin biosynthetic pathways involve several coordinately regulated and functionally related genes physically grouped into clusters that can be co-expressed under specified conditions. Generally, these genes can be identified through the presence of four classes of enzymes: terpene cyclases (TCs), dimethylallyltryptophan synthases (DMATSs), polyketide synthases (PKSs), and nonribosomal peptide synthetases (NRPSs) [55, 62, 63], which catalyze the condensation or rearrangement of simple molecules to form more complex structures. Typically, the clusters contain also the core genes responsible for structural modifications of the initial metabolite, transporters for metabolite transport, and transcription factors for coordinated transcriptional regulation of genes in the cluster. These chemical products undergo multiple enzymatic modifications to form biologically active SMs and are transported to their site of activity [64, 65].

The release of the full genomic sequences of *F. fujikuroi* [62] and closely related *Fusarium* species, such as *F. verticillioides* [66], *F. mangiferae*, and *F. proliferatum* [63], revealed that the species have the genetic capacity of producing even more SMs than previously thought. Before the publication of the first *Fusarium* genomic sequence, the members of the entire genus were believed to produce about 40 structurally distinct families of SMs, while some groups, for instance, fumonisins and trichothecenes, contain tens of different analogs [67, 68]. Regardless of this metabolic diversity within the genus, single species and isolates were reported to produce a relatively low number of metabolites, e.g., there is some evidence showing that *F. graminearum* produced eight secondary metabolite families, such as aurofusarin, butenolide, fusarins, trichothecenes, culmorin, cyclonerodiol, chlamydosporol, and zearalenones. However, the study of the *F. graminearum* genome sequence identified 16 PKSs, 19 NRPSs, and 8 TSs, which suggests that

a single species has the genetic potential to produce about the equal number of the SMs to that earlier claimed for the entire genus [65].

The in silico analyses of genomic sequences of a wide range of *Fusarium* species revealed surprisingly high level of differences in the distribution of secondary metabolite biosynthetic genes and, therefore, differences in the genetic potential of individual species to produce SMs [52, 66, 69]. Namely, the PKS gene *PGL1*, which is necessary for the production of a blackish perithecial pigment and a family of reddish mycelial pigments (fusarubins), was occurring in all *Fusaria* examined in multiple studies [52, 64, 70, 71]. Moreover, there are some reports indicating that the SM's biosynthetic gene clusters are well-conserved among organisms. From the evolutionary point of view, their maintenance could only be beneficial for the fungus if the final product would confer any advance to the producing organism, even if the effect of their action is subtle or not directly obvious [59, 60]. This statement applies for mycotoxins, like the narrowly distributed fumonisin and gibberellin gene clusters that are exhibited in only some species of the *F. fujikuroi* and *F. oxysporum* species complexes [72, 73]. Additionally, the fusarin biosynthetic genes, which are extensively spread in *Fusarium*, are occurring in all *F. oxysporum* isolates that have been analyzed [52].

5.1.1 Trichothecenes

Trichothecenes are the major group of mycotoxins produced by various *Fusarium* plant pathogens [61, 64, 74]. Due to their toxicity and economic significance, trichothecenes are among the best characterized mycotoxins. Structurally, they are sesquiterpenoid compounds with a tricyclic 12,13-epoxytrichothec-9-ene ring that can be chemically substituted at several positions, which result in multiple derivatives [75, 76].

There are over 200 trichothecene derivatives which can be grouped into four main groups: types A, B, C, and D. Type A trichothecenes characterized by hydroxyl, or ester substitution at C-8, contain diacetoxyscirpenol (DAS), T-2 toxin, HT-2 toxin, and neosolaniol, and T-2 toxin is the most toxic trichothecene in animals. Recently, a new chemotype has been discovered among type A trichothecenes and designed NX-2. Surprisingly, it can be produced by *F. graminearum*, which is a typical type B trichothecene producer [77, 78]. The most important producers of type A trichothecenes are *F. sporotrichioides*, *F. langsethiae*, *F. poae*, *F. sambucinum*, *F. armeniacum*, and *F. venenatum*. They may develop on variety of cereal grains especially in cold climate regions or during storage conditions [76, 79]. Type B trichothecenes contain a C-8 keto group and are produced by various *Fusarium* species, particularly from the *Fusarium graminearum* species complex: *F. graminearum* sensu lato, *F. culmorum*, *F. pseudograminearum*, and *F. cerealis*. The most common type B trichothecenes are deoxynivalenol (DON), nivalenol (NIV), and the DON-acetylated derivatives AcDONs. Type C trichothecenes are a minor group of toxins produces by several other genera of fungi, and type D includes compounds produced by *Stachybotrys* species that are considered as important indoor mold hazards [17, 74, 80].

Alongside this major metabolite, type B trichothecenes are among the most toxic mycotoxin compounds and best-studied virulence factors. The mechanism of action of this mycotoxin is based on the inhibition of protein synthesis in eukaryotes. Trichothecenes interact with peptidyl transferase enzyme binding the 60S ribosomal subunit, thus causing the inhibition of translation. Alternative mechanism of action involves the activation of numerous mitogen-activated protein kinases (MAPKs) [60]. Humans and animals that have consumed trichothecene mycotoxins present various symptoms, such as vomiting, dizziness, diarrhea, and spontaneous abortion [81]. Moreover, the potential of trichothecenes to act as virulence factors in plant-fungal interactions and elicit plant defense responses has been investigated [82]. While trichothecene production is not required for *Fusarium* to develop on the host and penetrate its tissues, they still are essential compounds for the exposure of the pathogen after initial colonization [60, 83].

The trichothecene biosynthetic (*TRI*) gene cluster is responsible for trichothecene biosynthesis and was first characterized in *F. graminearum* and *F. sporotrichioides* [84–86]. Trichothecene biosynthetic enzymes and direct regulatory proteins are encoded by 15 genes which are located at three different loci on different chromosomes: a 12-gene core *TRI* cluster [80, 87]; the two-gene locus, *TRI1* which encodes a cytochrome P450 monooxygenase and *TRI16* which encodes an acyl transferase; and a single acyl transferase gene *TRI101* locus that is responsible for esterification of acetate to the hydroxyl function at carbon atom 3 (C-3) of trichothecenes [88]. In *F. sporotrichioides*, the TRI1 enzyme catalyzes the hydroxylation of trichothecenes at C-8, and the TRI16 enzyme catalyzes esterification of a five-carbon carboxylic acid, isovalerate, to the C-8 oxygen [89, 90]. Analysis of the *TRI* loci in 16 species of *Fusarium* exposed that *TRI1* and *TRI101* are in the core *TRI* cluster in four species of *Fusarium* that are members of the *F. incarnatum-equiseti* species complex [91, 92]. It was shown that *TRI16* and *TRI10* are major transcriptional regulators of *TRI* expression [93].

The trichothecenes have a skeleton resulting from the farnesyl pyrophosphate (FPP) [94, 95]. The first step in the biosynthesis pathway is the conversion of FPP to trichodiene. This reaction is governed by *TRI5*-encoded trichodiene synthase [96]. Subsequently nine reactions follow, catalyzed by the enzymes encoded by *TRI4*, *TRI101*, *TRI11*, and *TRI3*, correspondingly, and leading to the formation of calonectrin [80]. All these steps are common for type A trichothecenes (T-2 toxin) and type B trichothecenes (NIV and DON) producing *Fusaria* [76, 80].

A comparative study showed that similar genes are functioning in *F. graminearum* and *F. sporotrichioides* [85]. For instance, *TRI7* and *TRI13* are functional only in *F. sporotrichioides* and in *F. cerealis* as well as in the strains of *F. graminearum* and *F. culmorum* producing NIV [86]. In *F. graminearum* DON producers, *FgTri7* and *FgTri13* are not functioning [86]; therefore, the biosynthesis continues directly from calonectrin with the products of *FgTri1* and *FgTri8* and leads to the formation of either 3AcDON or 15AcDON followed by DON [97]. In contrast, in NIV producers, the pathway proceeds with the product of *FgTri1* to generate 4AcNIV and the last step with *FgTri8* product giving NIV [76]. Alexander

et al. (2011) demonstrated that polymorphisms of *TRI8* resulted in the chemotype of AcDON [98]. Moreover, in *F. sporotrichioides*, which is a T-2 toxin producer, the biosynthesis pathway proceeds with the products *FsTri1*, *FsTri16*, and *FsTri8* [89, 97]. In most *F. graminearum* strains, *TRI1* is responsible for trichothecene oxygenation at both C-7 and C-8, which leads to the formation of variants like DON or NIV [99]. Nevertheless, in some *F. graminearum* strains, *TRI1* adds a hydroxyl group at C-7 only, leading to the formation of the T-2 toxin [77].

5.1.2 Fumonisins

Fumonisins are a group of mycotoxins primarily produced by *F. verticillioides*, *F. fujikuroi*, and *F. proliferatum*, worldwide pathogens of rice and maize but also found on a wide range of other agro-food crops [4, 12, 60]. Other species from the *F. fujikuroi* species complex also produce fumonisins, but they are of minor importance. Interestingly, there are also species that are fumonisin nonproducers, and for some the status is ambiguous, as at least some strains were found to produce fumonisins for *F. oxysporum*, *F. temperatum*, or *F. subglutinans* [4, 7, 8]. The synthesis of FBs in association with disease symptoms differs markedly depending on the host conditions and infected tissue type [100]. It has been shown that fumonisins produced by *F. verticillioides* have a slight impact on maize ear rot development and significant effect on maize seedling blight. The successful transformation of the fumonisin-producing genes into an endophytic, fumonisin-nonproducing *F. verticillioides* strain has converted this endophyte into a pathogen that causes seedling blight disease in maize [101, 102], strongly supporting the hypothesis that fumonisin is a pathogenicity factor during maize seedling infection [102].

At least 28 different analogs of fumonisins were described and divided into four main categories: A, B, C, and P series [103, 104]. The most important group are the B fumonisins, B_1, B_2, B_3, and B_4, mainly due to their toxicity to humans and animals. Fumonisin B_1 is also, apart from *Aspergilli*-produced aflatoxins, the most abundant and important contaminant of maize and maize-derived products. The structures of FBs were first described in 1988 and 1989 by the researchers in South Africa, New Caledonia, and France [105, 106]. The B series fumonisins have a 20-carbon polyketide backbone with terminal amine residue, several hydroxyl groups, and two propane-1,2,3-tricarboxylate esters at various positions. The A and P series fumonisins differ due to alteration or replacement of the terminal amine group, while the C series fumonisins have a 19-carbon backbone [1, 2, 17]. Fumonisins that are characterized by an unsubstituted primary amino group at the C-2, and structurally close to sphingolipids, actually can disturb the sphingolipid metabolism by inhibiting the enzyme ceramide synthase and consequently lead to the degeneration of the sphingolipid-rich tissues and disruption of cell membrane integrity [107].

Fumonisin biosynthetic (*FUM*) gene cluster has been first described in *F. verticillioides* belonging to the *Fusarium fujikuroi* species complex (FFSC), containing 17 genes encoding biosynthetic enzymes, a transcription factor, and an ABC transporter [4, 80, 103, 108]. The *FUM1* gene encodes a polyketide

synthase that catalyzes the synthesis of a linear polyketide that forms the backbone structure of fumonisins. Additionally, the *FUM8* gene runs the condensation of the linear polyketide with alanine to produce fumonisins [9], and *FUM21* encodes a Zn (II)2Cys6 DNA-binding transcription factor that positively regulates *FUM* expression [108]. The cluster also encodes an ABC transporter (*FUM19*) that provides a sort of self-protection by exporting the toxin from the cell and reducing its cellular concentration. The number, order, and orientation of genes within *FUM* cluster were specified to be similar for closely related *F. verticillioides* and *F. proliferatum* but also for *F. oxysporum*; however, only one fumonisin-producing strain O-1890 has been described in detail [6, 9, 103]. Nevertheless, the sequences flanking the *FUM* cluster seem to alter in *F. verticillioides*, *F. proliferatum*, and *F. oxysporum*, showing different genomic contexts of the *FUM* cluster in these three species and, possibly, also in other producers, like *F. nygamai* [7]. Proctor et al. (2003) determined the genomic context of the *FUM* cluster by the sequence analysis of the DNA regions flanking each side of the cluster. The analysis shows five different genomic context or genetic environments (GE), namely, GC1, GC2, GC3a, GC3b, and GC4. The one designed GC1 is devoted to the full *FUM* cluster in *F. verticillioides* [103] and for *FUM* cluster remnant in *F. musae* [109], where *ORF20* and *ORF21* represent pseudogenes, most likely the homologs of the *F. graminearum* gene FGSG_00274, and are flanking the *FUM21* side, whereas *ZBD1* and *ZNF1* are flanking the *FUM19* side. The GC2 was detected in all African clade species examined, where *ANK1* and *GAT1* are flanking the *FUM19* side and *ZBD1* and *MFS1* are flanking the *FUM21* side. The GC3a and GC3b were shown in American-clade species *F. anthophilum* and *F. bulbicola*, respectively. They have a similar structure with three genes (*CPM1*, *MF2*, and *DOX1*) flanking the *FUM19* side, differing in the *FUM21*-flanking region: in the GC3a, *FUM21* is flanked by *CPM2* and *TSP1*, while in the GC3b, there was no evidence for these genes. The GC4 was observed in *F. oxysporum* (FRC O-1890 strain), where there was no full-length gene within the Σ2800 bp region upstream of *FUM21* and a homolog of *CPM1* was flanking the *FUM19* side [59].

The fumonisin biosynthesis starts when the *FUM1* product catalyzes the condensation of nine acetate and two methyl units to form a linear, 18-carbon-long polyketide. The polyketide should be identical or similar in structure to 10,14-dimethyl octadecanoic acid. However, it is possible that the polyketide does not exist as a free acid but remains covalently attached to the phosphopantetheinyl cofactor of the PKS instead [110]. In the second step, the *FUM8*-encoded protein Fum8p catalyzes the condensation of the linear polyketide and alanine to yield a linear molecule that is 20 carbons long and has an amine at C-2, a carbonyl at C-3, and methyl residues at the C-12 and C-16 [9, 111, 112]. A third step of the pathway is catalyzed by the *FUM6*-encoded Fum6p protein and consists of the hydroxylation of the polyketide-amino acid condensation product at the C-14 and C-15 [9, 113]. The fourth, fifth, and sixth steps are the following reactions: C-3 carbonyl reduction, C-10 hydroxylation, and C-14/C-15 esterification, respectively. Metabolic profiling of numerous *F. verticillioides* mutants indicated that each of these reactions can occur independently from the others. The C-3 carbonyl reduction

is catalyzed by Fum13p [114], fumonisin C-10 hydroxylation is most likely catalyzed by Fum2p [2], and esterification of the tricarballylic moieties to the hydroxyls at C-14 and C-15 of fumonisins is catalyzed by Fum14p [115]. Although Fum14p catalyzes the C-14/C-15 esterification, analysis of gene deletion mutants indicated that Fum7p, Fum10p, and Fum11p also contribute to the formation of the tricarballylic esters [116]. The final step in the fumonisin biosynthesis is the Fum3p-catalyzed hydroxylation of the fumonisin backbone at the C-5. Fum3p is predicted to encode a dioxygenase, and its role in fumonisin biosynthesis was confirmed using enzyme assay in which the purified protein catalyzed the C-5 hydroxylation [117].

Phylogenetic discord of the *FUM* gene-based and primary metabolism gene genealogies was demonstrated, and it coincides with the differences in the *FUM* cluster genomic context, whereas it was not compatible with fumonisin chemotype differences [59]. Proctor et al. (2013) proposed that combination of a variety of dynamic processes, such as cluster duplication and loss, balancing selection, shifts in functional contrast, translation, and horizontal transfers, has shaped the evolution and distribution of some secondary metabolite biosynthetic gene cluster, as well as contributed to the metabolic diversity in fungi [59, 87, 118–121].

5.1.3 Zearalenone

Zearalenone (ZEA) is a phenolic resorcylic acid lactone mycotoxin with low acute toxicity that does not cause fatal toxicosis. It is associated mainly with maize but also occur in wheat, barley, and sorghum. Moreover, it can cause reproductive problems in farm animals, particularly in pigs. Zearalenone was first purified from a culture of *F. graminearum* and originally was designated as fermentation estrogenic substance F-2. Then, it was structurally characterized and named zearalenone [122]. ZEA is produced by several *Fusarium* species that usually also produce type B trichothecenes, and therefore it is found together with DON and NIV. Fungi belonging to the *F. graminearum* species complex are the most significant ZEA producers; however, there are other species that have been reported to produce ZEA, such as *F. culmorum* [123] and *F. cerealis* [124]. Fungi from the *F. oxysporum*, *F. solani*, and *F. fujikuroi* species complexes are not able to produce ZEA [1]. *F. equiseti-incarnatum* species complex is an exception here, as these fungi are able to produce ZEA but produce type A trichothecenes instead of type B [45, 125].

ZEA may undergo various modifications in the organisms of plants, fungi, and animals by phase I and phase II metabolism. Modified forms of ZEA found in animal feed include its reduced phase I metabolites (e.g., α-zearalenol, β-zearalenol, α-zearalanol, β-zearalanol) and its phase II conjugate forms with glucose, sulfate, and/or glucuronic acid [60]. Early chemical studies have proposed that ZEA is derived from the acetate through the polyketide synthesis pathway [126]. More recent research contributed to the development of the zearalenone biosynthetic gene cluster with two polyketide synthases, *PKS4* and *PKS13*, which have been characterized later [123, 127]. Despite the fact that biological functions of these genes have still been relatively poorly understood and some strains do not produce ZEA while still carry at least one of the genes, some evidence has been reported that the *PKS* genes

have accumulated enough intraspecific polymorphisms to be explored as promising targets for phylogenetic studies [3, 125].

5.1.4 Enniatins and Beauvericins

Enniatins (ENNs) and beauvericin (BEA) belong to a structurally and genetically related group of nonribosomal cyclic hexadepsipeptides consisting of alternating D-2-hydroxyisovaleric (d-HIV) acid and *N*-methyl-L-amino acids. The subunits are linked by peptide bonds and intramolecular ester (lactone) bonds, forming a cyclic depsipeptide [128, 129]. In the type A and B enniatins, these building blocks are typically either aliphatic *N*-methyl-valine, *N*-methyl-isoleucine, or a mixture of these amino acids [130]. In canonical beauvericin molecule, the three amino acid substituents are all aromatic *N*-methyl-phenylalanines instead of aliphatic residues [129, 131]. Also the identified three analogs of beauvericin (A, B, C) contain one, two, or three groups of 2-hydroxyisocaproic acid (HMP) instead of HIV group, respectively [132].

To date, 29 naturally occurring enniatin analogs have been identified. The most frequent variants detected in foods and feeds, especially in cereals, are enniatin A, A_1 (ENN A_1), B (ENN B), B_1 (ENN B_1), and B_4 (ENN B_4), together with smaller amounts of enniatins C, D, E, and F [128]. Enniatins are of high interest, because of their wide range of biological activities. Structural differences related to the *N*-methyl-L-amino acid are responsible for the different bioactivities of these mycotoxins. A mixture of ENNs can cause cytotoxic effects of various severities at low concentrations and on different types of cells [133]. Affected cells frequently include human cancer cells, implicating the potential use of ENNs as anticancer drugs [134]. In particular, ENNs A_1 and B_1 induce apoptotic cell death and disrupt the extracellular signal-regulated protein kinase's (ERK) activity associated with cell proliferation. This bioactivity has long been assumed to be associated with their ionophoric properties [135]. Today, the depsipeptides are known to incorporate into cell membranes and form pores with a high affinity for K^+, Na^+, Mg^{2+}, and Ca^{2+} [136]. ENNs also exhibit different biological properties, such as insecticidal and antibiotic activity against *Mycobacterium* sp. and *Plasmodium falciparum.* Of particular interest is the proven action of identified ENNs as inhibitors of major drug efflux pumps in *Saccharomyces cerevisiae* [137–140].

The chemical properties of depsipeptide compounds allow for their application in pharmaceutical products with anti-inflammatory and antibiotic properties in targeted treatment of diseases of the upper respiratory tract [141]. A mixture of enniatins was shown by Gaumann et al. (1960) to act synergistically as complex phytotoxin in causing wilt and necrosis to leaves of plants affected by *Fusarium* [142]. Pertinently, enniatins are often found in cereal grain at high concentrations, as a result of fungal infection. This fact has yet unknown implications for human and animal health, which leads to depsipeptide perception as emerging mycotoxins [143, 144].

Beauvericin (BEA) is a cyclodepsipeptide ionophore transporting monovalent cations across membranes as a free carrier uncoupling oxidative phosphorylation. BEA displays a diverse array of biological activities in vitro [145] and is one of the most potent cholesterol acyltransferase inhibitors of microbial origin. It shows

moderate antibiotic and antifungal activities; the combined use of beauvericin with ketoconazole (an antifungal drug) was found to enhance the antifungal effect, suggesting the potential use of beauvericin as a co-drug for antifungal infections in human [146, 147]. BEA has shown strong cytotoxicity to various human cancer cell lines and induced the apoptosis of some cancer cell lines by activating calcium-sensitive cell apoptotic pathways [148]. It also inhibits the directional cell motility (haptotaxis) of cancer cells at subcytotoxic concentrations [147].

ENN production is catalyzed by large multidomain protein (M = 347 kDa) – the nonribosomal peptide synthase (NRPS), known as enniatin synthetase (abbreviated as *Esyn1*) [149]. As a family of related enzymes, the fungal NRPSs are all modularly organized multienzyme complexes in which each module, located on the same protein chain, is responsible for the initiation, elongation, and termination of growing polypeptide (in this case – by ring closure). Each module of the NRPS system is composed of distinctly folded catalytic domains with highly conserved core motifs, important for their catalytic activities. A minimal (inexactly) repeated unit consists of three core domains in succession: an adenylation (A) domain which recognizes and activates the substrate via adenylation with ATP and a thiolation/transferase (T) or peptidyl carrier protein (PCP) domain which binds the activated substrate to a 4′-phosphopantetheine (PP) cofactor via a thioester bond and transfers the substrate to a condensation (C) domain which catalyzes peptide bond formation between adjacent substrates on the megasynthase complex. Several other specialized C-terminal domains involved in chain termination and release of the final peptide product have also been identified. Optional domains include methyltransferase (M), epimerization (E), heterocyclization (Cy), and oxidation (Ox) domains, which may alter the enzyme-bound precursors or growing peptide intermediates at various stages of the process. The full-length NRPS product is normally released by a thioesterase (TE) domain giving rise to free acids, lactones, or lactams. Eukaryotic NRPSs that synthesize cyclooligomer peptides assemble oligopeptide monomer intermediates by the programmed iterative reuse of their modules, which differs from the classical NRPS paradigm described in bacteria, and the resultant monomers are frequently employed in further recursive oligomerization and cyclization process [129, 150–152].

The *Esyn1*-encoded protein was previously purified and characterized by Zocher and co-workers (1982) from *Fusarium oxysporum* [153]. Biosynthesis proceeds through the condensation of three dipeptidol units followed by ring closure. The ENNs are synthesized from their primary precursors, i.e., valine, leucine, or isoleucine, D-2-hydroxyisovaleric acid, and S-adenosylmethionine. The NRPS domain architecture is composed of three functional modules: C-A-T-M (C, condensation domain; A, adenylation domain; T, thiolation/transferase domain; M, methyltransferase domain). The two adenylation domains are responsible for the specific activation of the primary substrates D-2-hydroxyisovaleric acid and L-amino acid as acyl adenylate intermediates [153–158].

A genomic locus containing the gene cluster related to beauvericin (BEA) biosynthesis in the entomopathogenic fungus, *Beauveria bassiana*, has also been cloned. Beauvericin synthetase (*bbBEAS*) consists of a single polypeptide chain with

a molecular mass of about 351 kD [159]. Similar to enniatin biosynthesis, beauvericin is also produced by a thiol template mechanism [160, 161]. However, the two depsipeptide synthetases differ in their substrate selectiveness. Beauvericin synthetase preferably accepts *N*-methyl-L-phenylalanine and some other aliphatic hydrophobic amino acids. The efficiency of incorporation into the cyclodepsipeptide framework decreases with the length of the side chain: *N*-methyl-L-phenylalanine was easily replaced by ortho-, meta-, and para-fluoro-substituted phenylalanine derivatives, as well as by *N*-methyl-L-leucine, *N*-methyl-L-norleucine, and *N*-methyl-L-isoleucine residues [149]. Consequently, significant sequence homologies to some of the *Fusarium* enzymes were found [150], establishing a common genetic background to depsipeptide biosynthesis. Previously, some *Fusarium* species like *Fusarium poae* have been reported to produce ENNs and BEA simultaneously [162, 163], which is justified by the fact that both mycotoxins share a common metabolic pathway and the co-occurrence of ENNs and BEA in field samples infected by *Fusarium* spp. has been observed [164, 165]. Previous works demonstrate high probability that even a single PCR-based *esyn1*-specific marker can detect potential producers of both toxins among *Fusarium* isolates originating from contaminated plant material [130, 163].

5.1.5 Fusaric Acid

Fusaric acid (FA) is a picolinic acid derivative which was isolated for the first time from *Fusarium heterosporum* strains. Further research have proven that other *Fusarium* species, e.g., *F. verticillioides*, *F. fujikuroi*, and *F. oxysporum*, are also able to produce this mycotoxin [166, 167]. FA shows moderate impact on mammalian health, but its high toxicity to plants is documented. It is responsible for "fusarium wilt" development through the lipid peroxidation, increase of reactive oxygen species, and, finally, host cells' death [168]. FA also causes bakanae disease in rice seedlings and has a strong antimicrobial activity, inhibiting *quorum sensing* in Gram-negative bacteria [169, 170].

Biosynthetic pathway of fusaric acid remains largely unexplored; however, the gene cluster responsible for encoding of proteins involved in this process has been identified in *F. verticillioides*. Initially, only 5 genes were described in fusaric acid biosynthetic (*FUB*) gene cluster, but just a few years later, additional 7 contiguous genes were located 14.6 kb upstream of the previous five genes identified [171, 172]. These genes are conserved in genomes of all FA-producing *Fusarium* strains, and no significant differences in cluster organization between the species have been found [167]. Functions of all 12 *FUB* genes were predicted using BLAST analysis (Table 2) [172, 173].

Fusaric acid synthase encoded by *FUB1* is responsible for the synthesis of six-carbon polyketide chain using three acetyl-CoA molecules. Fusion of polyketide chain, oxaloacetate, and amino group is catalyzed by amino acid kinase (*FUB3*). Hydrolase encoded by *FUB4* transform the product of this reaction to fusarate [171]. *FUB1* gene (designed also as *PKS21* according to the nomenclature proposed by Hansen et al. [55]) plays a significant role in FA biosynthesis. Orthologs of this gene were found in *F. fujikuroi*, *F. verticillioides*, *F. oxysporum*, *F. circinatum*, and *F.*

Table 2 Fusaric acid biosynthetic gene cluster structure – genes and their predicted functions [according to Ref. 172]

Functional gene name	Predicted function
FUB1	Polyketide synthase (PKS)
FUB2	Unknown protein
FUB3	Aspartate kinase
FUB4	Serine hydrolase
FUB5	Homoserine *O*-acetyltransferase
FUB6	NAD(P)-dependent dehydrogenase
FUB7	*O*-Acetylhomoserine (thiol-)lyase
FUB8	Nonribosomal peptide synthetase (NRPS)-like enzyme
FUB9	FMN-dependent dehydrogenase
FUB10	Fungal-type $Zn(II)_2Cys_6$ transcription factor
FUB11	Major facilitator superfamily transporter
FUB12	Fungal-type $Zn(II)_2Cys_6$ transcription factor

mangiferae [171, 173]. Deletions of *FUB1*, as well as *FUB4*, cause complete cessation of FA biosynthesis, while *FUB3* and *FUB5* silencing results in 20 to 40% drop in FA production [171, 174]. Although the processes related to FA biosynthesis are still not sufficiently understood, it is known that *FUB6*, *FUB7*, and *FUB8* genes are also crucial for the process [173].

FUB gene cluster contains two genes (*FUB10* and *FUB12*) responsible for the expression of $Zn(II)_2Cys_6$-pathway-specific transcription factors (TFs) which control the FA biosynthesis. The *FUB10* TF is directly linked to FA production, while *FUB12* TF is involved in effective FA conversion into fusarinolic acid and dehydrofusaric acid. Deletion of any of the TF genes results in decreased production of FA and its derivatives [171, 173, 174]. This process is also controlled by the global regulators, which build up complex regulatory network controlling life processes including SM biosynthesis [175]. For instance, culture medium of pH = 8 acts like a positive regulator of *FUB1* [PacC regulator], while copper, zinc and iron are negative regulators [176]. FA belongs to the nitrogen-induced SMs. High nitrogen concentrations affect the GATA-type TFs (AreA and AreB) which cause *FUB1* overexpression and, hence, increase in FA production [177]. The *Sge1* gene is another global regulator important in nitrogen-dependent FA biosynthesis. The function of *Sge1* differs between *Fusarium* species, for example, *FoSge1* regulates the conidiation and pathogenicity of *F. oxysporum*, while *F. fujikuroi FfSge1* is required for SM biosynthesis [178, 179]. *ΔSge1* mutants show reduced FA production [173, 175, 180]. *Fusarium velvet*-like complex is also involved in the regulation of the differentiation as well as the pathogens' virulence and FA biosynthesis. *Vel1*, *Vel2*, and *Lae1* genes are primary components of this complex. In *ΔVel1* and *ΔLae1* mutants, FA production was significantly lower than in the wild-type strains [180, 181]. Some reports suggest the epigenetic modifications like histone acetylation to influence these processes. Deletions in *Hda1* and *Hda2* genes, which are responsible for the expression of histone deacetylases, cause reduced FA biosynthesis in *F. fujikuroi* [180, 183].

5.1.6 Fusarins

Fusarins A, C, and D are a group of SMs built of a polyene chain linked to the 2-pyrrolidone ring. Additionally, fusarin C contains an epoxide group on the pyrrolidone ring, unlike fusarins A and D. First report on fusarin C produced by maize pathogen *F. moniliforme* (now *F. verticillioides*) has been published in 1981 in North America [184]. These mycotoxins are also produced by other *Fusaria*, e.g., *F. fujikuroi*, *F. graminearum*, and *F. venenatum* [167, 171, 185, 186]. Toxicity of fusarin C was not very widely investigated, but it was recognized by the International Agency for Research on Cancer as possible carcinogenic for human [187, 188]. Its mutagenic effect is probably related to the interaction of the epoxide group with DNA [189].

Enzymes involved in fusarin C biosynthesis are encoded by nine genes included in the *FUS* cluster. There are two versions of the *FUS* cluster organization. The first scheme is represented by *F. fujikuroi*, *F. verticillioides*, and *F. graminearum*, where the *FUS1-FUS9* genes are arranged one after another. The second one occurs in *F. solani* and in *F. circinatum* (which does not produce fusarins), where the genes *FUS9-FUS6* and *FUS2-FUS5* are separated by *FUS1* [167]. Predicted gene functions were presented in Table 3.

Fusarin C gene cluster consists of nine genes, but only four (*FUS1*, *FUS2*, *FUS8*, and *FUS9*) are essential for fusarin C biosynthesis [182]. *FUSS* was the first gene participating in fusarin biosynthesis identified, and it was described in *F. venenatum* and *F. verticillioides*. Its orthologs, *GzFUS1* and *fusA*, were identified in *F. graminearum* and *F. fujikuroi*, respectively [167, 171]. The *FUSS*-encoded protein is a combination of the polyketide synthase (PKS) and nonribosomal peptide synthetase (NRPS), an enzyme which plays a key role in fusarin biosynthetic pathway. The PKS-NRPS uses malonyl-CoA, six moieties of acetyl-CoA, and homoserine as substrates which are transformed into prefusarin [190]. Subsequently, prefusarin is oxidized by monooxygenase (*FUS8*) to form 20-hydroxy-prefusarin which undergoes epoxidation by α-/β-hydrolase (*FUS2*) to 20-hydroxy-fusarin. This product also undergoes oxidation by monooxygenase to the 20-carboxy-fusarin. Methyltransferase encoded by *FUS9* is responsible for the last substrate methylation and obtaining final product – fusarin C [167, 182].

Table 3 Fusarin biosynthetic gene cluster – gene designations and predicted functions [according to Ref. 182]

Functional gene name	Predicted function
FUS1	Polyketide synthase-nonribosomal peptide synthetase (PKS-NRPS10)
FUS2	α-/β- Hydrolase
FUS3	Glutathione S-transferase
FUS4	Peptidase A1
FUS5	Serine hydrolase
FUS6	Major facilitator superfamily (MFS) transporter
FUS7	Aldehyde dehydrogenase
FUS8	Cytochrome P450 monooxygenase
FUS9	Methyltransferase

So far, no fusarin pathway-specific transcriptional factors have been identified, but the impact of some global regulators on fusarin biosynthesis has been well-established [180]. The expression of the *FUS* genes is pH-dependent and is upregulated in acidic conditions, but PacC TF is not involved in this process. *FUS* expression is also nitrogen-dependent. The expression of the *velvet*-like complex is increased in response to high nitrogen concentrations. *Δvel1*, *Δvel2*, and *Δlae1* mutants produce significantly lower amounts of fusarins compared to the wild-type strain. The deletion of a glutamine synthetase transcriptional factor *gln1* dramatically decreases *FUS* genes' expression [182]. On the other hand, the epigenetic modifications of histones like acetylation positively influence the expression of *FUS* gene cluster [182].

5.1.7 Moniliformin

In 1973, Cole and co-workers have isolated a compound from *F. moniliforme* cultures (later properly identified as *F. proliferatum*) which they called moniliformin (MON) [191]. MON has a very simple chemical structure (3-hydroxycyclobut-3-ene-1,2-dione) and is biosynthesized also by other *Fusarium* species, e.g., *F. avenaceum*, *F. oxysporum*, *F. fujikuroi*, and *F. subglutinans* [192, 193]. This SM shows moderate toxicity toward plants and animals [144]. Moniliformin biosynthesis is a very short and simple process. Condensation of two units of acetate leads to the formation of cyclobutadione moiety, which after oxidation and dehydration results in MON synthesis [194]. Presumably due to the uncomplicated biosynthetic pathway, until now all attempts to identify specific gene cluster devoted to MON biosynthesis, as well as the pathway-specific regulators, have failed.

5.2 Pigments

Fusaria produce a wide range of pigments, with the colors from pink, through carmine red, to purple, but some species may also produce yellow and brown pigments. Pigments can be best seen during the incubation of the fungus on rich microbiological media on the plate reverse. Colors of fungal pigmentation depend on the applied medium, its composition and pH.

Most of the *Fusarium*-produced pigments are naphthoquinones and javanicin, anhydrojavanicin, fusarubin, anhydrofusarubin, bikaverin, bostricoidin, novarubin, and naphthoquinone dimer – aurofusarin belong to this group. Many of these compounds have antifungal and antibacterial properties which sometimes inhibit the development of laboratory cell lines (e.g., HeLa). In this section we present the most common *Fusarium*-produced pigments with known gene clusters: carotenoids, bikaverin, and fusarubin [195].

5.2.1 Carotenoids

Carotenoids are characteristic yellow and orange pigments produced by plants, algae, bacteria, and fungi including *Fusaria*. These pigments are tetraterpenoids

Table 4 The designations and predicted functions of the *car* gene cluster and enzymes involved in carotenoid synthesis [according to Ref. 197]

Functional gene name	Predicted function
carX	Oxygenase
carRA	Cyclase
carB	Desaturase
carO	Rhodopsin
ggs1	Geranylgeranyl pyrophosphate synthase 1
carT	Oxygenase
carD	Oxygenase
carS	Unknown

and play a role in photosynthesis, photoprotection, and plant signaling, but no other significant function besides pigmentation has been found in fungi. Carotenoids were identified for the first time in cultures of *F. aquaeductum* but later also in *F. fujikuroi* and *F. oxysporum*. *Fusarium* species are able to produce *β*-carotene, lycopene, and neurosporaxanthin thanks to the *car* gene cluster encoding enzymes involved in carotenoid biosynthesis [196, 197].

Carotenoid biosynthesis is basically a continuation of the mevalonic acid biosynthesis because of the use of geranylgeranyl pyrophosphate (GGPP) as a first substrate. *carRA* and *carB* were the first genes involved in carotenoid biosynthesis that were discovered [196]. The cyclase encoded by *carRA* catalyzes the transformation of two GGPP units into 15-*cis*-phytoene which is converted into neurosporene by desaturase encoded by *carB*. Then, this compound serves as a substrate for the *γ*-carotene formation. Two intermediate products of this reaction are possible, and the outcome depends on which enzyme (cyclase or desaturase) acts first. If desaturase is the first acting enzyme, the intermediate product will be lycopene, and *β*-zeacarotene is a product of the cyclase. Carotenoidogenesis may diverge into two ways at this point. Using the first, cyclase converts *γ*-carotene into *β*-carotene which can be transformed by oxygenase (encoded by *carX*) into two retinol units. Using the second route, *γ*-carotene is desaturated into torulene by the first oxygenase (encoded by *carT*) into *β*-apo-4′-carotenol which is finally converted into neurosporaxanthine thanks to the oxygenase action (encoded by *carD*) [196, 197]. The summary of the c*ar* gene cluster and their predicted functions is presented in Table 4.

In carotenoidogenesis light-dependent and light-independent regulators can participate. Long-lasting exposure to light stimulates expression of *carRA*, *carB*, *carO*, *carX*, and *carT* and led to pigment accumulation, while *carD* gene is insensitive to photoinduction. In turn, high nitrogen conditions repress carotenoids biosynthesis. There seems to be a significant impact of *carS* on nitrogen-dependent regulation. *ΔcarS* mutants produce higher amounts of carotenoids than wild-type in media containing high amounts of nitrogen, but this mechanism is yet not clear [197].

Table 5 Designations and predicted functions of the genes from the *bik* gene cluster responsible for the biosynthesis of bikaverin [according to Ref. 198]

Functional gene name	Predicted function
bik1	Polyketide synthase
bik2	FAD-dependent monooxygenase
bik3	*O*-Methyltransferase
bik4	NmrA-like transcriptional regulator
bik5	Fungal-type $Zn(II)_2Cys_6$ transcription factor
bik6	Major facilitator superfamily transporter

5.2.2 Bikaverin

Bikaverin is a red pigment of polyketide structure produced by a number of *Fusarium* species (*F. oxysporum*, *F. solani*, *F. fujikuroi*, *F. proliferatum*, and *F. verticillioides*), and *F. oxysporum* was the first species from which bikaverin was isolated. As with most pigments, it acts as a stress protection, for example, against UV light. Bikaverin gene cluster has been found and characterized for *F. fujikuroi* [198]. It consists of six genes, among which only three are essential for bikaverin biosynthesis. Acetyl-CoA units are condensed into prebikaverin by multifunctional polyketide synthase encoded by *bik1*. Transformation of this compound into nor-bikaverin is catalyzed by FAD-dependent monooxygenase and *O*-methyltransferase. Rework of *O*-methyltransferase leads to the final product – bikaverin. The *bik* cluster contains a gene *bik4* responsible for the expression of pathway-specific NmrA-like transcription factor [198, 199]. The organization of the *bik* cluster was presented in Table 5.

Bikaverin biosynthetic pathway is another one regulated in a nitrogen-dependent way. During nitrogen starvation, the bikaverin biosynthesis is stimulated at first, but after a few days, this process is abolished. Experiments with *ΔareA* and *ΔpacC* mutants deficient in these global regulators did not show any significant effect on the bikaverin biosynthesis in *F. fujikuroi*, suggesting the existence of other regulatory mechanisms for this process [198, 199].

5.2.3 Fusarubins

Red pigments fusarubins are produced by *F. verticillioides*, *F. graminearum*, *F. fujikuroi* as well as other *Fusaria*. Few works on fusarubin are available, and only biosynthesis of 8-*O*-methylfusarubin is clear [71]. The other compounds synthesized in the course of this biosynthetic pathway include 8-*O*-methylnectriafurone, 8-*O*-methyl-13-hydroxynorjavanicin, 8-*O*-methylanhydrofusarubinlactol, and 13-hydroxynorjavanicin and require extensive further research.

Fusarubin gene cluster contains six genes among which *fsr1–fsr3* play essential roles in 8-*O*-methylfusarubin biosynthesis. The condensation of seven acetyl-CoA units results in the formation of a heptaketide which is transformed into 6-*O*-demethylfusarubinaldehyde. These reactions are catalyzed by a polyketide synthase encoded by *fsr1*. The resulting substrate undergoes further transformation

Table 6 Fusarubin biosynthetic gene cluster's organization [according to Ref. 71]

Functional gene name	Predicted function
fsr1	Polyketide synthase
fsr2	*O*-Methyltransferase
fsr3	FAD-dependent monooxygenase
fsr4	Alcohol dehydrogenase superfamily
fsr5	Short-chain dehydrogenase/reductase
fsr6	Fungal-type $Zn(II)_2Cys_6$ transcription factor

to 8-*O*-methylfusarubin by FAD-dependent monooxygenase. Unfortunately, molecular mechanisms of fusarubin biosynthesis regulation remain unrevealed. It is only known that this process is stimulated under alkaline pH and nitrogen limitation conditions [71]. *Fsr* cluster genes were presented in Table 6.

5.3 Antimicrobials and Hormones

Fusarium SMs affect plant, animal, and human health. They very often show antimicrobial properties as well (both antifungal and antibacterial), for example, DAS, DON, and T-2 toxin. Some mycotoxins have only antibacterial effect. Beauvericin, enniatins, and fusaric acid belong to this group [200]. The role, biosynthetic pathways, and gene clusters of the abovementioned mycotoxins have been described in previous subsections. Similarly, naphthoquinones such as bikaverin and fusarubins and their derivatives having antimicrobial activities were discussed previously. Javanicin and anhydrofusarubin are antibiotics against Gram-positive bacteria, e.g., *S. aureus* and *Corynebacterium poinsettiae*. Gram-negative bacteria and filamentous fungi are resistant to naphthoquinones [201, 202]. Chemical properties and biosynthesis of these pigments were described in previous subsection. Its antibacterial properties probably are caused by electron-releasing group substitution at 2 or 3 position of the moiety [202]. Here, other metabolites with antibacterial activities were considered: antibiotic Y, equisetin, and gibberellins.

5.3.1 Antibiotic Y

Unfortunately only few reports from 1980s about the antibiotic Y are available. Antibiotic Y was isolated from *F. avenaceum*, and, hence, also avenacein Y is known [203]. The activity of antibiotic Y was investigated toward *Bacillus subtilis* and *Erwinia carotovora*, and the results show very strong antibacterial activity especially at slightly acidic pH = 6.2. This activity was even stronger than the activity of streptomycin against *Staphylococcus aureus* [204]. Notably, only minor inhibition of other fungal genera, like *Alternaria*, *Penicillium*, *Aspergillus*, and *Botrytis* was reported [205].

Table 7 *Eqx* genes and their names according to the *eqx* gene cluster as well as genes' predicted functions [according to Refs. 210, 213]

Functional gene name	*Eqx* gene cluster name	Predicted function
eqxS	*fsa1*	Polyketide synthase-nonribosomal peptide synthetase (PKS-NRPS10)
eqx3	*fsa2*	Diels-Alderase
eqxC	*fsa3*	*Trans*-acting enoylreductase
eqxD	*fsa4*	Methyltransferase
eqxR	*fsa5*	C6 transcription factor
eqxF	*fsa6*	C6 transcription factor
eqxG	*fsa7*	Major facilitator family (MFS) transporter
eqx9	*orf1*	Von Willebrand factor type A
eqxH	*orf2*	Cytochrome P450
eqx11	*orf3*	Short-chain reductases family protein
eqx11	*orf4*	Unknown protein
	orf5	Cell wall protein

5.3.2 Equisetin

Equisetin has been isolated for the first time from *F. equiseti* which gave rise to its name. This mycotoxin with antimicrobial properties inhibits the development of Gram-positive bacteria [206]. So far, equisetin was also found in cultures of *F. solani* and *F. heterosporum*. Equisetin is a phytotoxin causing seed deterioration, which simultaneously has an important pharmacological importance for human [207]. The interest in this compound increased dramatically because of its ability to inhibit the enzyme responsible for human DNA infection by human immunodeficiency virus type 1 (HIV-1) [208, 209].

Equisetin is a tetramic acid composed of octaketide linked with serine moiety. In 2005, Sims and colleagues described *eqx* gene cluster responsible for equisetin biosynthesis [210]. Eight units of malonyl-CoA are condensed and transformed by the *eqxC*-encoded enoylreductase and PKS-NRPS hybrid, encoded by *eqxS* gene. Heptaketide formed in these reactions undergoes a Diels-Alder cyclization carried out by *eqx3*-expressed protein. Further conversions are accomplished by PKS-NRPS and lead to the formation of trichosetin, which after N-methylation becomes equisetin [210–212]. Ten years after the results of Sims et al. were published [210], Kato et al. reported equisetin as an intermediate substrate in fusarisetin production and proposed new genes' designations [213]. As in other examples of PKS-NRPS-dependent biosynthetic pathways, also in this case, PKS-NRPS hybrid plays a key role for equisetin production, as *ΔeqxS* mutants completely lose their ability to produce equisetin [210, 212]. Unfortunately, there are no reports on detailed data on the regulation of *eqx* cluster are available. *Eqx* genes and their names according to the *eqx* gene cluster as well as genes' predicted functions were presented in Table 7.

5.3.3 Gibberellins

Gibberellins (GAs) are a group of well-known growth-promoting phytohormones, which are also secondary metabolites produced by some bacteria and filamentous fungi including *Fusarium*. GAs are tetracyclic diterpene acids containing 20 or 19 carbons in the cases where lactone bridge is present. Despite the fact that gibberellins are essentially plant hormones, for the first time, they were identified in *Gibberella fujikuroi* (*F. fujikuroi*), and this is where their name came from. Other *Fusarium* species have also the capacity to synthesize GAs, for instance, *F. circinatum*, *F. mangiferae*, and *F. oxysporum* produce abundant amounts of GAs. From the economical point of view, the most important gibberellins are GA_1, GA_3, GA_7, and GA_{14}, all produced by *F. fujikuroi* – the strain used most frequently in biotechnological production of GAs [62, 73, 214].

GA biosynthetic gene cluster was identified in *F. fujikuroi*, and it consists of seven genes (Table 8). The presence of this cluster was explained as a horizontal gene transfer from host plant to the pathogen [73, 215, 216]. Gibberellin biosynthesis starts from farnesyl pyrophosphate arising from the mevalonic acid biosynthetic pathway. This compound is transformed into geranylgeranyl diphosphate and then into *ent*-kaurenoic acid. These reactions are catalyzed by the enzymes encoded by *GGS2* as well as bifunctional *CPS/KS* and *P450-4* genes, accordingly. GA_{14} synthase leads to the formation of GA_{14} which is a substrate for C-20 oxidase, which forms GA_4. In turn, GA_4 may be further transformed into two ways. The first reaction is catalyzed by 13-hydroxylase and results in GA_1 production. The second one is catalyzed by desaturase and optionally followed by 13-hydroxylase, which leads to the formation of GA_7 and GA_3, respectively [73, 179, 214]. GA gene clusters of *Fusarium* species differ from each other. *F. fujikuroi*, *F. circinatum*, and *F. mangiferae* have the whole GA cluster consisting of all seven genes. Some strains of *F. oxysporum* contain also complete cluster, while in others some genes have been deleted (e.g., *P450-2*, *GGS2*, *CPS/KS*, and *P450-3* in II5 strain) or pseudogenes are present (*P450-2* pseudogene in PHW815 strain) [62].

Gibberellin biosynthesis is regulated in many ways. High nitrogen concentrations repress the production of GAs through decreased expression of nitrogen-dependent global regulators *areA*, *nmr*, and *meaB* [177]. Global regulator *Lae1* belonging to the v*elvet*-like complex is essential for GA biosynthesis. *Δlae1* mutant has abolished GA production, but, interestingly, the overexpression of histone acetyltransferase gene *HAT1* restores the GA biosynthesis in *Δlae1* mutants [180, 181]. Additional research is needed to explain this issue.

Table 8 Gibberellin biosynthetic gene cluster's organization in *F. fujikuroi* [according to Ref. 73]

Functional gene name	Predicted function
DES	Desaturase
P450-4	*Ent*-kaurene oxidase
P450-1	GA_{14} synthase
P450-2	C20 oxidase
GGS2	Geranylgeranyl diphosphate synthase 2
CPS/KS	*Ent*-copalyl diphosphate synthase/*ent*-kaurene synthase
P450-3	13-hydroxylase

6 Population and Chemotype Shifts

Combined analyses of multilocus genotyping and neutral molecular markers permit a large-scale analysis of the diversity, mycotoxigenic potential, and population structure among *Fusarium* species [217–223]. For instance, such analyses have exposed two dominant populations of *F. graminearum* in North America – NA1 and NA2 populations. The NA1 population is genetically diverse and comprises of native isolates which typically represent the 15-AcDON chemotype, whereas the NA2 population characterizes an invasive population that has undergone a bottleneck and is related with the 3-AcDON chemotype [24]. Recently, isolates possessing a novel NX-2 chemotype have been found in *F. graminearum* populations in southern Europe and in the north of the USA, which are sympatric with the NA1 and NA2 populations [77, 78, 223]. *F. graminearum* with NX-2 chemotype has undergone toxin diversification in response to the variations in selection pressure acting on the cytochrome P450 enzyme which is encoded by *TRI1* [24]. Kelly et al. (2016) suggested that adaptive constrains on the molecular evolution of trichothecene biosynthetic genes might be population- or niche-specific and, moreover, have shown that the variation of particular mycotoxins might be significant in niche adaptation [78].

Extensive research has provided tremendous insight into the genetic basis of the chemotype variation among *Fusarium* strains. On one hand, chemotype variation relates to the differences in the presence and/or absence of biosynthetic genes. For example, *TRI16* is present and functional in T-2 toxin-producing *Fusarium* species (*F. sporotrichioides* Sherb.), whereas it is not occurring or pseudogenized in the species producing NIV or DON. Similarly, the presence or absence of a functional *TRI13* is responsible for the DON and NIV chemotype polymorphism observed in *F. graminearum* and associated species [85, 86]. However, on the other hand, trichothecene chemotype variation results from the differences in function of allelic variants of the same *TRI1* gene [77]. In some *F. graminearum* strains, *TRI1* adds a hydroxyl group both at C-7 and C-8, resulting in the formation of DON and NIV [99], whereas in *F. sporotrichioides*, *TRI11* adds a hydroxyl group at C-8 only, leading to the formation of the T-2 toxin [89, 90].

The particular mycotoxin variant (chemotype) produced by an unknown isolate or a novel *Fusarium* species can readily be inferred using DNA-based methods. For instance, the *TRI5* gene which encodes trichodiene synthase [74] was one of the first ones to be used in designing the "generic trichothecene" marker [224]. Based on this knowledge, gene-specific markers were designed for identifying the particular chemotype variants of *F. culmorum*, *F. cerealis*, and *F. graminearum*. *TRI3*, *TRI7*, and *TRI13* genes were the targets in designing chemotype-specific markers which are helpful in detecting the DON, 3-AcDON, 15-AcDON, and NIV chemotypes, as well as the *TRI5* and *TRI4* for the discriminating type A versus type B trichothecene producers [224–228]. Moreover, the zearalenone chemotype has been detected in *F. culmorum* and *F. equiseti* populations using *PKS4* and *PKS13* genes from the ZEA gene cluster [125, 229, 230]. Additionally, the fumonisin chemotype was identified based on *FUM1* and *FUM8* gene-based markers among *F. verticillioides*, *F. anthophilum*, *F. fujikuroi*, and *F. proliferatum* species [2, 7, 12, 102, 231]. The

FRC O-1890 *F. oxysporum* strain has been used for the cloning and sequencing of the *FUM* gene cluster [9], although it is supposed to be the only strain of the species proven to produce fumonisins. Generally, *F. oxysporum* genotypes are regarded as able to produce fumonisins in low amounts [232, 233]; nevertheless, Stępień et al. [7] indicated that it was not possible to confirm the presence of *FUM* genes in any of the strains originating from natural *F. oxysporum* populations.

7 Conclusions

Fusarium genus appears to be very diverse, flexible, and dynamic group of fungi, able to grow and spread to new environments which includes infecting new hosts. Moreover, when climatic changes are taken into account, the population shifts and colonizing new areas become even more obvious. This unique ability depends often on the secondary metabolites produced by the fungi under specific conditions. Although the ecological roles of many of the SMs are still blurred or completely unknown, more and more researchers show their interest in revealing these issues. Apart from pure scientific curiosity, one has to keep in mind the possible use of the SMs in biotechnology, pharmacy, and medicine.

The SM biosynthetic gene clusters are an excellent model for evolutionary studies. Numerous reports on the divergence of the main pathways (e.g., trichothecenes, fumonisins, zearalenone) show that their history may be quite independent of the primary metabolic processes, implicating horizontal transfers, functional differentiations, and other rearrangements in adapting the microorganism to changing external conditions. Also, the discovery of new mycotoxin analogs is a proof for the dynamics that drives the *Fusarium* populations to develop and spread. Finally, the regulatory mechanisms of the SMs' biosynthesis are becoming much clearer each year, improving our understanding of fungal biology and biochemistry, which is particularly important in the context of the host-pathogen interactions on genetic and molecular levels. All this aspects make the future research of fungal secondary metabolism even more exciting and promising.

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Variation in Leaf-Surface and Leaf-Tissue Secondary Metabolites: Pyrrolizidine Alkaloids

11

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Contents

Abstract

Pyrrolizidine alkaloids (PAs) represent a class of typical SMs, which are constitutively formed in plants containing them and mediating plant-herbivore interactions. More than 400 PAs have been identified from approximately 6000 angiosperm species. Great diversity of PAs was found in many plants, especially in *Jacobaea* and *Senecio* plants. Leaf-tissue PA variation was found between plant species, individual plants of the same species, and even among different organs within one plant. This variation was determined by genetics, but also influenced by environmental factors. A few studies have been conducted to investigate the leaf-surface PA variations. According to the previous work on *J. vulgaris* plants and the *Jacobaea* hybrid plants, leaf-surface and leaf-tissue

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PA profiles of a particular genotype were different from one another: a number of PAs that were present in the leaf tissue at relatively high concentrations were absent from the leaf surface.

Nevertheless, positive correlations were found for the concentration of all PAs, that of the free bases, as well as that of a number of individual PAs between the leaf surface and leaf tissue. Moreover, the total amount of PAs present on the surface of the leaves was less than 0.01% of the total amount present in the leaf tissue. This makes it clear that the relationship between the leaf-surface and leaf-tissue SMs can offer an important new angle to study the insect-plant interaction mediated by plant SMs.

Keywords
Jacobaea vulgaris · *Jacobaea aquatica* · Secondary metabolites · Diversity

1 Introduction

At the end of the nineteenth century, Julius Sachs, one of the founders of plant physiology, realized that plants contained metabolites with no obvious function. Plant physiologist Albrecht Kossel designated the term "secondary" for the low-molecular-weight and seemingly nonfunctional metabolites occurring within plants [1, 2]. Meanwhile, others, such as Anton Kerner von Marilaun, Ernst Stahl, and Leo Errera, found that secondary metabolites protected plants from attack of animals [1, 3]. These so-called secondary metabolites are usually regarded to include compounds such as glucosides, saponins, tannins, alkaloids, essential oils, organic acids, and others, which are different from primary chemicals (primary metabolites, PMs) with respect to function and occurrence. SMs are not directly involved in the growth, development, or reproduction of the plant. Very often they occur in specific taxons [4].

Plants produce a high diversity of secondary metabolites (SMs). The number of compounds which are identified exceeds 100,000 [5], and the structure of at least 47,000 SMs has been described [6]. Within a particular species, or individual plant, a number of major SMs are usually accompanied by several derivatives as minor components [7]. For instance, 34 glucosinolates were found in *Arabidopsis thaliana* [8], and more than 20 indole alkaloids were produced in hairy root culture from *Rauwolfia serpentina* [9]. Besides the structural diversity, SMs often show a large variation in concentration. A good example is the variation in the total concentration of the Met-derived glucosinolates in leaves of the ecotypes of *A. thaliana*, which varied nearly 20-fold in accumulation of glucosinolates [8]. Qualitative and quantitative variation of SM in plants is determined by genetics [8, 10–13], the environment, and their interaction [14–16].

Besides SMs in leaf tissue, SMs and PMs on the leaf surface most likely play an important role in accepting a host plant [17, 18]. This has been most well studied for glucosinolates, a group of SMs present on leaf surface and in leaf tissue of cruciferous plants and function as defense chemicals against generalist herbivores,

but they are also used for host-plant recognition and selection by specialist insect herbivores such as the diamond back moth (DBM, *Plutella xylostella*) and the small white butterfly (*Pieris rapae*) [19]. Plants therefore face a dilemma with respect to the signaling of the concentrations of SMs on the leaf surface: signaling a high concentration may deter the generalist herbivores while attracting the specialist ones. For both generalist and specialist herbivores, it is important whether or not the signaling is honest, i.e., reflects the true concentration and composition of chemicals inside the leaves. Glucosinolates have been detected on the leaf surface of the cress species *Barbarea rupicola*, *B. verna*, and *B. vulgaris*, and the concentrations found on the surface were sufficient to be used by DBM as oviposition cues. However, glucosinolates were not detected on the leaf surface of other crucifers such as *Brassica napus* and *Nasturtium officinale*, although glucosinolates are present in the leaf tissue of these species at levels comparable to the three *Barbarea* spp. [20]. Very recently, Shroff et al. [21] reported that the glucosinolate profile on the leaf surface revealed differences from that in leaf tissue.

Hence, the relationship between chemical profiles of the leaf surface and leaf tissue may be species-specific, and this could offer a new angle to the study of insect-plant interactions mediated by plant SMs. The groups of SMs such as alkaloids, terpenes, flavonoids, and phenolics present on surface involve in plant chemical defense [17]. However, little is known about the relationship between these SMs on plant surface and the corresponding SMs inside plants.

2 Pyrrolizidine Alkaloids (PAs)

Pyrrolizidine alkaloids (PAs) represent a class of typical SMs, which are constitutively formed in the plants containing them and mediating plant-herbivore interactions [22]. More than 400 PAs have been identified from ca. 6000 angiosperm species [23], of which more than 95% belong to four families: Asteraceae, Boraginaceae, Fabaceae, and Orchidaceae [24].

PAs can occur in plants in two forms: tertiary amine (free base) and *N*-oxide [25–27]. Hartmann and coworkers showed that PAs are produced as *N*-oxides and are dominantly present as *N*-oxides in *Senecio* plants. The reduction from *N*-oxides to corresponding tertiary amines can happen spontaneously during alkaloid extraction, and then the high amount of tertiary PAs in the samples is an artifact [28, 29].However, recent research shows that not all PAs are exclusively present as *N*-oxides in the plants of *Jacobaea vulgaris* and hybrids between *J. vulgaris* and *Jacobaea aquatica*. In *J. vulgaris* and in hybrids between *J. vulgaris* and *J. aquatica*, some jacobine-like PAs occur in up to 50% as tertiary amines. Moreover the variation in ratio between the tertiary amines and *N*-oxides is genotype-dependent [25]. Pelser et al. [30] reported that 26 PAs (as tertiary amines) were present in 24 species of sect. *Jacobaea*. With more sensitive analytical methods for the detection, more structural PA variants can been found in such species as *J. vulgaris* and *J. aquatica* [25].

In *Jacobaea* species, all PAs except senecivernine are derived from senecionine *N*-oxide; senecionine *N*-oxide is synthesized in the roots, transported to the shoots via the phloem, and diversified into other PA structures in the shoots [28, 31]. Aside from structural diversification, PAs do not undergo any turnover or degradation [32].The diversity from senecionine *N*-oxide to other PAs comprises simple one-step or two-step reactions such as hydroxylations, epoxidations, dehydrogenations, and *O*-acetylations, as well as the more complex conversion of the retronecine into the otonecine base moiety [32]. The first specific compound of PA biosynthesis was identified as homospermidine, which is turned into a basic PA molecule with the enzyme homospermidine synthase (HSS) [33]. It was shown that the HSS-encoding gene originated by gene duplication [34], independently in unrelated angiosperm families [35]. The enzymes responsible for the PA diversification are not identified yet. It suggested that the genes encoding PA pathway-specific enzymes are regulated by a transient switch-off and switch-on mechanism rather than gain and loss, since PA distribution appears to be largely incidental in Senecio species [30]. Structures of PA detected in the *Jacobaea* hybrid system used in this study and a schematic diagram representing putative PA biosynthetic pathways are shown in Figs. 1 and 2.

PA accumulation in a particular tissue is caused by a number of interacting processes: (i) synthesis of senecionine *N*-oxide in roots, (ii) continuous long-distance translocation of senecionine *N*-oxide into shoots, (iii) differential senecionine *N*-oxide transformations in different plant organs, (iv) continuous allocation of PAs in the plant, and (v) tissue selective vacuolar storage of PAs [reviewed by 32]. In *Jacobaea erucifolia* (syn. *Senecio erucifolius*), a closely related species of *J. vulgaris*, PA biosynthesis occurs mainly in the root apex and thus coincides with the site of active root growth [37]. This coincides with the finding that in young *J. vulgaris* plants, the total PA amount in plants was positively correlated to root biomass but negatively correlated to shoot to root ratio, which suggested that PAs are produced by roots at a root-biomass-dependent rate, and the greater the shoot to root ratio, the greater the overall dilution of alkaloids [36]

3 Leaf-Tissue PA Variation in the *Jacobaea and Senecio* Plants

3.1 Interspecies Variation

Large variations of PA profiles were found among *Senecio* species [24]. PA profiles are species-specific [32]. For instance, jacobine-like PAs are very rich in some plants of *J. vulgaris*, and erucifoline-like PAs dominate in *J. erucifolia*. However, PA profiles do not represent the phylogenetic relationships between the *Senecio* and *Jacobaea* species [24, 30]. The difference between these findings on the evolutionary compared to an ecological time scale indicates that PAs probably are under selection and that it is easy to change the PA profile of plants.

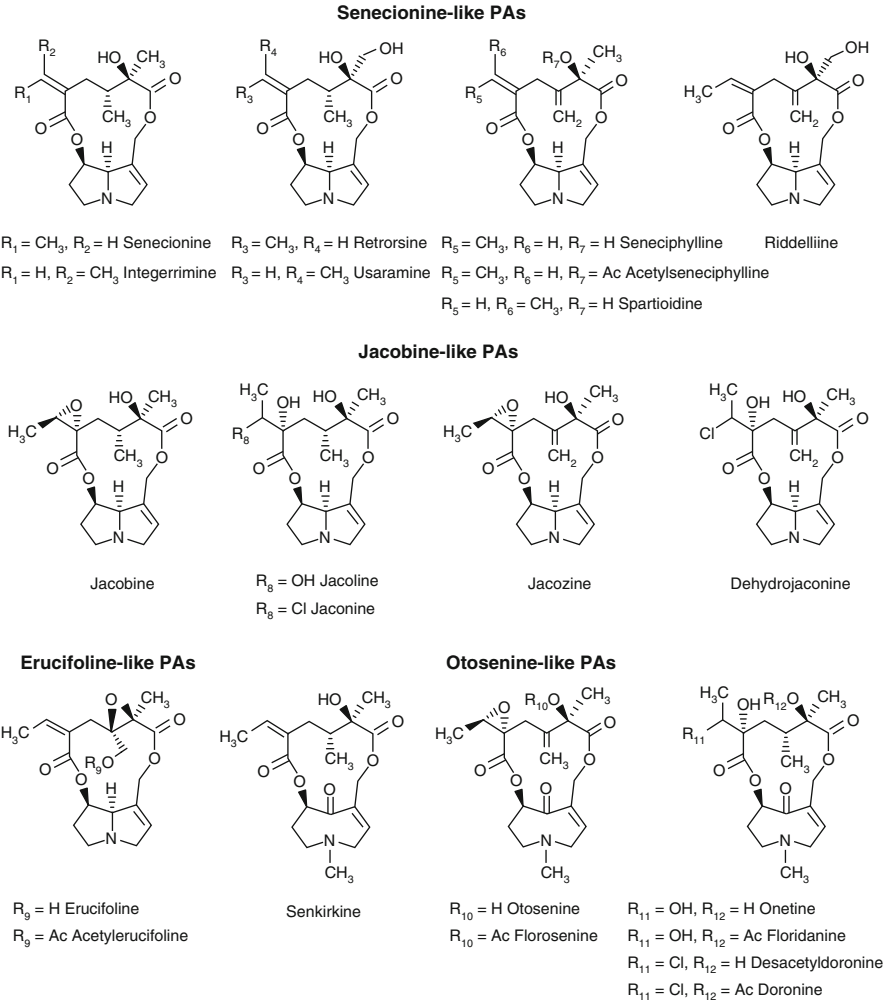

Fig. 1 Structures of the pyrrolizidine alkaloid (PAs) found in shoots and roots of *Jacobaea aquatica*, *Jacobaea vulgaris*, and F_1 and F_2 hybrids between these two species

3.2 Intraspecies Variation

PA profiles also vary within species. The famous example for the intraspecies PA variation is the presence of chemotypes of *J. vulgaris* and *J. erucifolius*. According to the evaluation of PAs of more than 100 *J. vulgaris* populations in Europe, it was found that there were two different chemotypes present: "jacobine chemotype" dominated by jacobine and its derivatives as major PAs and "erucifoline chemotypes" dominated by erucifoline-like PAs [38]. Except these two chemotypes, later on, "senecionine chemotype" (with senecionine-like PAs as dominating PAs)

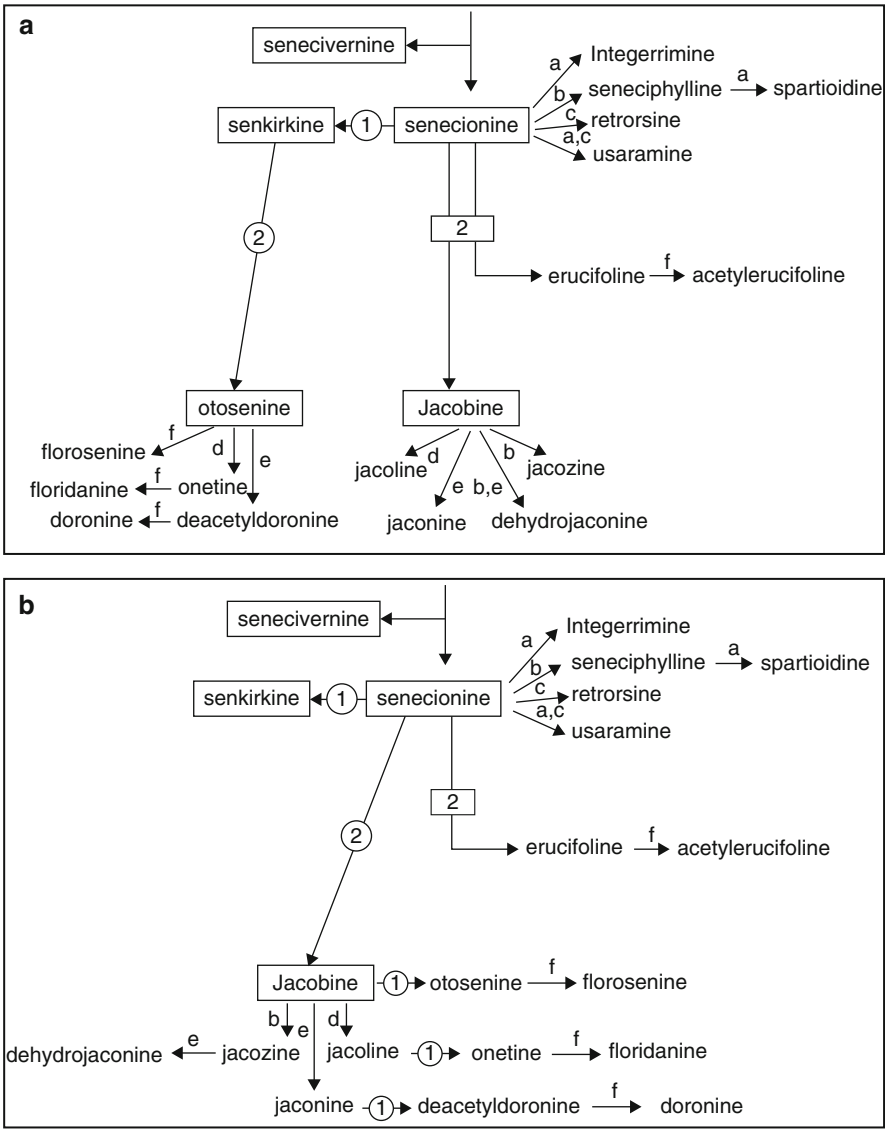

Fig. 2 Putative biosynthetic pathways for diversification of PAs in the *Jacobaea* section. With the exception of senecivernine, senecionine is the common precursor of all other PAs. Since the substrate specificity of the enzymes involved is not known, two scenarios are illustrated: (**a**) = senkirkine is assumed to be a common precursor of all otonecine derivatives; (**b**) = the otonecine derivatives originate independently from the respective retronecine derivatives. Two main reactions exist: conversion of retronecine to otonecine (reaction 1) and site-specific epoxide formation (reaction 2). Further structural diversification requires six simple one-step reactions marked by letters a–f: (**a**) Z/E isomerization at C20; (**b**) 13, 19-dehydrogenation; (**c**) site-specific hydroxylations; (**d**) hydrolysis of 15,20-epoxide; (**e**) chlorolysis of 15,20-epoxide; and (**f**) site-specific O-acetylations. (Adapted from Pelser et al. [30])

and "mixed chemotype" (with both jacobine- and erucifoline-like PAs as dominating PAs) were described [11]. The distribution of the chemotypes showed a geographic pattern: jacobine chemotypes mostly occur in the coastal areas and erucifoline chemotypes mainly in the inland of Europe [11, 38, 39]. Plants from same population often belong to the same chemotype but have variation in relation to PAs. For instance, the plants from Meijendel (Wassenaar, the Netherlands) contain mainly jacobine, but the percentage of jacobine ranged from 41% to 100% of total PA, and the percentage of erucifoline ranged from 0% to 19% of total PA [11].

3.3 Intraplant Variation

The PAs do not distribute equally over the organs of individual plants. PAs are stored in vacuoles and typically accumulate in the inflorescences and the peripheral stem tissues, i.e., epidermal and subepidermal cell layers in the plants of *Senecio vulgaris* [31]. The total concentration of PAs in vegetative *J. vulgaris* plants was found to decrease with leaf age [40], and inflorescences often have a higher concentration of PAs than leaves in reproductive vegetative *J. vulgaris* [38].

PA composition differs in the root and shoot of the vegetative plants of *J. vulgaris*, *J.* aquatica, and the F2 hybrids: generally, shoots have more variation in the composition and more jacobine-like PAs compared to the roots [10, 25]. Within a reproductive *J. vulgaris* plants, leaves have less senecionine-like PAs but more jacobine-like PAs or erucifoline-like PAs. In erucifoline chemotype the proportion of acetylerucifoline was much higher in leaves than in inflorescences [38].

PA amount on the leave surface of *J. vulgaris* plants is much lower (less than 1% of that of whole leaf) compared to that inside leaves, the concentration on leaf surface was marginally correlated with PA concentration of the total leaf tissues, and PA spectrum on the leaf differed from the PA spectrum of the total leaf [41].

3.4 Genetic Control and Environmental Influence on PA Variation

It is estimated that 50–100% of the variation in total PA concentration is due to genetic variation under climate chamber conditions [13]. PA measurement results of replicated genotypes illustrated that the PA concentration and composition were genotype-dependent [11, 25]. PA accumulation in plants is also affected by abiotic environmental factor such as nutrients and water. It was found that in drought or nutrient stress environment, the *J. vulgaris* plants tend to have higher concentration of PA than those in normal condition [42]. Increasing nutrients lead to a significant reduction in total PA concentration in shoots of *J. vulgaris* plants [43]. Hol et al. [43] postulated that the decreasing level of total PA in shoots under rich nutrient treatment may be resulted from a dilution effect: increasing nutrient supplies favor a relative increase of shoot biomass over root biomass, and as PA production increases with root growth, plants in nutrient-rich conditions relatively produce less PAs. Some genotypes of *J. vulgaris*, *J. aquatica*, and the hybrids between them produce

different PA concentrations and compositions under different nutrient and water treatments, so it seemed that PA expression was affect by genotype by environment interactions [15].

4 Leaf-Surface PA Variations of the *Jacobaea* and *Jacobaea* Hybrid Plants

4.1 Total Concentration of the Leaf-Surface PAs

Vrieling and Derridj [41] have investigated the leaf-surface PA content of eight genotypes from the wild *J. vulgaris* plants grown in a greenhouse and found that low concentrations (10–76 ng cm^{-2}) of leaf-surface PAs, corresponding to 0.10–0.89% of the total amount PA present in the leaf tissue in of natural grown *J. vulgaris* plants. The average total PA amount present on the surface of the leaves of hybrid plants from a crossing between *J. vulgaris* and *J. aquatica* was 8.0 ng (range 0.5–57.3 ng). Corrected for the leaf-surface area, the average total PA concentration was 349 pg cm^{-2}, with a range from 12.2 to 2835 pg cm^{-2} (Table 1). Expressed per leaf, the total amount of leaf-surface PAs was 8 ng (range: 0.5–57 ng), corresponding to 0.0064% (range: 0.0004–0.060%) of the total PA amount in the leaf tissue. Evidently, only a minute proportion of the PAs is deposited on the outside of the leaves [44].

4.2 Composition of the Leaf-Surface PAs

On average 77% of the total PA content present on the leaf surface of hybrid plants from a crossing between *J. vulgaris* and *J. aquatica* was in the *N*-oxide form, although a wide range (27–98%) was observed. The five major PAs found on the leaf surface were free base of jacobine and the *N*-oxides of four PAs (seneciphylline, senecionine, jacobine, and erucifoline, Table 1). Together these major PAs accounted for 83% (290 pg cm^{-2}) of the total PA concentration. Jaconine, jaconine *N*-oxide, dehydrojaconine, dehydrojacoline, and usaramine *N*-oxide were detected in most of the leaf-tissue samples at somewhat higher concentrations, but nevertheless were absent or virtually absent in the leaf-surface samples (Table 2). The results for jaconine and jaconine *N*-oxide are in agreement with the study of [41], which was unable to detect jaconine on the leaf surface.

5 Correlation Between Leaf-Surface and Leaf-Tissue PAs

Compared to the concentrations of individual PAs on the leaf surface with those in leaf tissue of hybrid plants from a crossing between *J. vulgaris* and *J. aquatica*, it was found that several otosenine-like PA (otosenine, onetine, florosenine, and floridanine) and jacobine-like PAs (jacobine, jacobine *N*-oxide, jacoline,

Table 1 Average relative concentration of the leaf-surface and leaf-tissue pyrrolizidine alkaloid (PAs) from 37 F_2 hybrid individuals of a cross between *Jacobaea aquatica* and *Jacobaea vulgaris*

Group	Pyrrolizidine alkaloid	Leaf-surface PAs		Leaf-tissue PAs	
		Relative concentration[a]	TA %[b]	Relative concentration	TA %
Senecionine-like PAs	Senecionine	15	2	4	1
	Integerrimine	3	3	3	0.43
	Retrorsine	0.46	31	0.57	2
	Usaramine	n.d.	n.d.	0.85	1
	Eruciflorine *N*-oxide	0.11	n.d.	0.01	0.00
	Seneciphylline	29	1	19	1
	Spartioidine	1	0.00	0.09	0.00
	Riddelliine	1	33	1	0.00
	Acetylseneciphylline	2	4	3	0.00
Jacobine-like PAs	Jacobine	31	31	29	29
	Jacoline	2	33	3	38
	Jaconine	0.03	n.d.	0.00	0.00
	Jacozine	3	54	6	47
	Dehydrojacoline	n.d.	n.d.	0.44	100
	Dehydrojaconine	n.d.	n.d.	0.26	81
Erucifoline-like PAs	Erucifoline	10	4	18	8
	Acetylerucifoline	1	n.d.	0.15	35
Otosenine-like PAs	Otosenine	1	100	1	100
	Onetine	0.17	100	0.12	100
	Desacetyldoronine	n.d.	n.d.	0.10	100
	Florosenine	1	100	0.21	100
	Floridanine	0.09	100	0.04	100
	Doronine	n.d.	n.d.	0.09	100
	Total PA free bases	23		20	
	Total PA *N*-oxides	77		80	
	Senecionine-like PAs	51	2	32	1
	Jacobine-like PAs	36	33	40	35
	Erucifoline-like PAs	11	4	26	6
	Otosenine-like PAs	2	100	2	100
	Total PA	349 (pg cm^{-2})		3142 (μg g^{-1})	

Adapted from Cheng et al. [44]
n.d. not detected
[a]Relative concentration = concentration of an individual or a group of PAs/total PA concentration × 100
[b]TA% = the tertiary amine concentration/(tertiary amine concentration + the corresponding *N*-oxide concentration) × 100

jacoline *N*-oxide, and jacozine) were significantly positively correlated. The other jacobine-like PAs, most notably jaconine and jaconine *N*-oxide, and the other otosenine-like PAs (desacetyldoronine and doronine) were absent on the leaf surface,

Table 2 Correlations between concentration of leaf-tissue and leaf-surface pyrrolizidine alkaloids (PAs) of 37 F_2 hybrid plants of a cross between *Jacobaea aquatica* and *Jacobaea vulgaris*

Group	Pyrrolizidine alkaloid	r/rs	Adjusted P[b]
Senecionine-like PAs	Senecionine[a]	0.11	ns
	Senecionine *N*-oxide	0.16	ns
	Intergerrimine[a]	0.01	ns
	Intergerrimine *N*-oxide	0.19	ns
	Retrorsine[a]	0.19	ns
	Retrorsine *N*-oxide[a]	0.17	ns
	Eruciflorine *N*-oxide[a]	0.62	**
	Riddelliine[a]	0.39	ns
	Riddelliine *N*-oxide[a]	0.03	ns
	Seneciphylline	0.37	ns
	Seneciphylline *N*-oxide	0.11	ns
	Acetylseneciphylline	0.47	ns
	Acetylseneciphylline *N*-oxide	0.22	ns
	Spartiodine *N*-oxide	0.38	ns
Jacobine-like PAs	Jacobine	0.74	***
	Jacobine *N*-oxide	0.69	***
	Jacoline	0.60	**
	Jacoline *N*-oxide	0.61	**
	Jacozine	0.78	***
	Jacozine *N*-oxide	0.47	ns
Erucifoline-like PAs	Erucifoline	0.44	ns
	Erucifoline *N*-oxide	0.52	*
	Acetylerucifoline *N*-oxide	0.20	ns
Otosenine-like PAs	Otosenine	0.69	***
	Onetine[a]	0.54	**
	Florosenine[a]	0.93	***
	Floridanine[a]	0.68	***
	PA free bases	0.75	***
	PA *N*-oxides	0.41	ns
	Sum concentration of senecionine-like PAs	0.19	ns
	Sum concentration of jacobine-like PAs	0.80	***
	Sum concentration of erucifoline-like PAs	0.51	*
	Sum concentration of otosenine-like PAs	0.72	***
	Sum concentration of all PA	0.50	*

Adapted from Cheng et al. [44]

[a]PAs with concentrations that were not normally distributed, for which Spearman correlation tests were carried out, while Pearson correlation tests were carried out for all other PAs

[b]P-values of the correlation testes were adjusted by Sequential Bonferroni method Significance codes: ns not significant

* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$

while jacozine *N*-oxide concentrations did not show a correlation between leaf surface and leaf tissue. Furthermore, of the senecionine-like PAs, only one minor compound (eruciflorine *N*-oxide) correlated, and of the erucifoline-like PAs, only

erucifoline *N*-oxide showed a weak correlation between leaf surface and leaf tissue. A positive correlation was also found for the total concentration of total PA, total free bases, while such a correlation was absent for senecionine-like PAs or *N*-oxides of PAs (Table 2).

There are only a few other studies that have demonstrated differences between the profiles of leaf-tissue and leaf-surface SMs. For instance, Brooks and Feeny [45] suggested that the different patterns in the leaf-tissue and leaf-surface chemical profiles of *Daucus carota* could be related to the seasonal variation. Badenes-Perez et al. reported that glucosinolate and saponin profiles differed between foliage and leaf surface of the *Barbarea* spp., *B. napus*, and *N. officinale* [20].

Moreover, the glucosinolate profile on the leaf surface revealed differences from that in leaf tissue of *A. thaliana* plants [21].

6 Conclusions

Great diversity of PAs was found in many plants, especially in *Jacobaea* and *Senecio* plants.

Leaf-tissue PA variation was determined by genetics, but also influenced by environmental factors. Few studies have been conducted to investigate the leaf-surface PA variations. According to the previous work on *J. vulgaris* plants and the *Jacobaea* hybrid plants, leaf-surface and leaf-tissue PA profiles of a particular genotype were different from one another: a number of PAs that were present in the leaf tissue at relatively high concentrations, such as jaconine and usaramine *N*-oxide, were absent from the leaf surface. Nevertheless, positive correlations were found for the concentration of all PAs, that of the free bases, as well as that of a number of jacobine- and otosenine-like PAs between the leaf surface and leaf tissue. Moreover, the total amount of PAs present on the surface of the leaves was less than 0.01% of the total amount present in the leaf tissue. This makes it clear that the relationship between the leaf-surface and leaf-tissue SMs can offer an important new angle to the study the insect-plant interaction mediated by plant SMs.

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Interactions of *Trichoderma* with Plants, Insects, and Plant Pathogen Microorganisms: Chemical and Molecular Bases

12

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Abstract

Trichoderma spp. are free-living fungi common in soils from different ecosystems, but can also establish endophytic associations with plants, roots, and seeds. *Trichoderma* are economically important due to their production of secondary metabolites of great interest in medicine, biotechnology, and agriculture. Fungal metabolites comprise nonvolatile and volatile compounds that include alcohols, aldehydes, organic acids, esters, hydrocarbonated compounds, ketones, and nitrogen- and sulfur-containing metabolites as the cyclic molecules indole-3-acetic acid and gliovirin, respectively. Fungal metabolites have been identified as natural products, and consequently, some compounds of interest have been obtained by chemical syntheses. In a natural scenario, a number of *Trichoderma* secondary metabolites have key roles regulating plant growth and development or affecting the proliferation of plant pathogenic microorganisms in the soil due to their production of antibiotics or siderophores. In this work, we consider the chemical basis for how *Trichoderma* spp. exert directly or indirectly beneficial effects on plants and control plant pathogenic microorganisms.

Keywords

Trichoderma · Secondary metabolites · Plant-microbe interactions · Biocontrol

1 Introduction

Trichoderma fungi occur as free-living organisms on the soil surface, in the soil core, or in association with belowground parts of living plants or organic material derived from dead plants and animals [1]. Since at least the 1920s, the fungi became famous for their ability to act as biocontrol agents against plant pathogens, protecting several major crops [2, 3]. Today it is well known that *Trichoderma* also has the ability to directly promote plant growth and development by the production of secondary metabolites, which play a central role in their interactions with other biota. Species like *T. atroviride*, *T. asperellum*, *T. citrinoviride*, *T. gamssi*, *T. harzianum*, *T. longibrachiatum*, *T. parareesei*, *T. reesei*, *T. viride*, and *T. virens* are the species most frequently studied due to their effect on plants and their natural products with potential application in medicine and agriculture [4–7].

The interaction strategies of *Trichoderma* with plants have been studied at various levels: (1) when the inoculum is near the root, so the fungal diffusible compounds play an important role during plant growth; (2) when the mycelium reached the plant root and both organisms physically interact, (3) when *Trichoderma* interact with plants only through the emission of volatiles, and (4) in multitrophic interaction systems, in which the beneficial effects of the fungal inoculation or its individual compounds have been tested for biocontrol purposes against plant pathogens or herbivores.

To understand the effect of the fungus on plants, it is first necessary to know the response of the fungus at different stimuli. In natural conditions, *Trichoderma*

species sense the environment and, in response to it, produce and release different kinds of molecules to cope with the stress causing profound changes in other organisms [2, 8, 9].

The chemical identification of fungal compounds has been crucial to explain the molecular mechanisms that *Trichoderma* modulate in other organisms [10–13]. Commonly, fungal metabolites have been analyzed using a combination of different analytical techniques. In general, secondary metabolites of *Trichoderma* include organic acids, esters, ethers, hydrocarbons, ketones, peptides, polyketides, pyrones, sulfur, and nitrogen-containing compounds [14].

In recent years, increased attention has been paid to secondary metabolites from *Trichoderma*. Next, we describe fungal compounds that play an important role in the interactions with plants and other microorganisms.

2 Plant-Fungus Interaction

Trichoderma spp. establish natural associations with a number of plants [8]. In a recent study addressing the endemic fungal biomes of *Trichoderma* from the Northwest Africa to New Zealand via the European Alps and Madagascar, important differences of fungal populations associated with endemic plants of those regions were reported. Particularly, the cosmopolitan plant maize (*Zea mays*) shared the majority of fungal strains (65.5%). Furthermore, for the studied regions,

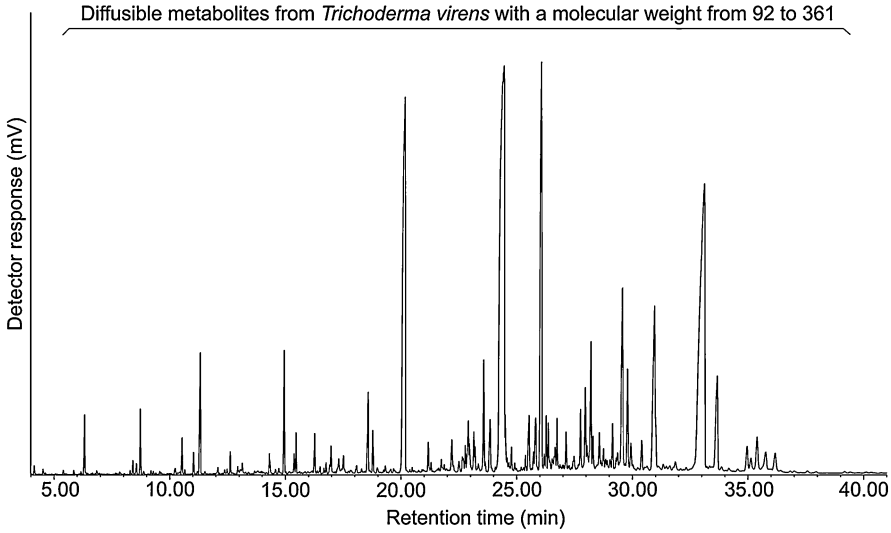

Fig. 1 Profile of secondary metabolites produced by *T. virens* Gv29-8. An inoculum of 10^6 spores was added to 1 L of potato dextrose broth (Difco®) and grown for 3 days at 28 °C with shaking at 200 rpm. Metabolites were extracted with ethyl acetate and methylated with acetyl chloride in methanol. Notice the abundance of fungal compounds in the chromatogram

Trichoderma koningii and *Trichoderma koningiopsis* presented a global fungal core community [15]. In the rhizosphere, *Trichoderma* species release constitutively a blend rich in secondary metabolites that are involved in the different plant beneficial effects that take part in the rhizosphere [7]. Figure 1 shows a metabolomic profile of *T. virens* (Gv29-8) obtained by GC-MS, which illustrates the richness and abundance of low molecular weight compounds.

Trichoderma spp. compounds include non-ribosomal peptides (NRPs), siderophores, anthraquinones, daucanes, pyrones, koninginins, trichodermamides, viridins, viridiofungins, nitrogen heterocyclic compounds, trichodenones and cyclopentenone derivatives, acoranes, azaphilones, harzialactones and derivatives, butenolides, trichothecenes, isocyano compounds, setin-like metabolites, bisorbicillinoids, diketopiperazines, ergosterol derivates, peptaibols, cyclonerodiol derivates, statins, koningic acid (heptelidic acid), and derivates [16].

Some compounds can promote plant growth, and others activate systemic resistance against plant pathogens. In the *Trichoderma*-plant interactions, it has been observed important changes in the modulation of fungal enzymes that participate in the biosynthesis of secondary metabolites. For example, *T. virens* Gv29-8 encodes the gene *TvCyt2* (a homologous protein of the p450 monooxygenase) that is downregulated at the beginning of the fungal-plant interaction. GC-MS analysis revealed that TvCyt2 is involved in the production of the compounds viridiflorol, tau-muurolol, and α-cadinol and pyrazine [1,2-a] indole-1, 4-diene, 2,3-dihydro-2-methyl-3-methylene, and those compounds triggered plant defense responses [17].

When *Trichoderma* interact with plant roots, it causes profound and substantial changes at the biochemical level, which, depending on the kind of metabolite regulated *in planta*, affect plant physiology, defense, and stress responses [18–21].

In plant tissues considerable changes in the phytohormone content after *Trichoderma* spp. inoculation have been detected [22, 23]. In the case of melon plants (*Cucumis melo*) inoculated with *T. harzianum*, significant increases in the contents of zeatin, indole-3-acetic acid (IAA), abscisic acid (ABA), 1-aminocyclopropane-1-carboxylic acid (ACC, an ethylene precursor), jasmonic acid (JA), and salicylic acid (SA) were detected in the shoot [24]. Figure 2a, b shows the growth pattern of *Arabidopsis thaliana* in control and *T. virens*-inoculated plants, respectively. Root growth promotion was correlated with the increased expression of the gene *CycB1::GUS*, a reporter of the cell division in the phase G2/M of the cell cycle (Fig. 2c, f). Here, inoculated plants presented a phenotype that resembles the effects induced by the plant growth regulator auxin (IAA) [11]. In fact, the content of phytohormones modulated by *Trichoderma* spp. in melon plants is directly related with their phenotype [25].

2.1 *Trichoderma* Root Colonization

In the beginning of the *Trichoderma*-root interaction, plants under different environmental conditions (stressed or non-stressed) release signaling molecules

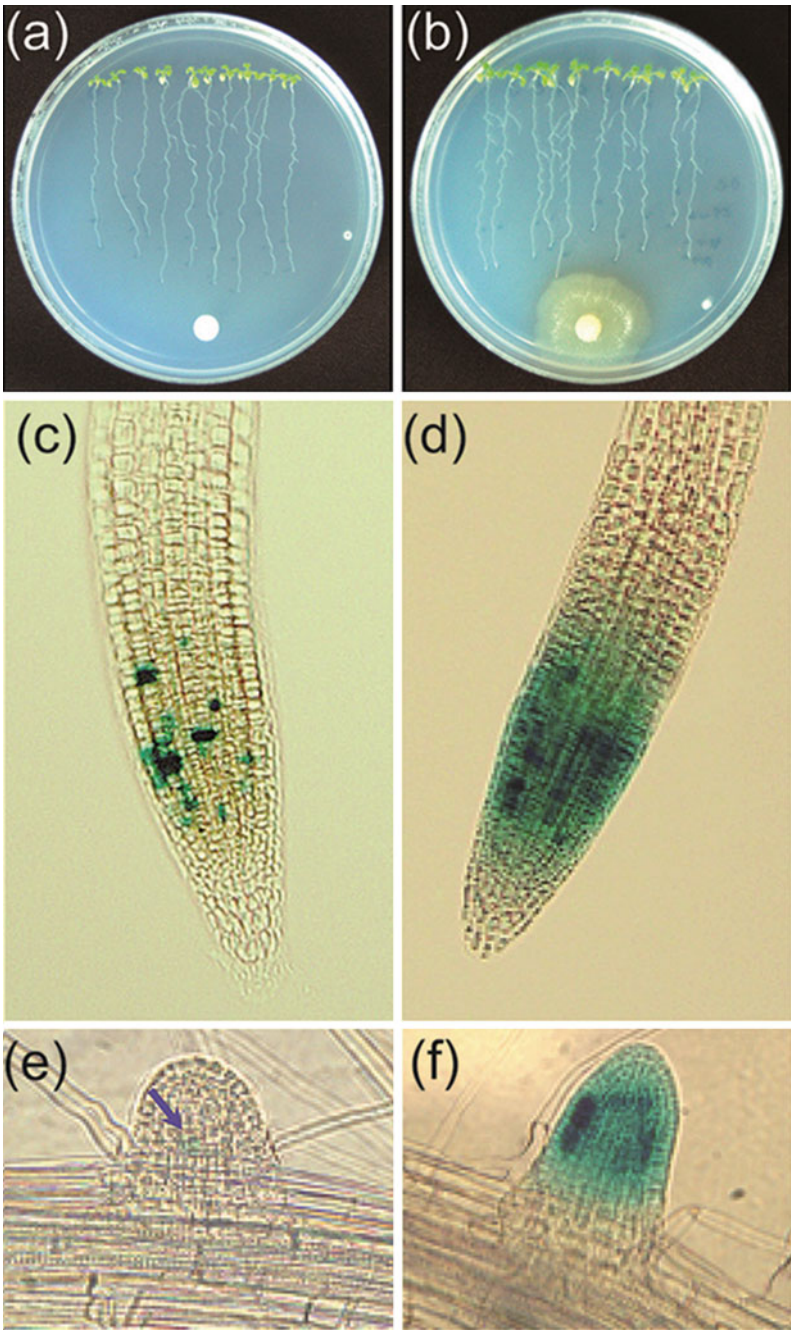

Fig. 2 *Trichoderma*-plant interaction. In vitro interaction system between *T. virens* Gv29-8 and *A. thaliana* ecotype Columbia-0. Plants were germinated and grown for 4 days on 0.2 × MS medium and then inoculated with 10^6 fungal spores and cocultured for an additional period of

that attract the fungus toward the root [26]. Sucrose derived from plants seems to be a key metabolite in the *Trichoderma*-root association [27, 28]. It is known that ThPG1 from *T. harzianum* T34 is a plant cell wall-degrading enzyme required for fungal root colonization [29]. In the fungus-root association process, these fungi penetrate the epidermis and the first cortical cell layers in the root [30, 31]. Commonly, *Trichoderma* growth is limited to the apoplast among root cells [32]. In the early stage of root colonization, the plant limits the endophytic colonization of *Trichoderma* through the cell wall reinforcement and accumulation of both antimicrobial compounds and reactive oxygen species [33].

On the other hand, it was observed that when *T. virens* colonizes maize roots through the root apoplast, the fungus releases several proteins that are likely involved in the suppression of the plant immunity, which facilitate the fungal root colonization. Fungal proteins secreted in the apoplast corresponded with cell wall hydrolysis, scavenging of reactive oxygen species and secondary metabolism [32]. *Trichoderma*-secreted enzymes that must facilitate the root colonization involve glycoside hydrolases, glycosyltransferases, polysaccharide lyases, carbohydrate esterases, and carbohydrate-binding proteins. Among these proteins glycoside hydrolases are the most abundant enzymes in *T. atroviride* IMI 206040, *T. virens* Gv29-8, *T. reesei* QM6a, *T. reesei* Rut C-30, *T. guizhouense* NJAU 4742, *T. harzianum* T6776, *T. parareesei* CBS 125925, and *T. gamsii* T6085 [31].

Plant hormones have been reported play a key role in regulating *Trichoderma* root colonization. For example, *T. harzianum* T-78 increased the root colonization of the *A. thaliana sid2* mutant that accumulates lower amount of SA compared with its background, the wild-type Columbia-0, suggesting that SA is a key regulator of *Trichoderma* root colonization [34]. Root colonization of *A. thaliana* by *T. asperelloides* T203 resulted in the substantial alteration of the plant transcriptome with marked changes in the expression of defense response-related genes [35]. When these fungi colonize roots, different plant processes at chemical, biochemical, and molecular levels are activated and can cause plant growth promotion, increased nutrient uptake, and inductions of local and systemic defense [7, 23, 36]. Concerning nutrient uptake, it is known that *T. asperellum* T42 improves tobacco plants with nitrogen utilization efficiency, which directly improved plant growth [36].

Trichoderma species also induce a beneficial impact on plants that have been grown in soils polluted with toxic elements such as sodium (Na^+) or arsenic (As). In the experimental case of *A. thaliana* seedlings grown in detrimental concentrations of salt (100 mM NaCl), *T. virens* and *T. atroviride* improved plant growth

Fig. 2 (continued) 4-days. (**a**) Control plants, (**b**) plants inoculated with *T. virens* Gv29.8. Notice the abundance of lateral root and shoot size in inoculated plants. Expression partner of *CycB1::GUS*, a transgenic marker of the phase G2/M in the division cell cycle in the root tip of the primary root of (**c**) a control plant and (**d**) a plant inoculated with *T. virens* as indicated above. (**e** and **f**) show the expression of the *CycB1::GUS* marker in lateral roots of control and *T. virens*-inoculated plants, respectively. Notice that *CycB1::GUS* is upregulated in the presence of the fungus, which suggests a modulation of the division cell cycle

through the activation of the auxin signaling and biochemical changes that included the accumulation of ABA, the antioxidant compound ascorbic acid, and the osmolyte L-proline [20]. In the case of *Eucalyptus globulus* grown in the presence of As, an element that decreases also plant growth, *T. harzianum* promoted *E. globulus* growth and induced the accumulation of chlorophyll, and in this scenario, *T. harzianum* increased As accumulation in *E. globulus* roots, thus showing As bioremediation potential [37].

2.2 Fungal Metabolites Involved in Plant Growth

Microbial synthesis of the phytohormone auxin has been known for a long time. This property is best documented for fungi that interact with plants because fungal auxin can interfere with many plant developmental processes [38]. Based on the occurrence of IAA intermediates described in plants, different pathways that share L-tryptophan (L-Trp) as a common precursor have been reported in microorganisms [39]. Production of IAA through the indole-3-pyruvic acid (IPA) pathway was identified in the fungus *Colletotrichum acutum* [40]. HPLC analysis and chromogenic stains after a fluorescence TLC separation unambiguously identified IAA, indole-3-ethanol (IEt), indole-3-acetaldehyde (IAAld), and IPA from cultures supplemented with L-Trp. Interestingly, increasing L-Trp concentrations drastically increased the levels of IEt but not IAA [40].

The ability of *Trichoderma* to produce indole auxins in pure culture has been demonstrated when L-Trp was provided in the medium [41]. Contreras-Cornejo and coworkers [11] reported that culture filtrates of *T. virens* Gv29-8 contained IAA and its concentrations increased from 13.48 ± 0.97 to 233.64 ± 3.06 μg l^{-1} when 100 mg l^{-1} of L-Trp were added to the culture medium. Furthermore, *T. virens* also produced the indole-derived compounds IAAld, IEt, and indole-3-carboxaldehyde (ICAld), likely involved in the biosynthetic and catalytic pathways of IAA. Other fungi such as *Amanita muscaria*, *Paxillus involutus*, *Suillus luteus*, *Suillus bovinus*, and *Rhizopogon luteolus* isolated from *Pinus sylvestris* also produce auxins [42].

Pharmacological analysis revealed that plants have different sensitivity to indole-derived substances during *Trichoderma*-plant interactions [11, 23]. For example, IAAld induced lateral root and root hair formation in *Arabidopsis thaliana*, but ICAld induced adventitious root formation [11, 23]. *T. asperellum* promotes maize seedlings growth and produces IAA in concentrations of 72.52 ± 15.14 μg/g of dry weight. In addition, *T. asperellum* increased the IAA content in the shoot and root of maize plants. Most likely, *T. asperellum* promoted the maize growth by activating the plasma membrane H^+-ATPase [43]. Figure 3 shows chemical structures of indolic compounds identified in *T. virens* and *T. atroviride* and the proposed biosynthetic pathway for IAA.

T. virens and *T. atroviride* also produce ethylene (ET) a gaseous hydrocarbonated compound derived from L-methionine [44]. In plants, ET induces root hair formation and controls root branching in a cross-talk mechanism with IAA [44]. More recently, it was identified that *T. virens* and *T. atroviride* also produce *cis*,

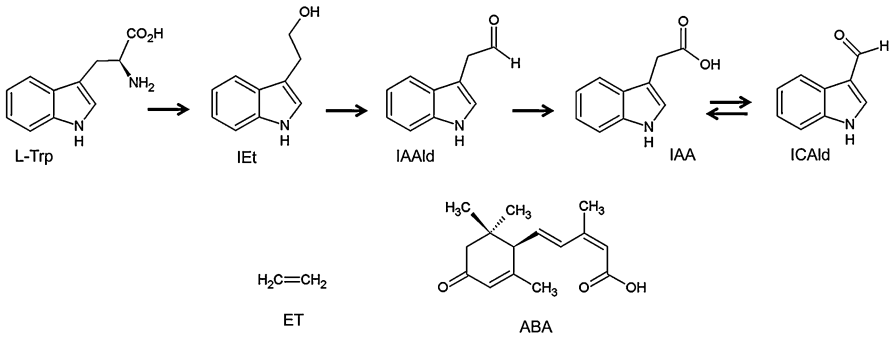

Fig. 3 Fungal compounds identified in *Trichoderma* that regulates plant growth. IAA, ET, and ABA are classical phytohormones. Different indole-derived compounds show the potential pathway for IAA biosynthesis through L-Trp

trans-abscisic acid (ABA) an isoprenoid compound, which in plants acts as hormone regulating mainly the aperture of stomata [45]. Chemical structures of ET and ABA are shown in Fig. 3.

Trichoderma species also produces different metabolites that have been considered as classical plant growth regulators like auxins, ET and ABA, which have very different molecular structures and chemical identities (Fig. 4). Some *Ascomycota* fungi also produce the simple pyrone 6-pentyl-2*H*-pyran-2-one (6-PP), which is a flavoring agent responsible for the coconut aroma associated with *T. harzianum*, *T. viride*, and *Trichoderma koningii*. In *T. atroviride* IMI206040, 6-PP is a product derived from linoleic acid (LA), and in the biosynthetic mechanism, LA is oxidized to 13-hydroperoxide-diene (13-HPOD) followed by the formation of 5-hydroxy-2,4-decenoic acid by β-oxidation and isomerization, and a final esterification then results in 6-PP [1, 46]. Four analogues of 6-PP have also been isolated from *Trichoderma* species: the 6-(1′ pentenyl)-2H-pyran-2-one produced by *T. harzianum*, the hydro-derivatives massoilactone and δ-decanolactone produced by *Trichoderma* spp., and the viridepyronone isolated from *T. viride* [16].

Cyclonerodiol is a sesquiterpene isolated from *T. koningii* and *T. harzianum* and has been shown to inhibit growth of etiolated coleoptiles of wheat plants [47]. *T. harzianum* and the strain F-1531 also produce harzianic acid, a compound that presents a pyrrolidinedione ring with the ability to regulate tomato growth [1, 48–50]. Other fungal compounds that also can alter plant growth in a dose-dependent manner are harzianolide, a butenolide-derived compound; harzianopyridone, a penta-substituted pyridine cyclic compound produced by *T. harzianum*; koninginins A, B, D, and E chemically identified as complex pyranes isolated from some species of *Trichoderma*; and trichocaranes A, B, C, and D considered as daucane sesquiterpenes or caronates [51].

Moreover, production of gluconic, citric, and fumaric acids by *Trichoderma* decreases soil pH, which might favor solubilization of phosphates, and mineral cations as iron, manganese, and magnesium [7, 10, 16, 51]. In contrast, trichosetin,

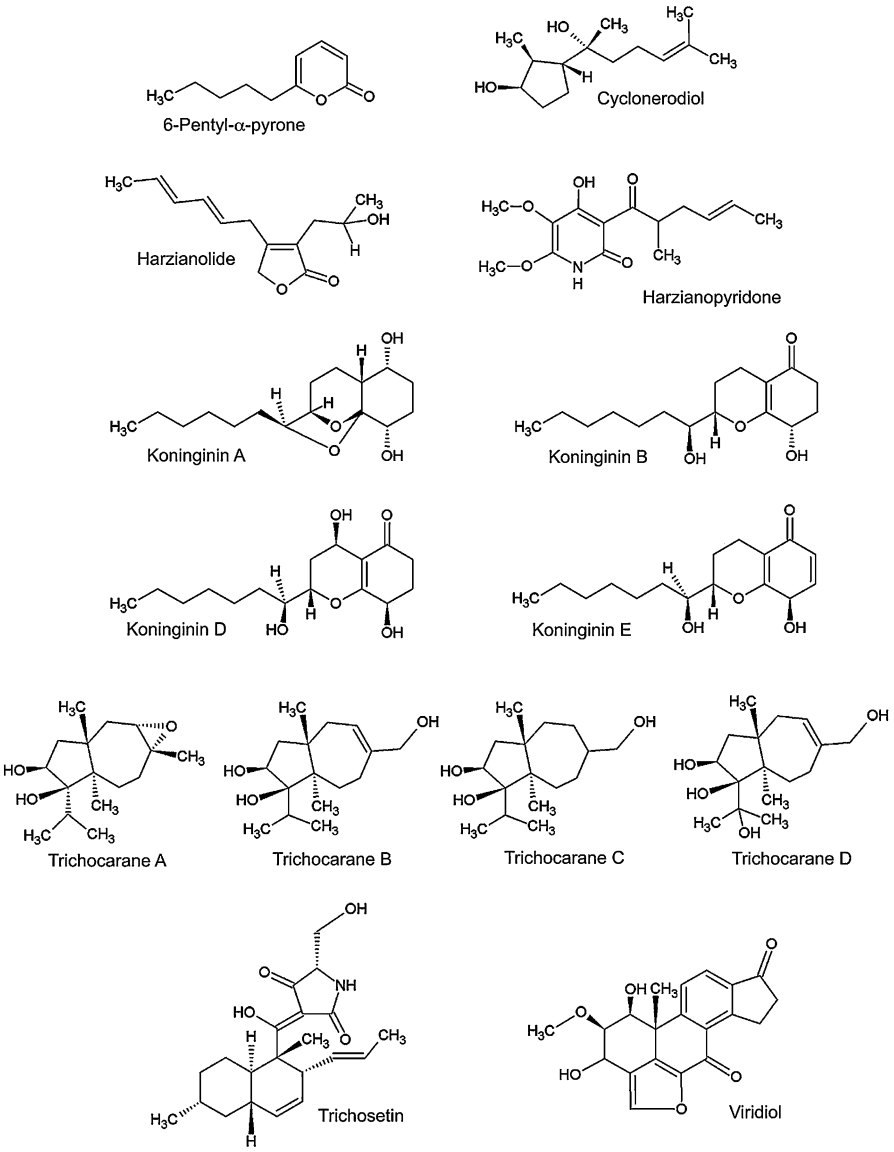

Fig. 4 Secondary metabolites from *Trichoderma* that alter plant growth. Notice that these compounds have very different molecular structures among them and with the canonical phytohormones

a setin-like compound identified in the cocultive of *T. harzianum* and *Catharanthus roseus*, inhibited the plant growth of *Oryza sativa*, *Vigna radiata*, *Medicago sativa*, *Capsicum frutescens*, and *Lycopersicum esculentum* [52]. Similarly, viridiol, a steroidal compound produced by *T. viride*, has phytotoxic activity [16].

2.3 Plant Immunity Enhanced by *Trichoderma* Species and Their Fungal Metabolites

Trichoderma can induce an enhanced defensive capacity in plants that provide protection against a broad spectrum of plant pests [5, 7, 34]. Fungal molecules that trigger plant defense responses are known as elicitors. In general, glycoproteins, carbohydrates, fatty acids, peptides, and extracellular microbial enzymes are non-specific elicitors [12, 53].

The signal molecules involved in the establishment of plant defense responses elicited by *Trichoderma* are just beginning to be identified [7, 12, 16]. Several *Trichoderma* species produce trichothecenes, most notably trichodermin and harzianum A (HA) [54, 55]. Recently, Malmierca and coworkers [56] reported that disruption of the gene *tri4* of *Trichoderma arundinaceum* IBT 40837 (Ta37), which encodes a cytochrome P450 monooxygenase that oxygenates trichodiene to give rise to isotrichodiol, reduced the antifungal activity against *B. cinerea* and *R. solani* and the ability to induce the expression of SA- and JA-responsive genes in comparison with the wild-type strain, indicating that HA plays an important function in the sensitization of Ta37-pretreated plants against pathogens. Furthermore, trichodiene is able to elicit the expression of *Botrytis* genes involved in the synthesis of botrydial and also induces the terpene gene expression in *Trichoderma* strains [57].

Djonovic and coworkers [58] analyzed the pattern of proteins secreted by *T. virens* strain Gv29-8. Electrophoretic analysis of protein extracts revealed a remarkable abundance of a low molecular weight protein. The protein was designated as Sm1 (small protein). The amino acid composition of Sm1 revealed a high percentage of hydrophobic residues (40%), including four cysteines and three tryptophans, and its characteristics were consistent with those reported to fungal elicitors. The exogenous application of the proteinaceous elicitor Sm1 of *T. virens* in cotton (*Gossypium hirsutum*) roots induced the expression of defense-related genes such as *GLU* (β-1,3-glucanase), *CHT* (chitinase), *POD6* (peroxidase), and *GhLOX1* (lipoxygenase1). Furthermore, 0.5 nmol of Sm1 in cotton cotyledons was able to induce resistance against the foliar pathogen *Colletotrichum* sp. Sm1 also triggered defense responses in maize plants [59]. Similarly, *T. atroviride* secretes a Sm1-homologous protein, Epl1, in the presence of maize roots which is released as dimer, but in the monomeric form triggers effective defense responses against the pathogenic fungus *Colletotrichum graminicola* [60]. *Trichoderma formosa* also produces a small peptide elicitor of plant defense homologous similar to the cerato-platanin Epl1. Epl1 from *T. formosa* is a 12 kDa peptide [61]. Ruocco and coworkers [62] reported that *T. longibrachiatum* MK1 and other fungal strains produce a hydrophobin type II that has 71 amino acids and a molecular weight of 7218 Da, which is able to activate plant defense and enhance root branching in tomato seedlings.

Different *Trichoderma* strains also produce non-ribosomal peptides (NRPs) that activate plant defense responses and have antibiotic properties against different types of fungi [12, 30]. NRPs result from fusion of at least two amino acids by multi-modular mega-enzymes, called non-ribosomal peptide synthetases (NRPSs) outside

the ribosome, and in some cases followed by secondary modifications, peptabiotics, and epidithiodioxopiperazines are some kind of NRPs from *Trichoderma* [1].

Species of the genus *Trichoderma* are prolific producers of peptaibols which may contain 7–20 amino acids and characteristically have an acylated N-terminal group, C-terminal amino alcohol, and a high content of 2-amino-isobutyric acid (Aib) [16, 63]. The first identified peptaibol of this class is known as alamethicin from *T. viride* [64]. This peptaibol has antimicrobial activity against gram-positive bacteria and also is a potent elicitor of volatile compounds in lima bean (*Phaseolus lunatus*). Other peptaibols are suzukacillin A, trichorovins, trichodecenins I and II and trichocellins from *T. viride*, trichokonins V–VIII from *T. koningii*, trichobrachin A I–IV and B I–IV from *T. longibrachiatum*, atroviridins from *T. atroviride*, etc. [16, 65–68]. Other characterized peptaibiotics are 14 12-residue trichocryptins B, 12 11-residue trichocryptins A, 19 11-residue trichobrevins A and B, 6 10-residue trichoferins, and 17 8-residue trichocompactins [69]. The trichogins A from *T. longibrachiatum* and the trichodecenins from *T. viride* are an example of lipopeptaibols [65, 70].

Viterbo and coworkers [71] described that *T. virens* Gv29-8 produces at least three lengths of peptaibols (11, 14, and 18 residues long). It was found that synthetic 18mer peptaibols, TvBI (Ac-UGAVUQUAUSLUPLUUQV-OH) and TvBII (Ac-UGALUQUAUSLUPLUUQV-OH) as *T. virens*, elicited cucumber (*Cucumis sativus*) defense responses that were effective against the leaf pathogen *Pseudomonas syringae* pv. *lachrymans*. Similarly, the peptaibols 11mer and 14 mer also from *T. virens* seem to be involved in the activation of defense responses in *A. thaliana* via SA [12]. Clearly, this information is evidence that NRPs play key roles in the *Trichoderma*-plant chemical communication to stimulate plant immunity.

On the other hand, the low molecular weight compound 1-octen-3-ol, a typical oxylipin from fungi, activated jasmonic acid/ethylene-dependent and wound-dependent defense genes such as *AOS*, *HPL*, and *PDF 1.2* (*PLANT DEFENSIN1.2*) and enhanced resistance against *B. cinerea* in *A. thaliana* [53]. Plant defense activation by *Trichoderma* also includes the induction and accumulation of phytoalexins. Camalexin is the main phytoalexin of *A. thaliana* that can be stimulated after infection with bacterial and fungal plant pathogens [23]. A number of aldehydes possess the ability to react with cysteine to form the corresponding thiazolidine carboxylic acid [72]. It has been reported that the synthesis of camalexin may proceed through the condensation of ICAld with L-cysteine followed by a two-step oxidation and decarboxylation [73]. Interestingly, a study showed that *T. virens* and *T. atroviride* or the application of ICAld increased camalexin accumulation in *A. thaliana* seedlings [23].

Nitric oxide (NO) is a key molecule for regulation of plant defense responses, and it is rapidly generated after plant-microorganism interaction [74]. There is evidence that *T. asperelloides* suppresses the NO generation stimulated by the plant pathogen fungus *Fusarium oxysporum* in *Arabidopsis thaliana* roots, most likely to prevent toxic effects caused for this reactive species [75]. Supporting this reasoning is the finding that *T. harzianum* T-22 enhanced the antioxidative mechanisms involving higher activity of ascorbate, glutathione, superoxide dismutase catalase, and

ascorbate peroxidase enzymes in tomato (*Lycopersicum esculentum* L. cv. Jubilee) seedlings grown under abiotic stress conditions [76].

3 Fungal Metabolites Involved in the Biocontrol Activity of *Trichoderma* Species

Plant beneficial microorganisms can provide an initial barrier against pathogen attack on the root. The protection by *Trichoderma* spp. has been reported for several plants against several plant pathogens [77]. There are several mycoparasitic species that are able to attack and lyse plant pathogenic fungi such as *Alternaria alternata*, *Botrytis cinerea*, *Rhizoctonia solani*, *Sclerotinia sclerotiorum*, *Pythium* spp., and *Fusarium* spp. [30, 78]. For example, *T. ovalisporum* DIS 70a, *T. stilbohypoxyli* DIS 259j, and *T. theobromicola* DIS 376f delayed the disease development caused by *P. capsici* in hot pepper (*Capsicum annuum*). Figure 5 shows the repression of *B. cinerea* and *P. cinnamomi* by *T. virens* Gv29-8 under in vitro conditions.

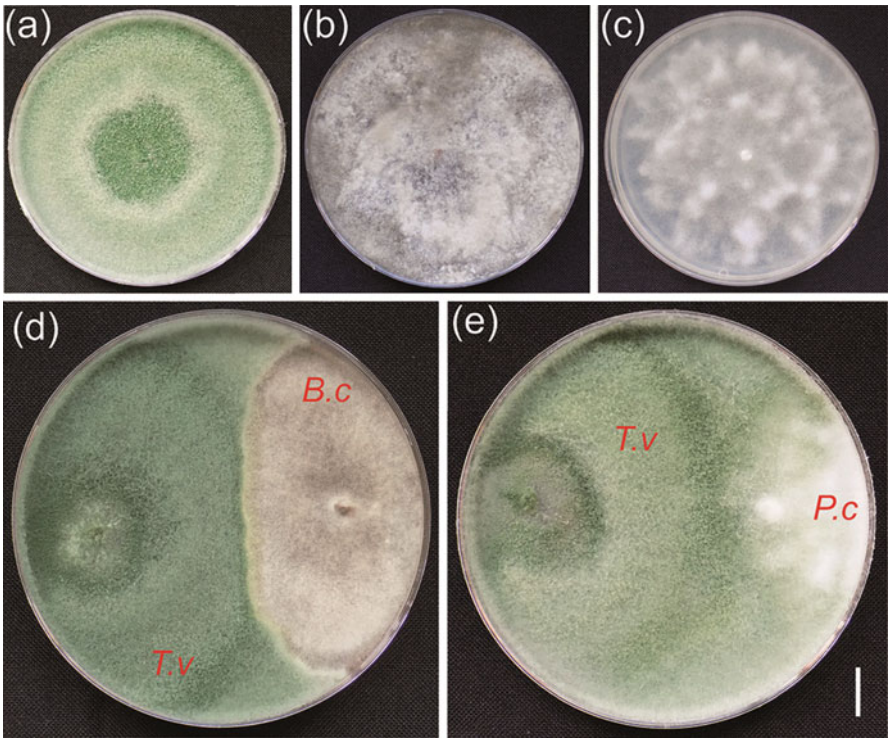

Fig. 5 Biocontrol of *T. virens* Gv29-8 on phytopathogenic microorganisms. Images from a to e show the microbial growth on commercial potato dextrose agar after 10 days of monocultive or cocultive. (**a**) *T. virens (T.v)*, (**b**) *B. cinerea (B.c)*, and (**c**) *P. cinnamomi (P.c)*. Confrontation between (**d**) *T. virens* and *B. cinerea* and (**e**) *T. virens* and *P. cinnamomi*. Notice that the growth of both phytopathogenic microorganisms is restricted by *T. virens*. This growth repression involves several processes like mycoparasitism, antibiosis, and competence for space and nutrients. Bar = 1 cm

Direct biocontrol mode of action in *Trichoderma*-plant pathogen interaction includes mycoparasitism and antibiosis [2, 70, 79, 80]. Other *Trichoderma* mechanisms to exert biocontrol against plant pathogenic microorganisms involve also competition by nutrients and space [28, 81]. Mycoparasitism involves host recognition, attachment to and coiling around the host hyphae. This mechanism requires tropic growth of the biocontrol agent toward the targeted fungi, lectin-mediated coiling of *Trichoderma* hyphae to the pathogen, and finally the attack [79]. Furthermore, during mycoparasitism, secretion of antibiotic metabolites also takes place, resulting in disarming the pathogen and killing it [77, 78, 82]. For example, the anthraquinone pachybasin, identified in *T. harzianum*, increases the number of coils of the biocontrol agent against *R. solani* [83]. During mycoparasitism production and release of atpenins, potent and specific inhibitors of mitochondria metabolism in the parasite have also been reported [84].

Mycoparasitism has been also studied at the genetic level. The transcriptome response of the biocontrol agent *T. atroviride* with the plant pathogens *B. cinerea* and *R. solani* revealed that some genes of *T. atroviride* were upregulated in the early stage of the physical contact between microorganisms. The upregulated genes involved those for nitrogen metabolism, stress response, signal transduction, and lipid catabolism [85]. Undoubtedly, mycoparasitism is a complex process that is fine-tuned by *Trichoderma* to coordinate the gene expression and production of effective secondary metabolites against its prey.

3.1 The Role of Released Fungal Siderophores in the Rhizosphere

Trichoderma produce several compounds that chelate iron and form Fe (III) complexes. Then, the microorganisms can reutilize sequestered iron in a physiological mechanism where the charged siderophore is taken up by ferric-chelate transporters [51, 86]. Coprogen, coprogen B, ferricrocin, and fusarin are well-known fungal siderophores (Fig. 6). A recent study has shown that *T. atroviride*, *T. asperellum*, *T. gamsii*, *T. hamatum*, *T. harzianum*, *T. virens*, *T. polysporum*, and *T. reesei* produce coprogen, fusigen, fusarin A, and ferricrocin [87]. Fungal siderophores are catalyzed by NRPS, generally from L-ornithine-derived *N*5-acyl-*N*5-hydroxy-L-ornithine with different possible acyl groups whereby the NRPS covalently links these units via ester or peptide bonds to linear or cyclic oligomers that later can be modified to give different siderophores [1, 87, 88]. The production of siderophores can stop the growth of plant pathogen microorganisms by depriving them of iron. These compounds can solubilize unavailable iron for plants [51]. More recently, it was reported that HA also is a metabolite that binds iron with good affinity [89].

3.2 Antibiotic Production by *Trichoderma*

Trichoderma species are known due to their ability to produce metabolites with antibiotic activity (Fig. 7). Of these, alkyl pyrones, isonitriles, polyketides,

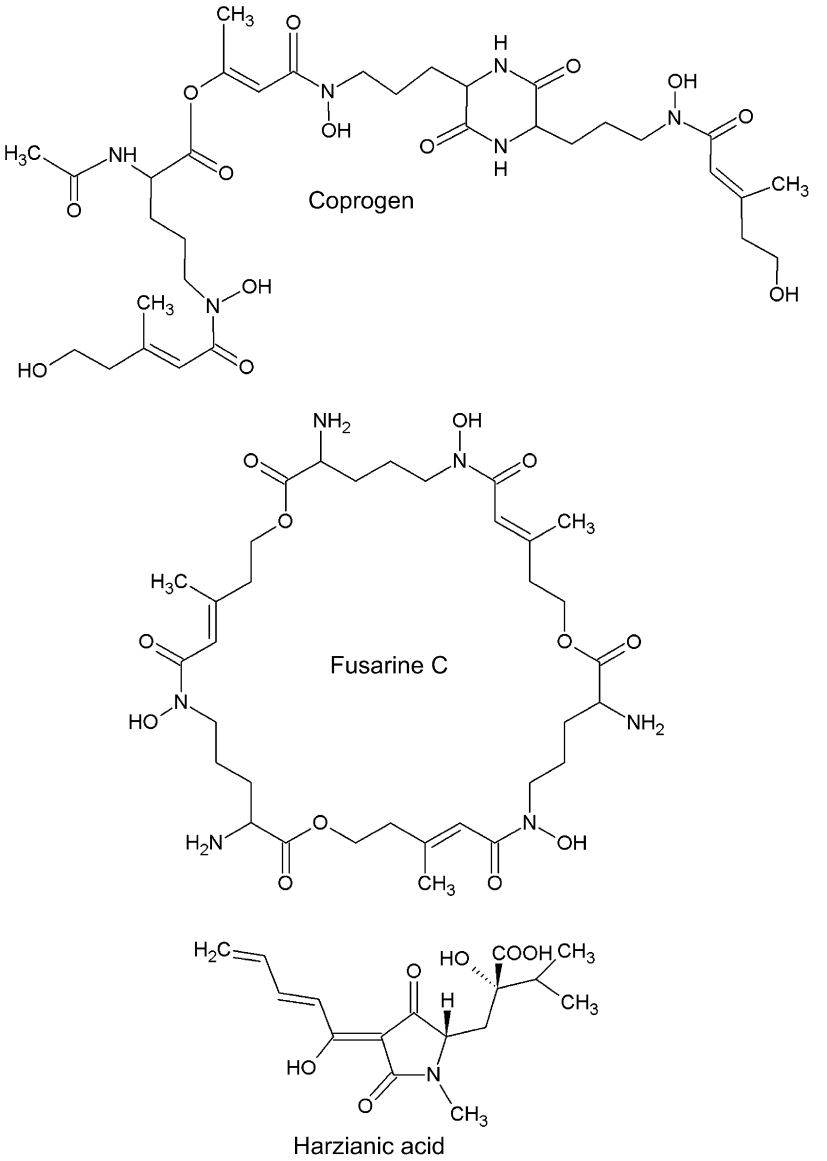

Fig. 6 Chemical structures of *Trichoderma* siderophores. These compounds have key roles in interactions with plants and iron deprivation to other rhizospheric microorganisms. Notice the position of the oxygen in the fusarin C that easily can chelate iron in the center of the molecule

peptaibols, diketopiperazines, sesquiterpenes, and steroids are fungal metabolites with antibiotic properties frequently associated with their biocontrol activity [79, 90]. For example, addition of 0.3 mg/ml of 6-PP to agar medium caused a 69.6% growth reduction in *R. solani* and a 31.7% reduction in *Fusarium oxysporum* f. sp.

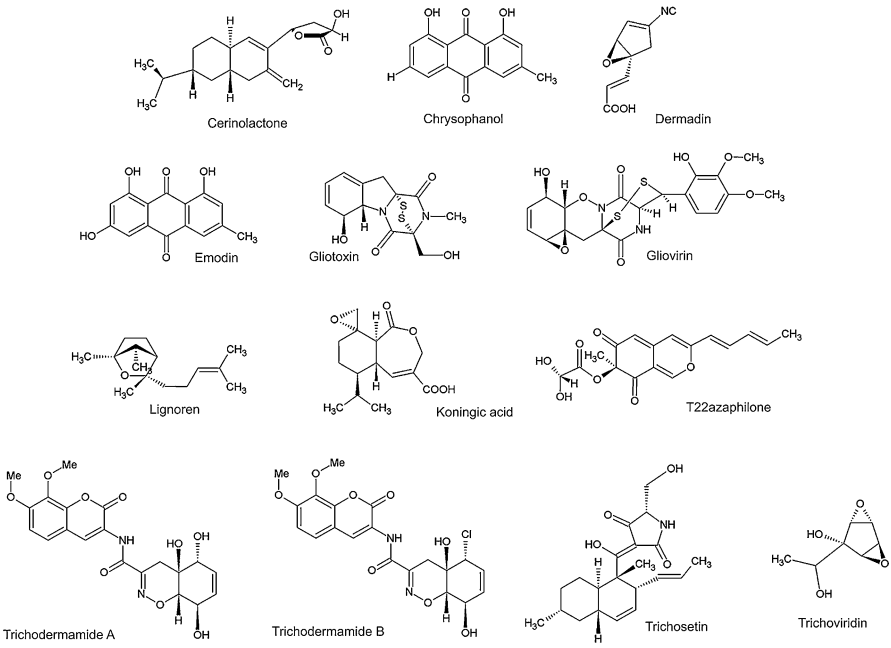

Fig. 7 Fungal compounds with antibiotic activity. *Trichoderma* metabolites are active against a broad spectrum of pathogen microorganisms for plants and humans. This kind of metabolites comprises a heterogeneous group of molecules that could be considered as biomarkers to specific *Trichoderma* strains

lycopersici after 2 days. When used in spore germination tests, 0.45 mg/ml was found to completely inhibit the germination of *Fusarium* spores [16]. The biological effects of 6-PP are numerous, reduced production of the mycotoxin deoxynivalenol by *Fusarium graminearum* and antifungal properties by reducing the mycelial growth rate of *R. solani* and *F. oxysporum* f. sp. *lycopersici* [91, 92].

The sesquiterpene koningic acid was found in the filtrate culture of three different strains of fungi isolated from soil samples; these strains were identified as *Gliocladium virens* (*T. virens*), *Chaetomium globosum*, and *T. viride*. Koningic acid shows specific activity against the anaerobic bacterium *Bacteroides fragilis* [93]. Harzianic acid is a metabolite with multiple biological functions among them antibiotic activity and inhibition of the protein phosphatase type 2A (PP2A) [48, 49]. The butenolide harzianolide has fungicide activity against *Gaeumannomyces graminis* var. *tritici*, *R. solani*, and *Pythium ultimum* [16, 94].

Hydroxyl-lactone cerinolactone produced by *Trichoderma cerinum* has been reported to have antifungal activity against *P. ultimum*, *R. solani*, and *B. cinerea* [81]. Chrysophanol is an anthraquinone that acts against *Aspergillus fumigatus*, *Candida albicans*, *Cryptococcus neoformans*, and *Trichophyton mentagrophytes* [95]. The antibiotic dermadin (code name U-21, 963) is an isocyano cyclopentene compound produced by *T. koningii* and *T. viride* [16, 96]. *T. viride* also produces emodin an antibacterial anthraquinone isolated from the roots of *Cassia occidentalis* [97].

The gliotoxin is a diketopiperazine produced by *T. viride*, *T. hamatum*, and *T. virens* that is very effective against *R. solani*; this compound also has properties as an antiviral and antibacterial [16, 98]. *T. virens* also produces gliovirin, a heterocyclic nitrogen- and sulfur-containing compound of the diketopiperazine class, which is effective against *Pythium ultimum* [99]. Lignoren is a cyclonerodiol-derived compound produced by *Trichoderma lignorum*, with moderate antibacterial activity against *Bacillus subtilis* and *Pseudomonas aeruginosa* but no fungistatic nor fungicide activity against *Candida albicans*, *Fusarium culmorum*, or *Penicillium notatum* [100]. *T. harzianum* produces the antibiotic T22azaphilone, which possess an oxygenated bicyclic core, inhibiting the growth of *Gaeumannomyces graminis*, *P. ultimum*, and *R. solani* [101]. Trichosetin also has antibacterial activity against *Bacillus subtilis* and *Staphylococcus aureus* [102]. *T. koningii* and *T. viride* produce trichoviridin, a cyclopentyl isocyanide compound [103–105]. Trichodermamides A and B are two modified dipeptides produced by *T. virens* [16].

4 Multiple Functions of Fungal Secondary Metabolites

Rhizospheric fungi produce a diversity of VOCs, and the majority of those metabolites are hydrocarbons comprising dozens of carbon skeletons that can form oxygenated sesquiterpenes [6, 106]. Volatile sesquiterpenes are 15-carbon isoprenoids and constitute a structurally diverse family of natural compounds with different regio- and stereochemistry (Fig. 8). Due to the wide variety of biochemical functions in organisms, such as antimicrobial, antifungal, herbicidal, and hormonal activities, many of these compounds have been found to be useful for medicines, pesticides, fragrances, and flavors [107, 108]. A recent comparative study among the VOCs produced by *T. atroviride* IMI 206040, *T. reesei* QM6a, and *T. virens* Gv29-8 revealed substantial differences in the chemical composition [6]. In that work, fungal species mainly produced oxylipins and terpenes. Volatile sesquiterpenes from *T. atroviride* P1 have been detected, among them α-farnesene, β-farnesene, nerolidol, γ-curcumene, α-zingiberene, β-bisabolene, and α-bergamotene [109]. More recently, it was reported that *T. virens* produces a rich blend of isoprenoid terpenes such as β-caryophyllene, (-)-β-elemene, germacrene D, τ-cadinene, α-amorphene, τ-selinene, δ-cadinene, etc. [6, 110]. Figure 8 shows some volatile terpenes produced by fungi, and the majority of them have been reported in several *Trichoderma* strains.

The chemical profile of VOCs from *T. viride* revealed that the fungus produces the aldehydes 2-methylpropanal, butanal; the isomers 2- and 3-methylbutanal and pentanal; and the terpenes limonene, β-himachalene, farnesene, and aromadendrene. Interestingly, *Arabidopsis thaliana* seedlings exposed to *T. viride* VOCs increased both the shoot and root biomass, and that effect was correlated with the accumulation of total chlorophyll [111]. More recently, it was reported that VOCs from *T. asperellum* T-34 and *T. harzianum* T-78 increased the expression of the transcription factor *MYB72*, which plays a dual role in the

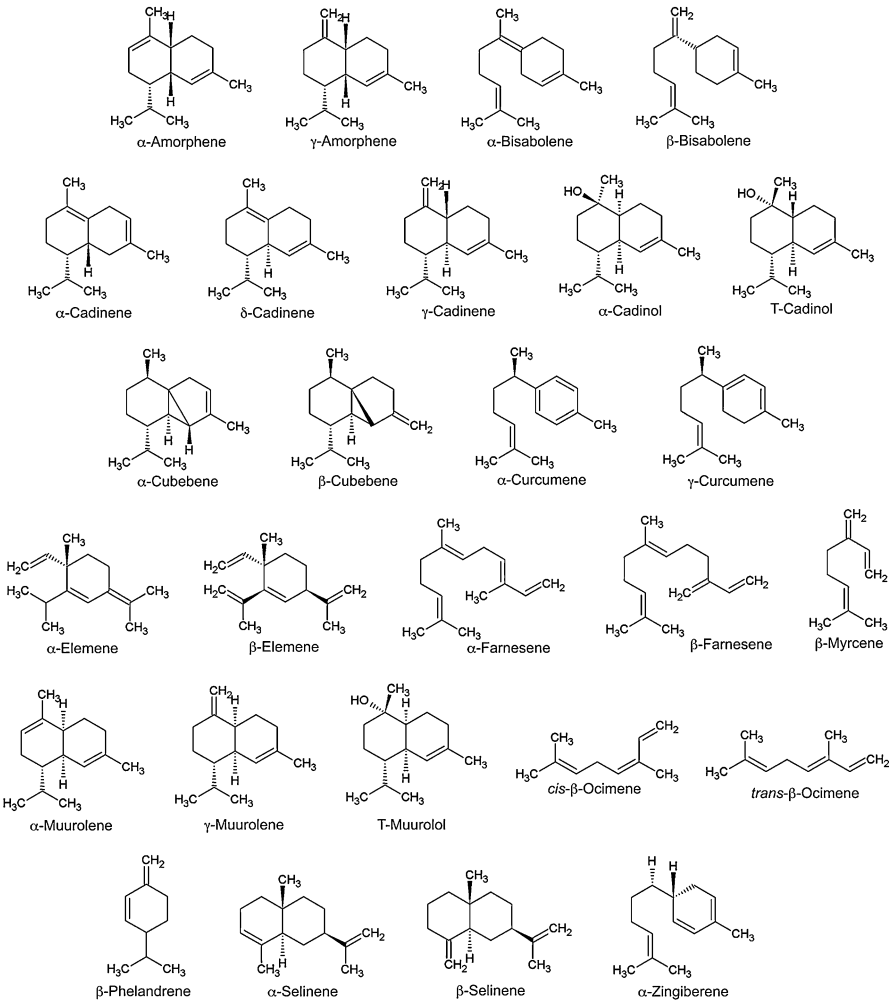

Fig. 8 Volatile compounds identified in fungi. *Trichoderma* strains produce a number of isoprenoid mono- (C_{10}) and sesquiterpenes (C_{15}). Notice the close relation of the chemical structure among sesquiterpenes. Frequently, chemical typification is difficult due to their mass spectra similarity

induction of defense responses and the activation of Fe uptake in the model plant *A. thaliana* [34].

4.1 Ecological Functions of *Trichoderma* Metabolites

T. atroviride produces several volatile C_8 compounds, such as 3-octanone, 1-octen-3-ol, and 3-octanol (Fig. 9). These metabolites are the end products of fatty acid

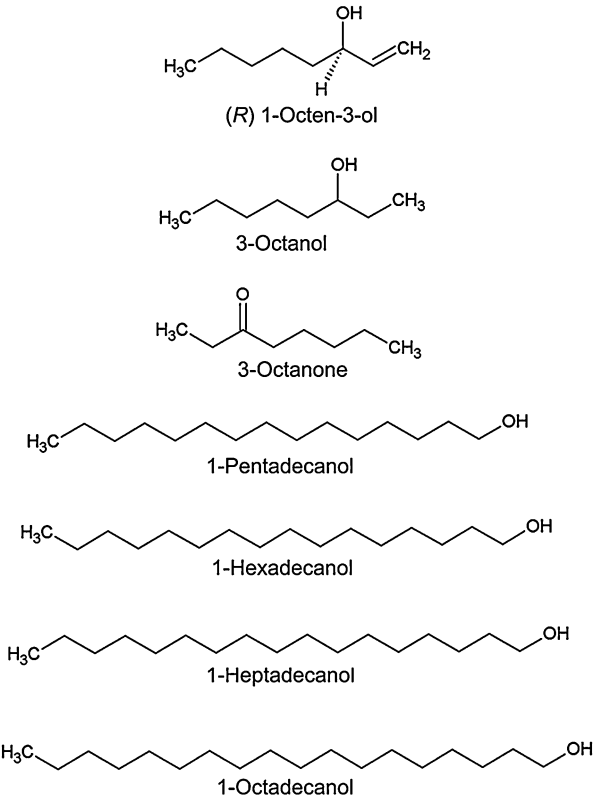

Fig. 9 C_8 compounds and aliphatic alcohols produced by *Trichoderma*. 1-octen-3-ol is an oxylipin-derived compound commonly found in fungi. 1-octen-3-ol has different functions as autoregulator for the self-fungus in interactions with plants and insects

metabolism, sharing acetyl-CoA as precursor [109, 112]. There is evidence that 1-octen-3-ol acts as a sexual hormone and also attracts numerous insect species [113]. In truffles, 1-octen-3-ol might attract the fly *Suillia pallida* or the beetle *Leiodes cinnamomea* to fruiting bodies [114]. More recently it was reported that 1-octen-3-ol and 6-PP, both compounds produced by *T. atroviride*, reduced the attack of the fall armyworm *Spodoptera frugiperda* in maize leaves [115]. Furthermore, it was reported that *T. atroviride* associated with maize roots and infested with *S. frugiperda* in the leaves, likely released some plant or fungal VOCs that attracted female wasps of *Campoletis sonorensis*, the natural enemy of *S. frugiperda* [116]. In that work, a key role for the fungal metabolite 6-PP was found by attracting to *C. sonorensis* wasps and thereby increasing the number of parasitized larvae [116].

Trichoderma citrinoviride ITEM 4484 produces long-chain alcohols 1-pentadecanol, 1-hexadecanol, 1-heptadecanol, and 1-octadecanol; these molecules are unbranched, unsubstituted with linear aliphatic group and have not chiral centers (Fig. 9). Such fungal alcohols have deterrent activity against the bird cherry-oat aphid *Rhopalosiphum padi* [117]. *T. longibrachiatum* associated with tomato roots altered the profile of plant host VOCs (*Z*)-3-hexenol, α-pinene, longifolene, and β-caryophyllene resulting in improved attractiveness to the aphid parasitoid *Aphidius*

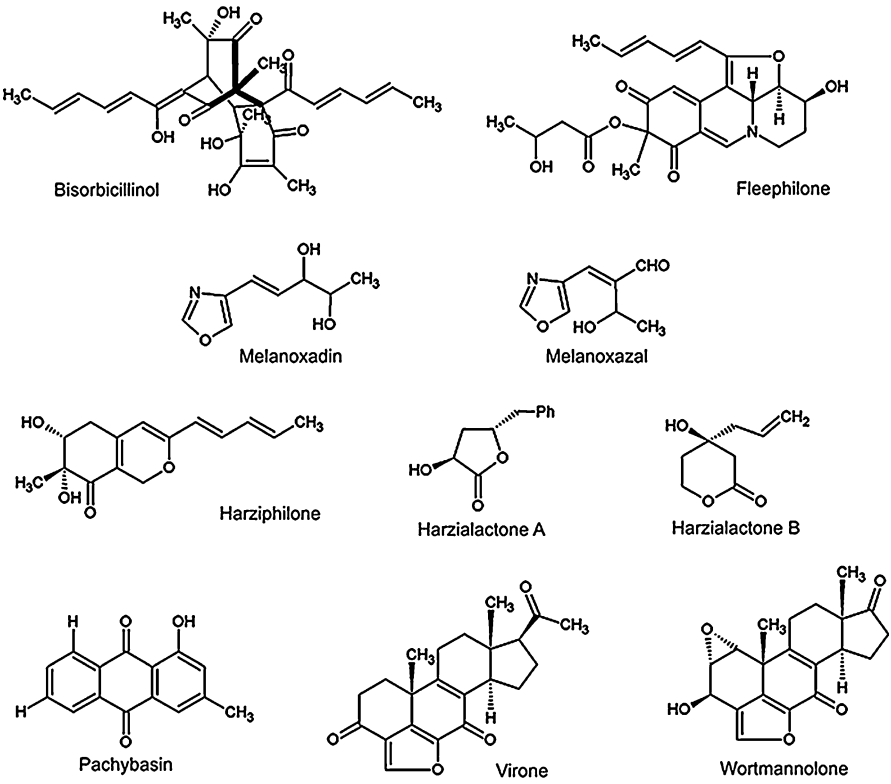

Fig. 10 Fungal metabolites with potential application. Some of these compounds could be used as therapeutic compounds

ervi and the aphid predator *Macrolophus pygmaeus* [118]. Fungal metabolites also can act as autoregulatory substances of morphogenetic processes. For example, 1-octen-3-ol, 3-octanol, and 3-octanone enhanced the condition response in *T. atroviride* [119]. It has been reported that *T. viride* and *Trichoderma aureoviride* produce pachybasin an anthraquinone likely involved with the fungal pigmentation [120, 121]. Finally, melanoxadin and melanoxazal are two nitrogen heterocyclic molecules and specifically are oxazole-derived compounds produced by the *Trichoderma* strain ATF-451; both compounds can inhibit melanin production in the larval hemolymph of the silkworm *Bombyx mori* [16].

4.2 Pharmaceutical and Medical Impact of Fungal Metabolites

There are some metabolites from *Trichoderma* of interest in the medicine (Fig. 10). For example, bisorbicillinol a compound isolated from *Trichoderma* sp. strain USF-2690 has antioxidant properties [122]. Harziphilone and fleephilone are two

azaphilones identified in extracts from *T. harzianum* with inhibitory activity against the binding of regulation of virion expression-proteins to RRE RNA [123]. Harzialactones A and B are two hydroxylactones isolated from *T. harzianum* [124]. *T. virens* produces virone, a steroidal antibiotic compound that possesses a furan ring; such metabolite also can inhibit the phosphatidylinositol 3-kinase [125, 126]. Wortmannolone is also produced by *T. virens*, and such compound can inhibit the phosphatidylinositol 3-kinase; this compound could be used in neoplasms, in humans [16, 125]. Undoubtedly, *Trichoderma* fungi produce a plethora of secondary metabolites of different classes and are of great interest in the field of the medicine. For this reason it is crucial the discovery and characterization of fungal-derived molecules.

5 Conclusions

Chemical and pharmacological studies of secondary metabolites from different *Trichoderma* species have shown that these fungi produce a number of compounds with potential application in medicine, biotechnology, and agriculture. Generally, these chemical variations are correlated with the fungal lifestyle. In terms of *Trichoderma*-plant interactions, the fungi can alter plant growth, activate defense responses, and solubilize unavailable soil nutrients. Such effects might be induced through direct or indirect mechanisms involving classical plant growth regulator auxins, ABA and ET. Moreover, siderophores released by *Trichoderma* also play key roles in plant growth and can control plant pathogens by depriving iron to other rhizosphere microorganisms. Proteinaceous compounds as sm1 or Epl1 from *T. virens* and *T. atroviride*, respectively, are potent elicitors of systemic resistance. However, it remains to be seen what is the biological function of a myriad of *Trichoderma* secondary metabolites. The use of analytical techniques will be useful to elucidate new compounds to be used in the medicine or agriculture.

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Legume-Rhizobium Symbiosis: Secondary Metabolites, Free Radical Processes, and Effects of Heavy Metals

13

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Contents

Abstract

Leguminous plants are able to establish symbiosis with a group of nitrogen-fixing soil bacteria called collectively rhizobia. This symbiosis leads to the formation of root nodules, specialized structures within which bacteria carry out nitrogen

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fixation. Both rhizobia and host legumes exhibit a strong specificity, which can be a result of their coevolution. Symbiotic specificity is provided by the complex exchange of signals between both symbiotic partners. To initiate symbiosis, legumes produce a cocktail of flavonoids that trigger synthesis and secretion of bacterial lipochgitooligosaccharide molecules called Nod factors. Nod factors together with surface polysaccharides and secreted proteins are proposed to be major rhizobial determinants of host specificity. Much evidence suggests that reactive oxygen species (ROS) play a key role in the formation and functioning of legume-rhizobium symbiosis. Elevated levels of heavy metals in soils can affect rhizobial growth and host legumes as well as impair legume-rhizobium symbiosis, in particular due to enhanced ROS production. On the other hand, if plants form symbiosis with rhizobia, heavy metals are accumulated preferentially in nodules that can be one of the possible ways to reduce toxic effects of heavy metals to legumes.

Keywords
Rhizobium · Chemotaxis · Nodule development, Nod factors · Surface polysaccharides · Phytohormones · ROS · Oxidative stress · Bioremediation

Abbreviations

EPS	Extracellular polysaccharide
IAA	Indole-3-acetic acid
Lb	Leghemoglobin
LPS	Lipopolysaccharide
RNS	Reactive nitrogen species
ROS	Reactive oxygen species

1 Introduction

Nitrogen is an essential element for all living organisms, including plants. It is a component of main cellular macromolecules, such as proteins and nucleic acids, and low-molecular mass compounds like chlorophylls, amines, and vitamins. On Earth, most of nitrogen was found in the inaccessible form of atmospheric nitrogen gas (N_2). Biological fixation plays an important role in the conversion of chemically inert N_2 into metabolically active ammonia (NH_3), which can be utilized by plants in different ways. The ability to convert N_2 to NH_3 has evolved only among prokaryotes called collectively diazotrophs. The latter include both free-living (e.g., azobacteria and cyanobacteria) and symbiotic nitrogen fixators (rhizobia) [1–3].

During evolution, some plant species, especially from *Fabaceae* family, have developed a complex relationship with rhizobia to receive benefits under nitrogen-limiting soil conditions. Formation of nitrogen-fixing nodules on the plant root seems to be a result of coevolution of legumes and rhizobia. Plants, obviously, influenced more evolution of nodule bacteria, than bacteria did. Bacterial genetic plasticity may be indicative of the large capacity of rhizobia to adapt to

legumes [4, 5]. Within root nodules, the rhizobia are developed into specialized symbiotic forms, bacteroids, which fix N_2 into ammonia by using nitrogenase enzyme complex and supply it to the host plant. This relationship provides nutrient benefits for both partners, a plant cell supplying carbon sources to bacteria and receiving, in response, NH_3 for growth [6–8]. Symbiosis of legumes with rhizobia covers over 60% of plant needs in nitrogen [9] and accounts for 20% of the estimated biological nitrogen fixed each year on Earth [10]. There are species of agronomic importance among legumes – common bean (*Phaseolus vulgaris*), alfalfa (*Medicago sativa*), soybean (*Glycine max*), pea (*Pisum sativum*), and lentil (*Lens culinaris*). The establishment of successful legume-rhizobium symbiosis can increase plant biomass and crop yield and contribute to nitrogen enrichment of the soil [7, 8].

To establish the effective symbiosis, two symbiotic partners require being compatible with each other. Compatibility depends on mutual recognition via chemical signals releasing from both the host plant and nodule bacteria [8, 11, 12]. However, bacteria frequently can invade incompatible plants. In these cases, bacteria are not able to form nodules or form nodules that cannot fix molecular nitrogen [8, 9, 12, 13]. Chemotaxis of soil rhizobia to root exudates plays an important role in competitive nodulation [3, 14–16]. Specialized metabolites (or secondary metabolites) produced by legume roots attract rhizobia that adhere to the wall of the root hair cells [11, 13, 14, 16, 17]. Root exudates are complex mixtures of low-molecular mass organic compounds with flavonoids being the most important in the initiation of symbiosis with rhizobia as benefit partners [3, 18–20]. Plant flavonoids activate bacterial transcriptional factors NodDs that trigger the expression of nodulation genes (*nod* genes) [1, 17, 20, 21]. The products of *nod* genes are proteins involved in synthesis and export of specific lipochitooligosaccharides called Nod factors. Bacterial Nod factors serve as signaling molecules that initiate nodule formation in root cortex [1, 8, 12, 18, 22, 23, 27]. Recent studies suggest a crucial role of Nod factors in the regulation of host phytohormone balance as a prerequisite for successful nodule formation [16, 24–27]. The interaction between bacterial surface polysaccharides and plant lectin receptors is also involved in the recognition process and successful colonization of root hairs [6, 20, 28–31].

Rhizobial infection intensifies oxidative processes in plants, leading to increased production of reactive oxygen species (ROS) and reactive nitrogen species (RNS) [10, 32–37]. There is increasing evidence that ROS/RNS and antioxidant system play a key role in the formation and functioning of legume-rhizobium symbiosis [10, 38–47]. Uncontrolled changes in levels of these reactive species impair either the formation of root nodules or N_2-fixing activity of bacteroids [10, 38, 40, 42, 48]. A number of studies reported that elevated levels of heavy metals in soils can disturb redox balance either in legume plants rhizobia affecting their growth and decreasing efficacy of legume-rhizobium symbiosis [48–56]. Under cultivation of legumes on the soils with the high level of heavy metals (Cd, Cr, Cu, Pb, etc.), the root nodules can be the major accumulators of heavy metals from soil [57–59].In this context, rhizobia are actively studied as one of the suitable tools for effective soil bioremediation with reducing toxic effects of heavy metals to legumes [48, 51, 58–62]. At the same time, legume-rhizobium

symbiosis seems to be also sensitive to heavy metals, and its protective effects against metal toxicity are not fully clear.

This chapter summarizes recent data on the role of signal secondary metabolites in the initiation and functioning of symbiosis between rhizobia and their hosts, *Fabaceae* family plants. Furthermore, we analyze the involvement of ROS/RNS in both the establishment of effective legume-rhizobium symbiosis and the toxicity of heavy metals to legume-rhizobium symbiosis. Perspectives of using rhizobia for remediation of metal contaminated soils are also discussed.

2 The Establishment of Symbiotic Interaction Between Rhizobia and Leguminous Plants

2.1 Nodule Development

Development of legume-rhizobium symbiosis occurs through a few coordinated stages, including (Fig. 1) *i*) preinfection stage, in which both partners produce chemical signals for mutual recognition and as a result bacteria are attached to the cell wall of a root hair; (*ii*) infection process leading to root hair curling and development of infection threads within the root hair for transport of bacteria to nodule cells; (*iii*) formation of nodules, specialized root organs, within which the bacteria further differentiate into nitrogen-fixing bacteroids; and (*iv*) functioning the mature nodules, their senescence and necrosis [3, 7, 12, 19–21, 44]. Each stage in legume-rhizobium symbiosis is regulated by signals from both the nodule bacteria and their host plant [1, 5, 14, 17, 19, 20, 44, 63].

The host plant and rhizobia first establish contact with each other at the surface of the growing tip of a root hair. Rhizobia can persist at low levels as free-living, soil saprophyte bacteria in the absence of a suitable host plant. If the appropriate host appears, the legume-rhizobium symbiosis starts with a complex signal exchange between the host plant and its symbiotic bacteria [12, 23, 64]. Chemical compounds secreted by both partners play the main role in this early stage of communication. In particular, plant roots secrete flavonoids, which induce synthesis of rhizobial Nod factors – a specific group of lipochitooligosaccharides. The latter serve as signaling molecules that should be appropriately recognized by the host plant [1, 8, 63, 65]. If the initial contact is successful, the root hair curls to trap a small number of bacteria. This is accompanied by local hydrolysis of the cell wall of the root hair to allow bacteria to infect plant root cells [66, 67]. From this trap site, the root hair begins an inverse tip growth, forming a long and narrow passage, called the infection thread, in which the bacteria “travel” by continuously dividing at the leading edge [65, 68, 69]. Infection threads are progressive ingrowths of plant cell membranes containing a matrix composed of plant cell wall material [31].

While the bacteria enter the root hair, host cells in the root cortex restore properties of stem cells, which undergo active division [7, 70]. It increases a population of newly generated cells (nodule cells), which form a new root organ, the nodule (Fig. 2).

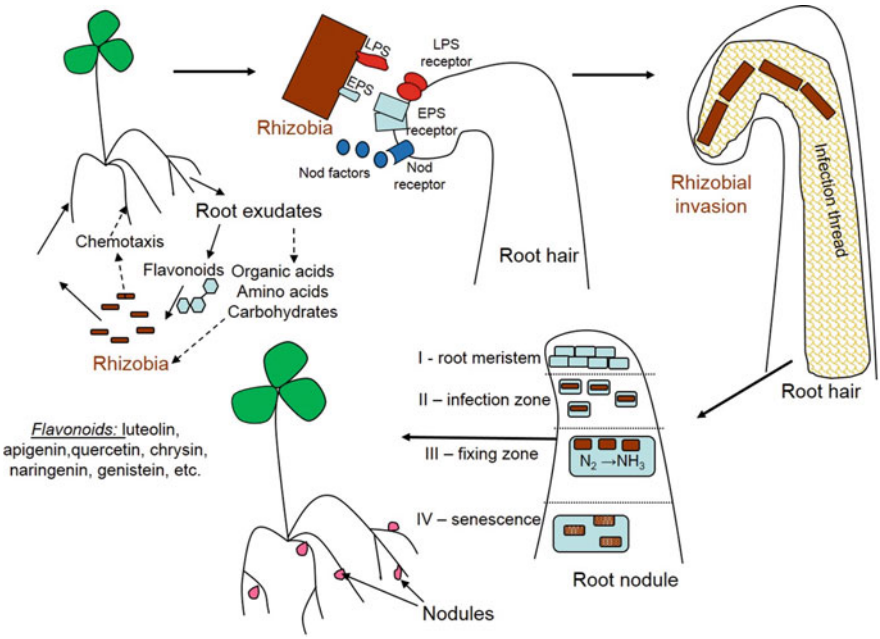

Fig. 1 Development of legume-rhizobium symbiosis. Scheme describes the formation of indeterminate nodules

Fig. 2 Mature nodule on pea roots

The key stage of infection is the transfer of bacteria from the infection thread to the cytoplasm of the nodule cell. This process occurs through endocytosis. The individual bacteria are surrounded by a membrane of plant origin, within which they further differentiate into N_2-fixing bacteroids [63, 65, 69, 71]. The root cells rapidly replicate, then stop the division, and begin to increase in size, forming a nodule tissue [7, 70]. After the bacterial invasion in nodules, vascular-fibrous bundles from the procambium of the central cylinder and then the leading elements of the

xylem and phloem branch penetrate the nodules. Hence, nodules are newly plant organs, which contain infection bacterial zone, where molecular nitrogen fixation occurs; conducting fabrics, in which vegetative photoassimilates arrive to bacteria and products of nitrogen fixation are transported to plants; and meristem, which is responsible for the growth of nodules [72]. At the final stage of nodule formation, the bacteria intensively multiply in the cytoplasm of the nodule cell. They are surrounded by additional membranes and lose the flagella, gradually being pulled out and gaining the appearance of girdle sticks. In this state, they continue to multiply and turn into different forms of bacteroids [73]. Transformation of bacteria into nitrogen-fixing bacteroids completes nodule formation [7, 70].

2.2 Chemotaxis of Rhizobia to Root Exudates as an Early Event in Symbiotic Initiation

The host plant and rhizobia exhibit a strong mutual specificity. Since many microorganisms, including pathogenic and symbiotic ones, are present in the soil, the host plant must first identify rhizobia as beneficial partners [1, 5, 8]. To initiate communication, plants secrete large amounts of different low-molecular mass compounds; some of them are used by microorganisms as carbon and energy sources (carbohydrates, amino acids, organic acids), whereas others can be signal molecules for attraction of the homologous (compatible) bacteria (flavonoids) [3, 18, 20]. Root and seedling exudates induce directed movement (chemotaxis) of nodule bacteria to the host plant that is an early stage in the interaction between micro- and macrosymbionts [11, 19]. Reacting to root exudates, bacteria are concentrated in the rhizosphere zone at a width of ~ 100 microns, and their number increases by 2–3 orders of magnitude [11, 74]. Bacteria can recognize the host plant via nonspecific and specific chemotaxis reaction. The result of the first one is the movement of bacteria to simple molecules (carbohydrates, organic acids, and amino acids); the result of the second is the movement to large molecules (hormones, lectins, and enzymes) [14, 20]. In the laboratory, chemotaxis activity can be assessed by measuring growth zone (chemotaxis zone) of rhizobia after inoculation onto agar plates containing different chemical compounds [15] (Fig. 3).

Rhizobia are positively chemotactic to various legume epidermal exudates, including carbohydrates, amino acids, dicarboxylic and hydroxyaromatic acids, and many phenolic compounds [18, 20, 75, 76]. The composition of root exudates can be different between plant species and allows the selective requirement of specific groups of microorganisms [77]. For example, pea plants select their symbiont *Rhizobium leguminosarum* by the excretion of homoserine into the rhizosphere [78]. Root exudates also play an important role in plant defense through the secretion of phytochemicals that can inhibit the growth of certain microbes. The ability to tolerate these chemicals can play an important role in the ability to colonize the plant [3].

Flavonoids are the most important compounds in legume root exudates [18, 79, 80]. They act as signal molecules and induce a specific symbiotic response in

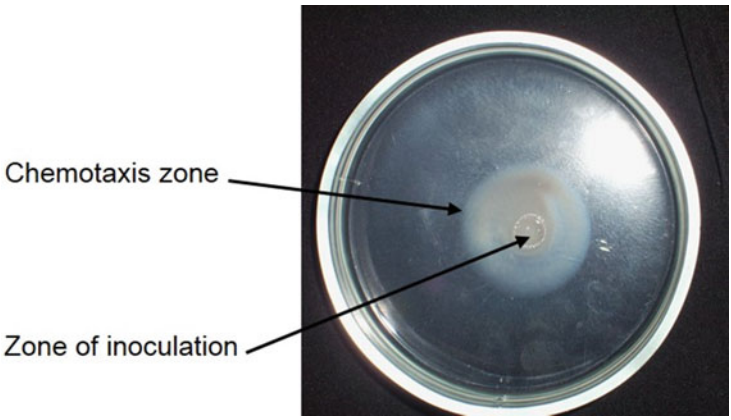

Fig. 3 Positive chemotaxis of *R. leguminosarum* to L-serine. Chemotaxis activity is assessed by measuring bacterial growth zone on agar plates

compatible bacteria. At present time, over 10,000 different flavonoids have been identified in plants. Flavonoids are low-molecular mass secondary metabolites, which are synthetized via the central phenylpropanoid and acetate-maleic acid pathways [18–20]. Structure of flavonoids is based on flavone backbone. Different modifications of this basic structure yield the following subgroups of flavonoids: flavones, flavonols, flavanones, isoflavones, isoflavans, pterocarpans, proanthocyanidins, and chalcones [18, 75]. Flavonoids can be produced as either aglycons or glycosidic conjugates [20, 75]. In the form of glycosides, flavonoids are more water-soluble and can diffuse easily from the root surface into the rhizosphere, where they may undergo hydrolysis to the aglycon form by rhizobia. Moreover, the bacteria are able to affect the hydrophobicity of flavonoids, as it was observed for *R. meliloti,* which produces cyclosophoraoses forming complex with luteolin and enhancing its solubility [18, 81].

Flavonoids have been involved in many functions in plants, including pigmentation, protection against ultraviolet light, free radical scavenging, pollen fertility, regulation of auxin transport, and defense against pathogenic bacteria and fungi [17–20, 75]. In legumes, flavonoids also have a crucial role in the initiation of the symbiosis acting as principal signals recognized by compatible rhizobia [20, 79].

2.3 Flavonoids as Plant Signal Molecules Activating Bacterial NodD Factors

Under nitrogen-limiting conditions, legume roots or seeds secrete a cocktail of different compounds, mostly flavonoids, into the soil. These compounds can passively diffuse across the bacterial membrane. These compounds play an important role in legume-rhizobium symbiosis, first as chemoattractants for compatible species of rhizobia and then as primary plant signals that regulate expression of many

rhizobial genes. Exactly which flavonoid in the rhizosphere a compatible bacterium perceives can be difficult to determine since plants secrete a complex mixture of flavonoids [1, 7, 71]. In the bacterial cell, flavonoids induce NodD-mediated expression of bacterial nodulation (*nod*) genes, which encode the enzymes required for the synthesis of bacterial Nod factors, a family of lipochitooligosaccharides essential for initiation and development of symbiotic interaction in most legumes [17, 65].

In the response to plant flavonoids, compatible rhizobia can elicit qualitative and quantitative composition of these compounds in root exudates of the respective host plants [18, 82]. In particular, root exudates of *Ph. vulgaris* inoculated with *R. leguminosarum* bv. *phaseoli* contained higher amount of the flavonoid phytoalexin coumestrol and its isoflavonoid precursor daidzein than did exudates of sterile plants [83]. Other study showed the increased quantities of daidzein, naringenin, liquiritigenin, and isoliquiritigenin in root exudates of *P. vulgaris* after inoculation with homologous rhizobia [84]. It was shown that rhizobia stimulate production of flavonoids via increasing activities of phenylalanine ammonia lyase and chalcone synthase involved in the plant phenylpropanoid biosynthesis pathway [85]. Most flavonoids function as *nod* gene inducers in nanomolar and micromolar concentrations. Mixture of flavonoids seems to be more efficient in induction of *nod* gene expression than a single type [86, 87]. At the same time, different flavonoids can have distinct roles in nodulation process as was observed in *Medicago truncatula* inoculated by *Sinorhizobium meliloti* [80]. Rhizobia are able to degrade plant flavonoids with formation of a number of flavonoid derivatives and other phenolic metabolites; some of them may act as *nod* gene inducers [11, 20]. Certain flavonoids may act simultaneously as inducers or inhibitors of *nod* gene expression depending on rhizobial species, as in the case of genistein and daidzein [86]. Both these compounds activate *nod* genes in *Bradyrhizobium japonicum* and *Rhizobium* sp. NGR234 and are repressors of *nod* gene expression in *R. leguminosarum* bvs. *trifolii* and *viciae* [79].

Plant flavonoids penetrate the bacteria and activate bacterial NodD proteins, which are members of the LysR family of transcriptional activators. NodD proteins are encoded by *nodD* genes constitutively expressed in bacterial cells [7, 67, 88]. Flavonoid-activated NodD proteins bind to conserved DNA sequences (*nod*-boxes) in the promoters of inducible nodulation genes (*nod* genes) with forming a bend in DNA at the binding site [19, 89]. This binding triggers the expression of responsive *nod* genes. NodD proteins from different rhizobial species respond to different sets of flavonoids. For example, the daidzein and genistein, isoflavonoids of soybean, induce *nod* gene expression in *B. japonicum*. At the same time, daidzein prevents production of Nod factors in the noncompatible *Sinorhizobium meliloti*, which responds positively to the flavone luteolin and does so in a NodD-dependent manner [1, 90].

Rhizobia species may contain one to five homological NodD proteins, which can be activated by flavonoids or several non-flavonoid compounds like jasmonates [19]. The different NodD proteins determine, at least partially, the bacterial specificity to the host, and they are adapted to recognizing defined flavonoid compounds produced by different legumes [91]. In *R. meliloti*, the different *nodD* genes (*nodD1*,

nodD2, and *nodD3*) affect the rate at which this bacterium nodulates different host plants. For example, trigonelline and stachydrine, major components in seed exudates of *M. sativa* L., induce *nod* gene transcription in *R. meliloti* by activating the regulatory protein NodD2, but not the homologous NodDl protein [92].

2.4 Nod Factors, Surface Polysaccharides, and Secreted Proteins as Rhizobial Determinants of Host Specificity

2.4.1 Nod Factors

The products of rhizobial *nod* genes are involved in synthesis and secretion of specific lipochitooligosaccharidic molecules called Nod factors. Nod factors serve as signaling molecules that are essential for bacterial invasion and initiation of the nodule formation in the root cortex [7, 21, 93, 94]. Nod factors are the most important signals in the symbiotic development; without them rhizobia cannot enter legume roots [19]. Nod factors are oligomers that consist of usually four or five β-(1,4)-linked *N*-acetyl-glucosamine residues, to which a fatty acyl chain with varying length and varying degrees of unsaturation is attached at the nonreducing terminus [20, 22]. Within rhizobia, Nod factors are structurally diverse and specific for individual rhizobial strains [11, 18, 86, 87, 90, 91]. Different rhizobial species produce various Nod factors, which have chemical substitutions on the reducing and nonreducing monosaccharides in the backbone chain and variations in the structure of the acyl chain. The broad range of Nod factors produced by rhizobia appears to be important for the selection of host range and specific nodulation. For example, each species of *Rhizobium* has a certain set of *nod* genes that determine the length of the lipochitooligosaccharide skeleton and make the Nod factors specific to the host plant [19].

Rhizobia have common and specific *nod* genes. The first groups of *nod* genes (*nod*ABC) encode the core Nod structure that is common to all rhizobia species [22, 67]. *NodA* gene encodes an acyltransferase that binds an acyl chain to the nonreducing end of the oligosaccharides; *nodB* encodes a deacetylase, which removes the *N*-acetyl moiety from the nonreducing terminus of these oligosaccharides; and *nodC* encodes *N*-acetyl-glucosaminyltransferase that polymerizes UDP-*N*-acetyl-D-glucosamine into oligosaccharide chains [67]. The second group of *nod* genes (e.g., *nod*PQ, *nod*H, *nod*EF, *nod*X) has a strong species specificity [71]. They control the modification of chemical structure of Nod factors by changing the size and saturation of the acyl chain or adding to the terminal sugar units with acetyl, methyl, carbamoyl, sulfuryl, or glycosyl groups [12].

Nod genes were shown to be highly conserved even between distantly related lineages of rhizobia, suggesting that they might have a monophyletic origin and could have been transmitted to different groups of nonsymbiotic bacteria by horizontal transfer [22]. Genes encoding enzymes involved in the Nod factor synthesis and genes of symbiotic nitrogen fixation (*nif* and *fix* genes) are either located on one of the megaplasmids, called a symbiotic plasmid (pSym) (e.g., in *R. leguminosarum*,

S. meliloti, *R. etli*), or grouped in a large chromosomal region called a symbiotic island (e.g., in *M. loti* and *B. japonicum*) [20, 71].

Nod factors initiate specific signaling cascades in root hairs and root cortex resulting in expression of the early genes of symbiotic interaction. The products of these genes cause deformation of the root hairs followed by root hair curling to trap bacteria and induce formation and growth of infection threads to transport the bacteria to the root cortex [29, 94]. Nod factors also promote nodule formation a result of stimulation of cell proliferation in the cortex root due to changes in phytohormone levels and induction-specific plant genes (nodulins) [63, 72, 75, 94]. Nod factors are perceived by plant Nod factor receptors (e.g., NFR1 and NFR5 in *Lotus japonicus*), which are LysM-domain-containing receptor kinases [12, 95]. Direct binding of Nod factors to the extracellular LysM domains of the receptor complex leads to activation of the downstream nodulation signaling pathways [96]. In particular, after Nod factor perception by plant receptor kinases, Ca^{2+} oscillation occurring in root hair cells initiates downstream signaling events [20, 95]. Specificity in Nod factor binding is thought to be critical for recognition between the prospective symbiotic partners.

2.4.2 Surface Polysaccharides

Many studies reported that rhizobial invasion of the host nodule via the infection threads is strongly influenced by a complex variety of bacterial polysaccharides in addition to Nod factors [28, 30, 97–99]. Rhizobia produce at least seven different types of cell-surface polysaccharides: extracellular polysaccharides, lipo-polysaccharides, capsular polysaccharides, gel-forming polysaccharide, K-antigen polysaccharides, cyclic glucans, and high-molecular mass neutral polysaccharides (glucomannans) [30, 31, 91, 97]. Polysaccharides contribute to various stages of symbiotic development including root colonization, host-plant recognition, infection thread formation, and nodule invasion. Bacterial polysaccharides are also important for the evasion of plant immune responses and as protectants against ROS [94, 97, 100].

Bacterial polysaccharides are recognized by specific plant receptors called collectively lectins. Lectins are glycoproteins, which are abundant in legume seeds and present on tips of growing root hairs. Lectins have no enzymatic activity; however, binding carbohydrate residues, they facilitate the attachment of bacteria to the host plant and modulate some processes of symbiosis [20, 28, 101].

Rhizobial polysaccharides lightly connected with the bacterial surface and secreted in large amounts into the soil are named exopolysaccharides (EPSs). EPSs are a major component of the cell surface and play a significant role in secondary attachment. Rhizobial EPSs are chemically diverse species- or strain-specific heteropolymers and homopolymers that are composed of linear or branched repeating units containing monosaccharides (D-glucose, D-mannose, D-galactose) and D-galacturonic acid, substituted with noncarbohydrate moieties (e.g., acetyl, pyruvyl, succinyl, etc.) [20, 28, 31]. These molecules demonstrate highly variable compositions between strains and species [76, 91], but low-molecular mass fractions were the most active in the infection process [102]. The role of EPSs was the best

illustrated in *Sinorhizobium-Medicago* symbiosis. It was shown that succinoglycan, a major surface EPS in bacteria *S. meliloti*, is required for the initiation and elongation of infection threads, and increased succinoglycan production enhances nodulation capacity [98, 99]. However, the symbiotic role of EPS is more complicated in the *Mesorhizobium-Lotus* interaction [103]. Several EPS mutants of *M. loti* R7A formed uninfected nodule primordia on roots of *L. japonicus* and *L. corniculatus*, whereas other mutants formed effective nodules [103]. It was proposed that EPSs are able to modulate the host immunity and its ability depends on the length of EPSs. Full-length EPSs and EPS minor mutants can suppress plant innate immunity allowing infection, whereas significant modified EPSs trigger plant defense responses resulting in block of infection [12, 103]. In addition, the expression of predicted defense-related genes significantly increased in *M. truncatula* inoculated with a succinoglycan-deficient mutant compared with the control strain producing succinoglycan [98].

Lipopolysaccharides (LPS) are typical components of the outer membrane in the gram-negative bacteria. Rhizobial LPSs consist of three structural regions: an O-chain polysaccharide that is attached to a core oligosaccharide, which is attached to an acylated saccharide known as the lipid A. Lipid A is a hydrophobic component, which anchored LPS into the phospholipid layer of the outer membrane [30, 31]. LPSs also play an essential role in legume-rhizobium symbiosis, but in later steps of this interaction (i.e., differentiation of bacteria into bacteroids) [12, 30, 97]. LPSs from different strains of *R. leguminosarum* had the same chemical structure regardless of the symbiotic properties of bacteria. However, the content of LPSs may vary in different *R. leguminosarum* strains [104]. The structures of lipid A in different rhizobial species have a variation in the glycosyl component of its backbone and acylation pattern [12]. Depending on the structure, lipid A can have no effects or either activate or inhibit the host innate immune response [105]. As a result, lipid A can differently affect nodulation process [12, 30, 97].

Rhizobia also can synthesize several types of basic cyclic glucans, which contain 15–30 glucose residues, depending on species of microorganisms and the type of glucan (β-(1,2)-, β-(1,3)-, and β-(1,6)- glucans) [31]. Free-living rhizobia and bacteroids can synthesize cyclic polysaccharides, which contain about 13 glucose residues linked by β-(1,6)- and β-(1,3)-glycosidic bonds [106]. Cyclic neutral β-(1,2)-glucans are located in the periplasmic space and play an important role during hypoosmotic adaptation and plant infection [31]. Cyclic glucans are able to increase the solubility of legume flavonoids and thus to make nodulation more effective. These oligosaccharides can also serve as host-specific determinants of rhizobia, since irrespective of the nodule type formed by the host plant, when rhizobia lack cyclic glucans [12].

2.4.3 Secreted Proteins

In addition to the non-proteinaceous host specificity determinants described above (Nod factors and surface polysaccharides), a third class of rhizobial signals that affect symbiosis consists of secreted proteins [64]. Rhizobia have several secretion

systems (type I, type III, type IV, and type VI) which can transport specific proteins affecting formation of symbiosis [91]. Rhizobia can produce different proteins that influence host range or suppress plant defense reactions. The first secreted rhizobial protein for which a role in symbiosis was shown was *R. leguminosarum* bv. *viciae* NodO. NodO is a calcium-binding protein that is released by a type I secretion system [107]. This protein is encoded by *nodO*, a flavonoid- and NodD-inducible gene, and promotes development of infection thread in root hairs [19]. Among other proteins that are secreted via the type I secretion system, there are several adhesins. Adhesins seem to play rather a role in attachment and biofilm formation than in infection process [108]. Rhizobial type III secretion system plays the most important role in transport of secreted proteins involved in legume-rhizobium symbiosis. In particular, type III secretion system is responsible for transport of nodulation outer proteins or Nops. The Nop may be delivered into host-plant cells via pili on the bacterial surface [19, 109]. Expression of rhizobial genes of type III secretion system is induced by flavonoids and depends on NodD [109]. Some of secreted proteins promote symbiosis on certain legumes, whereas other proteins either have no effect or can significantly reduce symbiotic proficiency in legumes [110]. The role of secreted proteins is rhizobia symbiosis and nodule formation is not fully clear and needs to be studied in details.

2.5 Role of Phytohormones in Legume-Rhizobium Symbiosis

The development and functioning of nitrogen-fixing nodules require a complex regulation of rhizobial infection and root nodule organogenesis. In recent years, the role of phytohormone signaling pathways has been evidenced in the establishment of legume-rhizobium symbiosis. Plant hormones (phytohormones) are known to be major regulators of cell proliferation, differentiation, and senescence; thus, they control plant growth and organogenesis, ripening of fruits and seeds, and plant death [24, 26, 111]. During legume-rhizobium symbiosis, levels of phytohormones are significantly changed. Modulation in phytohormone levels may be achieved in two ways: through direct synthesis of phytohormones by rhizobia and through indirect effect of bacterial Nod factors on the phytohormone balance in the plant [24]. The majority of soil microorganisms, including rhizobia, can produce a number of phytohormones (auxins, gibberellins, cytokinins, ethylene, and abscisic acid) [24, 112, 113]. Phytohormones synthesized by rhizobia enhance symbiotic efficacy but do not appear to be necessary for nodule formation [24]. In addition, many studies suggest that Nod factor-induced changes in the host phytohormone balance have a crucial role for successful nodule formation [24, 25, 27, 111]. Cytokinin, strigolactones, and local accumulation of auxin can promote nodule development. However, ethylene, jasmonic acid, abscisic acid, and gibberellic acid negatively regulate infection thread formation and nodule development [111]. Effects of some hormones can depend on their concentration, as it was found for indole-3-acetic acid (IAA). At low levels, IAA is required for root hair infection in rhizobia-legume symbiosis, but IAA at high concentrations inhibits nodule formation, in

particular, due to stimulation of ethylene synthesis [20, 60, 112, 113]. Details of the role of phytohormones in legume-rhizobium symbiosis and mechanisms of their regulation are available in several recent excellent reviews [24, 25, 27, 111].

2.6 Nodule Functioning and Senescence

Nodules can be classified into two main groups according to their mode of development. Determinate nodules have a short-lived root meristem; they initiate from the outermost one or two layers of cortical cells and grow by plant cell expansion and division, progressing through well-defined developmental stages. Determinate nodules usually adopt a globular shape and are formed on *Lotus* sp., *Phaseolus* sp., *G. max*, and a number of tropical legumes. The mature nodules contain a homogenous central tissue composed of infected cells fully packed with N_2-fixing bacteroids and some uninfected cells. Senescence in these nodules occurs radially, beginning at the center and extending to the periphery [7]. Indeterminate nodules have a persistent meristem and elongate, to become cylindrical. New nodule cells are gradually infected by rhizobia residing in the nodule; this produces more cylindrical mature nodules separated into distinct developmental zones (Fig. 1): zone I is made of meristematic cells; zone II is where cells are infected by bacteria which differentiate into bacteroids; zone III is where bacteroids reduce N_2 into ammonia which is exported to the plant; and zone IV is characterized by the disruption of the partnership and the onset of senescence [45, 114]. *Medicago* sp., *Vicia* sp., *Trifolium* sp., *P. sativum*, and *Astragalus* are typical legumes with indeterminate nodules [31, 76]. In contrast to bacteroids in determinate nodules, those from indeterminate nodules have lost their capacity to reproduce [7].

In mature nodules, compatible rhizobia differentiate into bacteroids that express the enzymes of the nitrogenase complex and begin to fix nitrogen. N_2 reduction by the bacteroid nitrogenase is the core reaction of the symbiotic process [63]. Incompatible host-strain interactions can also lead to formation of nodules, but the latter are defective in nitrogen fixation [111]. N_2-fixation defective phenotype was not due to a lack of infection but caused by bacteroid degradation after differentiation [9, 12].

Bacteroids receive carbon as dicarboxylates from legumes, and in exchange, they fix N_2 in a low O_2 environment and secrete ammonia to the plant. To effective N_2 fixation, bacteroids must balance electron flow to nitrogenase, lipids, polyhydroxybutyrate, and O_2 and coordinate this process with reductant production by the tricarboxylic acid cycle [74]. In nodules, bacteroids are provided with microaerobic environment required for expression of enzymes of the nitrogenase complex, which is located on the internal membrane of bacteroids. The nitrogenase reaction is complex and energetically expensive, since the reduction by nitrogenase of 1 molecule of N_2 to 2 molecules of NH_4^+ requires 16 molecules of ATP and 8 electrons [6, 10]. Paradoxically, despite the N_2-fixation process requirement of high O_2 levels, the nitrogenase is an O_2-sensitive enzyme. Maintaining a very low concentration of free O_2 is achieved by the presence of leghemoglobin (Lb), a plant-

produced oxygen-binding protein. Leghemoglobin accumulates to millimolar concentrations of O_2 in the cytoplasm of infected cells prior to nitrogen fixation and buffers the free O_2 concentration at around 7–11 nM, while maintaining high O_2 flux for respiration [7]. Ammonia synthetized in nitrogenase reaction reacts with intracellular keto acids, such as α-ketoglutaric, pyruvic acid, or oxalic acids in dehydrogenase- and transaminase-catalyzed reactions forming respective amino acids, such as glutamine, alanine, or asparagine [115]. Nitrogen-containing substances in the form of free ammonia, amino acids, or amides are transported from nodules to the roots and, then, to the aboveground parts of plants [37].

In all nodule types, the N_2-fixation period is optimal between 4 and 5 weeks after infection. Beyond this period, first reductions of N_2-fixing bacteroid capacity are detectable, and a senescence process occurs in the N-fixing nodule zone. Generally, dynamics of the senescence process in nodules include decrease of N_2-fixing activity and leghemoglobin content, modifications in the nodule components of regulating redox state, and an increase of proteolytic activity, ultimately leading to the death of infected cells [7].

3 Role of Free Radical Processes in Legume-Rhizobium Symbiosis

3.1 Reactive Oxygen/Nitrogen Species as Components of Plant Aerobic Metabolism and Plant Immunity

Production of reactive oxygen species (ROS) is a part of normal aerobic cellular metabolism of living organisms, including plants. These reactive species include singlet oxygen ($^1O_2$), superoxide anion radical $\left(O_2^{\bullet -}\right)$, hydrogen peroxide ($H_2O_2$), and hydroxyl radical ($HO^{\bullet}$). The most short-lived ROS is the hydroxyl radical ($HO^{\bullet}$), whereas $O_2^{\bullet -}$ and H_2O_2 are more stable compounds. Hydroxyl radical is the most reactive ROS, and H_2O_2 is the least reactive among other reactive species. In the organism, conversion of less reactive ROS into more reactive compounds is possible. Thus, $O_2^{\bullet -}$ can be a starting compound for the synthesis of other ROS: hydrogen peroxide, peroxynitrite ($ONOO^-$), singlet oxygen, and hydroxyl radical $HO^{\bullet}$ [10, 116–119]. In leaves, chloroplasts and peroxisomes are the main ROS producers in the presence of light [120]. Conversely, in non-green plant tissues or in the darkness, the mitochondria appear to be the main ROS producers [10, 117]. ROS can also be produced in some enzymatic reactions in plants; in particular NAPDH oxidase located in plasma membrane is considered as an important source of $O_2^{\bullet -}$ in the cell [121]. Besides ROS, plant cells produce reactive nitrogen species (RNS) with $^{\bullet}NO$ being the most important among them [41, 116]. The main source of $^{\bullet}NO$ is a reaction catalyzed by the plant NO synthases, which convert L-arginine to $^{\bullet}NO$ and L-citrulline [116, 122]. In addition to NO synthases, nitrate reductases also contribute to NO production in plants [41, 122]. NO is a ubiquitous signaling molecule in plants, controlling physiological processes as diverse as

flowering, iron homeostasis, drought response, or resistance against pathogens [41, 116, 122].

Due to high reactivity, ROS/RNS possess strong damaging properties. To avoid oxidative damages, plant cells have evolved a number of antioxidant mechanisms that aid to maintain low steady-stable levels of ROS/RNS, which are sufficient to performing their signaling functions [116, 117]. The production of ROS/RNS is significantly increased when plants are exposed to adverse abiotic factors or attack of pathogens [42, 89, 117]. Enhanced ROS production followed by oxidative stress development is considered as a component of host-plant immunity to combat with pathogens, including microbial infection. In this context, the inability of incompatible rhizobia to form productive nodules is explained by death of bacteroids and inactivation of nitrogenase due to intense oxidative stress induced by plant cells [46]. It seems that a small increase in ROS/RNS levels is required for successful rhizobial infection and nodule formation. At low levels, ROS/RNS are proposed to be involved in signal transduction cascades during nodule development [44–46, 93]. However, large amounts of ROS and RNS generated during the interaction between rhizobia and legumes can potentially cause development of oxidative/nitrosative stress followed by nodulation defects; therefore, concentrations of these reactive species must be tightly regulated by antioxidant enzymes and metabolites from both the host and microsymbiont sides.

3.2 Role of ROS/$NO^{\bullet}$ in Early Steps of Symbiotic Interaction

There is much evidence that ROS and antioxidant defense play an important role in the establishment of an effective legume-rhizobium symbiosis [32–35, 38, 40, 41, 123]. Similarly to respond to pathogen invasion, the infection of legumes with rhizobia causes an intensification of oxidative processes in plant cells, promoted by increased production of ROS and $^{\bullet}NO$. However, apart from the response to pathogenesis, production of ROS and $NO^{\bullet}$ may not be a plant defense response to the rhizobia but rather a process that is needed for the development of a symbiosis [28, 44–46, 93].

Early differentiation during legume-rhizobium symbiosis involves the structural modification of root hairs and formation of infection threads, which allows root infection by the bacteria. In parallel, root cortex cells dedifferentiate to generate nodule meristem. The molecular communication between plant and bacteria involves the modification in ROS and RNS production by the plant partner. Changes of ROS and RNS accumulation have been detected during the symbiotic interaction from the first hours following the initial interaction up to the ceasing of the interaction during nodule senescence [93].

As in the case of pathogen attack, the root cells respond to rhizobia infection with increased production of $O_2^{\bullet -}$ and H_2O_2. Production of H_2O_2 during symbiosis was detected in infection threads and root nodules of *M. sativa* and *P. sativum* [38]. It was shown that changes in $O_2^{\bullet -}$ and H_2O_2 levels in *P. sativum* roots under symbiosis

development depend on the efficacy of rhizobial strains [33]. Significantly increased levels $O_2^{\bullet -}$ and H_2O_2 were found in the pea roots after inoculation by incompatible strains of bacteria *R. leguminosarum* bv. *phaseoli* [33]. This may indicate ROS involvement in protection against infection of the pea roots with incompatible rhizobia. The inoculation of pea roots by compatible *R. leguminosarum* bv. *viciae* strains also increased $O_2^{\bullet -}$ and H_2O_2 levels with simultaneous stimulation of antioxidant enzymes in pea seedling epicotyls. The latter suggests that the plants have certain mechanisms to prevent bacterial infection in organs that cannot form nodules [33]. It is supposed that limitation of rhizobial infection is connected with triggering a reaction similar to the systemic acquired resistance in phytopathogenesis [34] or systemic induced resistance as in the case of infection by nonpathogenic microorganisms [33]. ROS can upregulate expression of genes encoding hydrolytic enzymes, stress-protective proteins, enzymes involved in synthesis of phenolic compounds, phytotoxins, and other substances required for development of acquired resistance to pathogens [124]. Thus, ROS generation is among key components of the plant response to infection with both compatible and incompatible bacteria.

The elevated levels of ROS were found to be necessary for the effective penetration of bacteria into plant tissues, since the decrease of ROS and $^{\bullet}NO$ levels prevented formation of bacterial infection thread and delayed nodule formation [34, 46]. Mutant strain of *S. meliloti*, which degrades H_2O_2 very efficiently (owing to the overexpression of a catalase gene), demonstrated altered infection properties and induced the formation of a reduced number of nodules on roots of symbiotic plant *Medicago* [94]. H_2O_2 was found to be necessary for the optimal propagation of infectious bacterial threads inside root hairs and membranes of plant cells [41]. In addition, ROS and $^{\bullet}NO$ were found to be involved in the induction of early nodulin gene expression and the repression of plant defense, thereby favoring the establishment of the symbiosis [44–46, 93]. Moreover, H_2O_2 appears to control a key step of the interaction, since H_2O_2 is relatively long-living ROS and can easily diffuse via biological membranes and act at distant places. An *S. meliloti* strain, overexpressing a catalase gene, showed a delayed nodulation phenotype associated with aberrant infection threads [41].

At the initial stages of symbiosis, an oxidative burst occurs in the place of bacterial infection [38]. Oxidative burst can have a dual function in legume-rhizobium symbiosis. First, temporal oxidative burst inhibits the protective reactions of plants on penetration of compatible bacteria. On the other hand, intense oxidative burst can activate protective mechanisms of plants under incompatible conditions for symbiosis [125]. During the infection process, production of $O_2^{\bullet -}$ and H_2O_2 was localized in infection threads and infected cells [38]. In *P. vulgaris*, a transient increase of ROS was detected at the tip of root hairs within seconds after addition of Nod factors [35]. However, after several minutes H_2O_2 production appears to be inhibited by Nod factors [32]. It was suggested that ROS production is necessary for infection initiation, but prolonged and elevated levels are detrimental to nodulation [46]. Bacterial Nod factors were found to stimulate oxidative burst by blocking

the induction of *nod* genes in plants when the interaction between symbionts was incompatible [37, 125].

Under bacterial infection, legume NADPH oxidases play a pivotal role in production of $O_2^{\bullet -}$ and H_2O_2 and, in turn, have a crucial role in different stages of nodulation [47]. The inhibition of ROS production by the NAD(P)H oxidase inhibitor diphenyleneiodonium [126] and the correlation between ROS accumulation and transcript accumulation of two NADPH oxidase genes in response to Nod factors in *M. truncatula* roots [32] support for the involvement of NADPH oxidases in ROS generation. The involvement of other potential enzymatic ROS sources cannot be excluded. The source of H_2O_2 is a number of plant peroxidases and other oxidases [127]. Production of ROS in legume-rhizobium symbiosis also occurs during the reductive processes required for nitrogen fixation. Many compounds that act as electron donors for nitrogenase (e.g. ferredoxin) can undergo auto-oxidation with $O_2^{\bullet -}$ formation. ROS production may also be promoted by leghemoglobin, which facilitates O_2 transport to the bacteroids at a low but constant flux, thus preventing O_2 inactivation of nitrogenase [42]. In the presence of O_2, leghemoglobin can undergo auto-oxidation, and as a result, $O_2^{\bullet -}$ is generated with further dismutation to H_2O_2 [42, 94]. The interaction of leghemoglobin with H_2O_2 leads to the formation of a highly oxidized ferric-porphyrin cation radical, which further can oxidize protein molecules with formation, for example, tyrosine radicals [128]. H_2O_2 can be released from leghemoglobin and promote $HO^{\bullet}$ generation via Fenton reaction [128].

Together with ROS, RNS are now considered as major components of oxidative burst and redox regulation [41]. RNS, such as nitric oxide ($^{\bullet}NO$) and peroxynitrite ($ONOO^-$), can be formed in nodules and other plant organs. There are several possible pathways of $^{\bullet}NO$ synthesis, which can be divided into oxidative (NO synthase, polyamine-mediated, hydroxylamine-mediated) and reductive (plasma membrane-bound nitrite NO reductase, mitochondrial electron transport chain, xanthine oxidoreductase) pathways [46, 123]. To date, there is no evidence for an involvement of the bacterial partner in NO production during symbiosis establishment. As with ROS, uncontrolled formation of RNS is potentially dangerous and may cause cellular damage, but low concentrations of RNS, especially of $^{\bullet}NO$, are critical in many plant processes, stress responses, and nodule formation [39, 123]. Transcriptomic analysis at an early stage of the symbiosis showed that $^{\bullet}NO$ is potentially involved in the repression of plant defense reactions, favoring the establishment of the legume-rhizobium interaction [45]. Various genes involved in the developmental program of the root hair during nodulation (kinases, receptor-like kinases, and transcription factors), in carbon metabolism (sucrose transport, sucrose synthase, or malate dehydrogenase), as well as in proteasome-dependent proteolysis were upregulated by $^{\bullet}NO$. Genes involved in the control of the cellular redox state, such as glutathione (GSH) and H_2O_2 metabolism, are also regulated by $^{\bullet}NO$ [45]. Redox signaling mediated by RNS is realized via posttranslational modification of antioxidant proteins or transcription factors. RNS can lead to nitrosylation (addition of an NO group) or nitration (addition of an NO_2 group) of

cysteine or tyrosine residues, respectively [46]. In nodules of *M. truncatula*, $^{\bullet}NO$ has been shown to activate two genes encoding proteins involved in H_2O_2 metabolism (peroxidase and germin-like oxalate oxidase), suggesting a cross talk between ROS and RNS signaling [123, 129]. Peroxynitrite ($ONOO^-$) is a signaling molecule formed when $^{\bullet}NO$ reacts with $O_2^{\bullet -}$. Its function may be mediated by the selective nitration of tyrosine residues in a small number of proteins [46].

Nitrogenase complex in bacteroids is very sensitive to ROS attack; therefore, it is not surprising that legume nodules have efficient mechanisms to maintain proper redox balance and low ROS levels. Because of susceptibility of N_2 fixation to oxidative damage, legume nodules have evolved a complex and wide range of defense mechanisms aimed at destroying ROS or preventing their formation [42]. Nodules possess a powerful antioxidant system, which includes antioxidant enzymes (superoxide dismutase, catalase, and various peroxidases), enzymes of the ascorbate-glutathione cycle, and low-molecular mass antioxidant metabolites such as ascorbate, glutathione, and tocopherols [40, 123]. The capacity of nodule antioxidant system affects largely nitrogen-fixing efficiency; in particular, nodules may not function without ascorbate-glutathione cycle [123].

3.3 Redox Balance and Nodule Senescence

The lifespan of the rhizobia-plant symbiotic relationship is relatively short, and the disruption of this symbiosis affects the yield of the crop. Average nodule lifespan is 10–12 weeks, but their N_2-fixing capacity starts to decline 3–5 weeks after initiation, and this decline is caused by nodule senescence [130]. Puppo et al. [130] concluded that nodule senescence is an active and programmed process in development, in which ROS, antioxidants, hormones, and proteinases have key roles. A typically visible sign of nodule senescence is its color, changing from pink to green because of disruption of Lb activity. In aging soybean nodules, green Lb arises from heme nitration, underlining the critical role of RNS in the senescence process [45].

Nitrogen-fixing nodules are particularly rich in antioxidant defense mechanisms, which are sufficient to cope with ROS toxicity. During the natural senescence of nodules, levels or activity of some antioxidants (glutathione, catalase), but not others (ascorbate peroxidase, α-tocopherol), significantly decreases [131]. The redox imbalance followed by oxidative stress promotes oxidation of lipids and proteins and the degradation of membranes. Aging nodules accumulate oxidized thiols, lipids, proteins, and DNA. Aging was shown to cause a 50% decrease of homoglutathione in soybean and bean nodules and an 82% decrease of glutathione in pea nodules [131].

In mature nodules, $^{\bullet}NO$ was shown to inhibit N_2 fixation and to trigger nodule senescence [45]. *S. meliloti* degrades $^{\bullet}NO$ in *Medicago* nodules, leading to a delay in nodule senescence [94, 132]. $O_2^{\bullet -}$ and H_2O_2 were also supposed to act as signaling molecules involved in senescence of legume-rhizobium symbiosis [44]. At the later stages of symbiotic formation, when the amount of rhizobia in the roots reaches

a certain level, the host plant may also induce mechanisms of ROS generation to regulate further nodule formation [39]. Furthermore, during nodule senescence high ROS levels have been detected in senescing symbiosomes suggesting ROS involvement in this process [36].

Developmental nodule senescence is a complex and programmed process, which induces a decrease of nitrogen-fixing activity and leghemoglobin content, modifications in the nodule antioxidant components, and an increase of proteolytic activity, ultimately leading to the death of infected cells. Leghemoglobin, which plays a fundamental role in nodule functioning, is an important physiological marker for following the progression of nodule senescence [7]. Content of leghemoglobin progressively decreases with the onset of senescence. In turn, this decreases O_2 availability to bacteroids and nitrogenase, releasing free iron to produce ROS via Fenton reaction. The auto-oxidation of the active form of leghemoglobin to ferro-Lb-O_2 is associated with $O_2^{\bullet -}$ and H_2O_2 generation and the degradation of the heme group of leghemoglobin by H_2O_2 that likely allows the release of the catalytic Fe. The latter may enhance the production of $HO^{\bullet}$ through the Fenton and the Haber-Weiss reactions [7]. In senescent soybean nodules, the high level of H_2O_2 detected in the cytoplasmic and apoplastic compartments of the infected tissue was associated with an enhanced expression of cysteine protease gene suggesting a link between oxidative stress and proteolytic activities under nodule senescence [7, 133].

In conclusion, ROS and RNS are involved in the regulation of legume-rhizobium symbiosis. However, they may have different roles, as they are involved in the establishment and the functioning of the nodule on the one hand and in the regulation of the nodule number and the onset of senescence on the other hand [93].

4 Toxic Effects of Heavy Metals on Legumes and Legume-Rhizobium Symbiosis

4.1 Toxicity of Heavy Metals in Plants: Overview

Plants receive mineral elements from the soil primarily in the form of inorganic ions [55]. Mineral elements can be divided into two groups: essential nutrients and toxic non-nutrient elements. The first group includes the macronutrients such as nitrogen (N), potassium (K), calcium (Ca), magnesium (Mg), phosphorous (P), sulfur (S), and silicon (Si) and the micronutrients – chlorine (Cl), iron (Fe), boron (B), manganese (Mn), sodium (Na), zinc (Zn), copper (Cu), nickel (Ni), and molybdenum (Mo). These elements are essential components of plants, and their absence or deficiency may cause adverse biochemical perturbations leading to morphological changes and inhibition of plant growth and reproduction. Micronutrients (Cu, N, Zn, Mo, etc.) are required in very small quantities. Many anthropogenic activities lead to excessive accumulation of microelements in the soil that makes them hazardous to the majority of plant species. Other minerals called heavy metals like cadmium (Cd),

mercury (Hg), lead (Pb), chromium (Cr), arsenic (As), silver (Ag), and antimony (Sb) are toxic to plants even at low concentrations [55, 134].

Heavy metals are the main group of inorganic pollutants, which contaminate large soil areas, and many of them cause serious risks for agricultural plants and, respectively, human health [135]. The most important sources of heavy metals in the environment are human activities such as mining, smelting procedures, metallurgical industry, chemical industry, traffic, intense using of pesticides and detergents, etc. [134]. Heavy metals are non-biodegradable; therefore, they can be accumulated in the soils [135, 136]. Plants, including legumes, are able to uptake heavy metals from soils that commonly have a negative impact on their physiological and biochemical processes. Plant responses to heavy metal exposure are dose-dependent. For essential metals, these responses have several dose-dependent phases – from deficiency-sufficiency at low doses of the metal, tolerance at moderate doses, and to toxicity at high doses. For nonessential metals, only tolerance and toxicity stages take place [137]. The adverse effects of heavy metal include inhibition of seed germination and seedling development, reduction in root and shoot biomass, mutagenic effects, accelerated senescence and death of plants, and decreased quality of flowers and crop yield [134, 137–139]. Many of these effects are caused by the ability of heavy metals to directly modify many proteins and DNA and to inhibit biosynthesis of chlorophylls and proteins [140–142]. As a result, progressive chlorosis and necrosis and decreased protein content are typical features of heavy metal toxicity in plants [143].

Heavy metals (Cd, Cr, Ni, Hg, etc.) are present in the soils as free metal ions, soluble metal complexes (sequestered to ligands), exchangeable metal ions, organically bound metals, and precipitated or insoluble compounds such as oxides, carbonates, and hydroxides, or they may constitute a part of the structure of silicate materials [144]. The primary toxicity mechanisms of the metal ions may be different due to their chemical properties, especially valence, ion radius, and capacity to form organic complexes. Metal toxicity is also greatly influenced by the coexistence of other metals in the soil, which could have both synergic and antagonistic effects depending on the relative concentrations and other soil properties (i.e., presence of nutrient elements). For example, Ca^{2+} strongly inhibits the uptake of Ni in *Arabidopsis bertolonii*, whereas the opposite effect was observed in *Berkheya coddii* [55, 145]. Heavy metals can inactivate directly many metal-containing proteins via substitution of the primary metal or causing protein denaturation. In particular, chromium ions (VI) inhibit such enzymes as nitrate reductase [146, 147] and Fe^{3+} reductase in plant roots [148]. In plant mitochondria, Cr^{6+} can inhibit electron transport by replacing Cu and Fe ions in prosthetic groups of many mitochondrial redox carriers [145, 149].

4.2 Oxidative Stress as a Mechanism of Heavy Metal Toxicity

Similar to other biotic and abiotic stresses, heavy metal exposure also induces oxidative stress development in plant cells [117, 119, 145, 147]. Depending on the chemical properties and behavior of metals in biological systems, one of the

following mechanisms can cause their toxicity: *i*) interference with functional sites in proteins; (*ii*) displacement of essential elements in the enzymes, leading to loss of enzymatic activity; and (*iii*) increase in ROS levels [119, 150, 151]. Increased ROS production can be a result of inhibition of electron transport chains in chloroplasts and mitochondria, metal-induced denaturation of antioxidant enzymes, or exhaustion of a pool of reduced glutathione [55, 119]. Excess of heavy metals increase ROS production in subcellular organelles such as peroxisomes, chloroplasts, and mitochondria, which constitute together the predominant sources of ROS production in plants [116].

Some of heavy metals (Cu, Fe, Cr) belongs to transition metals with changeable valence; therefore, they can participate, in cellular redox reactions, affecting directly ROS production [119]. These metals at high doses may stimulate ROS production via participation in the Haber-Weiss reaction or Fenton reactions [55, 119, 151]. For example, Cr^{6+} is reduced by cellular reductants, such as glutathione, to Cr^{5+}, which can further react with H_2O_2 in Fenton reaction with $HO^{\bullet}$ formation [152]:

$$Cr^{6+} + O_2^{\bullet -} \rightarrow Cr^{5+} + O_2 \quad (1)$$

$$Cr^{5+} + H_2O_2 \rightarrow Cr^{6+} + HO^{\bullet} + OH^{-} \quad (2)$$

Enhanced ROS production has a negative impact on plant cells, since these species can interact with virtually all cellular components, namely, lipids, carbohydrates, proteins, nucleic acids, etc. When the levels of ROS are significantly increased, cells undergo oxidative stress [118, 119, 151, 153]. Oxidative stress is resulted in enhanced lipid peroxidation of membranes [55], oxidation of many proteins, various modifications of DNA bases, and changes in homeostasis of calcium and thiol groups [154]. Heavy metal-induced lipid peroxidation has one of the most deleterious effects in plants, since it alters membrane fluidity, and structure, and inhibits membrane-dependent processes such as electron flow in chloroplasts and mitochondria [55, 150].

To counteract oxidative stress, plant cells possess various defense systems, which consist of nonenzymatic and enzymatic antioxidants, metal chelators, and repair components [116, 153, 155]. Antioxidant system of plants includes *i*) the enzymes that directly scavenge ROS and other free radicals (superoxide dismutase, catalase, and different peroxidases); (*ii*) the nonenzymatic low-molecular mass antioxidants such as ascorbate, glutathione, α-tocopherol, carotenoids, and phenol compounds; (*iii*) the enzymes of ascorbate-glutathione pathway, which scavenge H_2O_2 in a coupled series of reactions by using NAD(P)H; (*iv*) the enzymes involved in the disulfide reduction, thioredoxin and glutaredoxin; and (*v*) the metal-binding proteins such as ferritin, phytochelatins, and metallothioneins [28, 116, 123]. Antioxidant system can overcome oxidative stress and oxidative damages, if cells are exposed to heavy metals at the low and moderate levels. However, high metal concentrations may induce higher intensity oxidative stress. In this case, the antioxidant system capacity may not be sufficient to cope with damaging effects of heavy metals, and even antioxidant enzymes can be inactivated [118].

4.3 Effects of Legume-Rhizobium Symbiosis on Heavy Metal Toxicity

Elevated levels of heavy metals in soils have deleterious effects not only for plants but also for soil microbiota. Heavy metals may cause changes in microbial composition and decrease beneficial activities of microsymbionts [156]. The decline in plant growth and symbiosis was found in white clover plants, which were grown in soils contaminated with cadmium, lead, and zinc [54, 157]. Many metals (Cu, Ni, Zn, Cd, Cr, As) were found to inhibit the growth, morphology, and activities of various symbiotic N_2-fixing bacteria like *R. leguminosarum*, *Mesorhizobium ciceri*, *Bradyrhizobium* sp. [53, 158–161]. A strong inhibitory effect of copper on growth and enzyme activities of *Bradyrhizobium* BMP1 strain was found [59]. Hirsch and coauthors showed that *R. leguminosarum* bv. *trifolii* population was significantly altered by long-term exposure to heavy metals, and this rhizobia lost the ability to establish functional symbiosis with white and red clover [162]. In addition to the toxicity of heavy metals on the growth and survival of *Rhizobia*, nodulation defects in legumes were also observed [163]. Effective *R. leguminosarum* bv. *trifolii* population did not survive during long-term incubation in soils containing 7.1 mg Cd kg^{-1} [62].

Heavy metals can disturb redox balance in both the host and rhizobia affecting their growth and decreasing efficacy of legume-rhizobium symbiosis [28, 49, 50, 52–56]. Several studies have reported that nitrogen-fixing bacteria can diminish the toxicity of heavy metals (Cd, Cr, Cu, Pb, etc.) on host plants, since the root nodules can be the major accumulators of heavy metals from the soil [57–59, 62]. At the same time, legume-rhizobium symbiosis seems to be also sensitive to heavy metals, and its protective effects against metal toxicity are not fully clear. It should be noted that resistance of the bacteria to heavy metals is both species- and strain-specific [59]. We can surmise that rhizobia with powerful protective systems can be successfully used for effective soil bioremediation [28, 51, 58, 60–62].

5 Conclusions and Perspectives

Legume-rhizobium symbiosis seems to be a great example of plant and bacterial coevolution. Root secretion and plant immunity are key factors determining interaction of rhizobia with plant roots. There are many members of the microbiota in the soil, and rhizobia must compete with them before infecting legumes and forming nitrogen-fixing bacteroids. The ability to respond to plant signals and chemoattractants and to colonize root surfaces is crucial for the competitive success of these bacteria. There tends to be strict species specificity between legumes and their compatible symbionts. Genetic and molecular mechanisms that regulate symbiotic specificity are diverse, involving a wide range of host and bacterial genes/signals with various modes of action. A variety of secondary metabolites released by both the host plant and bacteria are involved in mutual recognition and nodule development. In particular, the specificity is determined largely by the structure of

bacterial Nod factors, whose synthesis is induced by plant flavonoids. A variety of rhizobial cell-surface exopolysaccharides and lipopolysaccharides and secreted proteins appear to function as signals from the rhizobia to its host. Recent studies have just begun to disclose the underlying molecular mechanisms that regulate this specificity, and there are many challenging questions waiting to be answered.

Increasing evidence has shown that ROS/RNS, especially H_2O_2 and $^{\bullet}NO$, play an important signaling role in the establishment of legume-rhizobium symbiosis, the functioning and senescence steps in mature nodules. Changes in the levels of these reactive species in both partners impair either the development of the nodules or their N_2-fixing activity. At low levels, ROS/RNS activate expression of genes involved in progression of infection threads, nodule development, and differentiation of bacteroids. High levels of ROS/RNS lead to the development of intense oxidative stress and accelerated senescence of nodules. Since nitrogenase of bacteroids is very sensitive to oxidation, enhanced ROS/RNS levels may decrease N_2-fixing activity of bacteroids. Elevated levels of heavy metals in soils can increase significantly ROS production in both legumes and their microsymbionts. In legume-rhizobium symbiosis, rhizobia seem to be more stressed due to preferential accumulation of heavy metals in root nodules. Data available suggest that using rhizobia can be an effective approach to minimize toxic effects heavy metals have on agricultural plants. At the same time, the protective efficacy of nodule bacteria depends on many factors such as type and concentrations of heavy metals, compatibility of partners, bacterial virulence, adaptive capacity of both partners, N_2-fixing activity of bacteria, etc. Therefore, the study of effects of heavy metal on legume-rhizobium symbiosis and search of ways to enhance metal resistance of nodule bacteria are perspective directions for future research. The genetic construction of rhizobia strains better adapted to field conditions and with enhanced stress resistance and compatibility with legumes may be a great opportunity to increase the benefit from their use in bioremediation of soils polluted with heavy metals.

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Effects of Cyanobacterial Secondary Metabolites on Phytoplankton Community Succession

14

Ying Pei, Runbing Xu, Sabine Hilt, and Xuexiu Chang

Contents

Abstract

Allelopathic effects are one of the factors potentially influencing the succession of phytoplankton communities; however, their influence has often been neglected. This is especially true for cyanobacteria that often outcompete other phytoplankton species and form blooms causing severe problems. Allelopathic effects of cyanobacteria can play an important role for phytoplankton succession. In this chapter, we introduce the different ways how aquatic organisms are influenced by cyanobacterial allelochemicals; the mechanisms of their interaction from the

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aspects of chemical intermediates, target reaction, and target signals; and interfering factors and the ecological consequences of this process.

Cyanobacteria produce and excrete a variety of allelopathic compounds that affect other Cyanophyta, eukaryotic algae, bacteria, zooplankton, higher plants, and fish and mammalian cells. These effects are regulated by various abiotic and biotic conditions, such as nutrient availability, temperature, and light intensity but also cell density and growth phase of the source cyanobacterial community. The bioactive metabolites include cyclic peptides, alkaloids, terpenoids, and others which can have a variety of inhibitory effects on the different target organisms. Ecological consequences such as declines in biodiversity and accumulation of toxins in the food chain have been shown. However, most of these compounds have not yet been fully tested regarding their full range of effects on natural phytoplankton communities. A detailed elucidation of the influence of cyanobacterial allelochemicals is of key importance for understanding and managing the succession of natural phytoplankton communities.

Keywords
Cyanobacteria · Allelopathy · Secondary metabolites · Phytoplankton succession · Chemical ecology

1 Introduction

The seasonal succession of phytoplankton is an annually repeated process of community assembly in freshwater and marine ecosystems shaped by external factors and internal interactions. While the role of physical factors, grazing, and nutrient limitation has been known for long, several ecological interactions have become research foci more recently, such as overwintering of key organisms, the microbial food web, parasitism, and higher-order predators. These novel interactions revealed strong effects on species replacements as summarized in a review by Sommer et al. [1]. One mechanism affecting phytoplankton succession, however, has still been neglected despite early studies [2, 3] indicating its potential relevance: allelopathy.

The term allelopathy was first introduced by Molisch [4] to describe the process of ethylene accelerating fruit ripening, as an effect that one plant impacted another [4]. Rice [5] redefined this term as any direct or indirect effects of compounds produced and released by plants and microorganisms on other plants (microbes) in the negative or positive way. In 1996, the International Allelopathy Society (IAS) further developed this definition into a process involving secondary metabolites produced by bacteria, fungi, algae, and plants secondary metabolites that impact biological systems. Allelochemicals are the compounds produced and released by one organism that directly influences others [6]. Studies investigating allelopathic effects of terrestrial plants are abundant in the field of agriculture and forestry [7, 8]. However, allelopathic autotrophs are also a well-known phenomenon in aquatic ecosystems [9].

Already decades ago, allelopathic effects of cyanobacteria have been suggested as a major controlling factor for successions of phytoplankton communities [2, 3]. Cyanobacteria, prokaryotic bacteria with photosynthetic ability, and important autotrophic producers are reported in each continent except Antarctica [10]. Anthropogenic eutrophication has resulted in the occurrence of harmful cyanobacterial blooms in many marine and freshwater ecosystems that can cause severe problems [11, 12]. Certain cyanobacterial species outcompete other members of the phytoplankton communities and form thick mats in the upper layer of water bodies that can cause decline and deaths of other members of the aquatic community [11]. The reasons accounting for this phenomenon have not been fully understood [13]. Buoyancy regulation, the higher optimal temperature, and efficient nutrient uptake systems have been suggested to be involved [10, 14, 15]. Keating (1977, 1978) presented the first evidence for cyanobacterial allelopathy as a major controlling factor in bloom sequence determination [2, 3]. The huge variety of secondary metabolites produced by cyanobacteria has stimulated their isolation and characterization for potential commercial use, e.g., in pharmacy [16–19]. These studies have also advanced research on allelopathic interactions among phytoplankton.

In this chapter, we summarize the known effects of cyanobacterial allelochemicals on different aquatic organism groups, their specific mechanisms, regulatory factors, and ecological consequences of cyanobacterial allelopathic effects in aquatic ecosystems to evaluate the state of the art and detect major knowledge gaps.

2 Effects of Cyanobacterial Allelochemicals

Allelopathic activity includes both inhibitory and stimulatory effects of the donor on the acceptor [2, 20, 21]. The majority of studies published about the allelopathic effects of cyanobacteria in aquatic systems have been focused on negative impacts. Allelopathy of cyanobacterial species is considered as one of the reasons for their bloom formation in the early stages by outcompeting other autotrophs. On the other hand, allelochemicals mediating these effects are also toxic to organisms of other trophic levels.

2.1 Effects on Planktonic Phototrophs

All phytoplankton species compete with cyanobacteria for light and nutrients; among them green algae are often concerned by researchers as a target of allelopathic inhibitory effects by cyanobacteria. Allelopathic effects of cyanobacteria on Chlorophyta are species-specific. Figueredo et al. [22] found allelopathic effects of *Cylindrospermopsis raciborskii* cells on two species of Chlorophyta, *Coelastrum sphaericum* and *Monoraphidium contortum*. Even at low cell density, some cyanobacteria (e.g., species in genera *Oscillatoria* and *Cylindrospermopsis*) were shown to strongly suppress the growth of Chlorophyta, *Ankistrodesmus falcatus* and *Chlorella vulgaris* [23]. Bittencourt-Oliveira et al. [24] investigated the effects of

toxin production and non-toxin-producing strains of cyanobacteria, *Microcystis aeruginosa* and *Microcystis panniformis*, respectively, on different strains of Chlorophyta, *Monoraphidium convolutum* and *Scenedesmus acuminatus*. The results showed inhibitory effects of both cyanobacterial strains on both green algae and different strains of green algae exhibited differential sensitivity.

Diatoms are another important phytoplankton group which comprise about 25% of the world's net primary production and are essential to several biogeochemical cycles [25, 26]. Several studies indicate allelopathic effect of cyanobacteria on diatoms. Schagerl et al. [27] found three cyanobacterial strains, *Anabaena torulosa* and two *Nostoc* strains that allelopathically inhibited the growth of a naturally co-occurring diatom strain, *Fragilaria* sp., through agar diffusion tests. The sensitivity of diatoms varied depending on the donor species, the acting chemicals, and the diatom species. Wang et al. [28] could show that *M. aeruginosa* cells can have severe inhibitory effects on the target diatom *Cyclotella* sp. during their exponential growth phase. Repeated addition of cyanobacterial culture filtrates showed the strongest impacts on the diatom *Thalassiosira* sp. as compared to single additions [29].

Cryptophytes were also found to be affected by cyanobacterial allelochemicals. Suikkanen et al. [29] tested three cyanobacterial species (*Nodularia spumigena*, *Aphanizomenon flos-aquae*, and *Anabaena lemmermannii*) and found inhibitory effects on the cryptophyte *Rhodomonas* sp. for all. In subsequent studies, they found that not the hepatotoxin nodularin but unknown metabolites from the cyanobacterium *Nodularia spumigena* inhibited the growth of the cryptophyte species (*Rhodomonas* sp.) from the same habitat [21, 30]. Similar inhibitory effects were also found in a system including *Microcystis aeruginosa* (cyanobacteria) and *Cryptomonas ovata*; the Cryptophyta was heavily inhibited by *M. aeruginosa* with living cells showing the strongest effect [31].

Cyanobacterial species are also inhibited by other co-occurring cyanobacteria by producing and releasing inhibitory metabolites [32]. Keating [2] found that, in most cases, the dominant cyanobacterial species in a pond allelopathically inhibited their cyanobacterial predecessors and promoted their cyanobacterial successors. *Microcystis* sp. can affect the cell differentiation of the filamentous cyanobacterial genus *Trichormus* by decreasing their heterocyst and akinete forming [33]. The genus *Trichormus* also allelopathically inhibited several cyanobacterial strains, and this inhibitory effect was mostly stronger than that of Chlorophyta species tested [34]. The genus *Nostoc* is a filamentous and nitrogen-fixation group of cyanobacteria with published allelopathic abilities [35]. Schagerl et al. [27] documented strong allelopathic effects of *Nostoc* sp. on strains of *Anabaena* and *Microcystis*. Strains of filamentous *Oscillatoria* were documented to produce bioactive metabolites cyclic peptides portoamide A which inhibited the growth of *Cylindrospermopsis raciborskii* [36].

2.2 Effects on Other Aquatic Organisms

Secondary metabolites of cyanobacteria have also been shown to directly or indirectly influence other, non-phototrophic organisms of the aquatic food web. Events

of humans and livestock poisoned by cyanobacterial bloom have been documented since long [11, 12, 37]. Most studies have focused on microcystins, hepatotoxic cyclic peptides that have been restricted by WHO to 1 μg L^{-1} for drinking water [38]. However, cyanobacteria have also been observed to adversely affect other organisms by releasing microcystins but also other secondary metabolites.

2.2.1 Bacteria

Heterotrophic bacteria can be strong competitors of phytoplankton and thus also affect their succession, in particular in environments where phytoplankton production is limited by the availability of mineral nutrients (e.g., Bratbak and Thingstad [39]). Some cyanobacteria have been observed to suppress the growth of gram-negative and positive strains [40]. Metabolites from filamentous cyanobacteria *Lyngbya* sp. inhibited the model bacteria genus *Bacillus*, but no effects were found on other bacteria taxa, such as *Pseudomonas* and *Streptococcus* [41]. This result indicates that the inhibitory effects on bacteria are also target-specific. Allelochemicals from the cyanobacterium *Nostoc insulare* inhibited both gram-positive and –negative species of bacteria at the level of μg/mL [42]. The bioactive metabolites from *Phormidium* sp. strongly retrained the taxa of *Acidobacteria* subgroup 6 and *Gemmatimonadetes* when both groups formed natural community assemblages. They were totally vanished in the allelochemical treatment group while strains of Rhodospirillaceae and members of *Flectobacillus* considerably increased [43].

2.2.2 Macrophytes

Aquatic macrophytes can strongly affect phytoplankton abundance and thus potentially also their succession through several different direct and indirect mechanisms. These interactions can result in the stabilization of clear-water conditions and the occurrence of alternative stable states in different aquatic ecosystems [44–46]. Mohamed [47] recently reviewed the available literature on macrophyte-cyanobacterial allelopathic interactions and found studies on allelopathic activities of cyanotoxins affecting more than ten different emerged and submerged macrophyte species. He concluded that although most studies were conducted at concentrations beyond environmental relevance, there are still indications for harmful allelopathic effects of microcystins on macrophytes under realistic in situ conditions. Cyanobacterial allelochemicals have been found to affect seedling germination, seedling growth, and leaf photosynthesis of macrophytes. Photosynthesis of seedling leaves seems the most sensitive to cyanobacterial allelochemicals [48]. Some studies indicate interesting mutual allelopathic effects among cyanobacteria and macrophytes, e.g., Xu et al. [49] showing negative effects of exudates of *Microcystis* sp. on seedling vitality and growth of two variants of the submerged macrophyte species *Ottelia acuminata*, while the culture water of mature macrophytes promoted the growth of cyanobacteria.

2.2.3 Zooplankton

Grazing of phytoplankton by zooplankton is one of the main factors influencing seasonal phytoplankton succession [1]. Generalist and tolerant grazers may reduce cyanobacterial blooms [50], while selective and tolerant grazers are expected to

facilitate cyanobacteria by grazing on their eukaryotic phototrophic competitors [51, 52]. Direct ingestion of cyanobacteria by zooplankters has been shown, and interactions between *Daphnia* and cyanobacteria have been investigated extensively. Generally, cyanobacteria have toxic effects on *Daphnia* [53]. Rohrlack et al. [54] found that metabolites from *Microcystis* sp. caused a lethal molting disruption in *Daphnia* spp. The toxin microcin SF608 proved to inhibit the detoxification enzyme glutathione S-transferase (sGST) of *Daphnia* [55]. However, *Daphnia* was able to increase its tolerance if continuously exposed to cyanobacteria [56].

2.2.4 Higher Tropic Levels

Cyanobacterial toxins have also been found to target different aquatic vertebrates and amphibians that may indirectly affect phytoplankton succession by top-down control of zooplankton or their predators. Cyanobacterial exudates have teratogenic effects on amphibians and interference with their embryo growth, and these effects are similar to those caused by the famous teratogen retinoid [57]. Fish are often strongly affected by cyanobacterial toxins, especially during their early development. Zi et al. [58] found that cell-free filtrates of *M. aeruginosa* posed a malformation of the heart at the embryo stage of an endangered Chinese fish species (*Sinocyclocheilus grahami*). On the other hand, cyanobacteria decreased the mortality rates of fish by their antimicrobial properties against pathogen microbes [59]. Retinoid-like activities of cyanobacterial exudates caused diverse teratogenic effects and interference with the growth of zebrafish embryos, but microcystins were not responsible for any of the observed effects [60, 61]. Chronic toxicity on zebrafish larvae by exudates of the bloom-forming cyanobacterium *Cylindrospermopsis* was manifested by Zagatto et al. [62].

3 Cyanobacterial Secondary Metabolites and Their Mode of Action

3.1 Secondary Metabolites

Cyanobacteria produce a wide range of toxins including hepatotoxins, cytotoxins, neurotoxins, and dermatotoxins [10, 63]. The most famous toxins include microcystins, cylindrospermopsins, nodularins, anatoxins-a, and saxitoxins [63] which were intensely studied due to their potential effects on human health and commercial use. Each of them has a main structure core with diverse moieties to variants, and the metabolic pathways of their synthesis have been elucidated [63–65].

Microcystins (MCs) are the most well-known and widespread cyanobacterial toxins around the world. Their functions as feeding deterrent, metal-chelating agent, and signaling molecule have been described, but the role of microcystins as allelopathic compounds is still under discussion [66, 67]. From an evolutionary perspective, the development of microcystins has been long before the occurrence of eukaryotic phototrophs, and their initial role may not be related to allelopathic effects [66]. However, several studies clearly indicate that microcystins can affect

phytoplankton communities under natural or in situ-like conditions [33, 68]. In summary, the natural functions of microcystins need further consideration in future research.

Cylindrospermopsin (CYN) produced by genus *Cylindrospermopsis* and other strains are cytotoxic and hepatotoxic [63, 69]. To date, five different CYNs have been isolated with different shares of the total CYN concentration, but their physiological function is not clear [70]. Several studies support the role of CYNs as allelochemicals. CYNs inhibited the growth and caused cell necrosis in the target strain *M. aeruginosa*, and the production of CYNs increased under environmental stress [71, 72]. CYNs may thus support the dominance and expansion of CYNs-producing strains [70]. Another toxin produced by cyanobacteria is saxitoxin [70, 73], but its neurotoxic property has led to a research focus on mammal poisoning.

Overall, most studies on these well-known cyanobacterial toxins have focused on their toxic abilities against humans or animals, while research on their potential role in controlling phytoplankton communities and their succession are still rare.

Apart from those widely studied toxins, other toxic chemicals have also been separated from several cyanobacterial strains from the genus *Microcystis*, *Fischerella*, *Anabaena*, *Aphanizomenon*, *Cylindrospermopsis*, *Nostoc*, etc. The most common effective secondary metabolites include cyclic peptides, alkaloids, terpenoids, ketone/ester, and member of the phenyl family (Table 1). The potential role of these secondary metabolites as allelochemicals or pharmaceutical or for agricultural applications has been reviewed in several studies [16, 18, 74]. In this chapter, we describe selected bioactive chemicals, their source species, modes of actions, and target species.

Peptides produced by cyanobacteria are the best studied type of secondary and bioactive metabolites. The most famous example are microcystins (see above), while other cyclic peptides detected in cyanobacteria include fischerellin, hassallidin, and portoamide. These peptides have shown toxicity against cyanobacteria, green algae, bacteria, yeast, and crustaceans (Table 1).

Bio-alkaloids are commonly considered to be potent toxins [75, 76] and are also found in the cells of cyanobacteria (Table 1). They showed inhibitory effects on cyanobacteria, bacteria (model), green algae, and zebrafish embryos. The majority of these chemicals are indole moiety contained. Most of the studies relevant for aquatic ecosystems indicate that alkaloids are more likely to interact with the environment of cyanobacteria than peptides. However, the pathways of their synthesis have rarely been explained and need cross-disciplinary research.

Terpenoids are compounds existing in all living organisms and consist of diverse structural variants. Their synthesis in cyanobacterial cells via the methylerythritol-phosphate (MEP) pathway has been identified, and present knowledge has led to the manipulation of their synthesis [77]. Documented natural terpenoids from cyanobacterial sources are inhibitors of bacteria, cyanobacteria, eukaryotic algae, invertebrates, and vertebrates (Table 1). They are mostly low molecular weight compounds and their toxicity can be weakened by shaking or "disturbing" the cultures [78].

Table 1 Bioactive chemicals from various cyanobacterial strains and their action on different aquatic organisms

	Species	Chemicals	Actions	References
Peptides				
1	*Fischerella muscicola*	Fischerellin	Inhibition of photosynthesis of other cyanobacteria and chlorophytes	Gross et al. [84]
2	*Microcystis aeruginosa*	Kasumigamide	Suppression of the green alga *Chlamydomonas neglecta*	Ishida and Murakami [85]
3	*Microcystis aeruginosa*	Microviridin J	Lethal molting disruption in *Daphnia* spp.	Rohrlack et al. [54]
4	*Lyngbya* sp. strain 15-2	Pahayokolides A and B	Inhibition of *Bacillus*, yeast *Saccharomyces cerevisiae*, co-habited green algae, cancer cell lines	Berry et al. [41], An et al. [86]
5	*Anabaena* *Aphanizomenon* *Cylindrospermopsis raciborskii* *Nostoc* *Tolypothrix*	Hassallidin	Activity against parasitic fungi, *Candida albicans*	Vestola et al. [87]
6	*Oscillatoria* sp.	Portoamides A and B	Inhibition of green algae *Chlorella vulgaris*, cyanobacteria *Cylindrospermopsis raciborskii* LEGE 99045, Chlorophyta *Ankistrodesmus falcatus* *Chlamydomonas reinhardtii* CCAP 11/45	Leao et al. [36]
7	*Microcystis aeruginosa*	Aeruginazole	Mild antimicrobial activity	Adiv et al. [88]
8	*Genus Microcystis*	Piricyclamides	Not tested	Leikoski et al. [89]
9	*Microcystis* sp.	Microcin SF608	Inhibition of trypsin activity; stress reaction in water moss; in *Daphnia*: inhibition of detoxification enzymes	Wiegand et al. [55], Banker and Carmeli [90]
10	*Microcystis* sp.	Micropeptins SF909, SF995	Inhibition of activity of chymotrypsin and trypsin	Banker and Carmeli [90]

11	*Nostoc* spp.	Nostocyclamid M	Growth inhibition of *Anabaena* 7120	Jüttner et al. [91]
12	*Microcystis aeruginosa*	Aerucyclamides A and B	Toxic to the freshwater crustacean *Thamnocephalus platyurus*	Portmann et al. [92]
Terpenoid				
1	*Microcoleus lacustris*	Two abietane diterpenes (20-nor-3a-acetoxy- abieta-5,7,9,11,13-pentaene and 20-nor-3a-acetoxy-12- hydroxy-abieta-5,7,9,11,13-pentaene)	Antibacterial activity	Pérez Gutiérrez et al. [93]
2	*Calothrix* PCC 7507	Eremophilone Geosmin	Inhibition of insects and crustacea	Höckelmann et al. [94]
3	*Microcystis aeruginosa*	Terpenoid: β-Cyclocitral β-Ionone Sulfur compounds: Dimethyl sulfide Dimethyl trisulfide	Inhibition and damage *Microcystis aeruginosa* cells	Zhang et al. [95]
4	*Microcystis aeruginosa*	VOCs naphthalene *β*-Cyclocitral *β*-Ionone	Not tested	Walsh et al. [96]
5	*Microcystis* sp.	Microcarbonin A	Inhibition of internal carbonic anhydrase; cell death in dinoflagellate *Peridinium gatunense*	Beresovsky et al. [97]
6	*Cylindrospermopsis raciborskii*	Carotenoid glycosides	Toxic to zebrafish embryos developments	Jaja-Chimedza et al. [98]
7	*Tychonema bourrellyi*	β-ionone	Inhibition of cyanobacteria *Microcystis aeruginosa*	Shao et al. [78]
Alkaloids				
1	*Fischerella* sp. strain 52-1	Hapalindoles	Inhibition of photosynthesis of green alga *Chlamydomonas* sp. and growth of cyanobacteria	Gantar et al. [20]

(*continued*)

Table 1 (continued)

	Species	Chemicals	Actions	References
2	*Hapalosiphon welwitschii* *Fischerella ambigua* *Fischerella* sp. *Westiella intricata*	Hapalindoles family	Not tested	Micallef et al. [99]
3	*Nodularia harveyana*	Norharmane (9H-pyrido(3,4-b)indole	Inhibition of *Arthrospira (Spirulina) laxissima* and other microalgae	Volk and Furkert [42], Volk [79], Volk and Mundt [100], Volk [101]
4	*Nostoc spongiaeforme* TISTR 8169	Nostocine A	Growth inhibition of microalgae: for cyanobacteria, including strains in genera *Nostoc*, *Anabaena*, *Scytonema*, and *Oscillatoria*; for green algae, including strains in genera *Chlamydomonas*, *Chlorella*, and *Dunaliella*	Hirata et al. [102]
5	*Fischerella* sp.	12-*Epi*-hapalindole E isonitrile	Growth inhibition (gram- and gram+ bacteria, cyanobacteria; fungi; mammal cells); inhibition of RNA synthesis and consequently protein synthesis in *Bacillus subtilis*	Doan et al. [103]
6	*Calothrix* sp.	Calothrixin A	Growth inhibition (gram+ bacteria, mammal cells); inhibited DNA replication; inhibition of RNA synthesis and consequently protein synthesis in *Bacillus subtilis*; inhibition of RNA polymerase	Doan et al. [103], Rickards et al. [104]
7	*Fischerella* sp. CENA 19	Fischerellin A; 12-Epi-hapalindole F	Inhibition of growth of cyanobacteria *Synechococcus* sp. PCC 7942 and toxin-producing strains of *Microcystis* sp.	Etchegaray et al. [105]
8	*Fischerella* 52-1	12-Epi-hapalindole H isonitrile 12-Epi-ambiguine B nitrile	Inhibition of zebrafish embryos	Walton et al. 2014 [106]

Other categories				
1	*Microcystis aeruginosa*	Linoleic acid (ester)	Inhibition of growth of green alga *Chlorella vulgaris*	Song et al. [81]
2	*Scytonema hofmanni*	Cyanobacterin (halogenated; aromatic)	Growth inhibition of diatom *Nitzschia pusilla*	Abarzua et al. [107]
3	*Scytonema hofmanni*	Cyanobacterin	Inhibition of other cyanobacteria, such as certain trains in genera *Synechococcus*, *Anacystis*, *Microcystis*, *Anabaena*, and *Nostoc*	Gleason and Paulson [108]
4	*Scytonema hofmanni*	Cyanobacterin	Inhibition of cyanobacterium *Synechococcus* sp. and maybe more species of cyanobacteria and other phytoplankters	Mason et al. [109]
5	*Nostoc linckia*	Cyanobacterin LU-1	Inhibition of cell division and light-dependent oxygen evolution of *Synechococcus* sp.	Gromov et al. [80]
6	*Aphanizomenon ovalisporum*; *C. raciborskii* AQS; *A. ovalisporum* APH; *Anabaena* 66-2; *Microcystis* 81-11; *Nostoc* 23-2; *Pseudanabaena* 108-1	Polymethoxy-1-alkenes (PMAs) (polymethoxy alkene)	Toxic effects to zebrafish embryo	Jaja-Chimedza et al. [83, 110]
7	*Nostoc insulare*	4,4′-Dihydroxybiphenyl (aromatic)	Inhibition of *Arthrospira (Spirulina) laxissima* and other microalgae; anticyanobacterial activities, moderate antibacterial and antifungal activities; cytotoxicity	Volk and Furkert [42], Volk [79], Volk and Mundt [100], Volk [101]
8	*Nostoc insulare*	N,N′-(4,5-dimethyl-1,2- phenylene) bis-acetamide (aromatic)	Not tested	Volk and Mundt [100], Volk [101]

Cyanobacteria are also able to synthesize aromatic compounds. Aromatic compounds from the genus *Nostoc* have conspicuous algicidal activity to cyanobacterial species but moderate antibacterial and antifungal activities [42, 79]. Cyanobacterin is another putative allelochemical, and inhibitory activities were observed toward cyanobacteria, green algae, and a diatom (Table 1) with effects on oxygen evolution during photosynthesis as a possible mechanism [80]. High carbon number alkanes such as linoleic acid and polymethoxy-1-alkenes were also found to act as allelochemicals. These metabolic products inhibited the growth of green algae and the development of zebrafish embryos [81, 82]. A study by Jaja-Chimedza et al. [83] indicated that polymethoxy alkenes are widely distributed in various species of aquatic microalgae.

Many of the chemicals isolated from cyanobacteria have not yet been tested for inhibitory effects in aquatic ecosystems (Table 1) but seem worth paying attention to in future research.

3.2 Modes of Action

Cyanobacterial secondary metabolites exert their effects in target organisms by several modes of action. In general, these mechanisms include changes in the morphology (e.g., membrane destruction and cell lysis) and inhibition of processes involved in photosynthesis and oxidative stress.

The presence of cyanobacteria often inhibits the growth of target organisms due to multiple reasons. Observations through electron microscopes indicated damages on the membranes of some sensitive species [108]. In other cases, cell differentiation and heterocyst and akinete formation of filamentous algae were influenced by extracts of cyanobacterial cells [33]. Effects on photosynthetic activity were tested using different approaches. Cyanobacteria lowered the electron transport rate (ETR_{max}) of some target species [22, 78], inhibited the activity of photosystem II (e.g., Gross et al. [84]), or lowered their oxygen evolution [34, 80, 108, 111]. Allelopathic cyanobacteria can also cause oxidative stress inside of the affected species cells which has been tested by either measuring the occurrence of reactive oxygen species (ROS) or antioxidative enzymes. Mostly, the presence of cyanobacterial allelochemicals contributed to an increase in ROS and stimulated antioxidative enzyme systems and/or low molecular weight antioxidants [78, 112]. Certain compounds directly affected nucleotide synthesis. Doan et al. [103] found that compounds from two cyanobacterial genera inhibited the RNA synthesis of target bacteria and in vitro tests manifested that this resulted from effects on the RNA polymerase. Rzymski et al. [72] suggested that the alkaline phosphatase (ALP) activity was affected by a *Cylindrospermopsis* strain. Song et al. [81] were able to show that *M. aeruginosa* allelopathy had an impact on multiple metabolic pathways involved in energy generation and metabolism, including glycolysis, carbon fixation, and fatty-acid biosynthesis using proteomics and metabolomics analyses. Due to these different approaches to measure the effects of cyanobacteria on target species,

it is difficult to define unified criteria to compare the damage caused by cyanobacteria among different target organisms.

3.3 Impact of Signaling Molecules

Recent research indicates that signaling molecules might be important in regulating the production of secondary metabolites in cyanobacteria and that their allelopathic activity can be enhanced in the presence of the target species. Ma et al. [113] found that the allelopathic effect of *Microcystis* sp. was inducible by observing that co-cultured *Microcystis* strains have stronger inhibitory effects on *Aphanizomenon*. Detecting the signaling molecules, however, is difficult; only a study deciphered both signal molecules and allelochemicals in a dynamic system. Song et al. [81] found that nitric oxide (NO) acted as a signal molecule released from the target green alga *Chlorella vulgaris*. This molecule triggered the release of the allelochemical linoleic acid in *M. aeruginosa* cells.

4 Interfering Factors

Environmental factors can significantly change the growth conditions for cyanobacteria and thus their production and release of allelochemicals. In addition, allelopathic activities of cyanobacteria can vary depending on their growth phase and initial density.

4.1 Biotic Factors

Zhang et al. [114] found evidence for a strong impact of initial cell density and thus biomass on the allelopathic effect of *Microcystis* on macrophytes. The initial biomass ratio determined the outcome of competition tests between the two both bloom-forming cyanobacteria *Microcystis* sp. and *Anabaena* sp. [68].

Different growth phases of cyanobacteria populations resulted in the release of different secondary metabolites, and cells exhibited the strongest allelopathic potential in specific developmental periods. Volk [101] isolated three potential allelochemicals from two different growth phases of a *Nostoc* strain. The one secreted during the linear phase is non-toxic to eukaryotic cells, but the two chemicals isolated from the stationary phase possessed toxicity to a human amniotic epithelial cell line [100]. Other studies seem to confirm that allelopathic effects from the exponential growth phase of cyanobacteria were the strongest compared to other developmental stages independent of the target species [28, 29, 36, 115]. Interestingly, several studies indicated that some cyanobacterial strains stimulated the target organisms to some extent during the cyanobacteria decline phase [28, 115]. These results support earlier findings of Keating (1977) who found that different

cyanobacterial species inhibited their immediate predecessor but stimulated their successor [2].

Allelopathic effects of cyanobacteria certainly also depended on the sensitivity of the target organism. Different organisms exhibited various responses to different cell-free filtrates of cyanobacterial donors or their allelochemicals [29, 32, 43]. In principle, phytoplankton has been shown to develop local genetic adaptation to external stress such as grazing pressure [116]; however, for allelopathy, this has not yet been shown [117]. The presence of another competitor can also affect the sensitivity of cyanobacteria to allelochemicals. Chang et al. [118] tested whether *M. aeruginosa*, known to be sensitive to polyphenolic allelochemicals, remains suppressed when interacting with the less sensitive green alga *Desmodesmus armatus*. Interaction with the green alga turned the inhibiting effect of allelochemicals on the cyanobacterium into an enhancement resulting in increased growth rates and an increasing abundance of the cyanobacterium under allelochemical presence. *Microcystis* species decreased their microcystin production under the presence of cylindrospermopsin, an allelochemical released from other strains of cyanobacteria [119]. Pei et al. [120] tested allelopathic effects of *Microcystis* sp. on a common green alga under the influence of the macrophyte allelochemical, *N*-phenyl-2-naphthylamine (PNA). Allelopathic effects on the green alga were stimulated by PNA.

4.2 Abiotic Factors

The growth of phototrophs is influenced by several physicochemical conditions, including light, temperature, pH, and nutrient availability. Each cyanobacterial species or even strain has specific optimum requirements, and deviations from these will result in impaired growth with potential consequences for allelochemical production and release.

High water temperatures (above 25 °C) are considered to accelerate cyanobacterial bloom formation. Although the average optimal temperature of cyanobacteria species has no conspicuous difference to that of green algae [121], specific optimal values in various cyanobacteria in combination with other properties such as their buoyancy might contribute to their dominance. Field research has demonstrated that cyanobacterial blooms and the species succession were highly related to water temperature [122]. Several species outcompeted others under higher temperatures [123] and this phenomenon could be related to the toxin release of specific species. Ma et al. (2015) indicated that an increased water temperature will favor the bloom by toxic cyanobacteria [113]. For some cyanobacterial species, higher water temperatures supported the toxic strain rather than non-toxic strain despite higher growth rates of the non-toxic strain [124]. Hirata et al. (2003) could show that the release of a putative allelochemical (nostocine A) was enhanced by higher temperature, and this chemical showed strong toxic effects on green algae [102].

Light intensities affect the growth of cyanobacteria, and it has been demonstrated that under different light intensities, the inhibitory effect of *Cylindrospermopsis* sp. varied considerably with higher light intensities enhancing the effect [125]. This property is highly species-specific, and strains with a broader absorption spectrum had a competitive advantage under light limitation [126]. Chia et al. [119] found that *Microcystis* sp. under the influence of cylindrospermopsin increased their microcystin content when light was limiting and decreased it when light intensity was optimum. Not only the concentration of secondary metabolites but also their varieties are changing with changing light conditions. Walsh et al. [96] documented that volatile organic compounds (VOCs) of *Microcystis* sp. showed multiple patterns under various light and iron levels. In their study, light and nutrient level seemed to trigger the production of certain VOCs, but the functions of these compounds have not been thoroughly deciphered yet.

Preussel et al. [71] investigated the combined effect of light and temperature. Lower temperature increased the release of cylindrospermopsin with increasing light intensity, whereas at 25 °C, cylindrospermopsin release decreased with higher light intensities.

The pH of water has been shown to positively affect the secretion of an algicide by filamentous cyanobacterium *Oscillatoria* sp. [127], and nutrient depletion could also enhance the allelopathic activity of cyanobacteria. Under phosphorus limitation, *Cylindrospermopsis* sp. exhibited an enhanced inhibitory activity toward Chlorophyta [125]. In *Oscillatoria* sp. the release of algicides was increased by a depletion of magnesium and phosphorus [127]. Apart from the total amount of allelochemicals, their composition was influenced by nutrient limitation [34]. As a consequence, high nutrient levels facilitated toxic cyanobacterial strains [124].

5 Conclusions

Allelochemicals produced and released by cyanobacteria potentially provide them with a competitive advantage due to their inhibiting effects on other members of the phytoplankton community and due to indirect effects on organisms of different higher trophic levels that control phytoplankton via a top-down cascade.

A huge variety of chemicals are produced and released from cyanobacterial cells. Some of these substances, especially those that are toxic to humans and occur during bloom events such as microcystins and saxitoxins, have been studied intensively. However, many more potential allelochemicals such as peptides, terpenoids, alkaloids, phenyls, and others are produced by cyanobacteria. These play yet unknown roles in the succession of natural phytoplankton communities. The most commonly detected modes of action of these substances are photosynthesis inhibition and the formation of reactive oxygen species, while recent proteomics and metabolomics approaches also indicated an influence on several metabolic pathways. Several studies have manifested that the allelopathic effects of cyanobacteria are inducible. The presence of competitors or signaling molecules triggered the release of allelochemicals in cyanobacteria. All these processes are regulated by various biotic

and abiotic environmental conditions such as the initial cell ratio of donor and target organisms, their growth phase, and species- and strain-specific sensitivities of the target organism to allelochemicals as well as light, temperature, and nutrient supply.

Several studies have shown that cyanobacterial allelopathic effects can be involved in the succession of phytoplankton communities, and the available additional knowledge on substances produced and released by cyanobacteria suggests that this process is more relevant than currently acknowledged. Also models simulating phytoplankton and its succession in aquatic ecosystems have not yet incorporated this mechanism (see, e.g., Shimoda and Arhonditsis [128]). Future research should thus strive to decipher cyanobacterial allelopathy as a potentially highly relevant element in controlling phytoplankton succession. This knowledge is needed for both a better basic understanding of aquatic ecosystem functioning and to assure future water quality management.

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Tree-Leaf Chemicals and Feeding Behavior of Arboreal Mammals in Seasonal Environment

15

Mutsumi Ito and Fumio Hayashi

Contents

Abstract

Mammalian herbivores are considered to eat plants with high nutrition but to avoid those with harmful chemical components. Most species eat plants selectively, but some species develop tolerance to the harmful plant chemicals. Food selection occurs between tree species, conspecific trees, leaves within the same tree, and leaf parts. Leaf chemical components also differ in these hierarchical structures. However, the effects of plant chemicals on food selectivity are yet unknown in tree-leaf eating mammals such as arboreal primates, rodents, and marsupials, as compared with other mammals eating herbaceous plants, seeds, nuts, and fruits. Moreover, the effects of seasonal changes in leaf chemicals and the microscale distribution of chemicals within the single leaf have little examined. This chapter shows how tree-leaf chemicals affect feeding behavior of the

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arboreal mammals, also the Japanese giant flying squirrel as the case study, in their seasonal environments. This flying squirrel has a peculiar manner to eat only the central part of a single leaf. The measurements of the microscale distributions of chemicals within the single leaf and the seasonal changes in leaf chemicals of available trees in their habitat suggest that sugar concentration is an important factor affecting which species of trees they eat, and the total phenolic concentration affects which parts of the single leaf they eat.

Keywords
Glucose · Flying squirrels · Food choice · *Quercus* trees · Secondary metabolites · Total phenolics

1 Introduction

Mammalian herbivores eat plant materials and are forced to make optimal choices regarding their daily diets by selecting plants with higher nutritional value and by avoiding those with possible toxins to maintain and develop their bodies [1–3]. The main nutritional resources from plants are sugars, proteins, and minerals; some species have higher concentrations of these than others [3]. However, plants usually include fibers (e.g., cellulose, hemicellulose, and lignin), which are poor energy sources, and also develop secondary metabolites, such as toxins (e.g., phenolics and alkaloids) and digestion inhibitors (condensed tannins) [1–5]. Food selection by mammalian herbivores based on the abundance of valuable nutrients and plant defensive chemicals has been documented and, in most cases, they select food materials with substantial amounts of preferable nutrients over those with other components [2, 6–11].

Many mammals are considered to have four primary taste modalities (sweet, salty, bitter, and sour), with umami, a savory taste associated with specific amino acids, being a fifth modality, as in humans [12, 13]. Sweet and umami promote feeding behavior, whereas bitter and sour tastes reduce food intake, and the perceptions of sweetness may contribute to preferences for fruits, flowers, and other foods high in soluble sugars [12]. Clear preferences for sweetness and/or soluble sugar have been documented in animals ranging from relatively small mammals, such as frugivorous bats [14, 15], to large mammals, such as roe deer and primates [8, 9, 16]. In contrast, a significant number of plant secondary metabolites are bitter or otherwise unpleasant in taste [4]. The taste of bitterness may predict the deterrent effects of toxins and digestibility-reducing tannin compounds in fruits and leaves [17] and may be a principal cause of food rejection [18, 19]. Therefore, herbivorous mammals often show behavioral avoidance and/or tolerance of plant defensive secondary metabolites, such as high concentrations of tannins and related polyphenolics [1, 20–23], although several phenolics such as flavonoids and tannins also function as antioxidants and these compounds contribute exclusively to the browning reactions observed in injured or pathogen-invaded plant tissues [24, 25].

On the other hand, leaf defenses used against insects are targeted to microscale levels compared to those used against mammals [26]. For example, leaves of the evergreen shrub *Pseudowintera colorata* are protected from herbivorous insects by increased chemical defenses at leaf margins, which are usually red-pigmented [27]. Because many herbivores initiate feeding at the leaf edges, it is at least possible that the marginal anthocyanins function as a visual signal to indicate that the leaves contain unpalatable compounds [27]. Edge feeding is common among orthopteran, coleopteran, and lepidopteran larvae [28] and damages leaves through increased water loss and risk of infection [29]. Thus, defensive chemicals may be abundant at leaf margins, where herbivorous insects prefer to initiate feeding [27]. In the cottonwood *Populus angustifolia*, the total phenolic contents increase from the base to the top of the leaf, which may affect the establishment site selection by the stem mothers of the aphid *Pemphigus betae* [30]. Such a microscale distribution of leaf defensive chemicals may also affect feeding behavior of herbivorous mammals.

Tree leaves are more abundant and common food resources of herbivores than grasses, fruits, nuts, and seeds. However, the relationships between food selectivity and plant chemicals are not fully understood in tree-leaf eating mammals as compared with those graminivores (herbaceous plant eaters), frugivores (fruit eaters), and granivores (seed and nut eaters). Tree-leaf eating mammals are generally limited to arboreal primates, rodents, and marsupials. Grand-dwelling mammals sometimes use tree leaves as food resources. In this chapter, first, we review the effects of leaf chemicals on tree-leaf eating mammals in the four hierarchical structures, between tree species, conspecific trees, leaves in the same tree, and leaf parts. Second, we show how leaf chemicals and herbivore's feeding behavior change in the seasonal environments. In the temperate mixed forest, all tree leaves are available for leaf-eating mammals in summer, but only evergreen trees are available in winter (Fig. 1). The leaf chemicals also change with leaf ages. Finally, as a case study, we show the relationships between leaf chemicals and feeding behavior of Japanese giant flying squirrels *Petaurista leucogenys*. They have a peculiar leaf-feeding manner, eating a central part of a single leaf. The measurements of the microscale distributions of chemicals within the single leaf and the seasonal patterns of available trees differing in leaf chemicals suggest that sugar concentration is an important factor affecting which species of trees they eat, and total phenolic concentration affects which parts of the single leaf they eat.

2 Effects of Tree-Leaf Chemicals on Feeding Behavior

2.1 Which Species of Leaves They Eat

Effects of chemical components of tree leaves on food selection have been reported for several tree-leaf eating mammals such as primates, rodents, marsupials, and those feeding tree leaves in some occasions. Various chemical components (e.g., energy, sugar, carbohydrate, fibers, proteins, secondary metabolites, and other chemicals) are tested for their food selection (Table 1).

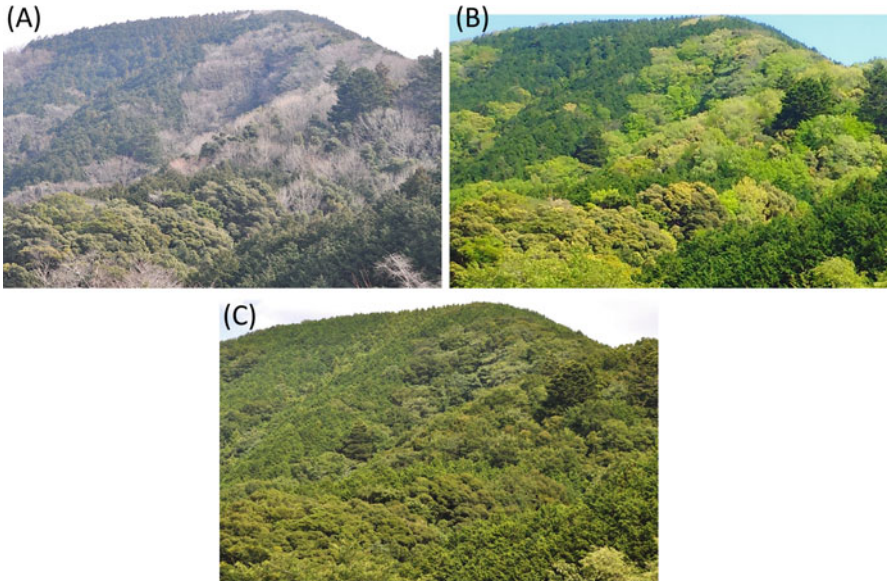

Fig. 1 Seasonal landscape changes in Japanese common forest mixing of deciduous and evergreen trees in (**a**) spring, (**b**) early summer, and (**c**) summer

Energy contents (calorie per unit weight) of tree leaves little affect food choice of herbivores (Table 1). Black colobus monkeys have a negative effect of energy on their food choice, but this is caused by negative correlation between energy and fiber contents, the latter of which is avoided by them [32].

Clear preferences for soluble sugar and/or sweetness have been documented in various mammals [8, 9, 14, 15]. They prefer to eat sweet food materials because sweetness is a reliable marker of energy (caloric) content [13, 52, 53]. Likewise, roe deer, black howlers, and giant flying squirrels prefer sweeter leaves when choosing from various tree species [16, 35, 39]. Although these three species show positive effects, four of seven have no effect of sugars when they select leaves (Table 1). Some mammals have significant preferences for specific types of sugars [54–56], whereas others do not [57, 58]. For example, the honey possum (*Tarsipes rostratus*), which feeds only on nectar and pollen, has a significant preference to sucrose, whereas the pygmy possum (*Cercartetus concinnus*), which feeds on a wide range of foods, does not [59].

The contents of acid and neutral detergent fibers, celluloses, hemicelluloses, lignins, acid detergent lignins, and their mixes have strongly negative effects (18 of 40 cases) on food selection (Table 1). Easily digestible leaves, estimated by the residue after pepsin/cellulose enzyme treatments, are preferred by herbivores in most (6 of 8) cases (Table 1). Strong relationships between fiber contents and digestibility suggest avoidance of fiber-rich leaves [2]. Leaf toughness also increases with fiber contents and, in general, herbivores avoid harder leaves and harder part of plants [47]. Hemicellulose contents are, however, positively correlated with food

Table 1 Effects of leaf chemicals on leaf selection among different species of trees by tree-leaf eating mammals. Effects are shown by the positive (+), no (0), or negative (−) relationship with leaf chemical concentrations

Chemical components	Order	Common name	No. of plant species	Effects	References
Energy	Carnivora	Asiatic black bear	8	0	[31]
	Primates	Black colobus	6–20	−	[32]
	Primates	King colobus	13–16	0	[33]
	Primates	Mexican black howler	24	0	[34]
	Rodentia	Rock cavy	10	0	[6]
Sugar	Artiodactyla	Roe deer	70	+	[16]
	Primates	Black howler	11	+	[35]
	Primates	Cao vit gibbon	20	0	[36]
	Primates	Mexican black howler	24	0	[34]
	Primates	Southern woolly lemur	57	0	[37]
	Primates	Verreaux's sifaka	48	0	[38]
	Rodentia	Japanese giant flying squirrel	2	+	[39]
Acid detergent fiber (ADF)	Artiodactyla	Greater kudu	14	0	[40]
	Artiodactyla	Roe deer	70	−	[16]
	Cetartiodactyla	Boer goat	14	0	[40]
	Cetartiodactyla	Impala	14	0	[40]
	Primates	Black colobus	12–20	−	[32]
	Primates	Cao vit gibbon	20	0	[36]
	Primates	Common langur	20	−	[7]
	Primates	King colobus	13–16	0	[33]
	Primates	Lesser weasel lemur	17	−	[41]
	Primates	Mantled howler	31	−	[42]
	Primates	Maroon leaf monkey	39	−	[43]
	Primates	Mexican black howler	24	0	[34]
	Primates	Red colobus	>12	0	[44]
	Primates	Red colobus	39	0	[45]
	Primates	South Indian leaf monkey	16	−	[46]
	Primates	Southern woolly lemur	57	0	[37]

(*continued*)

Table 1 (continued)

Chemical components	Order	Common name	No. of plant species	Effects	References
	Primates	Sumatran surili	56	–	[43]
	Primates	Verreaux's sifaka	48	0	[38]
Neutral detergent fiber (NDF)	Artiodactyla	Greater kudu	14	0	[40]
	Carnivora	Asiatic black bear	8	–	[31]
	Cetartiodactyla	Boer goat	14	–	[40]
	Cetartiodactyla	Impala	14	0	[40]
	Primates	Cao vit gibbon	20	0	[36]
	Primates	Chimpanzee	17	0	[19]
	Primates	Japanese macaque	13	–	[47]
	Primates	Mexican black howler	24	0	[34]
	Primates	Proboscis monkey	50	–	[48]
	Primates	South Indian leaf monkey	16	–	[46]
	Primates	Southern woolly lemur	57	0	[37]
	Primates	Verreaux's sifaka	48	0	[38]
Cellulose	Artiodactyla	Roe deer	70	–	[16]
Hemicellulose	Primates	Verreaux's sifaka	48	+	[38]
Lignin	Primates	South Indian leaf monkey	16	–	[46]
Acid detergent lignin	Primates	Cao vit gibbon	20	0	[36]
	Primates	Mexican black howler	24	0	[34]
	Artiodactyla	Greater kudu	14	0	[40]
	Cetartiodactyla	Boer goat	14	–	[40]
	Cetartiodactyla	Impala	14	0	[40]
Cellulose + hemicellulose + lignin	Primates	Mantled howler	13	–	[49]
ADF + NDF + lignin	Primates	Black howler	11	–	[35]
Digestibility	Artiodactyla	Roe deer	70	+	[16]
	Primates	Black colobus	14–20	+	[32]
	Primates	Mantled howler	31	+	[42]
	Primates	Maroon leaf monkey	39	+	[43]
	Primates	Proboscis monkey	50	+	[48]

(*continued*)

Table 1 (continued)

Chemical components	Order	Common name	No. of plant species	Effects	References
	Primates	Red colobus	>12	0	[44]
	Primates	South Indian leaf monkey	16	0	[46]
	Primates	Sumatran surili	56	+	[43]
Protein, crude protein, soluble protein	Artiodactyla	Roe deer	70	–	[16]
	Carnivora	Asiatic black bear	8	+	[31]
	Primates	Black howler	11	0	[35]
	Primates	Chimpanzee	21–37	0	[19]
	Primates	Common langur	20	+	[7]
	Primates	Lesser weasel lemur (wet season)	17	+	[41]
	Primates	Lesser weasel lemur (dry season)	17	–	[41]
	Primates	Mantled howler	13	+	[49]
	Primates	Mantled howler	31	+	[42]
	Primates	Mexican black howler	24	0	[34]
	Primates	Proboscis monkey	7–50	+	[10, 48]
	Primates	Red colobus	>12	0	[44]
	Primates	Red leaf monkey	7	+	[10]
	Primates	South Indian leaf monkey	16	0	[46]
	Primates	Southern woolly lemur	57	+	[37]
	Primates	Verreaux's sifaka	48	0	[38]
N	Artiodactyla	Greater kudu	14	0	[40]
	Cetartiodactyla	Boer goat	14	0	[40]
	Cetartiodactyla	Impala	14	+	[40]
	Diprotodontia	Greater glider	2	+	[11]
	Primates	Black colobus	6–20	+	[32]
	Primates	Cao vit gibbon	20	+	[36]
	Primates	King colobus	13–16	0	[33]
	Primates	Maroon leaf monkey	39	+	[43]
	Primates	Red colobus	39	0	[45]

(*continued*)

Table 1 (continued)

Chemical components	Order	Common name	No. of plant species	Effects	References
	Primates	Silver leaf monkey	28	0	[50]
	Primates	Southern woolly lemur	57	0	[37]
	Rodentia	Rock cavy	10	+	[6]
Protein/ADF	Primates	Black howler	11	0	[35]
	Primates	Lesser weasel lemur	17	+	[41]
	Primates	Mexican black howler	24	0	[34]
	Primates	Red colobus	>12	0	[44]
	Primates	Red colobus	39	+	[45]
	Primates	Verreaux's sifaka	48	0	[38]
Protein/NDF	Primates	Cao vit gibbon	20	0	[36]
Protein/(cellulose + hemicellulose + lignin)	Primates	Mantled howler	13	+	[49]
N/ADF	Primates	King colobus	13–16	0	[33]
N/(ADF + condensed tannin)	Primates	Black colobus	6–20	+	[32]
Crude fat	Primates	Chimpanzee	21–37	0	[19]
Lipid	Primates	Black howler	11	0	[35]
	Primates	Mexican black howler	24	0	[34]
	Primates	Verreaux's sifaka	48	0	[38]
Total phenol	Artiodactyla	Roe deer	70	+	[16]
	Primates	Black colobus	20	–	[32]
	Primates	Mantled howler	13	–	[49]
	Primates	Maroon leaf monkey	39	0	[43]
	Primates	Red colobus	39	0	[45]
	Primates	South Indian leaf monkey	16	0	[46]
	Primates	Sumatran surili	56	0	[43]
	Primates	Verreaux's sifaka	48	0	[38]
	Rodentia	Japanese giant flying squirrel	2	+	[39]
	Primates	Red colobus	>12	0	[44]

(*continued*)

Table 1 (continued)

Chemical components	Order	Common name	No. of plant species	Effects	References
Polyphenol	Artiodactyla	Greater kudu	14	0	[40]
	Cetartiodactyla	Boer goat	14	0	[40]
	Cetartiodactyla	Impala	14	+	[40]
	Primates	Southern woolly lemur	57	0	[37]
Tannin, condensed tannin	Artiodactyla	Greater kudu	14	0	[40]
	Cetartiodactyla	Boer goat	14	0	[40]
	Cetartiodactyla	Impala	14	0	[40]
	Primates	Black colobus	12–20	–	[32]
	Primates	Cao vit gibbon	20	0	[36]
	Primates	Chimpanzee	18–22	–	[19]
	Primates	Common langur	20	0	[7]
	Primates	Mantled howler	13	0	[42]
	Primates	Maroon leaf monkey	35	0	[43]
	Primates	Mexican black howler	7–24	0	[34, 51]
	Primates	Proboscis monkey	7	0	[10, 48]
	Primates	Red colobus	39	0	[45]
	Primates	Red leaf monkey	7	0	[10]
	Primates	South Indian leaf monkey	16	0	[46]
	Primates	Southern woolly lemur	57	0	[37]
	Primates	Sumatran surili	56	0	[43]
	Primates	Verreaux's sifaka	48	+	[38]
	Primates	Red colobus	>12	0	[44]
Hydrolyzable tannin	Primates	Red colobus	39	0	[45]
Saponin	Primates	Red colobus	>12	0	[44]
	Primates	Verreaux's sifaka	48	0	[38]
Alkaloid	Primates	Mantled howler	13	0	[42]
	Primates	Red colobus	>12	0	[44]
	Primates	South Indian leaf monkey	16	0	[46]
	Primates	Southern woolly lemur	45	0	[37]
	Primates	Verreaux's sifaka	48	0	[38]

(*continued*)

Table 1 (continued)

Chemical components	Order	Common name	No. of plant species	Effects	References
Cyanogenic glycoside	Primates	Red colobus	>12	0	[44]
Formylated phloroglucinol	Diprotodontia	Greater glider	2	–	[11]
Water	Primates	Black howler	11	0	[35]
	Primates	Chimpanzee	21–37	0	[19]
	Primates	Mantled howler	21	0	[42]
	Primates	South Indian leaf monkey	16	0	[46]
	Primates	Verreaux's sifaka	48	0	[38]
	Rodentia	Japanese giant flying squirrel	2	+	[39]
Ash, crude ash, minerals	Primates	Black colobus	6–20	+	[32]
	Primates	Cao vit gibbon	20	0	[36]
	Primates	Chimpanzee	21–37	+	[19]
	Primates	Mantled howler	31	0	[42]
	Primates	Mexican black howler	24	0	[34]
	Primates	Proboscis monkey	7	0	[10]
	Primates	Red leaf monkey	7	0	[10]
	Rodentia	Rock cavy	10	0	[6]
C	Primates	South Indian leaf monkey	5	0	[46]
	Rodentia	Rock cavy	10	0	[6]
Ca	Primates	South Indian leaf monkey	5	0	[46]
	Rodentia	Rock cavy	10	–	[6]
Cu	Primates	South Indian leaf monkey	5	0	[46]
Fe	Primates	South Indian leaf monkey	5	0	[46]
K	Primates	South Indian leaf monkey	5	0	[46]
	Rodentia	Rock cavy	10	+	[6]
Mg	Primates	South Indian leaf monkey	5	0	[46]
	Rodentia	Rock cavy	10	0	[6]
Mn	Primates	South Indian leaf monkey	5	0	[46]
	Rodentia	Rock cavy	10	0	[6]

(*continued*)

Table 1 (continued)

Chemical components	Order	Common name	No. of plant species	Effects	References
Na	Artiodactyla	Greater kudu	14	0	[40]
	Cetartiodactyla	Boer goat	14	0	[40]
	Cetartiodactyla	Impala	14	0	[40]
	Primates	South Indian leaf monkey	5	0	[46]
	Rodentia	Rock cavy	10	0	[6]
P	Artiodactyla	Greater kudu	14	0	[40]
	Cetartiodactyla	Boer goat	14	0	[40]
	Cetartiodactyla	Impala	14	0	[40]
	Primates	Black colobus	6	0	[32]
	Primates	South Indian leaf monkey	5	0	[46]
	Rodentia	Rock cavy	10	0	[6]
S	Rodentia	Rock cavy	10	0	[6]
Zn	Primates	South Indian leaf monkey	5	0	[46]
	Rodentia	Rock cavy	10	0	[6]

preference in sifakas although the reason remains unknown [38]. In some primates and rodents, nutrition from specialized cecal microbiota converts diverse plant materials such as fibers into absorbable nutrients [60, 61].

Protein (also N) is always limited for herbivores, and they must select protein-rich leaves particularly when average protein concentrations from leaves available in the habitat are low [62]. In fact, protein and N contents of leaves affect herbivore's preference positively in 14 of 28 cases. Herbivores usually prefer leaves including more proteins and less fibers [33, 37, 44–46, 62]. Therefore, the ratio of proteins and fibers are the most important criterion of their tree-leaf selections. Of 10 food selection studies based on the protein/fiber ratio of tree leaves, four are positive, six are no, and zero is negative effects (Table 1). Although herbivores can obtain sufficient protein by feeding a great amount of leaves, selective feeding of protein-rich leaves may be more adaptive for their long lives. However, the physiological mechanisms underlying choice of protein-rich leaves are still unknown.

Fat or lipid contents of tree leaves are seemed not to affect food choice of arboreal herbivores although only a few studies have examined so far (Table 1). In general, fat or lipid contents in the leaves are much less than in fruits, nuts, and seeds which are usually preferred by herbivores [34, 63].

The secondary metabolites are considered to be most important factor for food selection of herbivores [2, 3, 5, 22]. These components function partly in toxin and partly in inhibitors of protein and carbohydrate digestion [1, 3–5, 64] and are supposed to be avoided by herbivores. Mammals usually avoid leaves highly

containing secondary metabolites by their bitter or unpalatable taste through experiences of toxicity or postingestive effects [4, 22, 65, 66]. However, only a few (5 of 42) examinations show negative effects, but most (33 of 42) show no effects and some (4 of 42) show positive effects on food selection (Table 1). Clear negative effects are shown particularly in food selection by arboreal marsupials among conspecific trees variable greatly in their leaf phenolic contents (also see Sect. 2.2).

Thus, interspecific comparisons do not support the general prediction of the negative effects of the secondary metabolites on their food choice. This is probably because correlations between secondary metabolite and other leaf chemical concentrations mask the effects of each factor on herbivore's food selection. For example, there is a positive correlation between sugar and phenolic contents between *Quercus* tree species (see Sect. 4). If the herbivores select leaves with higher sugar concentrations, they also select leaves with higher phenolic concentrations, resulting positive selection by phenolics in Table 1 [39]. Besides such methodological difficulties, this is partly caused by herbivore's feeding behavior that they often consume a small amount of leaves from a variety of taxonomically distinct tree species. This type of feeding may minimize a risk of toxic damages by the leaves with unknown toxic substrates and/or have a role for continuous sampling to learn their toxicity [1, 67, 68]. Learning is emphasized to be important for herbivorous mammals to select appropriate food in the field because they live long and establish their home ranges in the spatially structured forest [5, 69, 70]. Repeated experiences enable them to learn appropriate feeding sites, leaf selection, and feeding manners. On the other hand, some mammals can produce salivary proteins as a defense against dietary tannins [64]. In black howler monkeys, they always secrete tannin-binding salivary proteins and therefore they obtain nutrients from leaves even containing high levels of tannins [51].

Leaves containing more water are preferable by giant flying squirrels, but this factor is not seemed to affect food selection of primates (Table 1). Water contents may be one of the indices of leaf softness, so that herbivores may prefer to eat leaves including more water if they avoid harder leaves [47, 48, 71]. The south Indian leaf monkey prefers young leaves containing more water to mature leaves. In this case, it is suggested that leaves with more water may include more water-soluble materials such as sugars and minerals [46]. The functions of leaf water in leaf selectivity will be carefully treated depending on the situation.

Ash concentrations affect food selection positively in colobus monkeys and chimpanzees, but do not in other herbivores (Table 1). Elemental analysis of leaves is unclear in its effect on leaf selectivity (Table 1).

2.2 Which Leaves They Eat Within the Same Species

Comparisons of the effects of leaf chemicals among different tree species in the previous Sect. 2.1 include many complicated factors for analyses. Leaf chemicals often co-vary and multispecies comparisons sometimes lead misunderstanding by ignoring phylogenetic constraints [72]. To be clear the effects of leaf chemicals on

Table 2 Effects of leaf chemicals on leaf selection between conspecific trees, young and mature leaves, marginal and central parts of a leaf, and blade and petiole parts of a leaf by tree-leaf eating mammals. Effects are shown by the positive (+), no (0), or negative (−) relationship with leaf chemical concentrations

Chemical components	Order	Common name	Between	Effects	References
Energy	Primates	Black colobus	Young and mature leaves	0	[32]
	Primates	King colobus	Young and mature leaves	0	[33]
Sugar	Primates	Southern woolly lemur	Young and mature leaves	−	[37]
	Primates	Black colobus	Young and mature leaves	+	[32]
	Primates	Mantled howler	Young and mature leaves	0	[49]
	Rodentia	Japanese giant flying squirrel	Center and margin of a leaf	0	[73]
Acid detergent fiber (ADF)	Primates	Black colobus	Young and mature leaves	−	[32]
	Primates	King colobus	Young and mature leaves	−	[33]
	Primates	Mantled howler	Young and mature leaves	−	[42]
	Primates	Red colobus	Young and mature leaves	−	[45]
	Primates	Red colobus	Young and mature leaves	0	[44]
	Primates	South Indian leaf monkey	Blade and petiole of a leaf	−	[46]
	Primates	South Indian leaf monkey	Young and mature leaves	−	[46]
	Primates	Southern woolly lemur	Young and mature leaves	−	[37]

(*continued*)

Table 2 (continued)

Chemical components	Order	Common name	Between	Effects	References
Neutral detergent fiber (NDF)	Primates	South Indian leaf monkey	Blade and petiole of a leaf	−	[46]
	Primates	South Indian leaf monkey	Young and mature leaves	−	[46]
	Primates	Southern woolly lemur	Young and mature leaves	0	[37]
Cellulose, hemicellulose, lignin	Diprotodontia	Ringtail possum	Trees	0	[74]
Lignin	Primates	South Indian leaf monkey	Young and mature leaves	0	[46]
	Primates	South Indian leaf monkey	Blade and petiole of a leaf	+	[46]
Lignocellulose	Diprotodontia	Greater glider	Young and mature leaves	−	[75]
Cellulose + hemicellulose + lignin	Primates	Mantled howler	Young and mature leaves	−	[49]
Digestibility	Primates	Black colobus	Young and mature leaves	+	[32]
	Primates	Red colobus	Young and mature leaves	+	[44]
	Primates	South Indian leaf monkey	Young and mature leaves	+	[46]
Protein, crude protein, soluble protein	Primates	Mantled howler	Young and mature leaves	+	[42, 49]
	Primates	Red colobus	Young and mature leaves	+	[44, 45]
	Primates	South Indian leaf monkey	Blade and petiole of a leaf	−	[46]
	Primates	South Indian leaf monkey	Young and mature leaves	+	[46]

(*continued*)

Table 2 (continued)

Chemical components	Order	Common name	Between	Effects	References
	Primates	Southern woolly lemur	Young and mature leaves	+	[37]
N	Diprotodontia	Greater glider	Young and mature leaves	+	[75]
	Diprotodontia	Koala	Trees	+	[76]
	Diprotodontia	Ringtail possum	Trees	0	[74]
	Primates	Black colobus	Young and mature leaves	+	[32]
	Primates	King colobus	Young and mature leaves	+	[33]
	Primates	Red colobus	Young and mature leaves	+	[45]
	Primates	Southern woolly lemur	Young and mature leaves	+	[37]
Protein/ADF	Primates	King colobus	Young and mature leaves	0	[33]
	Primates	Mantled howler	Young and mature leaves	+	[49]
	Primates	Red colobus	Young and mature leaves	+	[44]
N/(ADF + condensed tannin)	Primates	Black colobus	Young and mature leaves	+	[32]
Total phenol	Diprotodontia	Ringtail possum	Trees	0	[74]
	Primates	Black colobus	Young and mature leaves	−	[32]
	Primates	Mantled howler	Young and mature leaves	0	[49]
	Primates	Red colobus	Young and mature leaves	0	[45]
	Primates	South Indian leaf monkey	Blade and petiole of a leaf	0	[46]

(*continued*)

Table 2 (continued)

Chemical components	Order	Common name	Between	Effects	References
	Primates	South Indian leaf monkey	Young and mature leaves	0	[46]
	Rodentia	Japanese giant flying squirrel	Center and margin of a leaf	–	[73]
Polyphenol	Primates	Southern woolly lemur	Young and mature leaves	0	[37]
Tannin, condensed tannin	Diprotodontia	Ringtail possum	Trees	0	[74]
	Primates	Black colobus	Young and mature leaves	0	[32]
	Primates	Red colobus	Young and mature leaves	0	[45]
	Primates	South Indian leaf monkey	Blade and petiole of a leaf	0	[46]
	Primates	South Indian leaf monkey	Young and mature leaves	0	[46]
	Primates	Southern woolly lemur	Young and mature leaves	0	[37]
Hydrolyzable tannin	Primates	Red colobus	Young and mature leaves	0	[45]
Saponin	Primates	Red colobus	Young and mature leaves	0	[44]
Alkaloid	Primates	Red colobus	Young and mature leaves	0	[44]
	Primates	Southern woolly lemur	Young and mature leaves	0	[37]
Phenolic glycoside	Artiodactyla	Sheep	Trees	–	[77]
Cyanogenic glycoside	Primates	Red colobus	Young and mature leaves	0	[44]
Formylated phloroglucinols	Diprotodontia	Koala	Trees	–	[76]
	Diprotodontia	Ringtail possum	Trees	–	[74]

(*continued*)

Table 2 (continued)

Chemical components	Order	Common name	Between	Effects	References
Cineole, macrocarpal	Diprotodontia	Ringtail possum	Artificial diets	–	[74]
Sideroxylonal	Diprotodontia	Brushtail possum	Trees	–	[78–80]
	Diprotodontia	Greater glider	Trees	–	[23]
	Diprotodontia	Koala	Trees	–	[79, 81]
	Diprotodontia	Ringtail possum	Trees	–	[79, 82]
Flavanone, chrysin, pinocembrin	Diprotodontia	Brushtail possum	Artificial diets	–	[83]
Naringenin	Diprotodontia	Brushtail possum	Artificial diets	0	[83]
Terpene	Artiodactyla	Black-tailed deer	Trees	–	[84]
	Diprotodontia	Ringtail possum	Trees	–	[74]
Water	Primates	Mantled howler	Young and mature leaves	+	[42]
	Primates	South Indian leaf monkey	Blade and petiole of a leaf	+	[46]
	Primates	South Indian leaf monkey	Young and mature leaves	+	[46]
	Rodentia	Japanese giant flying squirrel	Center and margin of a leaf	+	[73]
Ash, crude ash	Primates	Black colobus	Young and mature leaves	0	[32]
	Primates	Mantled howler	Young and mature leaves	0	[42]
P	Primates	Black colobus	Young and mature leaves	+	[32]

herbivore's food choice, comparisons between conspecific trees, leaves of different ages and positions in the same tree, and the different parts of a single leaf are preferable. However, a few studies are useful at present and unfortunately most of them deal with the comparisons between young and mature leaves (Table 2).

Two studies examine the effects of energy contents (calorie per unit weight) of tree leaves but report no effects. Four studies report the effects of leaf containing sugars and the positive effect is detected only in the black colobus monkey.

The contents of acid and neutral detergent fibers, celluloses, hemicelluloses, lignins, and their mixes have a negative effect on food selection in most (11 of 16) cases. Easily digestible leaves are preferred in all of three examinations. Protein (also N) contents of leaves have usually positive effects (10 of 12 cases). Therefore, the ratio of preferred proteins to avoid fibers is also positive (3 of 4 cases).

Of 31 examinations of secondary metabolites, 13 have negative effects on food choice, suggesting this factor important for their food selection, although 18 have no effects. Particularly in arboreal marsupials such as koalas, possums, and greater gliders, clear avoidance of the trees whose leaves include high phenolic concentrations is demonstrated [23, 74, 76, 78–82]. In these marsupials, laboratory experiments using artificial foods with different phenolic concentrations also confirm their food selection according to particular chemicals [74, 83].

Leaves including more water are preferable in all the four examinations. Effects of ashes and P are not still examined for arboreal herbivores.

2.3 Which Parts of Leaves They Eat

Petioles are generally the toughest part of the leaf, followed by the midribs and laminae, in 11 species of trees [47]. For both midrib and lamina, there is a positive correlation between toughness and fiber contents, and Japanese macaques tend to eat the soft parts. Chemical contents are also compared between leaf laminae and petioles of *Cullenia exarillata* leaves as shown in Table 2 [46]. In this tree, however, the petioles have more water, less crude protein, and lower acid and neutral detergent fibers than the laminae. No alkaloid reagents are detected in the petioles. These results suggest the petioles' easy chewing for the leaf-monkey.

Leaf margins have significantly greater phenolic content than the central parts of the leaf in several plant species [27, 85] because many herbivorous insects initiate feeding at leaf edges [27, 28]. In the tree *Quercus acutissima*, total phenolic concentrations are often lower in the central part than the margin of the single leaf and giant flying squirrels prefer the center to the margin ([73], also see Table 2 and the following Sect. 4). In contrast, total phenolics are distributed homogenously in the single leaf of *Quercus sessilifolia* and the squirrels seldom eat the leaves at only the central part [39]. Many plants employ also structural defenses such as spines, hairs, and thickened leaves [3, 86], and some herbivores have developed counter-adaptations to spinescent plants. The caterpillar *Hyphantria cunea* consumes the central part of spinescent holly leaves [87], and the woodrat *Neotoma albigula* removes the spines when feeding on spinescent cactus leaves [88]. Spinescent tree species contain significantly less total phenolics and condensed tannins than spineless ones among six species of African savanna trees [89]. Thus, there is a trade-off between chemical and structural defenses.

3 Tree-Leaf Chemicals in Seasonal Environments

3.1 General Patterns

Most trees live long but may change in their physiological conditions seasonally or between dry and wet periods. The mixed forest in temperate zones consists of both deciduous and evergreen trees. In winter, deciduous trees fall leaves throughout autumn to the next spring, and herbivores use only the leaves of evergreen trees (Fig. 1). The newly expanded leaves from winter buds are available in spring and after that herbivores can use both deciduous and evergreen tree leaves. Seasonally fluctuating temperatures and humidity may alter leaf chemical components. If so, availability of profitable leaves for arboreal herbivores change greatly with seasons.

Although the data on the seasonal changes in tree-leaf chemicals are still limited, we can see some general patterns. First, young leaves appeared in spring include more chemicals than mature leaves, and once matured, their contents are kept nearly constant or gradually decreased.

Sugars are included more in young leaves than matures, and after maturation their contents are nearly constant in *Quercus robur* [90, 91], *Quercus agrifolia* [92], *Quercus suber* [93], *Quercus acutissima* [39, 73], and *Betula pubescens* [94]. However, all kinds of sugars do not follow this pattern. In *Quercus robur* leaves, maltose and oligosaccharide contents considerably fluctuate during early summer, but sucrose content shows a marked increase in early summer and keeps increasing until autumn [90]. Inositol (a sugar alcohol) is present in low concentrations throughout the summer, but almost disappeared from senescing leaves [94].

The protein content of *Quercus robur* and *Betula pubescens* leaves also decreases markedly according to the leaf expansion and maturation processes from spring to early summer [90, 94]. Protein levels of leaves (*Alnus incana*, *Tilia americana*, *Prunus serotine*, *Ulmus americana*, *Acer rubrum*, *Betula papyrifera*, *Populus deltoides*, *Quercus velutina*, *Quercus macrocarpa*, *Quercus alba*, *Carya glabra*, and *Juglans nigra*) decrease by 22% in mature leaves compared with immature leaves [95]. N content of leaves decreases to less than half of the initial level in summer in *Acer palmatum*, *Acer saccharum*, *Quercus crispula*, and *Betula alleghaniensis* [96, 97] and in many other species of trees [98]. N and P contents in *Quercus agrifolia* leaves are higher in new leaves compared with matures [92]. Also, N, P, K, and ash contents of *Quercus suber* leaves are highest in young leaves in spring, reaching the minimum during the hot and dry summer, and increase slightly during the rainy period of autumn-winter, although Na, Mg, and Ca contents are lowest in spring-early summer and increase during summer and autumn-winter [93]. N and P fractions in leaves of *Larix laricina*, *Betula papyrifera*, and *Alnus crispa* are highest in young leaves and declined in concentration through the season, probably because the concentration is diluted by increasing leaf biomass in the former case and because organic N and P fractions are hydrolyzed and inorganic P and amino acid N are translocated out of leaves in the latter [99].

The water content of leaves decreases from spring to summer because water is included at higher percentage in buds and young leaves than mature leaves [94]. This

tendency is observed in *Quercus robur* [90], *Betula pubescens* [94], *Acer palmatum* [97], *Quercus crispula* [97], *Quercus acutissima* [39, 73], and many other tree species [95].

Second, young leaves appearing in spring include less chemicals than mature leaves, and once matured, their contents are kept nearly constant or slightly increased. Structural compounds (e.g., cellulose) in *Quercus agrifolia* leaves rapidly increase with leaf age [92]. Total carbohydrate increases by 77% on the average in mature leaves (*Alnus incana*, *Tilia americana*, *Ulmus americana*, *Acer rubrum*, *Betula papyrifera*, *Populus deltoides*, *Quercus velutina*, *Quercus macrocarpa*, *Quercus alba*, *Carya glabra*, and *Juglans nigra*), with sugar and starch increasing by 67% and 62% on the average, respectively [95].

3.2 Secondary Metabolites

Seasonal changes in the secondary metabolites are somewhat complex. The seasonal trends differ among phenolic groups and also tree species. The first general pattern is that young leaves in spring include more secondary metabolites than mature leaves. Total phenolic (and astringency) concentrations are higher in new leaves of *Qeurcus agrifolia* [92] and *Quercus acutissima* [39, 73]. Phenolic concentrations in the youngest leaves of *Populus deltoides* are three times those in the oldest leaves [100]. Tannin concentrations of *Leea glabra* young leaves are also higher than that of both medium-aged and old leaves [101]. The concentrations of cell wall-bound proanthocyanidins, gallotannins, and flavonoid glycosides decline after an initial increase in young leaves of *Betula pubescens* [94]. Total phenolic and hydrolyzable tannin contents in *Acer saccharum* leaves tend to decline from spring to summer but no further decline after summer [102]. Young *Quercus robur* leaves are much richer in the dominant phenolic hydrolyzable tannins and flavonoid glycosides than old leaves, although the opposite pattern is observed for a minor phenolic proanthocyanidins [103].

The second general pattern is that the secondary metabolites increase with leaf growing from spring to summer and keep high during summer and autumn. Total phenolic content of *Moringa oleifera* leaves is lowest in the newly opened leaves and increases gradually with the maturation of the leaves [104]. It peaks by the summer and then remains relatively constant through autumn in *Acer saccharum* [96] and in *Quercus robur* [91]. Pirvu et al. [105] report an increase of total phenolics in *Fagus sylvatica* leaves, with overall maximum values in September, and Sati et al. [106] also report a similar increase in *Gingko biloba* leaves, higher in the fall than in spring. Concentrations of condensed tannins gradually increase as the leaves mature [92]. Condensed tannin levels are also lowest in spring and increase throughout the growing season for *Quercus robur* [107] and *Quercus velutina* [108]. The tannin content increases during summer in *Acer palmatum* and *Quercus crispula* [97], and soluble proanthocyanidins content increases through growing season in *Betula pubescens* [94]. In *Acer saccharum* leaves, condensed tannin contents cannot be detected in the spring samples but reach a plateau through summer [102]. Phenolic

contents remain fairly constant during the growing season, but the compositions of catechin, gallocatechin, and two leucodelphinidins (flavonoid) change seasonally in *Quercus robur* leaves [107]. Total phenolic content in *Ribes nigrum* leaves increases from June to August, but their compositions differ with seasons [109].

The first seasonal pattern of the secondary metabolites may be effective to avoid young leaves from herbivore's predation, because some insects suffer higher mortality and reduction of growth when fed young leaves [100]. The second seasonal pattern in which the secondary metabolites increase during the growing season may be also the successive defensive response to herbivore's feeding, because in some insects, the period of the highest attack on leaves corresponds to the time when phenolic contents are absent or minimum [107]. However, the evolutionary processes between plant defense and herbivore's attack with detoxification or tolerance are sometimes arms races.

In other cases, the level of chlorogenic acid in the leaves of *Olea europaea* increases markedly in winter (January) and decreases to a minimum level in spring (April), thereafter, the chlorogenic acid level gradually increases again and reaches the maximum level in summer (July–August) [110]. However, the level of caffeic acid is high from winter to spring (January to April) and reaches its highest value in spring, and it starts to decrease and reaches the minimum value in summer (June–August) [110].

Comparisons of leaf chemicals between dry and wet seasons of seven tree species (*Senegalia caffra*, *Vachellia karroo*, *Burkea africana*, *Combretum molle*, *Combretum zeyheri*, *Searsia lancea*, and *Terminalia sericea*) suggest that condensed tannins are little different between seasons [111]. Water limitation induced experimentally has little impact on overall leaf secondary metabolite concentrations of *Eucalyptus* leaves, although a few components of them decrease by limiting water [112]. The proportions of young leaves in forest also differ between dry and wet seasons. Young leaves are more available in the wet season, which contain more protein and lower fiber, and lessor weasel lemurs select protein-rich leaves [41]. In the dry season, however, chemical differences among available leaves become unclear.

4 A Case Study: Seasonal Changes in Leaf Chemicals and the Giant Flying Squirrel's Feeding Behavior

The giant flying squirrel (Fig. 2 left) is an exclusively arboreal, nocturnal, and large-sized herbivore and distributed on Kyushu, Shikoku, and Honshu Islands of Japan [113]. Adult squirrels reach weights of up to 1.3 kg [113]. The home range size is 0.4–5.2 ha, usually larger in males than females, with considerable overlap between the sexes and between males [114, 115]. This large body size may be maintained by nutrition from specialized cecal microbiota, which are known to convert diverse plant materials into absorbable nutrients in the congeneric species *Petaurista alborufus lena* [60].

Fig. 2 Japanese giant flying squirrel *Petaurista leucogenys* eating the oak leaves *Quercus acutissima* at night (photo by Makoto Uenishi). Three leaves with their feeding marks are shown in right. They often eat only the central part of the leaf by biting the corner of it after folding two times (see Fig. 3)

The study site was 50 ha in an isolated section of woods in Hachioji, Tokyo, Japan. The vegetation was mixed temperate broadleaf and coniferous trees [116]. One to five morning censuses were conducted every month along a fixed 2-km transect. The census was continued from April 2013 to November 2015 for a total of 87 times and a total of 2761 food items were identified, consisting of 1559 (56.47%) leaves, 734 (26.58%) seeds/fruits, 264 (9.56%) buds, and 204 (7.39%) flowers. The leaves were most important foods for them and almost all (97.95%) leaves were from *Quercus* trees: *Q. acutissima* (63.72%), *Q. sessilifolia* (33.73%), *Q. crispula* (1.24%), *Q. glauca* (1.11%), *Q. myrsinifolia* (0.13%), and *Q. variabilis* (0.07%). *Quercus* trees include deciduous and evergreen species, which enables us to compare food availability and selectivity in the seasonal environment. Most dominant food in our study site, *Q. acutissima*, is a deciduous tree, and leaf debris was found from May to October. Feeding was intensive from May to July. Leaf debris of just-expanded leaves in early May of this tree was never found. Another dominant food, *Q. sessilifolia*, is evergreen and leaf debris was found from October to the next June when the deciduous *Q. acutissima* leaves were fallen.

Interestingly, there were three types of feeding debris. Type A, eaten at apical part of a leaf, included both asymmetric cutting without leaf folding (Fig. 3A) and symmetric cutting after folding the leaf once (Fig. 3B). Type B, eaten at basal part of a leaf, also included both of these patterns (Fig. 3A, B). Type C, which occurred after the leaf was folded twice, usually displayed circular openings (Figs. 2 right, 3C). Feeding after folding the leaf three times left two connected circles in the leaf [73]. Of the 1001 total feeding marks on *Q. acutissima* leaves, 240 (24.0%) were Type A, 439 (43.9%) were Type B, and 322 (32.2%) were Type C. Of the 520 total marks on *Q. sessilifolia* leaves, 474 (91.2%) were Type A, 36 (6.9%) were Type B,

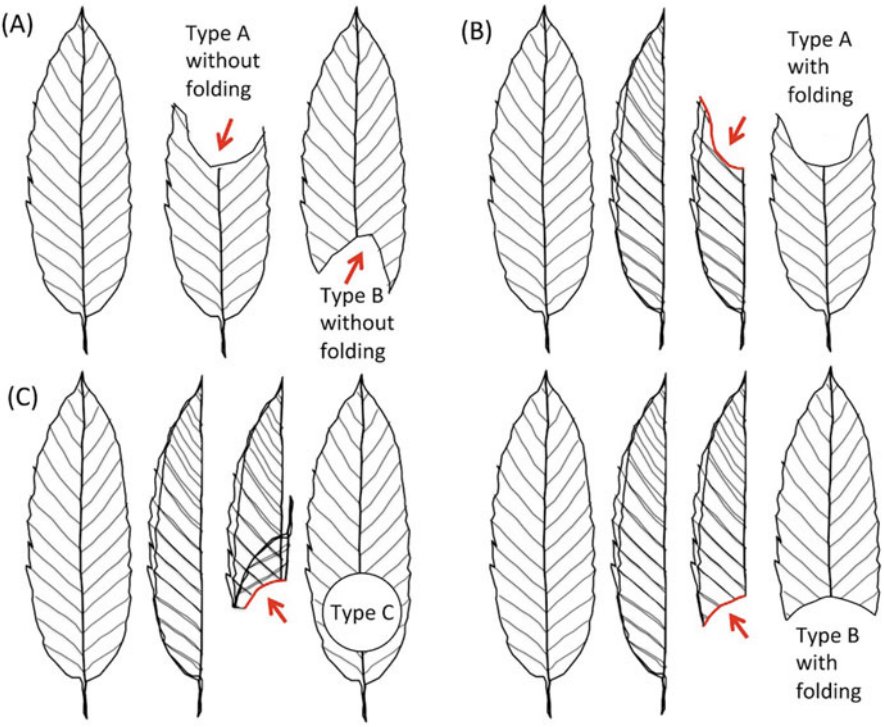

Fig. 3 Feeding patterns by Japanese giant flying squirrels on the oak leaves *Quercus acutissima*. Arrows indicate the feeding location by squirrels. Types A, B, and C show leaves with apical, basal, and central eating marks, respectively. (**a**) The squirrels eat a leaf apically (Type A) or basally (Type B) without leaf folding. (**b**) If the leaf is folded once as shown, the eating patterns are symmetric in right and left both in Types A and B. (**c**) If the leaf is folded twice as shown, the eating part is open at the central part of the leaf (Type C)

and only 10 (1.9%) were Type C. There was no clear seasonal tendency in the proportions of these three types of feeding patterns, but the proportions of these three feeding types differed between *Q. acutissima* and *Q. sessilifolia* [39].

Leaf-folding behavior before eating it is a complex task and may be needed to learn before doing so. This behavior was never observed in 2 of 15 local populations examined [73]. Such a local variation in feeding behavior is one of the evidences that eating of the central part of leaves is maintained by learning.

In the deciduous *Q. acutissima*, foliation occurred during April, and the fully expanded leaves were still soft and light green in early May. Total phenolic content (gallic acid equivalent) was much higher in early May than in other months (Fig. 4). If these data in early May were excluded, the average total phenolic contents were 55.4 ± 8.7 SD ($n = 13$) mg g^{-1} dry weight. In the evergreen *Q. sessilifolia*, there were no such seasonal trends, and total phenolic contents were always lower than those in *Q. acutissima* (Fig. 4); the average was 34.9 ± 8.9 SD ($n = 17$) mg g^{-1} dry weight.

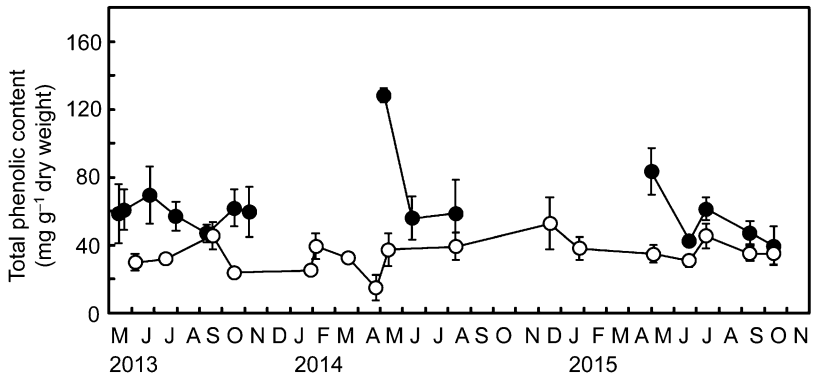

Fig. 4 Seasonal changes in total phenolic contents (mean ± SD) of the leaves in the deciduous oak *Quercus acutissima* (closed circles) and the evergreen oak *Q. sessilifolia* (open circles) in the habitat of Japanese giant flying squirrel *Petaurista leucogenys*

In *Q. acutissima*, the glucose contents of leaves collected at 10:30–11:00 a.m. were slightly higher in newly expanded leaves in early May than those in other seasons. In the evergreen *Q. sessilifolia*, however, such a trend was not detected, and glucose was always much lower than in *Q. acutissima* [39]. In *Q. acutissima*, the average glucose content was 71.7 ± 3.9 SD ($n = 5$) mg g^{-1} dry weight in the basal part of a leaf (excluding the values in early May), whereas in *Q. sessilifolia*, the glucose content was 24.6 ± 3.9 SD ($n = 8$) mg g^{-1} dry weight.

These measurements of the microscale distributions of chemicals within a single leaf of *Q. acutissima* and *Q. sessilifolia* suggested that sugar concentration is an important factor affecting which species the flying squirrels eat, and total phenolic concentration affects which parts of the single leaf they eat preferably. The deciduous *Q. acutissima* leaves were preferred for the flying squirrels to eat from spring to summer, despite the evergreen *Q. sessilifolia* leave were always available. In winter, however, *Q. acutissima* had no leaves and they ate the leaves of *Q. sessilifolia*. Sugar contents were homogeneously distributed within the single leaf both in *Q. acutissima* and *Q. sessilifolia*, but the former included three times more sugar than the latter. Thus, the flying squirrels may choose the sweeter *Q. acutissima* leaves from spring to summer. On the other hand, leaves of *Q. acutissima* contained 1.5 times more phenolics than those of *Q. sessilifolia*, suggesting that the flying squirrels do not reject such phenolic-rich (probably distasteful) leaves. In *Q. acutissima* leaves, however, the phenolic concentration was lower in the central part of the leaf than the margins, and thus the flying squirrels may eat preferably the central part of this leaf to avoid eating of phenolic-rich margins. In *Q. sessilifolia* leaves, there was no clear tendency of phenolic concentration within the single leaf. This may be the reason why the flying squirrels often eat the central part of *Q. acutissima* leaves, but eat *Q. sessilifolia* leaves from their apical or basal part.

For all species of *Quercus* trees found in our study site, four are deciduous while seven are evergreen. Leaf phenolic and glucose contents of these trees measured in

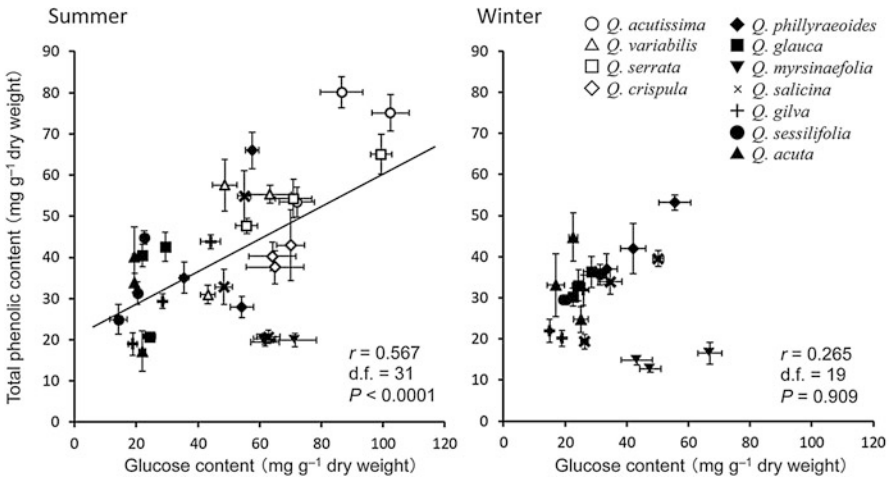

Fig. 5 Relationships between glucose and total phenolic contents in 11 (4 deciduous and 7 evergreen) *Quercus* species in summer and winter in the habitat of Japanese giant flying squirrel *Petaurista leucogenys*. The mean ± SE of three trees is plotted for each species

summer (2 June to 4 July) and winter (27 January to 24 February) suggested that the species with higher leaf glucose contents have higher leaf phenolics in summer, but no correlation between them in winter (Fig. 5). In general, deciduous species (*Q. acutissima*, *Q. variabilis*, *Q. serrata*, and *Q crispula*) have a higher glucose and phenolic contents than evergreen species (*Q. phillyraeoides*, *Q. glauca*, *Q. myrsinifolia*, *Q. salicina*, *Q. gilva*, *Q. sessilifolia*, and *Q. acuta*). In summer, therefore, leaves with a wide range of chemical contents are available for the flying squirrels, and more profitable (sweeter) leaves such as *Q. acutissima* can be selected clearly. In winter, however, they must select leaves among a narrow range of chemical contents and the selectivity of leaves may be unclear. Thus, leaf availability for herbivores is changed seasonally not only in tree species but also in leaf chemicals in their habitats. Such seasonal changes are repeated every year, and the herbivores living there for several years may learn which is the profitable food in that time in their home ranges.

The sweeter leaves of *Q. acutissima* had much higher levels of phenolics which are usually avoided. However, the flying squirrels tended to feed on *Q. acutissima* leaves. This situation may be similar to fruit-eating gorillas and other primates who tolerate higher concentrations of tannins if given with high-sugar solutions [9], because the perceived intensity of the tannic acid is decreased by sweetness [117]. The western lowland gorilla appears to choose fruit for sugar, with fiber and tannin as secondary concerns [18]. In most other mammals, however, the effects on food selection of sugar versus tannins and other phenolics remain unknown. It is unclear whether or not the flying squirrels are more tolerant of these chemicals as sugar concentration increases.

5 Conclusions

Tree leaves are much more abundant than other food resources of herbivores such as seeds, nuts, and fruits, but arboreal mammals do not eat tree leaves randomly. Leaf chemicals affect food choice and feeding behavior of them. They usually prefer sugar- and protein-rich leaves but avoid those including more fibers and secondary metabolites. Preference of sweeter leaves may be caused by its higher energy contents and by which they acquire sufficient amount of food within a short time. Protein (or N) is always limited for herbivores. Selective feeding of protein-rich leaves may be adaptive for their long lives, but the physiological mechanism how they select those leaves remains unknown. Fibers are generally avoided because of decreasing digestibility and increasing toughness of leaves. Secondary metabolites function in toxins (e.g., phenolics and alkaloids) and in digestion inhibitors (e.g., condensed tannins) and are usually avoided by their bitter taste or aversive post-ingestive feedback.

The effects of leaf chemicals on food choice of arboreal herbivores have been examined in four comparative categories; between tree species, between trees of the same species, between leaves of the single tree, and between the parts of a leaf. Most studies revealed that arboreal herbivores prefer leaves of particular tree species or young leaves. However, little information is available on selection between conspecific trees and partial eating of a leaf. Microscale distributions of chemicals may be an important factor to know which parts of a leaf they eat. When preferable or unpreferable chemicals are biased within the single leaf, they may eat partially. Chemical-mediated partial eating of a leaf is one of the future studies to be solved. On the other hand, leaf chemicals are not always constant but change seasonally. In the temperate zone or dry and wet periods, tree physiology and leaf chemicals change seasonally. In winter or dry seasons, leaves of deciduous trees are unavailable and arboreal mammals select leaves only within evergreen trees. In summer, however, they are surrounded by both types of trees in which a variety of leaves are available not only in tree species but also in leaf chemicals. Seasonal changes are repeated during individual lives, which enables them to learn appropriate feeding sites, leaf selection, and feeding manners based on temporally and spatially changing leaf chemicals.

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Deranged Physiology of Peach

16

Lyubka Koleva-Valkova and Adelina Harizanova

Contents

Abstract

Plants are a rich source of a large number of secondary metabolites (SM). These are compounds of varying structure, some of which have a low molecular weight but are generally considered to be of great importance for the survival of the plant. These compounds often accumulate in plants in smaller quantities than the main metabolites, and their synthesis strongly depends on the conditions of the environment and can change in the presence of a stress factor. Secondary metabolites are produced by plants in response to a signal and play an important role as protective chemicals, signal molecules, and attractants. Most of these substances are powerful antioxidants and serve to cope or reduce the effects of oxidative stress caused by various abiotic or biotic factors. For these reasons, secondary metabolites are important for human health too, and the plants that produce them

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are a valuable source. Fruit intended for fresh consumption is a suitable form for the procurement of these compounds as they retain their structure and activity.

Keywords
Peaches · Polyphenols · Secondary metabolites · Stress physiology

Abbreviations

ABA	Abscisic acid
AsA	Ascorbic acid
GSH	Glutathione
JA	Jasmonic acid
MD	Mandelonitrile
MDA	Malondialdehyde
PAL	Phenylalanine ammonium lyase
PPV	Plum pox virus
ROS	Reactive oxygen species
SA	Salicylic acid
SM	Secondary metabolites
TF	Transcription factors

1 Introduction

The plant kingdom has an enormous variety of chemical compounds. A significant part is the metabolites and products of primary metabolism. Another, no less significant, group of compounds that are different from those of primary metabolism vary greatly depending on the family and plant species. They are known as secondary metabolites (SM). According to some authors, secondary metabolites are compounds produced from plants that are not directly relevant for basic photosynthetic or respiratory metabolism [1]. The specificity, as well as the limited distribution of many such compounds, makes it possible to use them as taxonomic markers [2]. The plant kingdom offers a wide range of compounds that exhibit antioxidant properties. Essential oils and polyphenols such as tannins, flavonoids, and phenolic acids are considered excellent natural antioxidants. They are widespread and can be considered as the richest group of secondary metabolites in plants. As they have a positive effect on human health, the plants or fruits that hold them are of great interest to the food and pharmaceutical industry. *Prunus persica* (L.) belongs to the family Rosaceae and is grown in a huge area of Europe, India, North Africa, and West Asia. From all 3000 species belonging to the Rosaceae family, nearly 200 species are cultivated for their edible fruits and seeds [3, 4].

Peach is an important fruit and some of the major producers are Spain, Italy, China, and the United States (http://www.fao.org). Peaches and nectarines (both *Prunus persica*) are characterized by a wide range of different varieties, their healthy characteristics, color, and taste being important factors for consumer choice. The red color of the fruits has been the subject of most breeding programs. In particular, high

levels of red coloring are sought in varieties intended for fresh consumption. By contrast, the reduction of pigment content in any part of the fruit is the goal of most canning programs for the canning industry [5].

Plants have developed the ability to synthesize and store secondary metabolites as a means of protecting against herbivores, bacteria, fungi, and viruses, as well as other competing plants. Plants typically produce complex mixtures of SMs that can work in an additive or even synergistic ways. The mechanism of the protective action of secondary metabolites is not fully elucidated. Some protecting compounds are directed to a particular target, e.g., the neurotransmitter receptor or the ion channel of the animal pest; others have a broad spectrum of activity and show pleiotropic activity for several purposes. In addition to protective function, secondary metabolites also serve as signal compounds attracting pollinators and seeds spreading animals [6]. A characteristic feature of secondary metabolites is that their metabolism, especially synthesis and accumulation, strongly depends and is regulated by the conditions of the environment. The use of biostimulants can also have a positive effect on the biosynthesis of secondary metabolites, which increases the resistance of plants to various stress factors.

2 Secondary Metabolites: Classification and Function

Plants are a rich source of thousands of secondary metabolites. They consist of low molecular weight compounds that are considered crucial to the survival of the organism that produces them. These compounds are often accumulated by plants in smaller quantities than the major metabolites [7]. Secondary metabolites are produced by plants and play an important role as protective chemicals and signaling molecules. Alkaloids, flavonoids, essential oils, phenols, terpenes, etc. are included in this class of compounds [3, 8]. Signaling messages that regulate plant behavior are delivered from a wide range of chemical compounds. In some cases, they can facilitate communication between members of a species (e.g., pheromones) or between members of different species (e.g., allopathic substances) [9, 10]. These interactions have a largely negative effect on the germination, growth, development, propagation, and behavior of other organisms [7, 11, 12].

There are different classifications of secondary metabolites based on the content or absence of nitrogen in the molecules as well as their biosynthetic pathway or precursor. The most common classifications divide the secondary metabolites into two main groups: nitrogen-containing and non-nitrogenous compounds, each of which is subdivided into subgroups (Table 1).

Depending on the biosynthetic pathway, the secondary metabolites are divided into three main groups: (1) Terpenoids; (2) Flavonoids and concomitant phenolic and polyphenolic compounds; (3) Nitrogen-containing alkaloids and sulfur-containing compounds [14] (Fig. 1).

Terpenoids are the largest and most diverse family of natural products, ranging from linear to polycyclic molecule structures, and ranging in size from five-carbon (C5) hemiterpenes to natural rubber containing thousands of isoprene units (C5). All

Table 1 Classification of secondary metabolites in higher plants based on the content or absence of nitrogen in the molecule (by [13])

Type of secondary metabolite	Approximate numbers
Nitrogen-containing secondary metabolites	
Alkaloids	21,000
Nonprotein amino acids (NPAAS)	700
Amines	100
Cyanogenic glycoside	60
Glucosinolates	100
Alkamides	150
Lectins, peptides, polypeptide	2000
Secondary metabolites without nitrogen	
Monoterpenes including iridoids	2500
Sesquiterpenes	5000
Diterpenes	2500
Triterpenes, steroids, saponins	5000
Tetraterpenes	500
Flavonoids, tannins	5000
Phenylpropanoids, lignin, coumarins, lignans	2000
Polyacetylenes, fatty acid, waxes	1500
Anthraquinones and othes polyketides	750
Carbohydrates, organic acids	200

terpenoids are synthesized by condensation of isoprene units and are classified according to the number of five carbon atoms present in the basic structure [15]. Many aromatic molecules such as menthol, linalool, geraniol, and caryophyllene are formed from monoterpenes (C10) with two isoprene units and sesquiterpene (C15) with three isoprene units. Other bioactive compounds such as diterpenes (C20), triterpenes (C30), and tetraterpenes (C40) show very special properties [13].

A characteristic feature of phenolic compounds is the presence of at least one aromatic ring with one or more hydroxyl groups attached. There are more than 8000 phenolic compounds in the plant kingdom [16]. Phenols range from simple, low molecular, single aromatic rings to large and complex tannins and polyphenol derivatives. They can be classified based on the number and location of their carbon atoms and usually found conjugated to sugars and organic acids. Phenols can be classified into two groups: flavonoids and nonflavonoids [13].

Flavonoids are polyphenol compounds containing 15 carbon atoms with 2 aromatic rings attached through a triangle bridge. They are the most abundant phenolic compounds and are present in high concentrations in the epidermis of the leaves and the skin of the fruits. Their main function in plants is to participate in protective processes against high UV radiation, infections, oxidative stress. They also contribute to the pigmentation of plant parts, stimulate nitrogen-fixing microorganisms, and increase the resistance of plants to diseases [17]. The major subclasses of flavonoids

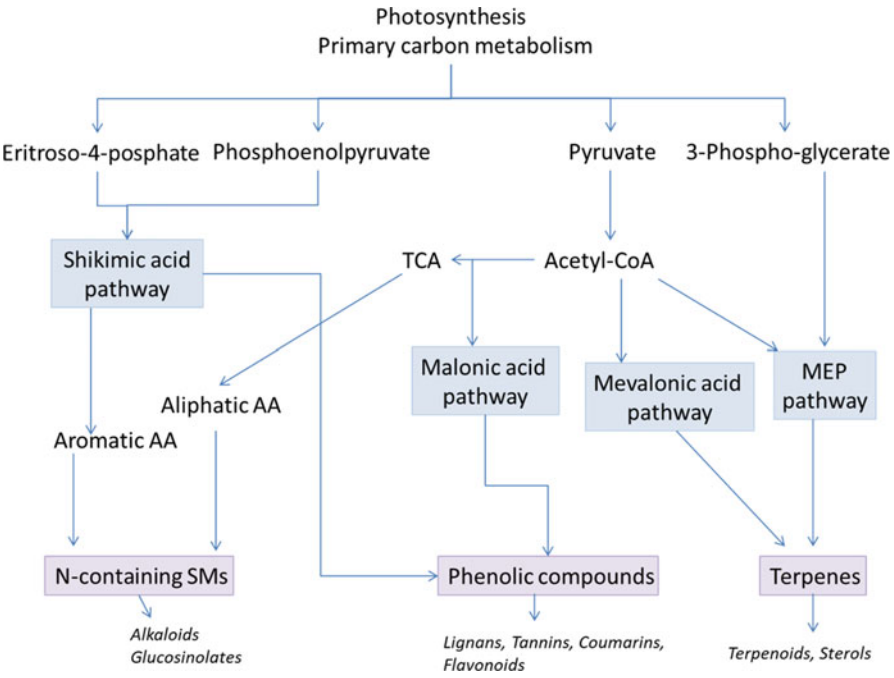

Fig. 1 Classification of secondary metabolites in higher plants based on their metabolic pathway of synthesis (by [14])

are flavones, flavonols, flavan-3-ols, isoflavones, flavanones, anthocyanidins, dihydroflavonols, flavan 3,4-diols, coumarins, chalcones, dihydrochalcones, and aurons. A variety of substitutes may be added to the primary flavonoid skeleton. Hydroxyl groups are typically present at 4, 5, and 7 positions. Sugars are very common in most flavonoids naturally occurring like glycosides. Both sugars and hydroxyl groups increase the water solubility of flavonoids, but other substituents such as methyl groups and isopentyl units make the flavonoids lipophilic. This, in turn, determines the sites for their accumulation [13].

Anthocyanins, flavonols, and flavan-3-ols play a central role in determining fruit quality [18]. Flavan-3-ols are the most complex subclass of flavonoids ranging from simple catechin and epicatechin monomers to oligomeric and polymeric proanthocyanidins, also known as fused tannins [13]. Proanthocyanidins give astringency to fresh fruits, fruit juices, and wine. They can be oxidized by forming brown pigments in the seeds and other tissues and can act as substances that inhibit the feeding of various pests in reproductive tissues and in developing fruits [5].

The major nonflavonoids are gallic acid, which is the precursor of hydrolyzable tannins, hydroxycinnamates, and their conjugated derivatives, and polyphenol stilbenes. Phenolic acids are also known as hydroxybenzoates, the main component being gallic acid. For the first time, it is isolated from the juice of specific bumps (gallae) formed in plants after an attack by parasitic insects. The tissue swelling is

due to the accumulation of carbohydrates and other nutrients that support the growth of insect larvae. The phenolic composition of the bumps consists of up to 70% of gallic acid esters [19].

Gallic acid is the major unit of gallotannins whereas gallic acid and hexahydroxydiphenoyl residues are both subunits of ellagitannins. Gallotannins and ellagitannins are called hydrolyzable tannins because they are easily degraded, releasing gallic acid and/or ellagic acid, while condensed tannins are not. Condensed tannins and hydrolysis tannins are capable of binding and precipitating collagen proteins in animal skins [13].

The main precursor to phenylpropanoids is cinnamic acid and its derivatives – hydroxycinnamates. The most common hydroxycinnamates are *p*-coumaric, caffeic, and ferulic acids, which are often accumulated as corresponding tartrate esters, caftaric, and fetaric acids. Conjugates of caffeic acid are common components of fruits and vegetables [13].

Members of the stilbene family having a C6–C2–C6 structure, such as flavonoids, are polyphenol compounds. Phytoalexins are compounds produced from plants in response to an attack by fungal, bacterial, and viral pathogens. Resveratrol is the most common stilbene [20].

Alkaloids are a large and structurally diverse group of compounds. Many are derived from amino acids, but others are the result of modifying different classes of molecules, including polyphenols, terpenes, or steroids. With some notable exceptions, alkaloids are the most soluble aqueous alcoholic solutions and commonly occur as salts (e.g., chlorides or sulfates) or as N-oxides in plants. Most of these have heterocyclic ring nitrogen or a ring system and a basic (alkaline) nature.

3 Distribution of Secondary Metabolites in Peach Tissues

The tissue distribution of secondary metabolites in peaches varies greatly and depends on varieties and environmental conditions. The main pigment responsible for the red coloration of peaches and nectarines is cyanidin – in particular, cyanidine 3-glucoside, one of the most common anthocyanin pigments in fruit. The hydroxycarboxylic acid derivatives, anthocyanins, flavonols, and flavan 3-ols are the most common phenols in peaches and nectarines. Peaches and nectarines contain cyanidin-3-O-glucoside, cyanidin-3-O-rutinoside, flavonols (quercetin-3-O-glucoside and quercetin-3-O-rutinoside), flavan 3-ols (catechin, epicatechin, and proanthocyanidins, including procyanidin B1), and others [5, 21, 22]. Apricots and peaches contain carotenoids mainly in the form of β-carotene [23]. Chlorogenic acids, caffeic acid, catechin, and procyanidin B3 (catechin- (4β-8) -catechin) are the major phenols in peaches. Chlorogenic and non-chlorogenic acids are the main derivatives of hydroxycinnamic acid, while procyanidin B1 (epicatechin- (4β-8) -catechin), catechin, and epicatechin are the predominant flavan 3-ols and flavonols found in peach skin compared to peach flesh. Anthocyanins are mainly found in peach and nectarine skin. Small amounts of pigments can also be found in the tissues near the stone. Cyanidine 3-glucoside and cyanidin 3-rutinoside are the main

pigments in nectarines and peaches. Some varieties may also contain cyanidine 3-acetylglucoside and cyanidin 3-galactoside. Quercetin 3-glucoside and quercetin 3-rutinoside are the major flavonols in nectarines and peaches and are found mainly in the skin [24].

Different phenolic compounds have been found in peach fruits. They are one of the richest in antioxidant substances. However, both the qualitative and quantitative profiles of these compounds vary considerably depending on the variety. In addition to phenolic compounds in peach fruit, a number of vitamins are also present, with significant amounts of ascorbic acid (vitamin C) and carotenoids (provitamin A). Different conditions before and after ripening of fruit can change the synthesis and emission of volatile substances from harvested plant products. This affects taste, ripening, and other factors that affect quality or storage potential. The peach content of volatile substances has been thoroughly studied. Up to now, more than one hundred volatile compounds have been identified. Some of the most common are linalool, benzaldehyde, ester terpenoids, norisoprenoids, ketones, and lactones. Color properties are predominantly determined by lactones and fewer aldehydes, alcohols, terpenoids. The chemical composition of the volatile compounds varies between the different parts of the fruit. In the mesocarp, closer to the skin, for example, the concentration of volatile substances such as norisoprenoids and benzaldehydes is higher than in the inner mesocarp close to the stone. Besides the composition during the ripening process, the chemical composition of the volatile substances is changing: the levels of the six carbon compounds are drastically reduced, while the content of lactones, benzaldehyde, linalool, norisoprenoids, and phenylalanine derivatives is increased. Volatile ingredients are also influenced by the conditions of fruit storage [25] (Fig. 2).

According to their biosynthetic origin, the secondary metabolites in plants can be divided into three main groups: terpenoids, nitrogen-containing compounds (alkaloids, glucosinolates, and cyanohydrins), and phenylpropanoids, also known as phenolic compounds [26]. One of the most important building blocks associated with the biosynthesis of secondary metabolites is obtained from acetyl coenzyme A, shikimic acid, mevalonic acid, and 1-deoxyxylose-5-phosphate. They participate, respectively, in the acetate, shikimate, mevalonate, and deoxyxylose phosphate pathways of biosynthesis [7, 26, 27].

4 Biosynthetic Pathways of Major Secondary Metabolites: Enzymes and Regulation

All plants have the capacity to produce secondary metabolites (SMs). The widest variety of them is found in the flower plants. The majority of these metabolites originate from five different precursors or metabolic pathways. These are acetyl coenzyme A (polyketides such as anthraquinones, flavonoids), active isoprene (various terpenoids), shikimic acid (aromatic amino acids, cinnamic acids, tannins, indole, and isoquinoline alkaloids), glycolysis (sugars, gallic acid), and TCA

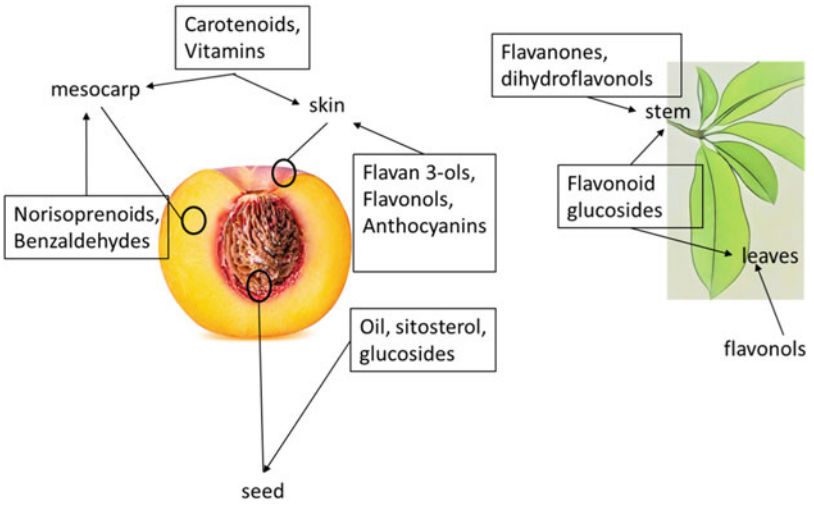

Fig. 2 Chemical compounds in peaches – fruits, leaves, and stems by [25] with modifications)

(alkaloids). These pathways, both individually and in combination, create enormous structural diversity, with around 200,000 currently identified.

This structural diversity is further enhanced by widespread glycosylation and esterification and also by the less frequent inclusion of other primary metabolites, such as certain nonaromatic amino acids and polysaccharides. Plants typically produce complex mixtures of SMs. The ingredients of these mixtures, which differ between plant organs and stages of development, generally belong to several classes of secondary metabolites; for example, terpenoids are often accompanied by phenols. In principle, a limited number of major secondary metabolites and several minor components are commonly found, which are often biosynthetically related to major constituents [28].

Plant secondary metabolites are synthesized by specific pathways. The sites of their synthesis can vary for both the type metabolite and the different plant species. In addition, some molecules can be synthesized in all plant tissues, while others are produced in a specific tissue or even in a cell-specific species [29]. The place of synthesis for SM is not always the place for their accumulation. Secondary metabolites, which are hydrophilic compounds, are predominantly stored in the vacuole, whereas lipophilic SMs are usually isolated in gum channels, oil cells, trichomes, or in the cuticle [7, 30].

Anthocyanins, flavonols and flavan 3-ols are synthesized through the flavonoid pathway, whose genetics and biochemistry are already well-studied. The process consists of several steps common to the synthesis of different flavonoids. Additionally, there are also branches of specific reactions that are specific to each type of flavonoid (Fig. 3). It is assumed that the flavonoid pathway is mainly regulated at the level of transcription of genes coding for enzymes from the pathway. Several transcription factors (TFs) from various plants that control this transcription have been isolated. In particular, the interacting TFs of the R2R3-MYB and bHLH form complex with

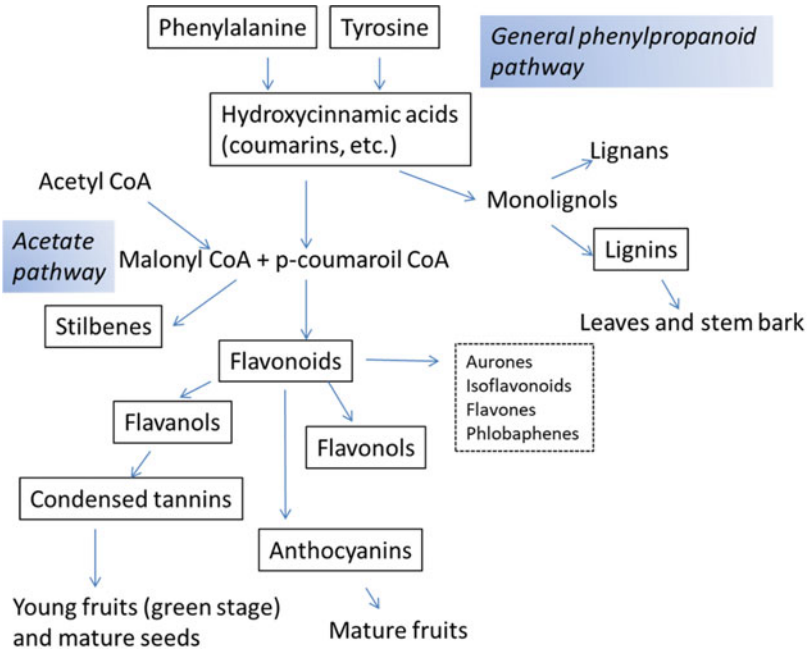

Fig. 3 Main pathways and branched reactions for biosynthesis of secondary metabolites in plants (by [24] with modifications)

WD40 proteins (called MBW complex) to activate the genes responsible for the anthocyanin and proanthocyanidin biosynthesis. The MBW complex usually regulates groups of flavonoid biosynthetic genes that vary between species. This regulation is via specific binding to motifs in the promoters of the pathway genes [5].

Phenolic compounds are one of the major classes of secondary metabolites in plants derived from phenylalanine and, to a lesser extent, in some plants also from tyrosine (Fig. 4). Chemically, the phenols can be defined as having an aromatic ring bearing one or more hydroxyl groups including their functional derivatives. The plants contain a wide variety of phenolic derivatives including simple phenols, phenylpropanoids, benzoic acid derivatives, flavonoids, stilbene, tannins, lignans, and lignins. Together with long-chain carboxylic acids, phenols are also components of suberin and cutin. These quite diverse substances are essential for the growth and reproduction of plants and also act as antinutritional and antipathogenic agents [31]. In addition, the phenols function as antibiotics, natural pesticides, symbiosis signaling agents for nitrogen-fixing bacteria, pollinator attractants, ultraviolet light protection agents, insulating materials that make cell walls impermeable to gas and water, and as structural materials that confer stability of the plants [24].

A key enzyme from the phenolic pathway is phenylalanine ammonium lyase (PAL), which catalyzes the deamination of phenylalanine and leads to the formation of a carbon–carbon double bond, resulting in trans-cinnamic acid. In some plants and grasses, tyrosine is converted to 4-hydroxycinnamic acid by the action of tyrosine

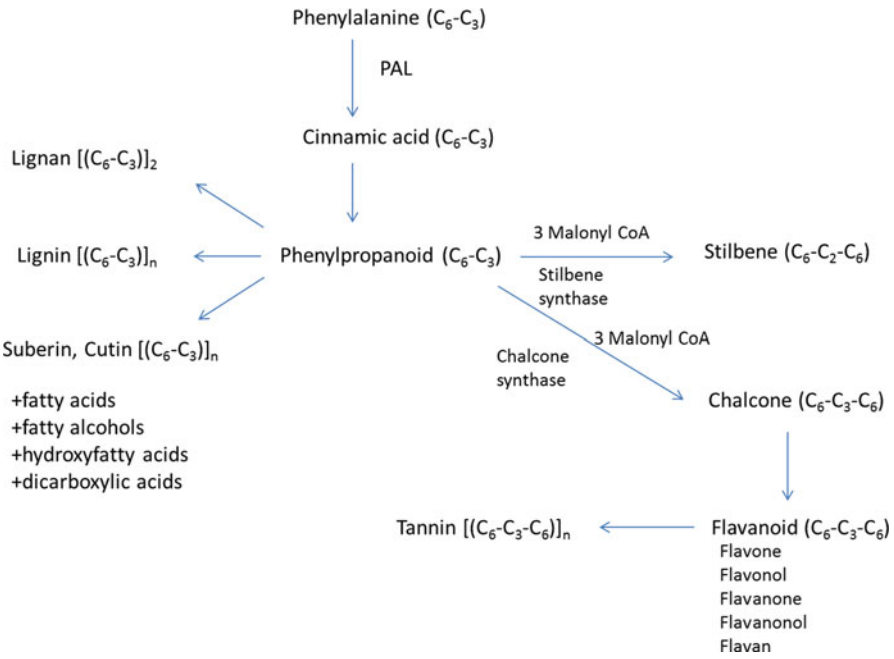

Fig. 4 Phenylpropanoid pathway for biosynthesis of secondary metabolites in plants (by [24] with modifications)

ammonium lyase (TAL). The introduction of a hydroxyl group in the para-position of the phenyl ring of cinnamic acid proceeds via catalysis with monooxygenase using cytochrome P450 as the oxygen binding site. The *p*-coumaric acid formed can be further hydroxylated in the 3 and 5 positions by hydroxylase and eventually methylated by O-methyl transferase with S-adenosylmethionine as a methyl donor; this results in the formation of caffeine, ferulic, and sinapic acids (Fig. 5). These compounds have a phenyl ring (C6) and a three-carbon side chain and are collectively called phenylpropanoids, which serve as precursors for the synthesis of lignins and many other compounds [24].

Benzoic acid derivatives are obtained by the loss of a bicarbonate residue from phenylpropanoids. Salicylic acid is a benzoic acid derivative and acts as a signal molecule (Fig. 5) [32]. After infection or ultraviolet radiation, many plants increase the salicylic acid content, which can induce biosynthesis of the protective substances. Similar to the phenylpropanoid series, the hydroxylation and eventual methylation of hydroxybenzoic acid lead to the formation of dihydroxybenzoic acid (protocatechuic acid), vanillic acid, syringic acid, and gallic acid. Hydroxybenzoic acids are usually present in the bound form in plants and are often a component of a complex structure such as lignins and hydrolyzable tannins. They are also in the form of organic acids and as sugar derivatives. However, there are exceptions in which they are mainly present in a free form [24].

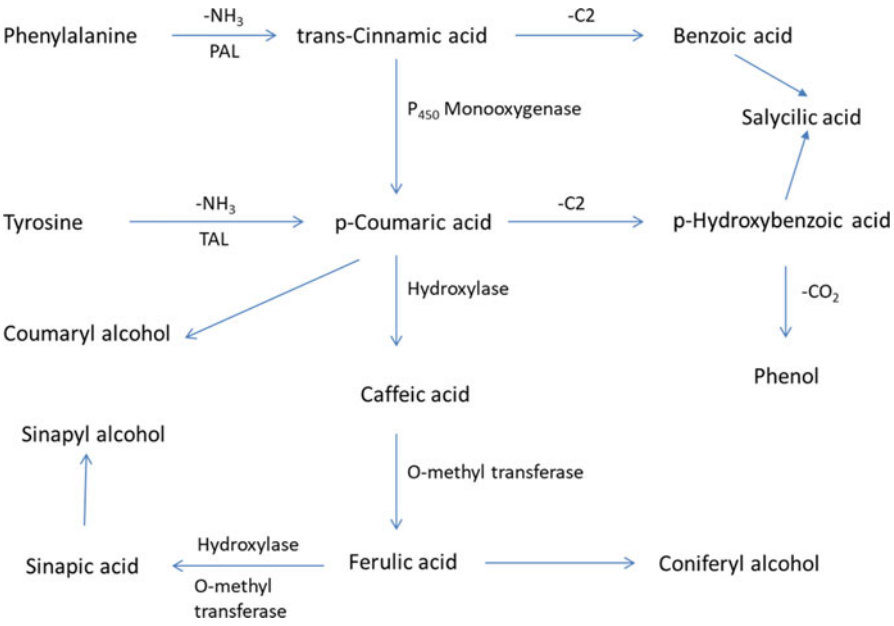

Fig. 5 Specific steps of phenylpropanoid pathway (by [24] with modifications)

Flavonoids, including flavones, isoflavones, and anthocyanidins, are formed by condensation of phenylpropane (C6–C3) and malonyl CoA molecules, resulting in the formation of chalcones which subsequently cyclize under acidic conditions (Fig. 6). Thus, flavonoids have the basic skeleton of diphenylpropanes (C6–C3–C6) with a different oxidation level of the central pyran ring. This also applies to stilbene, but in this case, after the introduction of the second phenyl moiety, a carbon atom of phenylpropane is separated. Stilbenes are powerful fungicides in plants, e.g., viniferine from vineyards. In the case of flavonoids and isoflavonoids, flavones, flavanones, flavonols, and flavanonols, as well as flavan 3-ols and related compounds may be formed depending on the substitution and unsaturation patterns. Flavones and flavonols occur as aglycons in foods. Till now about 200 flavonols and about 100 flavones have been identified in plants. Flavonols are different from flavones because they have a hydroxyl group in the 3-position and can be considered as 3-hydroxyflavones [24].

5 Change of Secondary Metabolites in Abiotic Stress

Plant stress is a state of tension caused by the changing conditions of the external and internal environment that cause a response from the affected organism. During the growing season, plants are often subjected to a stress of a different nature. Traditional abiotic stress factors for plants are low and high temperatures, drought, excess soil moisture, poor nutrition, etc., which greatly influence the formation of yields and

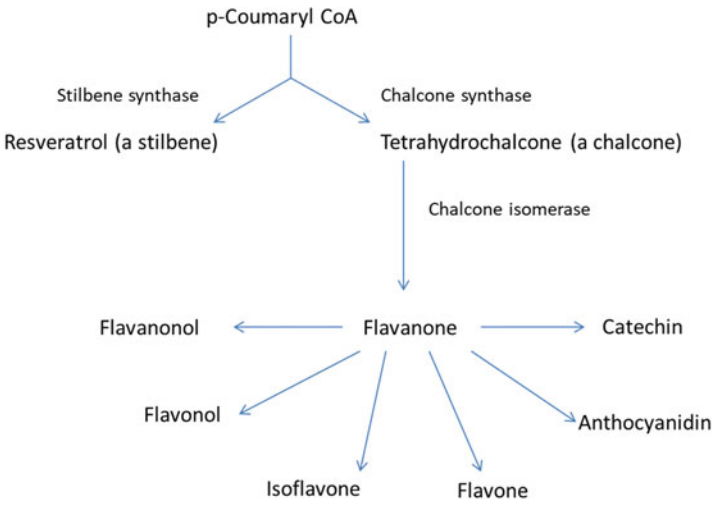

Fig. 6 Biosynthetic pathway of stilbenes and flavonoids (by [24] with modifications)

the quality of plant production. Under stress conditions, the normal metabolism of plants can undergo changes that lead to increased synthesis of some compounds and weakening of others (Fig. 7). Often these changes are related to the accumulation of compounds having a protective or regulatory function.

The content of phenolic compounds in peach fruit depends on many external and internal factors, including variety, the degree of maturity, environmental conditions, and storage conditions. Among them, light, temperature, oxygen, ethylene, growth regulators, nutrients, and pesticides have been shown to affect phenolic metabolism [33–35]. Plant phenols are readily oxidized by polyphenol oxidase (PPO), most commonly after tissue damage, as PPO is believed to act as a protective enzyme [36]. Endocarp lignification in the fruit of the peach is carried out in accordance with the separate induction of competitive flavonoid pathways in the mesocarp and the exocarp tissue layers. The induction of flavonoid biosynthesis is preserved among Rosaceae and possibly also in many other fruits, whereas the induction of lignin is not. The coordination of these two processes is likely to be critical to controlling a number of important agronomic situations in fruit and nuts. Furthermore, the development of peach and Arabidopsis endocarp seems to be controlled by very similar mechanisms, which include the regulatory transcription factors (which stimulate endocarp differentiation), negative regulator and factor that cause secondary wall formation, and lignin deposition [37].

5.1 Temperature and Oxidative Stress

Cold is one of several important environmental stresses affecting plant productivity and distribution. Tolerance to low but not freezing temperatures – the phenomenon is

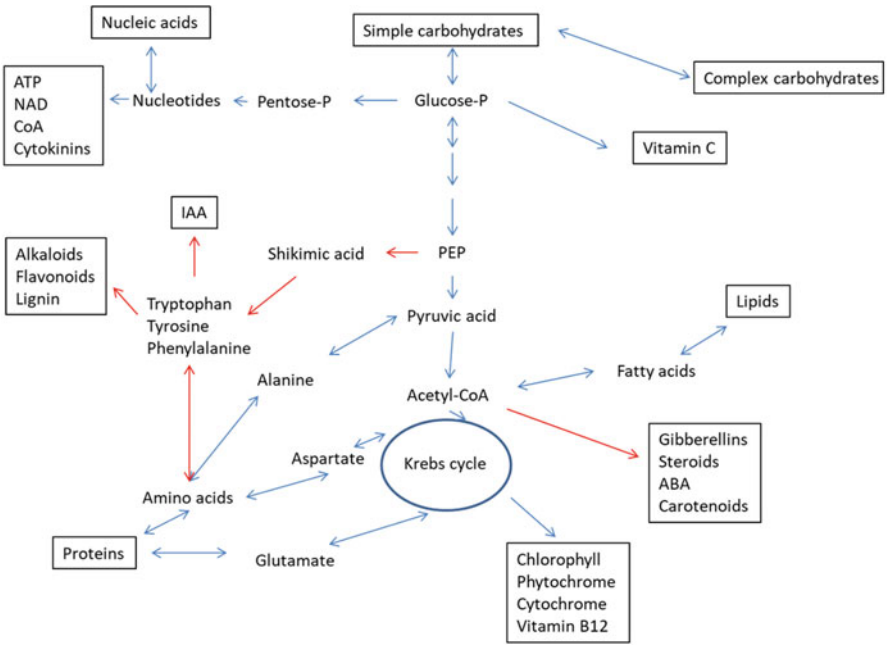

Fig. 7 Primary and secondary metabolism network

known as cold acclimatization – is a complicated response to stress, which involves a complex cross-link between signal transduction and gene expression. In *Arabidopsis thaliana,* cold acclimatization involves rapid, cold-induced expression of transcriptional activators, followed by expression of genes that are mobilized in response to cold stress. Most fruit species suffer from a negative low-temperature effect when stored in a refrigerator (0 to 7 °C). *Prunus* spp., including *Prunus persica* (L.) Batsch, are highly susceptible to chilling stress (overcooling). The main symptoms of overcooling injuries in peach fruits are dehydration (lack of juice), browning or redness of the mesocarp, sharpness or fleshiness of the flesh. Peach cooling symptoms develop during storage at room temperature after prolonged refrigeration storage [38].

Temperature is also one of the most important factors for maintaining the quality of peaches after harvesting. Some of the metabolic activities such as maturation and degradation of substances decrease by decreasing the temperatures. However, the injuries caused by the low temperatures deteriorates significantly the quality of the fruit during storage and the shelf life is limited. The emergence of cooling damage is often associated with oxidative stress due to increased production of reactive oxygen species such as superoxide anion (O^{2-}), hydrogen peroxide (H_2O_2), hydroxyl radical, nitric oxide, and peroxynitrite. Oxidative damage is considered an early response to sensitive tissues to cooling. If the production of ROS increases dramatically, as happens under stress in the environment, the hydroxyl radical reacts with membrane lipids, resulting in lipid peroxidation and membrane destruction.

Malondialdehyde (MDA) is a product of this lipid peroxidation and is used as a stress indicator in some tissues. To deal with ROS, the plants have developed an effective antioxidant defense system that reacts to oxidative stress and prevents the buildup of ROS and restores the oxidative damage. This system includes both lipid-soluble antioxidants (tocopherol and carotene) and water-soluble reductants, including ascorbic acid (AsA), glutathione (GSH), and enzymes such as catalase (CAT), ascorbate peroxidase (ARX), superoxide dismutase (SOD), and glutathione reductase (GR). The substance melatonin (N-acetyl-5-methoxytyptamine), which is a neurohormone secreted from the pineal gland in mammals, is found in plant tissues too. Melatonin has been reported to be involved in the growth, development, and response to stress in plants [39].

5.2 Water Deficiency and Oxidative Stress

Another important factor for the development of plants and in particular peach is the presence of sufficient water for irrigation. Water stress stimulates stinging and reduces CO_2 fixation, which can significantly reduce photosynthetic electron transport [40]. If water stress is prolonged and/or severe, part of the energy supplied by photons can be redirected to processes favoring the formation of reactive oxygen species (ROS) such as hydrogen peroxide (H_2O_2), which leads to oxidative damage to plant tissues [41]. However, the plants may activate ROS neutralizing enzymes and nonenzymatic systems including secondary metabolites such as phenolic compounds, alkaloids, isoprenoids, phenylpropanoids, and other antioxidants such as glutathione and ascorbic acid (AsA) to reduce oxidative damage [42, 43]. In addition, these systems can also play a very important role in the protection of cell membrane integrity [44, 45].

6 Biotic Stress

Except in abiotic stress, it has also been found that the level of phenolic compounds in plants increases as a response to infection by phytopathogens [46], consistent with the proposed role of these compounds in the protective plant mechanism. It has been found that infected plant tissues and resistant tissues are characterized by a general displacement of the metabolic model, which involves the activation of phenol-oxidizing enzymes and peroxidases. In fact, the degree of resistance is related to the number of phenolic compounds oxidized by phenolases [47].

The use of pesticides and fertilizers has been found to modulate the biosynthesis of phenols in plants [48–50]. Consequently, the increase in the polyphenols content observed in organically grown peaches and pears may support the hypothesis [48–50] that protective mechanisms against infects are related to an increase of endogenous polyphenols when there are no external pesticides that are widespread in conventional agriculture. Many plants show that the regulation of phenolic metabolism depends on several factors. Changes in the level of phenols and in the amount and

activity of oxidizing enzymes, especially phenol oxidase, are part of the mechanism of disease resistance that would be realized by inhibiting the polygalacturonase of the pathogen by oxidized phenols [47]. It is also possible that biochemical protections are present all the time in healthy plants, although observed variations in sensitivity to age seem to indicate that they can develop at certain stages [34, 47].

PAL is a rate-determining enzyme in the activation of the phenylpropanoid pathway, and the increase in PAL activity is associated with the biosynthesis of active metabolites such as phytoalexins, phenols, lignins, and salicylic acid in plant protection pathways [51]. POD participates in cell wall building processes, such as phenol oxidation, suberisation, and lignification, during the protective response against pathogenic agents [52]. PPO participates in the oxidation of polyphenols in quinones (antimicrobial compounds) and lignification of plant cells during the microbial invasion [53]. In addition, the accumulation of phenolic compounds is associated with disease resistance in a number of interactions between plants and a pathogen. The high level of phenolic compounds at the site of pathogen invasion may limit or slow down its growth [54, 55].

In a number of infectious diseases, the metabolism of the affected parts varies considerably under the influence of the pathogen. In leaf curl disease caused by *Taphrina deformans* (Berk.) Tul., it induces serious changes in the biochemical status of the infected plants, which are detectable not only in the tissues with observable symptoms but also in distally situated ones. These changes include the elevation of the activity of antioxidant enzymes (peroxidases), reduced polyphenols content and plastid pigments, alterations of antiradical activity, anthocyanin, and free proline concentrations [56]. The metabolism of the peach leaves affected by the pathogen resembles strongly the characteristic of the still immature leaves. A reduction in photosynthetic function is observed, and the import of sugars into the leaves is dominated by their exports. In addition, the content of both soluble carbohydrates and the enzymes involved in their metabolism is similar to that of young leaves, not mature (Fig. 8). Many of the effects of the disease on the metabolism of peach leaves are similar to those caused by other plant diseases on the metabolism of photosynthetic organs [57].

Like other crops, peach also is attacked by many plant pathogens such as fungi, bacteria, and viruses. Such pathogen-associated infections in plant tissues, particularly local and resistant (hypersensitive) infections, show a general metabolic change that involves the accumulation of amounts of secondary metabolites (phenols, flavonoids, coumarins, terpenoids, steroids, etc.). This change in the spectrum of secondary metabolites is mainly in response to the infectious agent or physiological stimuli and stress.

Besides playing a vital role in the normal development of healthy plants, the temperature is also a key factor in determining the nature of the interactions between plants and pathogens. Any major change in environmental conditions, especially temperature, will affect not only plants but also pathogens and therefore plant diseases [58]. Different temperature regimes are expected to have a direct impact on biochemical compounds in both healthy and infected plants and the most pronounced effect can be visualized in the total phenolic content (TPC). Polyphenols

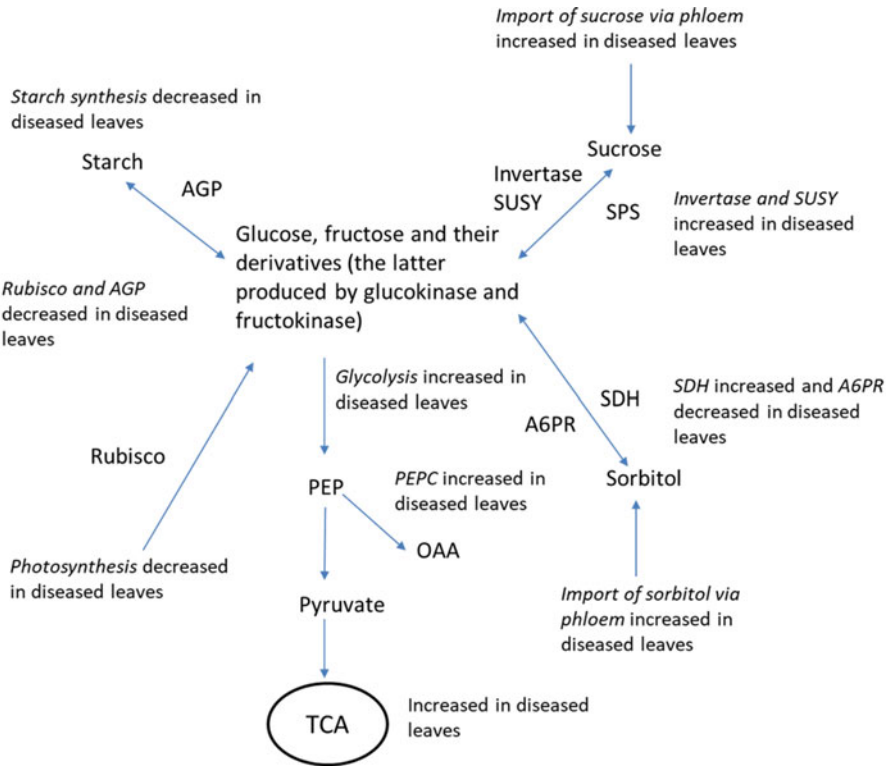

Fig. 8 The changes of metabolism caused by infection with PLC disease. The following enzymes are shown: glucokinase (EC 2.7.1.1), fructokinase (EC 2.7.1.4), and sucrose synthase (SUSY, E.C. 2.4.1.13); the invertases [both soluble and particulate acid invertase E.C.3.2.1.26, and neutral invertase (also known as alkaline invertase) E.C.3.2.1.27]; sucrose phosphate synthase (SPS, E.C. 2.4.1.14); NADPdependent aldose-6-phosphate reductase (A6PR, E.C. 1.1.1.200); sorbitol dehydrogenase (SDH,E.C. 1.1.1.14); ADP-glucose phosphorylase (AGP, EC 2.7.7.27); and phosphoenolpyruvate carboxylase (PEPC, EC: 4.1.1.31) (by [57])

have antioxidant and antimicrobial action [59]. The accumulation of polyphenol compounds in and around the local lesions in the plant is a reliable evidence of a hypersensitivity reaction. Naturally occurring antibiotic compounds that are found endogenously in healthy plants are embedded with chemical barriers to protect plants against attack by a wide range of fungal and bacterial pathogens. There are also viruses that cause peach diseases such as Plum pox virus, Prunus necrotic ringspot virus, and others. The viral infection causes necrosis of the cells at and near the site of the infection where the viral movement is often restricted. Otherwise, in the absence of necrosis, a systemic infection occurs and the reason for this is a significant change in the concentration of polyphenols due to viral infection. The extent of changes in the metabolism of virus-infected plants (respiration and photosynthesis) is often associated with the severity of symptoms and is greatest when tissues become necrotic [60].

The role of phytohormones in alleviating the adverse effects of both abiotic and biotic stress factors is well known. Among plant herbs, salicylic acid (SA) acts as a signaling and regulatory molecule in plant environmental stress responses by SA-mediated control of metabolic and molecular processes [61, 62].

Horsakova et al. [63] found that in the Plum pox virus infection in two peach varieties ("Symphony" and "Royal Glory"), the antioxidant activity (expressed by DPPH, ABTS, FRAP, DMPD, and Free Radicals) of all polyphenol compounds increases significantly. The increased antioxidant activity in the fruit of PPV-infected peach trees is probably due to the function of protective systems that regulate the production of reactive oxygen species and thus protect cells from oxidative damage. Peach fruits contain a whole range of natural substances that have a positive effect on human health. Carotenoids, vitamin C, and polyphenol compounds [64–67] are considered to be major antioxidants.

Free radicals are reactive oxygen species (ROS), namely atoms or molecules that, due to the absence of an electron, show high reactivity. Under normal circumstances, the production of ROS in the cell is low; however, the oxidative stress caused by PPV infection may lead to an increase in ROS [68], which in turn leads to a distortion of the balance between production and the elimination of ROS [69]. However, plants have developed very good protective mechanisms to neutralize ROS, thus protecting cells from oxidative damage. Protective antioxidant systems prevent the initiation of chain oxidation by removing partially reduced oxygen species such as superoxide and hydrogen peroxide [70]. Superoxide dismutase (SOD) catalyzes the conversion of the superoxide radical into hydrogen peroxide, which is subsequently converted by catalase (CAT) or ascorbate peroxidase (APX) into water [68]. Other processes occur in the so-called ascorbate-glutathione cycle when, during the ascorbate peroxidase catalysis, the hydrogen peroxide reacts with the ascorbate to form two molecules of water. At the same time, MDHA is formed which either is disproportionated to dehydroascorbate (DHA) and ascorbate or is reduced from NAD (P) H to ascorbate by dependent MDND. Dehydroascorbate is transformed into ascorbate during reduction with glutathione in a dehydroascorbate reductase (DHAR) catalyzed reaction. Oxidation of glutathione leads to the formation of disulfide (GSSG) between the cysteine residues of two glutathione molecules [71]. Oxidized glutathione is reduced by glutathione reductase using NADPH [72].

7 Influence of Biostimulants on the Content of Secondary Metabolites and Increase of Plant Tolerance in Stress Factors

Based on the biochemical mechanisms of plant cell protection and the importance of a number of metabolites for the detoxification of active oxygen species and radicals, a study has been developed to study the impact of biostimulants (substances without nutritional effect but affecting different processes) to increase plant tolerance to stress factors. Ascorbic acid and the ascorbate-glutathione cycle play an important role in the detoxification of ROS and the modulation of other fundamental functions

in plants under stress conditions [42, 73, 74]. Ascorbic acid is also the major nonenzyme antioxidant in the apoplast [75], where it also plays a key role in the perception of stressful environmental stimuli and stress signaling [76, 77]. Plant tolerance to environmental stressors can be enhanced by the exogenous use of useful molecules such as proline, amino acids, humic acid, and other antioxidants [78]. The physiological responses of herbaceous plants to the exogenous AsA have been extensively studied [79–82]. However, the effects of exogenous AsA applications on fruit tree species subject to water stress have been poorly studied, and there are currently no studies on the impact of exogenous AsA on water-stressed deciduous fruit trees and their responses after wetting. Water stress can inhibit the growth of young fruit trees and reduce the growth, yield, and quality of fruits of mature trees [83].

Ascorbic acid is the richest plant antioxidant [84] and is important for the photoprotection and regulation of photosynthesis by stomatal or nonstomatal factors [85, 86]. Foliar application of AsA in young peach trees can be a useful practice to overcome short periods of water scarcity. With regard to gas exchange, exogenous uses of AsA to young water-stressed peach trees significantly increased the assimilation of CO_2 in both varieties (Scarletprince and CaroTiger) to the control levels in a restorative watering step. Biosynthesis of AsA occurs on the internal mitochondrial membrane by the oxidation of L-galacto-1,4-lactone (L-GalL). The exogenous application of L-Gal, which is a precursor to ascorbate synthesis, increases CO_2 assimilation, photosynthetic electron transport velocity, and ultraviolet conduction [87]. Also, AsA plays a role in photosynthesis and donates electrons to photosystems I and II when the primary electron donor system is damaged [88]. Application of ascorbate results in increased photosynthesis, growth rate, and chlorophyll concentration in wheat plants under water stress compared to untreated plants [81]. This is of the utmost importance to alleviate the negative effects of water stress on the reduction of photosynthesis in young trees in commercial orchards experiencing a period of water stress (especially in areas where the current practice is to start irrigation after the second year); in young container-grown and field-grown trees in nurseries not only in drought periods but also when field trees are excavated and very fine roots are destroyed causing temporary water stress on trees while the roots are not recovering. Accumulation of osmolytes such as proline in water-stress plants can contribute to lower osmotic potential after wetting and allow water to move into cells [89].

In addition to ascorbate, other biologically active molecules also have a positive effect on a number of plants. Recently, melatonin has been shown to have a regulating effect on ripening and preventing disease. For example, pre-melatonin-treated grape berries exhibit a higher endogenous accumulation of melatonin, which not only increases grain size and weight but also enhances the synchronized grain maturation. The application of melatonin after harvesting effectively delays aging and maintains the quality of the peaches stored at ambient temperature. Exogenous melatonin pretreatment improves anthocyanin accumulation by regulating gene expression and increases antiradical activity in cabbage sprouts. Melatonin reduces injuries caused by low temperatures in peach fruits by increasing the protective

power in the fruit. However, the main physiological and molecular mechanism of inducing tolerance to low-temperature stress caused by melatonin remains unclear. As a positive regulator of the anti-ROS process, the data show that melatonin can not only directly purify some ROS but also modulates antioxidant enzymes and improves cellular antioxidant protection. Melatonin increases peach tolerance to cooling after harvest. Compared to control peaches, melatonin treatment slows down and reduces cooling injuries in fruit during storage in refrigeration chambers. Melatonin increases the expression of the genes involved in the antioxidant protective system, and also causes an increase in ascorbate and regulating genes involved in the ascorbate-glutathione cycle. AsA and GSH can directly detoxify ROS and thus contribute to the nonenzymatic ROS removal [39].

The role of phytohormones, alleviating the adverse effects of abiotic and biotic stress in plants, is widely described in the literature. Among the plant hormones, salicylic acid (SA) acts as a signaling and regulatory molecule in plant responses to environmental stresses by SA-mediated control of metabolic and molecular processes [61, 62].

There are different pathways for salicylic acid biosynthesis. One of them is found in peaches and its precursor is mandelonitrile (MD) [90]. In this pathway, MD acts as an intermediate molecule between the cyanogenic glycosidic cycle and SA biosynthesis [91]. The contribution of the different pathways to the total amount of SA varies according to plant species, their physiological status, and their rate of development [92–96]. For example, although it is generally accepted that the contribution of phenylalanine (Phe) ammonium lyase (PAL) pathway to the total amount of SA is small, this pathway becomes important during the interactions between the plant organism and the pathogen [62]. Furthermore, it has been found that treatment with MD increases the SA content and provides partial protection against the Plum pox virus (PPV) infection in peach plants [91].

The cyanoglucoside pathway (CNglcs) is involved, at least in part, in the biosynthesis of SA in peach plants, and MD acts as an intermediate molecule between SA biosynthesis and the CNglcs cycle [91]. It is known that SA is a signaling molecule in the plant protection response that can cause tolerance to various abiotic and biotic loads [61, 97]. Various authors have shown that SA can alleviate NaCl-induced injuries. This response, however, is somewhat controversial, and the results depend on plant species and their developmental phase in addition to the concentration of SA and the mode of administration [61, 98, 99]. In terms of biotic stress, peach plants GF305 are commonly used for plant–pathogen interaction studies with PPV, and it has been reported that PPV infection can cause oxidative stress at the subcellular level in these plants [92]. At least 10% of the total SA content in micropropagated peach trees was found to be due to the cycles of CNglcs by MD [91]. Under salt stress conditions, the increase observed in the concentration of SA in untreated (control) and Phe- treated micropropagated peaches correlated with elevated levels of SA precursor MD, whereas in PPV-infested shoots this correlation was observed only in control plants. Taken together, these results suggest that under stress conditions the major part of SA should come from isochorismate (IC) and PAL pathways [93, 94].

It is believed that the pathway of PAL is the main pathway for SA biosynthesis in saline stress [100] Nicotiana tabacum infected with tobacco mosaic virus [101]. In addition, CNglcs is believed to play a possible role in unfavorable environmental conditions [102], which is why MD can potentially play a role in the plant's responses.

SA content increased in both control and Phe-treated plants. Salt stress also increases the levels of ABA and JA in control and Phe-treated plants, but not in MD plants. In control plants, an SA/JA ratio increased as a result of the salinity stress, whereas in the MD treated, the SA/JA ratio was slightly decreased. This response correlates with the fact that sodium stress does not affect the development of MD-treated plants. ABA is a key modulator of the response to abiotic stress because of its important role in regulating the closure of the stomata. Furthermore, JA appears to act as a regulator of ABA biosynthesis [103]. Under physiological conditions, there was an increase in ABA levels in control plants and Phe-treated plants, which correlate with a significant increase in JA.

8 Conclusions

Knowledge of the properties and functions of the secondary metabolites as well as their biosynthetic pathways allows the use of different biostimulants in order to increase their biosynthesis and hence the resistance of the plants to various stresses factors. This can be successfully used in modern sustainable agriculture to reduce pesticide use.

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Fruit Scent: Biochemistry, Ecological Function, and Evolution

17

Omer Nevo and Manfred Ayasse

Contents

Abstract

Fruit scent plays an important role in human preference and has thus been studied primarily in the context of agricultural science. In wild species, fruit scent has long been speculated to play a role in mediating the mutualistic interaction between plants and fruit-eating animals that disperse their seeds. Yet until recently, empirical studies addressing this hypothesis have been all but absent. Studies in the past decade emphasized the ecological role of fruit scent as an animal attractant, as well as its evolution as a ripeness signal. But data are still limited and many questions remain open. This chapter summarizes recent developments in the study of the chemical ecology and evolution of wild fruit scent. It explores the chemistry and biochemistry of fruit scent, its use by various

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important seed dispersal vectors, its evolution, and other functions it may fulfill. We end with recommendation for future studies, in the hope that the next decade will be at least as fruitful as the previous one.

Keywords
Co-evolution · Constraints · Frugivory · Mutualism · Odor · Olfaction · Seed dispersal · Sense of smell

1 Introduction

Like all plant tissues, fruits are packed with secondary metabolites [1]. This diversity of secondary metabolites has been suggested to fulfill a plethora of ecological functions, from attraction of seed-dispersing frugivores (fruit-eating animals), through regulation of their interaction with the seeds, to repellance of fruit antagonists [2, 3]. In contrast, it has been suggested that fruit secondary metabolites are primarily defensive and in most cases similar to those present in nonreproductive tissue or unripe fruits, thus making them an extension of the plant's general line of defense [4, 5]. The assertion that fruit secondary metabolites are primarily the result of "leakage" from nonreproductive tissue has since been refuted [6, 7]. Nonetheless, among the myriad possible functions of secondary metabolites, defense against microbial, insect, and vertebrate antagonists has been in the focus of most studies [6–11]. This is not surprising given that in other plant tissues, the most explored and probably also prominent function of plant secondary metabolites is defense [12].

The prominence of defense in the discussion regarding fruit secondary metabolites can probably be attributed to the fact that most studies have focused on the large, nonvolatile, secondary metabolites. Indeed, many large, often water-soluble, secondary metabolites such as cyanogenic glucosides, glucosides, and polyphenols are defensive [12, 13]. But much less attention has been given to volatile secondary compounds (or volatile organic compounds: henceforth VOCs) – lighter and often more hydrophobic compounds that constitute what we would colloquially recognize as "scent" or "odor" [14, 15]. VOCs are ubiquitous in fruits and some are likely to play a role in fruit defense [16, 17]. Yet as recognized by the much more developed study of commercial fruit production, they are responsible for the aroma of fruits and their attractivity to human consumers [14, 18, 19]. Thus, another reason explaining the focus on the defensive rather than attractive function of fruit secondary metabolites is likely to have originated from the fact that VOCs of wild fruits have until recently rarely been studied [14, 15].

Over the past decade, the interest in wild fruit VOCs has increased substantially, in particular in their role as an attractant of animal seed dispersers [20]. This has been the result of a growing understanding that despite various constraints [21, 22], many fruit traits are likely to have evolved in response to animal seed disperser preferences [23–26]. Along with the proliferation of methods that allow analysis of scent [27, 28], the decades-old hypothesis that ripe fruit scent – i.e., VOC profile – is an evolved trait whose function is to attract seed dispersers [29] began receiving

support [20]. In that, the study of fruit traits and seed dispersal has followed the much more mature field of pollination ecology, in which the role of floral scent as a pollinator attractant has been recognized for decades [20, 23, 30, 31].

Yet despite the growing body of knowledge and understanding of the role of fruit scent as an attractor of seed dispersers, the number of studies that conducted chemical characterizations of wild fruit scent is still low, and is based on a narrow taxonomic coverage from only a few geographical regions, exclusively in the tropics. Moreover, the focus on fruit scent as an animal attraction mechanism risks downplaying other ecological roles which fruit scent may fulfill [16]. Thus, it is important to remember that the field is still in its initial stages and that there are many more open questions than definite answers [15].

This chapter will summarize the latest developments in the study of the ecology and evolution of wild fruit scent. It will first examine what chemicals tend to characterize fruit scent and describe the basic biochemical processes leading to their synthesis. It will then examine how fruit scent is used as a food detection and selection by various animals and present the supporting evidence for the adaptive hypothesis of fruit scent. It will end with a discussion of other factors determining fruit scent evolution, and conclude with recommendations for future studies.

2 Fruit Scent: Chemistry, Biochemistry, and Patterns of Emission

2.1 Chemistry and Biochemistry

Fruits of wild and cultivated species emit complex mixtures that can comprise dozens to hundreds of different VOCs [14]. Most of these belong to three prominent chemical classes, terpenoids, fatty acid derivatives, and aromatic compounds [32, 33], although some fruits also contain rarer compounds such as amino acid derivatives [14, 15].

2.1.1 Terpenoids

Terpenoids are the most diverse group of plant secondary metabolites [34, 35]. They are ubiquitous in leaves [13, 36] and flowers [30] and are also very common in unripe and ripe fruits of both wild and cultivated species [15, 37–40]. The most common volatile terpenoids are the C10 monoterpenes and C15 sesquiterpenes, along with their homoterpene or oxidized derivatives [34, 36, 41, 42]. Among many, common examples are limonene, α- and β-pinene, *cis*- and *trans*- β-ocimene, and β-myrcene (monoterpenes); β-caryophyllene, α-copaene, and α-humulene (sesquiterpenes); and linalool (monoterpene oxide) (Fig. 1, 1–3).

Terpenoids are synthesized via two separate biosynthetic pathways and are a construct of two or three basic C5 (isoprene) units, which are then modified to create the end product [35, 42]. The enormous diversity of plant terpenoids is a result of the latter stage, in which a narrow range of precursors are transformed into thousands of different end products by various members of the terpene synthase (TPS) family [42, 43].

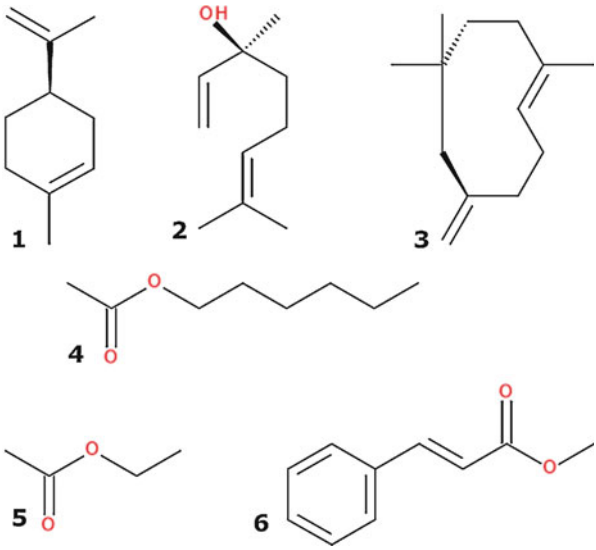

Fig. 1 Examples of common ripe fruits VOCs. 1. Limonene, a monoterpene. 2. linalool, monoterpene oxide. 3. β-caryophyllene, a sesquiterpene. 4. Hexyl acetate, an aliphatic ester. 5. Ethyl acetate, a highly volatile aliphatic ester. 6. *E*-methyl cinnamate, an aromatic ester

TPSs are highly non-specific, and thus terpenoids are always emitted in diverse mixtures [34, 44]. Terpenoids are involved in plant defense, either through direct toxicity or in indirect defense systems, as recruiting signals for natural enemies of antagonists [13, 34, 45]. At the same time, their presence in ripe fruit scent has been demonstrated to attract bats [38] and primates [46].

2.1.2 Fatty Acid Derivatives

Fatty acid derivatives such as saturated and unsaturated hydrocarbons and alcohols, esters, and aldehydes, ketones, and carboxylic acids are among the largest classes of volatile secondary metabolites in flowers [47]. Volatile fatty acid derivatives are primarily synthesized by degrading C18 linoleic and linolenic acids into C12 and C6 alcohols (e.g., n-hexanol, 2- or 3-hexenol), aldehydes (e.g., n-hexanal, 2- or 3-hexenal), and carboxylic acids (e.g., 3-hexenyl acetate) [33, 48–50]. These compounds and their derivatives are very common in plant green tissue and are often collectively called "green leaf volatiles" [13].

Fatty acid derivatives are highly common in fruits of wild and cultivated species [14, 15, 39, 40, 51]. Some fatty acid derivatives such as various green leaf volatiles can be found in both ripe and unripe fruits and are possibly involved in fruit defense [16]. In contrast, aliphatic esters tend to be more common in ripe [18, 51–53] and even more in overripe [54] fruits. Interestingly, while the synthesis of most plant VOCs is based on self-biosynthetic machinery and precursors, esters are at least partially synthesized by bacteria-produced precursors: Esters are synthesized by a condensation of a carboxylic acid and an alcohol, and alcohols are often the limiting factor in ester synthesis in fruits [53]. Ethanol, a precursor of ethyl esters, is a product of sugar fermentation by microbes [55], and treatment of fruits with antibiotics leads to a substantial reduction in ester emission [54] (Fig.1, 4–5).

2.1.3 Aromatic Compounds

Aromatic compounds are those which contain at least one conjugated planar ring. Like terpenoids and fatty acid derivatives, they are very common in flowers across plant families [32, 47] and are involved in pollinator attraction [56] and leaf defense [13]. Aromatic VOCs are common in ripe fruit [15, 38–40, 51] and constitute a significant portion of the scent emitted by wild fruits in Uganda and Madagascar [15]. The vast majority of volatile plant aromatic compounds are synthesized by a complex biosynthetic process whose precursors are aromatic amino acids synthesized via the shikimate pathway [33, 57, 58]. VOC synthesis is the result of deamination of the amino acid L-phenylalanine and reduction to C9 compounds [33, 49, 57]. One volatile product of this process is *trans*-cinnamic acid, which was identified in several Malagasy fruit species in its methyl ester form [51] (Fig. 1, 6). Further reduction of *trans*-cinnamic acid by removal of a C2 unit is the basis for synthesis of many other aromatic VOCs [49]. Other common aromatic compounds include methyl- and ethyl-salicylate, both esterized forms of the phytopheromone salicylic acid [59, 60]. While of lesser importance compared to other plant VOCs like terpenoids, aromatic compounds also play a role in both active and passive leaf defense [13, 58] and possibly play a role in fruit defense too [16].

2.1.4 Nitrogen- and Sulfur-Containing Compounds

While terpenoids, fatty acid derivatives, and aromatic compounds dominate the fruit scent profiles of most cultivated and wild species [14, 15, 51], fruits of some species emit less common compounds. Several wild fruits in Madagascar have been found to contain nitrogen- and sulfur-containing compounds [15, 51], although even in these species the relative contribution of these compounds to the scent profile was minor. However, compounds of these classes dominate the scent profiles of at least one wild species, the (in)famous durian (*Durio* sp.). Durian fruits, which are also cultivated in Southeast Asia, are known for their strong and distinct scent, although some cultivars are almost odorless [61]. While there has been some debate over which specific VOCs are responsible for the foul scent, it is known that Durian scent contains, in addition to more typical compounds like alcohols and esters, primarily sulfur-containing compounds [61–64]. Notably, as opposed to some past claims, the sulfur-containing compounds are synthesized by the fruit itself and not by bacteria inhabiting the flesh [65]. Interestingly, nitrogen- and sulfur-containing compounds are a product of protein metabolism [47]. This led to the speculation that their presence and amount in fruit scent could serve as an honest signal of protein content [15].

2.2 Patterns of Scent Emission

Although based on few cultivated model systems, it appears that at least in some species fruit VOCs are synthesized by specialized cells situated on the fruit's skin [66]. It is generally assumed that VOC release is predominantly passive through diffusion and that therefore it can only be regulated by up- or downregulating VOC synthesis [67]. Indeed, VOC synthesis is strongly regulated by the presence and

activity of the participating enzymes, i.e., regulated both by gene expression and the transcription level [68]. However, diffusion of largely hydrophobic VOCs at published rates would require extremely high concentrations which are potentially harmful, and it has therefore been proposed that plant VOCs are actively emitted using transmembrane structures [67] which are yet to be identified. Either way, emission of plant VOCs is a controlled process which is adjusted to the developmental stage of a particular tissue or even on smaller scales such as the circadian rhythm [68, 69].

2.2.1 The Ripening Process

The chemicals described above were documented in the scent of wild and cultivated ripe fruits. However, the data available per species is almost exclusively based on snapshots – records taken in a single moment, often in an unstandardized moment in the fruit's maturation process. However, fruit scent is not static. Many – but notably not all – fruits change their scent qualitatively and quantitatively once ripe [14, 37, 39, 40, 51]. In cultivated species, which have probably been artificially selected to increase their aroma, the amount of scent emitted by ripe fruits increases by a factor of up to 30 [14, 70]. In wild fruits the median increase in 19 species specializing on seed dispersal by primates was found to be 2.3 [51]. Notably, the same study found no increase in the amount of scent emitted in fruits consumed by sympatric bird-dispersed species, highlighting the not surprising similarity between human artificial selection and natural selection by our closest living relatives. An increase in the amount of scent emitted by ripe lemur-consumed fruits was found in other sites in Madagascar [71] and in two fig species from Panama [37]. Fruit scent also changes qualitatively upon ripeness, with increased emission of compounds that were present in low proportions or fully absent in unripe fruits [14, 37, 39, 40, 51].

Studies conducted in the wild [37, 39, 40, 51, 71] have aimed to record dichotomous behavior among animals while interacting with ripe and unripe fruits. This choice led in most cases to a comparison of two snapshots of fruit scent – one before the onset of ripening and the other around its peak. Thus, studies of wild species are not informative with regard to the process of change in fruit scent. A single exception is a study by Sánchez et al. [72], who reported a decrease in the amounts of ethanol and acetaldehyde in rusty figs (*Ficus rubiginosa*).

In contrast, several studies on cultivated fruits have tracked the changes in aroma compounds, along with changes in fruit quality and seed development [14, 73]. With regard to the ripening process, fruits can be roughly divided into two groups: climacteric and non-climacteric. Climacteric fruits are those whose final stage of development is characterized by increased respiration and ethylene production, and they tend to exhibit a rapid ripening process, while non-climacteric fruits mature more gradually [73, 74]. In climacteric cultivated species, the shift to ripe fruit scent is rapid. For example, in peaches, the major shift in fruit scent occurs abruptly, and once the seeds approach their final weight, as emission of three GLVs characteristic to unripe fruits decreases, while the emission of compounds typical to ripe fruits increases [75]. Similarly, another study of apricot and plum X apricot hybrids compared the volatile profiles of fruits in three late developmental stages: mature

green, commercial ripe, and tree ripe. In most cases, the latter two were similar, indicating that the shift in scent occurs abruptly and only when the seeds are mature [76]. In snake fruits (*Salacca edulis*), the prominent acids and alcohols increase more gradually during maturation, but ester emission skyrockets abruptly around the time the fruits become softer and mature [77]. Taken together, this is exactly the pattern expected in cases where fruit scent is used by animals to find or identify ripe fruits [37, 38, 46, 78], as plants are expected to be selected to begin attracting them only after the seeds are viable. This is similar to the patterns of floral scent emission, which tend to peak when the flowers become ready for pollination [49]. Yet while many wild fruits exhibit a rapid maturation process (Nevo, personal observation), it is rarely known whether wild fruits are climacteric.

2.2.2 Circadian Rhythm

In flowers, emission of VOCs can be constant or change rhythmically over the 24 h cycle, often the case in plants pollinated by nocturnal animals [30, 49]. Diel variation in fruit scent is far less investigated, and data are available for only two fig species from India. Mature syconia of *Ficus benghalensis*, a species dispersed by both diurnal birds and nocturnal bats, change their scent over the 24-h cycle: day VOC emissions are dominated by sesquiterpenes and fatty acid derivatives, while night emissions are substantially poorer in sesquiterpenes and contain more aliphatic esters and aromatic compounds [79]. In contrast, mature *Ficus racemosa* syconia do not show day-night differences in their scent profiles [79], even though they do show diel cycle variance in earlier stages of their development [69].

It is unknown whether fruits or mature syconia of other species alter their scent over the 24-h cycle. Yet it is likely to be common given the prevalence of this phenomenon in flowers [30, 49] and the fact that fruits tend to be more generalist than flower, i.e., they interact with a wider range of animal mutualists [80] and thus more likely to interact with diurnal, cathemeral, and nocturnal frugivores. For example, species like *Ficus maxima* are dispersed by both bats [38] and diurnal primates [81]. Bats and primates use their sense of smell differently: bats can rely on olfactory cues to detect and locate fruits [82], while primates do so only for selection over short distance [78]. Therefore, this and other similar species are excellent candidates to examine whether fruits[1] are selected to emit different olfactory signals at different times of the day.

3 Fruit Scent and Seed Disperser Attraction

The role of fruit scent as an attractant of vertebrate seed dispersal vectors has been the main focus of most work on fruit VOCs in recent years [20], although the idea that fruit scent is used by olfactorily oriented frugivores is decades old. Early works have integrated it into the framework of the Dispersal Syndrome Hypothesis,

[1]Including mature fig syconia, which are functionally equivalent.

according to which fruit characteristics have evolved in response to the traits of their primary seed disperser [29, 83, 84]. Yet empirical tests of the hypothesis that fruit scent has evolved as a signal for seed dispersers have until recently been absent, possibly due to the predominance of the view that fruit traits are not strongly selected by frugivores in the last 15 years of the last century [21, 22, 85, 86] – a timeframe in which the understanding of floral scent evolution has exploded [30, 32, 47]. Another factor has been that chemical communication between fruits and frugivores is more common in tropical regions, in which chemical sampling and analysis tend to be more challenging. Yet the growing support for the dispersal syndrome hypothesis [24, 26, 87] and increasing availability of techniques allowing chemical sampling and analysis [27, 28] have led to a renewed interest in the question. We first examine this question from the animal side, asking how and whether animals may use fruit scent to find and identify ripe fruits, i.e., whether fruit scent is a useful cue for frugivores. We then move on to examine whether in cases in which it is used by animals, fruit scent can be considered an evolved signal which is selected to fulfill this function.

3.1 Fruits Scent as a Cue

3.1.1 Bats

With 1200 known species, bats are the second-most diverse group of mammals [88]. Frugivory has evolved independently in the Old and New Worlds, and in both systems bats are important seed dispersers, contributing to early succession and, in the Old World, recruitment of canopy species [89]. As nocturnal animals, bats can rely on their vision less than diurnal species, although many retain dichromatic vision and may rely on vision more than previously considered [90]. Some bats lineages have evolved to use sonar [91] and can echolocate flowers and fruits which have presumably evolved specialized structures that reflect back their calls [20, 92–95]. But echolocation is not present in most Old World frugivorous bats [91], and thus a major sensory trajectory for frugivorous bats is olfaction [96]. Olfaction also plays a role in bat pollination, a relationship which is primarily facilitated by chemical communication [32] or a combination of olfaction and echolocation [97].

Reliance on olfaction has been demonstrated in behavioral tests that focused primarily on New World frugivorous bats. New World bats have been reported to be attracted to fig scent [98, 99], and early experiments showed attraction to the scent of bananas, which are consumed by local bats but are not wild and thus possibly not representative [100]. Thies et al. [101] showed that New World *Carollia perspicillata* and *C. castanea* use the scent of *Piper* fruits to identify ripe fruits. In a series of experiments, they showed that unripe fruits are rejected and that artificial fruits are approached only when impregnated with the scent of ripe fruits. *C. perspicillata* were also shown to possess high olfactory sensitivity to a series of esters, alcohols, and carboxylic acids common in fruits [102]. Similarly, in an experimental setting, New World *Artibeus watsoni* and *Vampyressa pusilla* showed

clear preference to ripe over unripe fruits, were attracted to experimental devices that emitted the scent of ripe fruits, and rejected dry-frozen fruits that retained the morphological features of ripe fruits but did not emit scent [82]. In contrast, it should be noted that in some bat-plant interactions (*Phyllostomus hastatus* feeding on *Gurania spinulosa*), fruit scent does not seem to play a role [95].

Experiments with Old World frugivorous bats have been rarer. Some tried but could not record reliance on olfaction in fruit foraging [72, 103]. However, these experiments focused primarily on ethanol and methanol, which are not typical plant secondary metabolites. In another study short-nosed fruit bats (*Cynopterus brachyotis*) were shown to prefer ripe fruits over unripe fruits of two fig species and to rely primarily on scent to find and identify ripe fruits in an experimental setting [37]. A follow-up study tested the response of the same Old World bat species and New World Jamaican fruit bats (*Artibeus jamaicensis*) to the scent of fig species from both habitats [38]. Both species were attracted to scent of figs from their respective habitats, but only the Neotropical species were attracted to the scent of unknown Paleotropical figs [38]. Since Old World frugivorous bats are older [89], show similar patterns of olfactory receptor evolution [96], and in most cases cannot echolocate [91], it is very likely that their ability and tendency to use their sense of smell for food detection and selection are comparable to that of New World bats.

All studies which tested the attraction of bats to fruit scent used intact fruits, fruit extracts, or synthetic mixtures. It is thus unknown whether any individual compound is particularly attractive to them. While bat-pollinated flowers tend to emit uncommon sulfur-containing compounds [32], bat-consumed fruits tend to emit common VOCs [37, 38, 40]. Monoterpenes are particularly common in the scent of both Paleotropical and Neotropical bat-consumed figs and have been proposed to play an important role in attracting them [38].

3.1.2 Primates

Along with bats and birds, primates are one of the biggest groups of seed dispersers in tropical systems [104]. Primate seed dispersal plays a pivotal role in a complex web of interactions between plants, primary and secondary seed dispersers [105, 106]. The fact that most sympatric frugivorous primates overlap in their diets but vary in their body size, movement patterns, and group size renders them, as a group, highly effective seed dispersers [107]. For example, since many primate species are large and arboreal, many species are unlikely to visit early-phase secondary forests in which trees are still too small to support them. But small-bodied primates like tamarins (*Saguinus* spp.) do venture into regenerating forests and thus fulfill a function similar to that of birds and bats by effectively dispersing seeds into secondary forests [108].

As opposed to bats, most primates are diurnal and, relative to other mammals, possess excellent color vision [109, 110]. As a result, visual cues play a role in the process of ripe fruit detection and selection [111–113], and primate color vision is likely to have exerted non-negligible selection pressures on the evolution of fruit color [114, 115].

However, primates are now recognized to possess an excellent sense of smell that is often on par with that of mammals like dogs and rodents [116], and it becomes increasingly clear that olfaction plays a major role in the feeding ecology of primates, primarily for discrimination between ripe and unripe fruits [15, 78]. Until recently, most studies that demonstrated reliance on olfaction for fruit selection did not consider the chemical properties of fruits [78]. Nevo et al. [46] showed that spider monkeys (*Ateles geoffroyi*) can discriminate between synthetic mixtures mimicking the scent of ripe and unripe fruits of two Neotropical fruit species. Notably, the monkeys can discriminate between the scents of ripe and unripe fruits even when individual compounds in the scent of unripe fruits are manipulated to match the concentration in ripe fruit scent. This indicates that identification of ripe fruits is not based on individual compounds and thus less sensitive to within-species variance in fruit VOC content, which has been found in studies that sampled multiple fruits per species [39, 51].

In the field, two recent studies quantified the relationship between fruit olfactory conspicuousness, defined as the difference between ripe and unripe fruit scent, and the tendency of primates to sniff fruits before ingesting or rejecting them. Red-bellied lemurs (*Eulemur rubriventer*) are more likely to sniff fruits of species which increase the amount of scent upon ripeness or change the chemical composition of ripe fruits [51]. In the neotropics, white-faced capuchins (*Cebus capucinus imitator*) increase the rate of sniffing when feeding on fruits of species in which the amount of scent emitted by ripe fruits is larger [117]. Another study found no relationship between sniffing behavior in brown lemurs (*Eulemur fulvus*) and the overall amount of scent emitted by ripe fruits [118], indicating that the determining factor is not scent per se but the olfactory conspicuousness of the fruit, i.e., how different it is from conspecific unripe fruits [15, 39]. However, scent in this study [118] was corrected for the surface area of the fruit. Thus, the variable analyzed was not the amount of scent available for the lemurs but the amount emitted by a unit of surface area. This procedure is meaningful when studying, for example, the costs of scent emission. But from an ecological perspective, in fruit selection, the animal is exposed to the scent emitted by a single fruit, which is therefore a more appropriate measurement.

Primate-consumed fruits tend to emit common VOCs: terpenoids, aromatic compounds, and fatty acid derivatives [15, 39, 51]. Lemur-consumed fruits in Madagascar – especially those which attract more olfactory investigation by lemurs – tend to be rich in aliphatic esters [51]. Some fruits also emit nitrogen- and sulfur-containing compounds [15, 16, 51].

3.1.3 Birds

Frugivorous birds are important seed dispersers across the tropics and are probably the most important animal seed disperser in temperate regions [119]. The most dominant group of frugivorous birds are the passerines, although frugivory is also present in non-negligible numbers among woodpeckers, parrots, and pigeons [120]. Birds possess an excellent color vision. Most species are tetrachromatic, i.e., possess one more pigment type than the best color-discriminating primates, among them

humans [109, 121, 122]. As a result, they tend to rely strongly on fruit color for detection and selection [24, 26, 123] and have exerted substantial selection pressures on fruit color [24, 26, 124].

It is a long-held notion that frugivorous birds tend not to rely on olfaction as strongly as mammals [29, 39, 40, 51, 125]. Evidence supporting this notion has been rather circumstantial and was based primarily on the relatively simple olfactory anatomy of many birds, especially passerines [126–128], and the reports that, unlike mammals, frugivorous birds do not sniff fruits before ingesting [125]. Bird-consumed fruits tend to emit lower amounts of VOCs [25] and change their scent profiles in ripeness less than mammal-consumed sympatric species [39, 40, 51]. This parallels the pattern observed in the bird pollination syndrome: in contrast to insect- and bat-pollinated flowers, bird-pollinated flowers tend to emit only scant amounts of scent [32]. In addition to the high reliance on vision, it is thus often assumed that frugivorous tend not to rely strongly on olfactory cues.

However, these notions should be taken with cause since it is possible that bird reliance on olfaction is simply less visible to human observers. Several studies demonstrated the ability of passerines to use chemical cues in various situations [128–131]. Performance in olfactory sensitivity and discrimination capacity tests is difficult to compare directly to other frugivores. The olfactory sensitivity of passerines is somewhat low but, in the range, relative to other birds and primates [128, 132]. In conditioning tests, blue tits (*Cyanistes caeruleus*) – a non-frugivorous passerine – learn to identify lavender oil, but their performance is rather low compared to, for example, primates [127, 133, 134].

Thus, the absence of evidence for reliance on fruit scent, along with the high visual capacities of birds and the fact that they tend to feed on less olfactory conspicuous fruits, indicates that frugivorous birds tend to rely less on fruit scent for food detection or selection. Yet given the many functions olfaction plays in the lives of many birds, including passerines [128], and the fact that non-frugivorous birds possess excellent olfaction [135], it is well likely that future studies would demonstrate that this notion is oversimplified.

3.1.4 Other Animals

As the primary agents of seed dispersal in most systems, bats, birds, and primates have received most of the focus in the study of the interaction between frugivory, sensory ecology, and seed dispersal. But other animals consume fruits and may use fruit scent to detect or identify ripe fruits. Elephants possess an excellent olfactory system [136, 137] which can be employed to find plant material [138], mate choice [139], and even identify human ethnic groups which hunt them [140]. *Balanites wilsoniana*, a species growing in continental Africa, is dispersed by elephants [141, 142] and emits a strong scent rich in aliphatic esters (Nevo, Valenta, Chapman, unpublished data), which the elephants are likely to use to find and select fruit.

Although birds are the most important animal seed disperser in temperate regions, some plant species receive dispersal services from mammals, many of them

nocturnal [143]. While less is known about the sensory ecology of less studied animals like hedgehogs, they possess very large main olfactory bulbs [144] and are very likely to rely on fruit scent when possible.

Finally, seed dispersal by invertebrates is fairly common. The most prominent invertebrate seed disperser is ants [145], which are attracted by fatty acids [146] present on the elaiosome – a lipid-rich appendage which serves as a reward and is functionally similar to fleshy fruits. Another invertebrate which occasionally provides seed dispersal services is slugs [147, 148], although neither their relative importance nor their reliance on scent has been investigated.

3.2 Fruit Scent: A Cue or an Evolved Signal?

While the previous section considered only the animal side of the interaction, i.e., how different animals may use fruit scent to find and identify ripe fruits, there is strong evidence that olfactory conspicuousness has evolved in some species to promote seed dispersal. The first is convergent evolution across taxa which share a dispersal vector. Comparing bat- and bird-dispersed figs, Hodgkison et al. [38] found that bat-dispersed figs have converged to emit monoterpenes, although, as they acknowledge, monoterpenes are not the dominant VOC class in a few other bat-dispersed figs [40, 79]. More qualitatively, Nevo et al. [51] found that ripe lemur-dispersed fruits are much more likely to emit aliphatic esters than do sympatric bird-dispersed species. In both studies, the fact that species which share a disperser at least partially converged in their ripe fruit scent chemistry is an indication that there is some selective pressure exerted by these seed dispersers.

A second line of evidence supporting the hypothesis that scent is an evolved signal to seed dispersal comes from a handful of studies which looked at the patterns of change in fruit scent upon ripeness and compared them between sympatric species that rely on mammal and bird seed dispersers. Studying three fig (*Ficus* spp.) species, Borges et al. [40] showed that at the dispersal stage, only bat-dispersed figs had a unique scent which probably drives attraction of seed-dispersing bats. Bat-dispersed fruits also tend to emit stronger scents than sympatric bird-dispersed fruits [25]. Taking a similar approach but going beyond the *Ficus* model system, Nevo et al. [39] showed that in a system of four Neotropical species, primate-dispersed fruits change their scent upon ripeness, while bird-dispersed species do not. This pattern was replicated on a much larger model system in Madagascar, where it was shown that species which specialize on lemur seed dispersal change their scent – qualitatively and quantitatively – significantly more than sympatric bird-dispersed species [51]. The pattern that emerges from these studies is that even though the ripening process is accompanied by much biochemical activity which may affect fruit scent, the change in fruit scent upon ripeness is much greater in species which interact with olfactorily oriented mammals.

3.3 Multimodality: Color and Scent

Animals rarely rely on a single sensory modality, and it would therefore be naive to think that any of the animals discussed above relies solely on scent to find fruits. Using different senses, animals respond to a combination of signals and cues which can be either complementary or redundant [20, 149]. In the former, different cues provide different information, while in the latter the information is the same and the function of the redundant signals is either to provide backup or to ensure that a wide range of frugivores, which emphasize different senses and receive the message.

In fruit foraging and selection, olfaction is often used along with vision or echolocation in bats. Thies et al. [101] found that olfaction and echolocation play complementary roles, as the former is used for longer-distance detection and the latter for more fine-scale final localization of fruits. In contrast, in primates, olfaction probably plays an important role in close-range selection within a patch [46, 51, 78], while visual cues have been hypothesized to be most relevant for identification of fruit patches over longer distances [111]. However, olfactory and visual cues can also be redundant. This can be demonstrated in polymorphous primate species, in which some individuals possess full trichromatic vision while the rest are dichromats, i.e., red-green color blind [109, 110]. While feeding in the same patch, dichromatic white-faced capuchin monkeys (*Cebus capucinus imitator*) sniff fruits significantly more than trichromatic group members [117, 150]. This indicates that dichromatic individuals compensate for the lesser access to visual cues by acquiring more information through olfaction. Interestingly, ripe fruit ingestion rates are higher in trichromats [113], possibly indicating that either visual cues are more accurate or, more realistically, that their acquisition is more time-efficient.

4 Other Factors Affecting Fruit Scent Evolution

While plants are under selection to advertise ripeness through fruit scent, other factors are likely to drive the evolution of fruit scent. These include other adaptive functions scent may fulfill, trade-offs, and various constraints.

4.1 Fruit Defense

Like other plant parts, ripe fleshy fruits are subjected to attack by vertebrate, invertebrate, and microbial antagonists [16]. VOCs are routinely involved in leaf defense [17] but are rarely considered in fruit defense. Nonetheless, since a major fraction of fruit VOCs such as terpenes, green leaf volatiles, and phenolics play some defensive role in other plant parts, they are likely to be involved in fruit defense too [16]. Thus, plants may be selected to alter the emission rates of individual or multiple VOCs, and hence change their scent, in response to antagonists. At the same time, even compounds which are defensive in other tissues or play a defensive role in fruits may primarily be selected due to their secondary function in attracting seed

dispersers. This would parallel the evolutionary pathway of floral scent, which has in many cases evolved secondarily out of VOC-based defense mechanisms [151]. For example, limonene, a monoterpene which dominates the scent of oranges and has been considered to play a defensive role, is in fact an attractant of vertebrate and invertebrate antagonists [152]. This may apply to many compounds that are considered to be defensive solely based on their broad chemical characteristics.

4.2 Developmental and Phylogenetic Constraints

Fruit scent composition may be affected by both phylogenetic and developmental constraints. Often defined in different ways, we refer to developmental constraints as the tendency of fruits to emit VOCs that are present in unripe fruits or other plant parts, not because of their fitness benefit in the fruits but because it is developmentally impossible, or too costly, for the plant to change the VOC profile of only ripe fruits. We refer to phylogenetic constraints as the tendency of a species to possess a trait not because of its fitness benefits to its own ecological circumstances but because it was inherited from an ancestor. The two are inherently connected, as, for example, developmental constraints would slow down adaptive change and hence render closely related taxa more similar.

4.2.1 Developmental Constraints

While hardly studied, the role of developmental constraints in determining ripe fruit scent is probably marginal. Nonvolatile ripe fruit secondary metabolites are independent of other plant parts and robust to changes in the abiotic environment, thus indicating that their presence is adaptive rather than a by-product of regulatory or biochemical processes originating outside the fruit [6, 7].

The strongest argument against a significant role of developmental constraints on ripe fruit scent is the significant change, qualitatively and quantitatively, in the scent of fruits as they mature [37, 39, 40, 51]. Since ripe fruits develop from unripe fruits, the fact that a countless number of species changes their scent profile drastically indicates that selection can effectively alter ripe fruit scent. Nonetheless, it should be remembered that different scents – i.e., different unique mixtures of VOCs – can originate from the same biochemical pathways [153]. For example, species such as *Micronychia macrophylla*, a lemur-dispersed species from Madagascar, change the scent of ripe fruits qualitatively and quantitatively, but do so primarily using terpenoids [51], which tend to originate from few biochemical pathways [34]. The tendency to emit chemically similar odorants may be the result of some constraints. But this is not universal: in the same system, *Ficus tiliifolia*, another lemur-dispersed species, changes its scent profile from a terpene-dominated VOC bouquet in unripe fruits to an aliphatic acid-dominated scent in ripe fruits [51].

Finally, another factor that could constrain the amount of scent emitted by a fruit is simply its size. Fruit size is one of the factors most malleable to selection by frugivores: in bird-dispersed species, there is an upper cap determined by bird gape width [154, 155], and in species that rely on larger and more energy-demanding

frugivores, fruits tend to be bigger [84]. This could have a strong effect on the potential of effective chemical signaling in fruits, as all else being equal, larger fruits would emit stronger scents. In cases where the olfactory signal is selected to allow animals to identify that an individual fruit is ripe [46, 78], an individual fruit needs to emit an amount of scent strong enough to be detected. As a consequence, the costs of olfactory signaling in small fruits would be much higher and might drive them not to signal ripeness through scent.

4.2.2 Phylogenetic Constraints

Phylogenetic constraints are hard to detect since they are a function of the time since speciation, selection pressures, and other constraints. Yet a common method to approximate them is to observe to what extent closely related taxa are similar. Although fruit scent is harder to compare between species due to its multi-dimensionality (a scent bouquet is composed of dozens, if not hundreds, of VOCs), a few studies addressed the question whether ripe fruits of closely related taxa tend to emit similar scents.

In a community of 30 species from Madagascar, Nevo et al. [51] found no effect of phylogeny on ripe fruit scent. In a cluster analysis, closely related taxa were found to be more similar to other species than to congeneric or confamilial species. These results have been replicated on a sample of 49 species from Uganda (Nevo et al., unpublished data) and South Germany [156]. Within the fig clade (*Ficus* spp.), a similar trend was found as on a global scale, as far-related taxa with similar ecology (bat dispersal) were found to be more similar to one another than they are to more closely related bird-dispersed species [38]. However, at a more local level, closely related taxa that are dispersed by bats did show clustering, indicating some phylogenetic conservatism in fruit scent [38].

An important point is that all these studies dealt with the different VOCs in fruit scent profiles as independent variables. In other words, they assume that a switch from compound A to B is equally likely to a switch from A to C. This assumption is problematic because some compounds are synthesized through the same pathways, and thus switches between them are more likely [153]. A recent study offers a method to address this issue by integrating the biochemical pathways to statistical analyses [153]. However, this approach is not easily implemented in large datasets in which many compounds are not fully identified, and hence not all biochemical pathways are known.

5 Conclusions and Future Directions

The understanding of the evolution and ecological functions of fruit scent has evolved tremendously in the past decade. The VOC profiles of ripe, and sometimes unripe, fruits of dozens of species from the neotropics, continental Africa, Madagascar, and Southeast Asia have been published and used to address various evolutionary and ecological questions. In combination with some behavioral studies, they have shown that fruits emit a tremendous diversity of scents which are used by

animals to detect and identify them and that animal behavior has in turn exerted selection pressures on some fruits to become olfactorily conspicuous. At the same time, much more is required to fully understand the selection pressures and constraints which shape the diversity of wild fruit aroma.

An aspect which clearly lags behind chemical characterization, but is equally important to answer both ecological and evolutionary questions, is behavioral essays. As discussed above, many studies examined either scent-based fruit foraging and selection or fruit scent chemistry, but only a handful [37–39, 46, 51] did both. Yet even as it becomes clear that several groups of animals rely on fruit scent, many questions remain open – most of them can only be addressed through systematic behavioral essays of the kind that has become ubiquitous in insect chemical ecology [157]: To what aspects of fruit scent do they respond? What information they seek? On which odorants they rely? Behavioral studies are also paramount to answering the question whether and to what extent frugivorous birds use fruit scent. It is a common assumption that they do not, or at least do so substantially less than mammals [39, 40, 51]. While there is evidence suggesting that this assumption is to some extent true, it should be verified and rigorously tested in behavioral tests.

Behavioral tests with vertebrates are particularly challenging: wild population densities are low, many animals avoid interactions with humans, and their intelligence and ability to learn complicate many experimental designs which have worked well with invertebrates. Some of these challenges can be met in more controlled experiments with captive animals, which are in turn hindered by the fact that captive animals are in some cases not good representative of wild behavior. Thus, a combination of wild and captive approaches is probably necessary to address many of the questions regarding the use of scent by animal seed dispersers.

Comparative studies of fruit scent would benefit greatly from an increased standardization in the field, which could allow syntheses based on the results of studies conducted by different groups and in different locations. At the moment, the use of different techniques renders most comparison between studies very unreliable [15]. Yet a global comparative approach is crucial to pinpoint the multiple selection pressures and constraints which resulted in contemporary patterns of scent released by fruits. At the same time, it is also important not to forgo higher-resolution studies of individual species or narrow lineages, which are more suitable for integrating factors like the biochemistry of fruit scent and other functions it may fulfill. The contrast between higher-scale lack of phylogenetic signal [38, 51] and its absence in lower scales [38] and the fact that both should be studied in the context of the biochemical pathways behind fruit scent [153] demonstrates this point. We are in hope that further integration in the field would take these steps so that the next decade will be at least as fruitful as the previous one.

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Part III

Allelochemicals in Plant-Plant Interaction

Horizontal Natural Product Transfer: A Novel Attribution in Allelopathy

18

Dirk Selmar, Sara Abouzeid, Alzahraa Radwan, Tahani Hijazin, Mahdi Yahyazadeh, Laura Lewerenz, Melanie Nowak, and Maik Kleinwächter

Contents

Abstract

Whereas the translocation of allelochemicals between plants is well established for many years, a corresponding transfer of common, typical natural products was unknown until recently. This phenomenon was unveiled when the potential sources of contaminations of plant-derived commodities by nicotine and pyrrolizidine alkaloids were analyzed thoroughly. According to this so-called "horizontal natural product transfer", alkaloids, which are leached out from decomposing alkaloid containing plant parts (donor plants), are taken up by the roots of acceptor plants. Meanwhile, it becomes evident that not only alkaloids are taken up by acceptor plants but also phenolic compounds such as coumarins or stilbenes.

In analogy to the widespread uptake of xenobiotics, the uptake of natural products is also generally due to a simple diffusion of the substances across the biomembranes and does not require a transporter. The uptake of certain substances only depends on their physicochemical properties.

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Contemporary analyses from co-cultivation experiments outlined that natural products are not exclusively transferred from dead and rotting donor plant material but also from living and vital plants. Moreover, the compounds imported are modified within the acceptor plants.

In this chapter, an actual overview on the phenomenon of horizontal natural product transfer is presented and its relevance for our understanding of plant-plant-interactions is discussed. The fact that common natural products are readily translocated from one plant into others will strongly change our understanding of allelopathy. Up to now, in plant-plant interactions, only "classical allelochemicals" had been taken into consideration, e.g., those compounds that reveal certain and definite significance by inhibiting the growth or the germination of potential competitors.

Keywords
Horizontal transfer · Natural products · Nicotine · Pyrrolizidine alkaloids · Xenobiotics · Alkaloids

1 Introduction

It is a matter of course that plants take up substances from the soil by the means of their roots. Apart from inorganic nutrients, such as nitrate, phosphate, or various metal ions, many other compounds are imported via the roots. Regarding chemical ecology, the uptake of active compounds, denoted as allelochemicals, which inhibit germination or growth of putative competitors, is of special interest [1]. In general, these substances are exuded from donor plants and exhibit their effect on the plants growing in their vicinity [2, 3]. Commonly, the allelochemicals are taken up by the acceptor plants [1, 4]. A corresponding uptake of substances is well known for xenobiotics, e.g., systemic herbicides and fungicides [5] or veterinary medicines [6]. Furthermore, salicylic acid, which is responsible for systemic acquired resistance, is known to be imported by roots [7, 8].

When considering the uptake of substances by the roots, one substantial issue has to be emphasized: whereas the import of most ionic nutrients like nitrate, sulfate, or metal ions requires specific transporters [9–11], most of the xenobiotics are taken up by just simple diffusion [5]. Due to their partially hydrophobic character, these molecules are able to diffuse passively through membranes [12–14]. Although these coherences were well established, corresponding reflections with respect to common natural products had not been considered. The situation changed with recent investigations, which were aimed to identify the potential sources of various contaminations of plant-derived commodities with nicotine [15] and pyrrolizidine alkaloids [16, 17]. Corresponding pot experiments [18] as well as extensive field trials [19] demonstrated that nicotine, which is leached out from dried tobacco plant material, i.e., discarded cigarette butts, is taken up massively by acceptor plants. Analogously, pyrrolizidine alkaloids (PAs), which are leached out from rotting PA containing weeds, e.g., *Senecio jacobaea*, are also taken up by

acceptor plants [20]. In the same manner as nicotine, also the PAs leached out from plant remains into the soil are subsequently taken up by other plants. Obviously, such transfer of alkaloids is – at least in part – responsible for the numerous and widespread PA contaminations of spice and medicinal plants reported [16, 17].

2 The Horizontal Natural Product Transfer: A Quite General Phenomenon

Inspired by the findings of the uptake of nicotine and PA by various acceptor plants, the concept of "*Horizontal Natural Product Transfer*" was introduced by Selmar et al. [21]: when natural products are leached out into the soil from decomposing plant parts – denoted as donor plants – these compounds could be transferred into the roots of acceptor plants and translocated into their leaves (Fig. 1). This, however, necessitates that the substances have to pass the plasmalemma of root cells of the acceptor plants. The corresponding uptake into the symplast could occur already within the rhizodermis, or, at the latest, when entering the cells of the endodermis. As outlined above, such uptake might be accomplished either by an active transport, facilitated by the action of carriers, or by passive diffusion through the biomembranes. Indeed, a large number of transporters are known so far (for review see [22–24]), and the involvement of such carrier proteins seems to be reasonable. But, we have to consider that a lot of substances are able to simply diffuse across biomembranes. Yet, a prerequisite for such diffusion is the solubility of the substance in aqueous as well as in organic fluids. In good approximation, this feature can be extrapolated from the distribution of the substance in a two-phase system comprising octanol and water. In consequence, membrane permeability can be evaluated and deduced from the corresponding distribution coefficient, the so-called K_{OW} value. In general, this term is listed as its decadal logarithm, i.e., the pK_{OW} value [25, 26], which frequently also is denoted as log*P* [27]. Substances revealing log*P* values between −1 and 3 generally are considered to be able to diffuse easily through biomembranes [5, 25, 28]. Nonetheless, although these deductions had been elaborated in studies dealing

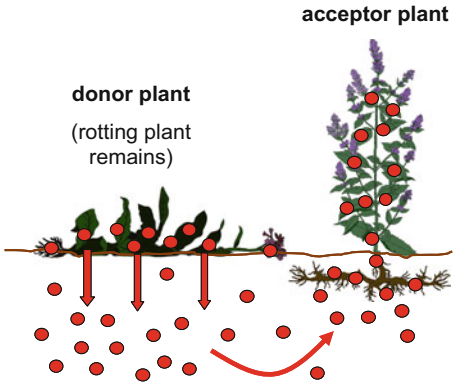

Fig. 1 Horizontal natural products transfer. According to [21], substances leached out from rotting plant materials (donor plants) into the soil are taken up by acceptor plants

with an uptake of xenobiotics, they are also relevant and suitable for all natural products, e.g., alkaloids. However, when considering the membrane permeability of alkaloids, an additional factor, i.e., the pH-dependent protonation of these natural compounds has to be considered [29]. In contrast to the free bases of most alkaloids, which are simply able to pass through membranes, their protonated forms – due to the positive charge – in general cannot penetrate biomembranes. This peculiarity is in accordance with the quite negative log*P* values of charged alkaloid salts. Thus, in addition to the log*P* of the various alkaloids, the pH of the medium also determines their membrane permeability. Consequently, the pH of the soil will massively influence the extent of the uptake of alkaloids from the soil. The higher the pH of the soil, the greater is the proportion of unprotonated alkaloids, and thus, their membrane permeability [29]. In contrast, in acidic soils, due to a high degree of protonated alkaloids, the uptake of alkaloids is drastically decreased [5].

Just recently, Yahyazadeh et al. [30] evinced that many different classes of alkaloids are also imported into putative acceptor plants. As prognosticated, all tested alkaloids revealing log*P* values between −1 and 3 are taken up. Consequently, in addition to nicotine and PAs, also tropane and purine alkaloids as well as benzylisoquinoline and indole alkaloids had been detected in the leaves of the acceptor plants. In contrast, when quaternary alkaloids such as coptisine, palmatine, or berberine had been applied to the soil, they were not taken up. The explanation for this difference is simple: these alkaloids – independently to the pH of the medium – always reveal a positive charge. Consequently, they are not able to pass through biomembranes. This notably is underlined by the quite negative log*P* values of these alkaloids [30].

Based on the nexus between the membrane permeability and log*P* values, it can be deduced that also substances representing other classes of natural products than alkaloids should be taken up in the same manner. In this sense, phenolic compounds, terpenoids, etc. should also be able to diffuse through biomembranes as long the hydrophilic as well as the lipophilic properties of the substance are within an appropriate range, i.e., when the log*P* values are between −1 and 3 [5, 25]. Nevertheless, up to recently, no experimental data on a putative transfer of natural products between donor and acceptor plants had been available. Notwithstanding, in the literature, various corresponding hints could be detected. In this sense, investigations of the uptake of so-called "*emerging organic contaminants*" (EOCs) revealed that indeed many different classes of organic compounds are able to pass biomembranes and are taken up by acceptor plants [31]. Moreover, in earlier studies dealing with the glucosylation of coumarins, it is stated that esculetin and scopoletin are able to pass the plasmalemma and are imported substantially by protoplasts from barley leaves [32]. Despite these indications, no investigations on the uptake of natural compounds representing other classes of natural products were available.

In order to visualize the phenomenon of horizontal natural product transfer, we applied colorants like betanidines to acceptor plants. Indeed, the green color of vital leaves overlays all other hues and the presence of these red dyes cannot be simply detected visually. However, when using etiolated seedlings, e.g., of peas or

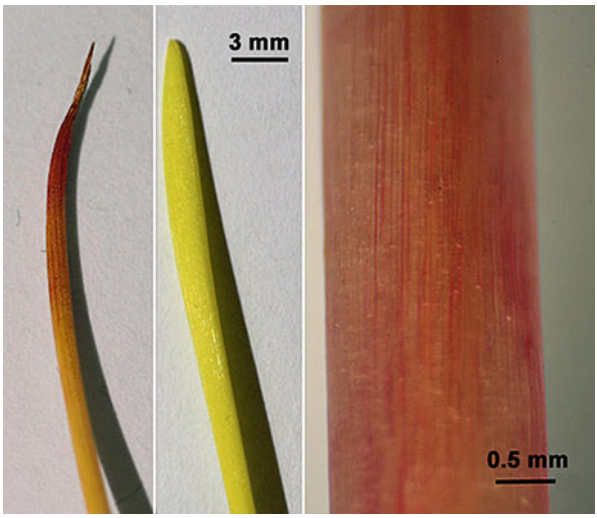

Fig. 2 Uptake of betalains by etiolated barley seedlings. An aqueous extract from red beet tubers containing various red dyed betalains was applied to the seedlings raised in a hydroponic system in the dark [33]. The control plants (middle) were raised without the addition of red beet extract

barley, in these chlorophyll free leaves, the presence of dyes became visible. In this sense, a crude extract from red beets was applied to barley seedlings [33]. The fascinating coloration (Fig. 2) unequivocally proves that the dyes are taken up by the roots of barley seedlings and translocated into their leaves. Nonetheless, there are various ambiguities which require further elucidation. It is well known that the crude extracts from red beet contain various red colored substances, e.g., betanin, isobetanin, and vulgaxanthin [34]. Many of these substances are quite unstable, especially in the presence of oxygen [35]. Moreover, in the course of extraction or at least in aqueous extracts, they will be hydrolyzed, e.g., to reveal betanidin. Accordingly, up to now, it is not known, which particular compounds have finally been taken up and translocated into the barley leaves. For further understanding of this fascinating phenomenon, in forthcoming experiments pure dyes have to be applied to etiolated seedlings.

Just recently, Hijazin et al. [36] verified that seedlings from various plant species, e.g., barley (*Hordeum vulgare* L.), radish (*Raphanus sativus* L.), pea (*Pisum sativum* L.), flax (*Linum usitatissimum* L.), and garden cress (*Lepidium sativum* L.), which were cultivated in hydroponic media, take up umbelliferone by their roots and translocate it into their leaves. Employing the same system, further analyses revealed that also resveratrol is taken up in the same manner. These findings demonstrate that the phenomenon of natural product transfer is not restricted to alkaloids but also pertains to phenolic compounds, such as stilbenes or coumarins.

When discussing the essentials of horizontal natural product transfer, apart from the physicochemical properties of the compounds, i.e., the membrane permeability, we always have to consider the persistence of the substances in the soil. In principle, the entire biomass from a rotting plant is completely decayed by microorganisms. Consequently, the relevant natural products also are degraded by microorganisms. Thus, when discussing the horizontal transfer of natural

products, we always have to be aware that the actual manifestation and extent of this phenomenon are determined by the outcome of several simultaneously occurring processes, i.e., the degradation of the substances by soil microorganisms, or the uptake by the acceptor plants. Unfortunately, up to now, no solid information on this topic is available.

3 Expanding the Concept of Horizontal Natural Product Transfer: Co-cultures

When deliberating the coherences and central issues of horizontal natural product transfer, the question will arise, whether – in analogy – to the leaching from dead, rotting plant material a corresponding transfer also might occur between living plants.

Allelopathy taught us that allelochemicals are released into the environment by various processes [37]. In addition to a leaching out of decomposing plant residues, allelochemicals also are actively exuded from living plants by their roots [2, 3] or by their leaves [37, 38]. Consequently, it seems to be quite reasonable to assume that analogously also common natural products might be released into the soil from living donor plants. Recently, so-called co-culture experiments have been conducted to verify such assumption: potential vital donor plants, e.g., pyrrolizidine alkaloids (PAs) containing *Senecio jacobaea* plants, had been cultivated in single pots together with potential acceptor plants, e.g., parsley. After 2 months of co-cultivation, the plants had been harvested and the PAs were quantified. Astonishingly, in all parsley plants co-cultivated with *Senecio jacobaea*, significant concentrations of PAs were present; the average content was more than 200 μg/kg d.w. [39]. Based on these results, further experiments and field trials had been conducted employing a wide array of acceptor plants, which had been co-cultivated with *S. jacobaea*. In all cases, considerable amounts of PAs, which previously had been synthesized in the donor plants, were present in the acceptor plants. Thus, there is no doubt that – at least in the case of PAs – the alkaloids are transferred from vital and living donor plants via the soil into acceptor plants. However, up to now, there is no indication about the mode of this transfer.

Considering the well-established active exudation of allelochemicals [2, 3, 37], it seems obvious to assume that the PAs – and maybe other alkaloids – might be exuded, either by the roots or by the leaves. However, we have to consider that all plants frequently are faced with attacks by herbivores and infections by pathogens. Accordingly, even vital and healthy plants exhibit numerous minor or larger injuries due to previous attacks. Moreover, due to regular mechanical interactions with the soil, frequently a certain number of root cells is destroyed, e.g., those of the calyptra. Therefore, it could not be excluded that the observed transfer of PA is related to natural injuries of the donor plant. To elucidate the actual mode of transfer, further research is required. Nonetheless, the fact that alkaloids are transferred from living and vital donor plants into acceptor plants necessitates a broadening of the concept of horizontal natural product transfer, which is displayed in Fig. 3.

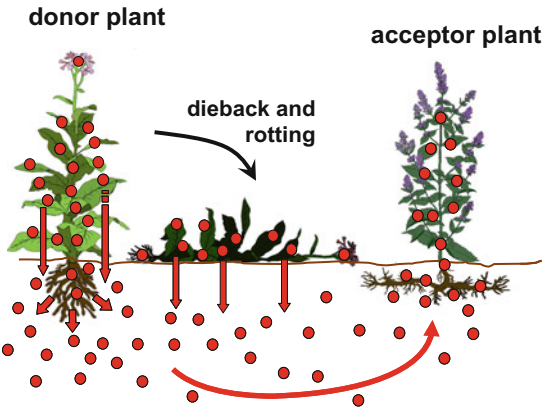

Fig. 3 Expanding the concept of horizontal natural product transfer according to Nowak et al. [50]. Co-cultures of acceptor and donator plants revealed that alkaloids also are transferred from vital and living donator plants to acceptor plants. Accordingly, apart from the leaching of rotting plant materials, further paths for the transfer have to be considered, i.e., the elution of natural compounds from small injuries of donor plants due to pathogen or herbivore attack, a leaching of dropped leaves or an active exudation

4 Modification of Imported Substances in the Acceptor Plants

A detailed evaluation of the contents of alkaloids imported into the acceptor plants displayed that the content of nicotine [18] as well as of the PAs [20] decreased by the time. In this context, it has to be considered that xenobiotics, which are taken up from the soil, frequently are modified within the acceptor plants, e.g., by oxidation, hydroxylation, and by conjugation with glucose [40, 41]. According to the so-called green liver concept, these reactions are proposed to be part of a deliberate detoxification of xenobiotics [40, 42]. Consequently, it is not far to conclude that the time-dependent decrease in the PA concentration in the acceptor plants maybe also caused by a modification of the imported alkaloids. Yet, in this particular case, it has to be noted that the quantification of PAs was performed by the employment of a standard LC-MS method, which is based on summing up the individual contents of 27 genuine PAs [20, 43]. In consequence, only the original, already known PAs – previously present in the donor plant – are determined and not their putative derivatives. Fortunately, an alternative method is available, i.e., the so-called sum parameter method. This approach is based on a HPLC-ESI-MS determination of the necine base [44]. Hence this method considers also the putative modification products of the imported PAs, too. Accordingly, the difference of the values between both procedures of quantification corresponds to the PAs, which putatively have been modified within the acceptor plants (Fig. 4). By doing so, it became apparent that the concentration of PAs in the acceptor plants is far higher than previously assumed [45]. In the case of parsley, 2 weeks after the mulching, more than three-

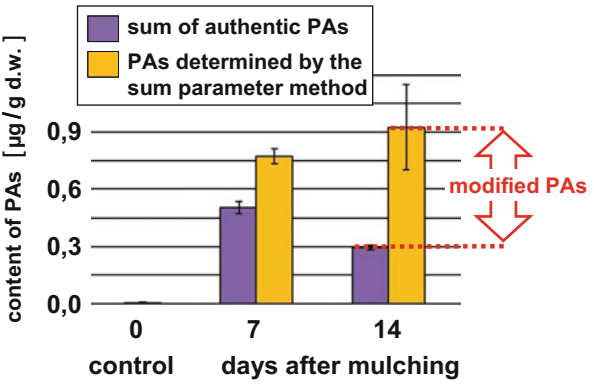

Fig. 4 Modification of PAs in the acceptor plants according to Nowak [45]. The PAs taken up by parsley plants had been quantified either classically by summing up all authentic PAs determined by LC-MS [20, 43] or by the sum parameter method [44]. In contrast to the classic approach, the latter method also considers all putative modification products. Consequently, the difference between both values corresponds to the PAs which putatively have been modified within the acceptor plants

quarter of the alkaloids taken up by the acceptor plants had already been modified, and thus being invisible for the standard quantification by LC-MS (Fig. 4). Undoubtedly, there is a tremendous demand for further research to elucidate the processes related to the modification of alkaloids. This especially accounts for the elucidation of the PA metabolites; since the amount of PAs in contaminated plant-derived commodities seems to be much higher than previously stated, a reliable estimation is required to evaluate the related health risk.

With respect to modifications of the imported natural products in the acceptor plants, in the case of phenolic compounds, already much more insights and details are available. The studies on the uptake of umbelliferone into various acceptor plants demonstrated that the metabolic fate of imported coumarin strongly depends on the plant species [36]. In acceptor plants like pea, radish, or flax, the umbelliferone is just accumulated in the leaves. By contrast, in barley umbelliferone is methoxylated to yield scopoletin, whereas in garden cress it is converted to esculin [36]. Moreover, our further studies on the uptake of resveratrol into barley seedlings outlined that this stilbene is glucosylated in this acceptor plant.

The oxidative conversion of secondary metabolites, e.g., the methoxylation or hydroxylation required for the conversion of umbelliferone to yield scopoletin or esculetin is frequently catalyzed by cytochrome P450 enzymes [46]. Yet, the determination of the activity of these enzymes is quite problematic, since cytochrome P450 enzymes require the close collaboration with an appropriate NADP-reductase and the availability of the specific substrate. An alternate approach to verify the involvement of P450 enzymes is based on the application of corresponding inhibitors or competitive substrates, respectively. In this manner, Hijazin et al. [36] applied naproxen, i.e., a widespread, efficient P450 inhibiting

agent [47], together with umbelliferone to the seedlings of barley and garden cress. In all naproxen-treated acceptor plants, the concentration of the hydroxylated derivatives of the imported umbelliferone was massively decreased. These results indicate that the hydroxylation of the umbelliferone imported into the barley and garden cress acceptor plants is catalyzed by a cytochrome P450 enzyme.

5 Conclusions

The insights displayed in this treatise outline vividly that the phenomenon of horizontal transfer of natural product is far more prevalent than initially assumed. Meanwhile, there is no doubt that in addition to alkaloids, also other natural products are transferred from donor to acceptor plants.

Moreover, the widespread uptake of natural products from the soil requires a reevaluation of the classical definition of xenobiotics. Up to now, xenobiotics are defined as "non-natural substances," which are "foreign to the plants" [48, 49]. Indeed, the substances taken up in the course of horizontal natural product transfer are "foreign" to the acceptor plants, but they represent natural products. Accordingly, a new definition, or at least a differentiation of the term xenobiotics, is required.

The perceptions that vital and living plants do incidentally release typical secondary metabolites into their environment, which subsequently are taken up from the soil by other plants, necessitate a reconsideration of our understanding of plant-plant-interactions. Up to now, only "typical allelochemicals," i.e., substances, which exhibit certain significance within plant-plant interactions, e.g., by inhibiting the growth or the germination of potential competitors, have been considered in this context. Now, we are aware that a random exchange of natural products between living plants – also between individuals of different species – is quite common. These insights should improve our understanding of the evolution of allelochemicals and thus, the interactions between plants in general.

A further aspect of the outlined transfer of natural products from living donor plants concerns several hitherto unexplained processes related to beneficial effects of crop rotations or the co-cultivation of certain vegetables. Based on the coherences outlined, the exchange of natural products between vital plants could be the bases for new explanations of these ambiguous phenomena.

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Plant Allelochemicals and Their Various Applications

19

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Abstract

Allelopathy has shown both inhibitory and stimulatory roles in plant processes such as on seed germination, overall growth, development, reproduction, disease/weed management, cell division, or biosynthesis of photosynthetic pigments of other plants by releasing some allelochemicals, mainly secondary metabolites. It is a multidisciplinary science, and their influences are noted in agriculture as well as forestry sectors. However, in several cases, a proper understanding of released chemical compounds or structure is desirable for the efficient positive application. It has been reported that metabolites, for instance, phenols, alkaloids, terpenoids, benzoxazinoids, glucosinolates, and isothiocyanates, are some important allelochemicals. This chapter is focused on the role of secondary metabolites as allelochemicals and their various applications.

Keywords

Allelochemicals · Agriculture · Forestry · Plant protection · Application

1 Introduction

Allelopathy (a biological phenomenon) term is detected from the Greek-derived compounds *allelo-* and *-pathy* (meaning "mutual harm" or "suffering"). In this phenomenon, one organism produces certain types of specific biochemicals which affect the germination, growth, survival, and reproduction processes of other adjoining or neighboring organisms. In plant system, these types of biochemicals (allelochemicals) are present in different parts of plants such as leaves, fruits, flowers, pollen, roots, and stems. These chemicals interact and affect the functions (respiration, photosynthesis, water balance, stomatal function, stem conductance of water, xylem element flux, membrane permeability, cell division/development, protein synthesis, enzyme activity alteration, and so on) of adjoining or neighboring plants and other species or richness of species [1–4]. Basically, they are non-nutritional secondary metabolites which are released by plants in different conditions and in different processes [5, 6]. In this phenomenon, due to interaction these allelochemicals control abundance and distribution of species within the plant community and also play a remarkable role in the success of invasive plants [7–11], for instance, water hyacinth [12, 13], spotted knapweed [14], and garlic mustard [15]. Allelopathy is also considered as one of the indirect factors of regular cropping hindrances in the agriculture sector. Thus, in recent past, agricultural production management plans and ecological restoration involving the application of allelopathy and allelochemicals are increasing [1]. In forest ecosystem, trees and

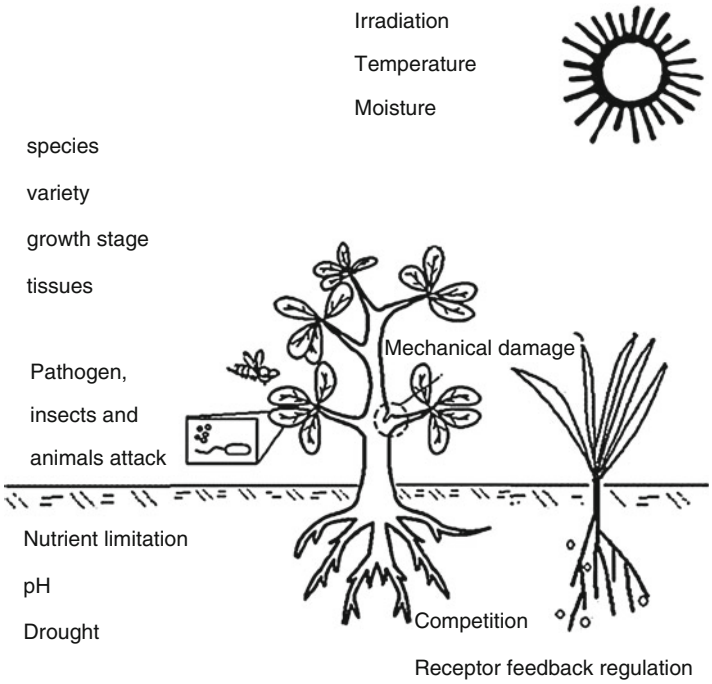


Fig. 1 Induction of allelochemical production by the plant itself (plant factors) and environmental factors. The plant factors include species, variety, growth stage, tissue type, etc. Environmental factors include abiotic factors (irradiation, temperature, nutrient limitation, moisture, pH) and biotic factors (plant competition, diseases, insects, animal attack, and receptor feedback regulation). (Adapted from Ref. [1])

understory plants also influence each other allelopathically, which leads to overall reduction in species richness and diversity. Studies have shown the reduction in understory species abundance and diversity, forest floor organic matter depletion, soil erosion and habitat degradation, and reduction in crop production due to allelopathy in many parts of the world [16]. Moreover, research study has showed the processes by which allelochemical comes into environment are volatilization from aerial parts, leaching, root exudation, and residual decomposition of dead plant parts [11]. However, allelochemical type and concentration released into the environment depend on the collective effects of the plant itself and environmental factors [1] (Fig. 1). This chapter describes major allelochemicals and their various applications and suggests some important points for further research.

2 Major Allelochemicals Present in Plants

Phytochemicals can be broadly organized into general categories starting from lipids, including the simple and fictionalized hydrocarbons as well as the terpenes. The plant natural products can be classified into two types – primary constituents and

secondary constituents – depending whether or not they have an essential role in plant metabolism and are universally present in all plants. Primary constituents include the common sugars, proteins, amino acids, the purines and pyrimidines of the nucleic acids, chlorophyll, and so on. Secondary constituents make up all the remaining plant constituents, which varied in their distribution from plant to plant. Allelochemicals are secondary metabolites produced by plant. There are various types of allelochemical.

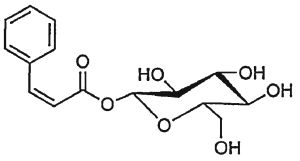
2.1 Phenolic Compounds

Phenolic compound contains hydroxyl group directly attached to aromatic ring. It contains aromatic phenol, tannins, some flavonoids, cinnamic acid derivatives, hydroxyl and substituted benzoic acids, and quinones. The most common allelochemicals of plant origin are benzoic acid and their derivatives [16]. One research report discussed the effect of aqueous extract of *Delonix regia* on the growth of lettuce (*Lactuca sativa*) and Chinese cabbage (*Brassica chinensis*) and found that aqueous extract inhibited the growth of plant. Chlorogenic acid, protocatechuic acid (3,4-dihydroxybenzoic acid), gallic acid, 3,4-dihydroxybenzaldehyde, p-hydroxybenzoic acid, caffeic acid (3,4-dihydroxycinnamic acid), and 3,5-dinitrobenzoic acid were the major compounds present in aqueous extract responsible for its allelopathy effect [17].

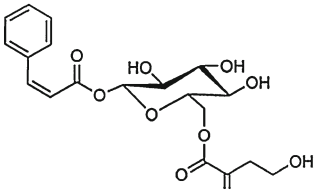
Research study revealed that phenolic compound from *Chenopodium murale* affects the growth and macromolecule content in chickpea and pea. Protocatechuic (12.8 5), ferulic (30.4%), p-coumaric (20.2%), and syringic acid (33.6%) are the four phenolic allelochemicals present in *Chenopodium murale* when analyzed by HPLC [18]. Two glucosides of cis-cinnamic acid, 1-*O*-cis-cinnamoyl-β-D-glucopyranose and 6-*O*-(49-hydroxy-29-methylenebutyroyl)-1-*O*-cis-cinnamoyl-β-D-glucopyranose, are present in the leaves of *Spiraea thunbergii* Sieb [19] and show plant growth inhibitory effects (Fig. 2).

The allelopathic influence of *Eucalyptus globulus* is due to generation of many volatile terpenes and phenolic acids [20, 21] and is responsible for the allelopathic effect on germination and seedling growth of various crops.

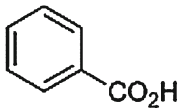
To determine the allelopathic chemical in different parts of *Eucalyptus tereticornis*, *E. camaldulensis*, *E. polycarpa*, and *E. microtheca*, a study was performed and confirmed the presence of phenolic compounds p-coumaric, gallic, gentisic, p-hydroxybenzoic, syringic, and vanillic acids and catechol [20]. Other reports are available which ensure the allelopathic effect of *Eucalyptus* species [22–25]. Study on allelopathic activity of fermented and unfermented wheat and corn straw extracts was performed [26]. Allelopathic activity was tested against seed germination of *Abutilon theophrasti*, *Asclepias syriaca*, and *Chenopodium album.* Crude straw extracts show more inhibition on seed germination and seedling growth as compared to fermented extract. The allelopathic effect of root exudates and residues of *Ageratum conyzoides* on *Oryza sativa* was studied which concluded



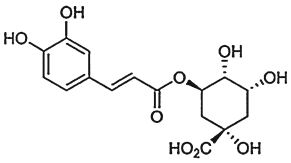
cis-cinnamic acid, 1-O-cis-cinnamoyl-β-D-glucopyranose



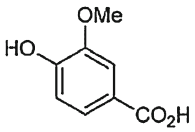
6-O-(49-hydroxy-29-methylenebutyroyl)-1-O-cis-cinnamoyl-β-D-glucopyranose



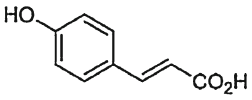
benzoic acid



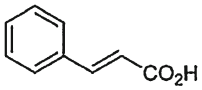
Chlorogenic acid



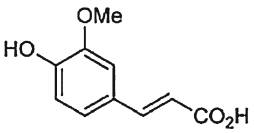
Vanillic acid



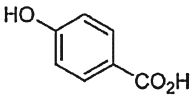
p-coumaric acid



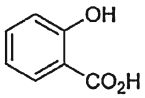
trans-cinnamic acid



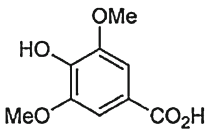
Ferulic acid



p-hydroxybenzoic acid



salicylic acid



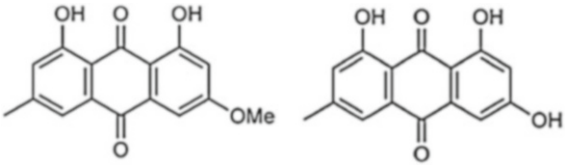
syringic acid

Fig. 2 Structure of allelochemical isolated from *Spiraea thunbergii* and *Eucalyptus* species

that phenolic allelochemicals released from root and residues are responsible for growth inhibition of *Oryza sativa* [27]. Four biological active compounds, namely, 4-hydroxy-3,5-dimethoxybenzaldehyde, (−) loliolide, 3-ß-hydroxy-5a,6a-epoxy-7-megastigmen-9-one, and 3-hydroxy-ß-ionone, have been isolated from Bangladesh indigenous rice (*Oryza sativa* L. variety, Boterswar) [4], and these four compounds synergistically suppressed the growth of *Echinochloa crus-galli* more strongly than the individual compounds.

Allelopathic activity and total phenolic content of water extract of shoot and root of foremost plant species which grow in 1-year-old and 2-year-old clear-cuts of scot *Pinus sylvestris* were studied [28]. Extracts of *Rumex acetosella* and *Calluna vulgaris* show the strongest allelopathic activity which inhibits seed germination

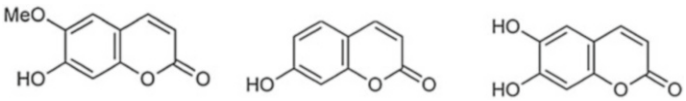
of *Pinus sylvestris.* The allelopathic activity is due to the highest phenolic accumulation. Also, there was higher phenolic content, and their variety in shoot extract was compared to the root extract of plant species.



Emodin Physcion

Two allelochemicals emodin and physcion (above figure) were detected from various parts (rhizomes, aerial parts, and fallen leaves) of *Polygonum sachalinense* Fr. Schm. [29].

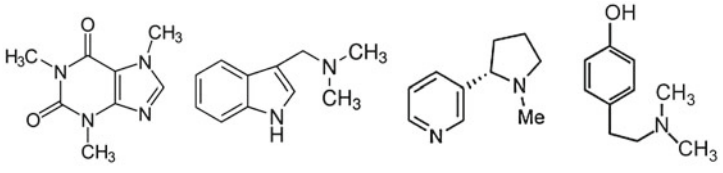
Coumarins and their glucosides are abundant and commonly present phytochemicals in the plants. Families, namely, Apiaceae, Rutaceae, Asteraceae, and Fabaceae, are known for the presence of coumarins. Some of the coumarins, scopoletin, umbelliferone, and esculetin, are known for their allelopathic effect. Some of the reports which support phytotoxic activity due to presence of coumarin are [30–33]. Imperatorin and psoralen are two other coumarins which show considerable allelopathic activity [34].



scopoletin umbelliferone Esculetin

2.2 Alkaloids

Many past and recent research reports revealed that alkaloids are also known for their allelopathic effect. Caffeine, gramine, and nicotine show the allelopathic effect. Caffeine is responsible for autotoxicity in coffee and tea plantations, while nicotine affects the seed germination at higher concentration [35]. Phytotoxicity of gramine on oat, wheat, rye, and weed *Lolium rigidum* was studied [36]. *Rhazya stricta* also contain alkaloids with allelopathic activity [37].



caffeine Gramine Nicotine Hordenine

Allelopathic effect of *Datura stramonium* was studied by many authors [38–41]. Effect of *Datura stramonium* on the growth and survival of various grass and legume species was performed [39]. Seed and leaf extracts of *Datura stramonium* have allelopathic effects on leaf chlorophyll content, root, and shoot length of *Cenchrus ciliaris* L. (grass) and *Notonia wightii* Am (legume) species [38]. Other reports support allelopathic effect of *Datura stramonium* [42, 43].

Research report is available [44] on allelopathic effects of *Melia azedarach* L. leaf litter and leaf aqueous extracts on germination, growth, and yield of *Vigna mungo* L. (black gram) and *Cicer arietinum* L. (chickpea). Both leaf aqueous extract and leaf litter inhibited the germination, initial growth, and biomass of black gram and chickpea. The chemicals responsible for allelopathic effect of leaf litter were phenolic acids, alkaloids, methyl ketones (volatile allelochemical), unsaturated fatty acids, benzofuran, propargyl acid, benzoxepine, fluorobenzoic acid, silicyclobutane, and palmitic acid analyzed by GCMS. Crude alkaloidal fraction of *Crotalaria retusa* (leaf and stem) was isolated from its methanolic extract and tested for its allelopathy in *Phaseolus vulgaris* (bean) at varying concentrations. Germination of bean seeds was reduced with increasing concentrations of alkaloid fraction. Study of mechanism for allelopathy shows crude alkaloid fraction cause oxidative stress that generate reactive of reactive oxygen species that initiated metabolic derangement in the bean seedlings [45].

2.3 Terpenoids

Terpenoids are known for their medicinal use since ancient times, but there are a number of allelochemicals present in terpenoids (Fig. 3). 1,8-Cineole and camphor are volatile monoterpenes which show inhibitory effect on plant growth [46]. One research reported that extraction procedure can improve the allelopathic activity of *Cynara cardunculus.* Different extracting solvents, water, methanol, ethanol, and ethyl acetate, were used, and all show allelopathic effect more than 50% when tested against six weeds *Amaranthus retroflexus*, *Portulaca oleracea*, *Stellaria media*, *Anagallis arvensis*, *Echinochloa crus-galli*, and *Lolium perenne*. Also four sesquiterpene lactones (cynaropicrin, cynaratriol, desacylcynaropicrin, and 11,13-dihydro-desacylcynaropicrin) and a lignan (pinoresinol) were isolated from the ethyl acetate fraction of the aqueous extract of *Cynara cardunculus*. All of them showed allelopathic effects, with cynaropicrin, desacylcynaropicrin, and pinoresinol having maximum activity [47].

One research reported ecological characteristics of terpenoids and their allelopathic effects on plants and conclude that terpenoids can cause inhibition, promotion, and autotoxic action on seed germination and seedling growth [48]. Toxicity of terpenoids may be due to different factors: (1) inhibition of ATP formation, (2) disruption of hormonal activity, (3) alkylation of nucleophiles, (4) complexation with protein, (5) binding with free sterols, and (5) inhibition of respiration [49]. Some terpenoids (essential oil and monoterpenoids) affect seed germination and plant growth [50, 51]. Allelopathic effects of leaf oil emulsion of *Eucalyptus grandis* × *Eucalyptus urophylla* were tested against proliferation

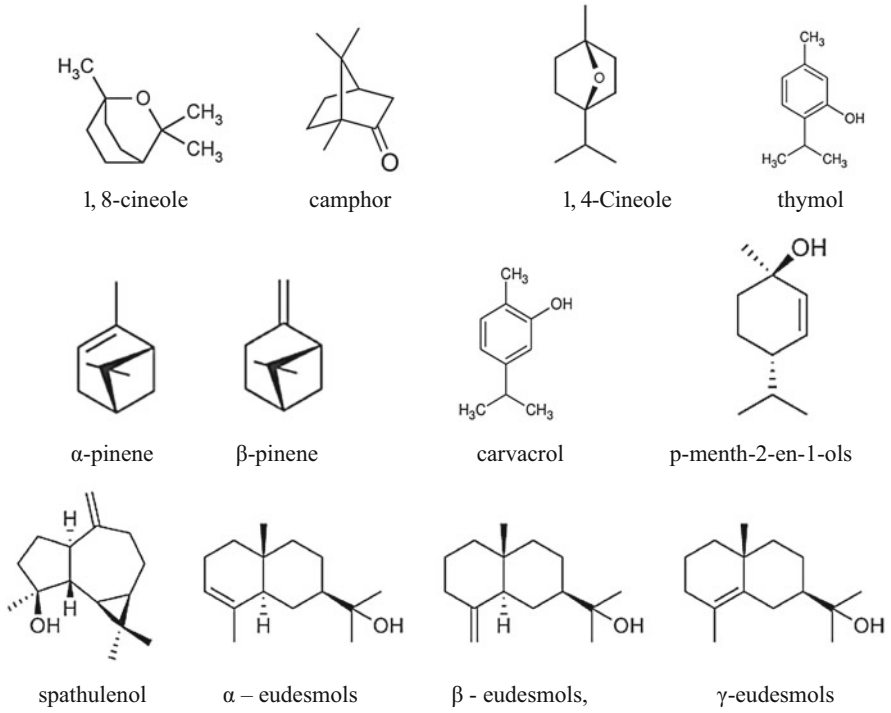


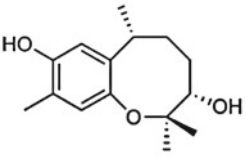
Fig. 3 Some terpenoids with allelopathic activity

of pathogenic fungi *Fusarium oxysporum*, *Pyricularia grisea*, *Gloeosporium musarum*, and *Phytophthora capsici*. The allelopathic effects of essential oil are due to presence of terpenoid (alloocimene 43.22%, α-pinene 13.63%, γ-terpinene 5.49%) [52]. Terpenes obtained from *Eupatorium adenophorum* and their allelopathic effects on seed germination of *Arabidopsis* were performed [53]. Eleven terpenes were isolated and identified from *Eupatorium adenophorum*. The structure of these isolated compounds was established by NMR (1D, 2D) and mass spectrometry. Isolated compounds were (−)-(1R∗,2S,∗4R∗,5S∗)-3,3-dimethyl-5-hydroxybicyclo [2,2,1]hept-2-ylmethanol (1); two new cadinane sesquiterpenes, (−)-(5S∗,6S∗,7S∗,9R∗,10S∗)-7-hydroxy-5,7-epidioxycadinan-3-ene-2-one (2) and (+)-(5S∗,6R∗,9R∗,10S∗)-5,6-dihydroxycadinan-3-ene-2,7-dione (3); and eight known terpene compounds (4, 6–12). Results suggest that out of all other terpenoids, cadinene-type sesquiterpenes are only part of the allelochemicals in *Eupatorium adenophorum.*

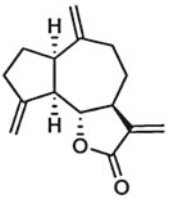
Allelopathic activity and phytotoxic activities of *Atriplex cana* essential oil were studied [54]. Seedling growth of *Amaranthus retroflexus* and *Poa annua* was inhibited by volatile organic compounds released by *Atriplex cana*. Experiment was performed in an airtight container in fresh leaves and stems of *Atriplex cana*. Previous studies reported a number of allelochemicals among the mono-,

sesqui-, and diterpenoids in plants. These plants have different volatile terpenoids with allelopathic activity [55].

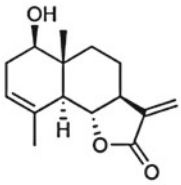
Other examples are 1,4-cineole and 1,8-cineole [46]. p-Menth-2-en-1-ols thymol, carvacrol, 1,8-cineole, α-pinene, and β-pinene were isolated as allelopathic monoterpenes from *Eucalyptus* species. Other allelopathic terpenes (sesquiterpenes) present in *Eucalyptus* are spathulenol and α-, β-, and γ-eudesmols [29]. Monoterpenes such as R-(+)-limonene show allelopathic activities on the germination and seedling growth of *Amaranthus tricolor* L. through a synergistic interaction with xanthoxylin [56]. Research study indicated that limonene, from *Juniperus ashei*, together with camphor and bornyl acetate, shows allelopathic effect on the germination and growth of *Bouteloua curtipendula* [57].



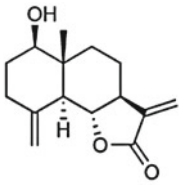
Heliannuol A



Dehydrocostuslactone



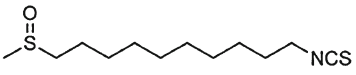
Santamarine



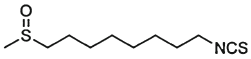
Reynosin

2.4 Glucosinolates and Isothiocyanates

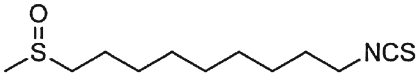
Glucosinolates are sulfur-rich compounds which upon hydrolysis are converted into isothiocyanates [58]. Isothiocyanates play an important role in defense against attack by insects/microorganism. Also, as they are volatile, they can be used for soil fumigation [59]. One research investigation reported that hirsutin, arabin, and camelinin are the allelopathic chemicals obtained from *Rorippa indica* Hiern (Cruciferae) roots [60] as shown below.



Camelinin

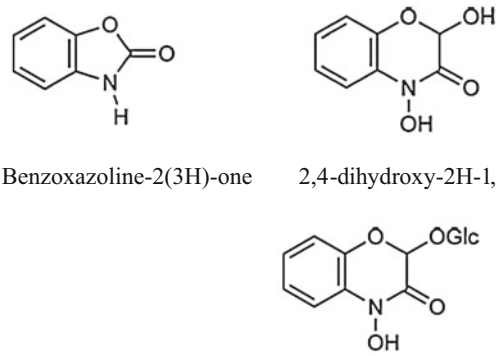


Hirsutin



Arabin

Aliphatic allyl isothiocyanate and aromatic isothiocyanates (ITCs) were tested for biological effect on *Arabidopsis thaliana* [61]. Result shows that treatment of aliphatic allyl isothiocyanate (allyl-ITC) reduces the root length, formation of lateral roots, and fresh weight in a dosage-dependent manner. Aromatic isothiocyanates



Benzoxazoline-2(3H)-one 2,4-dihydroxy-2H-1, 4-benzoxazin-3(4H)-one

2-β-D-glucoside 2,4-dihydroxy-2H-1, 4-benzoxazin-3(4H)-one

Fig. 4 Benzoxazinoids found in plants

also inhibit the growth, and growth inhibition was more than the aliphatic allyl isothiocyanate.

2.5 Benzoxazinoids

It is a class of allelochemicals that are derivative of indole. These allelochemicals act as natural insecticides, fungicides, and herbicides. These allelochemicals act as defense mechanism for *Zea mays* against numerous pathogens and pests [62]. According to research report, plants belonging to family Poaceae, Acanthaceae, Ranunculaceae, and *Scrophulariaceae* release benzoxazinoids. Some examples of benzoxazinoids are shown in Fig. 4.

Allelopathic potency of *Secale cereale* (rye) is due to the presence of benzoxazinone compounds [63]. In one research study, *Secale cereale* (rye) residue spread on the soil, and concentration of benzoxazinoids (BX) was determined in soil after definite interval of time. Two weeks later, concentration of methoxy-containing benzoxazinoid compounds was dominant. After rye applications, growth inhibition was recorded for lettuce and smooth pigweed species when treated soils were tested during the first 2 weeks [64].

2.6 Miscellaneous Compounds

A study was conducted to check the allelopathic effect of *Amaranthus retroflexus* aqueous extract (0% and 0.25%) on the wheat and cucumber plants [65]. Result indicates that extract effects on different processes (stomata opening, photosynthetic pigments contents, disrupted the membrane integrity, induction of oxidative stress) consequently reduced the growth and biomass production in treated plants. Docosane, triacontane, and ethoxytrimethylsilane are the

main allelochemicals present in aqueous extract of *Amaranthus retroflexus* analyzed by gas chromatography-mass spectrometry (GC-MS).

According to a research report, extract and exudates of *Microcystis aeruginosa* showed allelopathic effect on different processes of *Vallisneria natans* like physiological response, microbial stress of biofilm on leaves, and photosynthetic activity [66]. Results proved that allelochemical can cause physiological stress on the leaf biofilm and also affect surface topography of *Vallisneria natans* leaves. Microbial community in biofilm was more affected by exudate than extract. Evaluation of allelopathic potential and phytochemical analysis of five *Amaranthus* species: (a) *A. viridis*, (b) *A. hybridus*, (c) *A. deflexus*, (d) *A. retroflexus*, and (e) *A. spinosum*. Ethanolic leaf extract was prepared and tested against *Lactuca sativa* [67]. Phytochemical analysis showed the presence of steroids, carotenoids, and organic acids in all species. Extract concentrations that were used for testing showed an inhibitory effect on germination and the germination speed index of *Lactuca sativa* seeds in a dose-dependent manner.

Melaleuca cajuputi extract was prepared by using Soxhlet assembly and extraction of supercritical carbon dioxide and characterized by GC-MS and reported two major sesquiterpenes: caryophyllene and humulene [68]. The extract was tested against notorious paddy weeds which are known as barnyard grass to check its allelopathic effect. *Melaleuca cajuputi* extract proved allelopathic effect against weed. Also, *Melaleuca cajuputi* oil can be used as bioherbicides.

Leaf extract (aqueous) and leaf litter (aqueous) of *Melia dubia* Cav. were studied for its allelopathic potential against early growth, germination, and biomass of the *Solanum melongena* L. and *Capsicum frutescens* L. by pot culture bioassay in laboratory. GC-MS was used to analyze *Melia dubia* leaf litter which showed the presence of derivatives of phenolic acids and unsaturated fatty acids, omega-3 fatty acid, chromene, aromatic ketone, alkaloids, and methyl ketones. Both extracts inhibited the germination traits, but in early stage allelopathic effect was seen and it disappeared in later stage [69].

A study was conducted to check the extract of *Chaetoceros curvisetus* on the growth of *Skeletonema costatum*, and it showed allelopathic activity. The isolation and characterization of allelochemicals responsible for activity were performed using chromatography and HPLC-electrospray time-of-flight mass spectrometry (ESI-TOF-MS). 2-(2-Cyanophenyl) amino)-2-oxoethyl,3-cyclohexyl propanoate was an allelochemical present in *Chaetoceros curvisetus* [70].

Parthenium hysterophorus is also reported to have allelopathic effect [71, 72]. Leaf aqueous extract of *Parthenium hysterophorus* inhibits the germination and growth of barley, peas, corn, and wheat [73]. In a research investigation, the extract was prepared by using enhanced solvent extraction/supercritical CO_2, and allelopathic compounds were isolated from sunflower leaves by using high-pressure techniques [74]. Various types of compounds were isolated by this method including flavonoids, terpenes, heliannuols, and fatty acids from *Helianthus annuus* leaves. Most effective inhibiting effect is shown by tambulin, sesquiterpene 10-oxo-isodauc-3-en-15-al, heliannuol D, and pinoresinol. Effects of *Artemisia ordosica* leaves extract (aqueous) were tested against *Nostoc* sp. and *Chlorella vulgaris* [75].

Aqueous extracts contain humic acid and fulvic acid along with saccharides, alcohols, organic acids, and phenols. Study showed that different concentrations of extract have different effects on different activities. Low concentration improved *Chlorella vulgaris* chlorophyll fluorescence yield and growth rate, while high concentration has an inhibitory effect on the growth and photosynthetic effects of both soil microalgae. In a similar way, research study reported the allelopathic effect of different solvent extracts of *Pinus roxburghii* (pine tree) against various types of weeds such as *Euphorbia helioscopia*, *Avena fatua*, *Phalaris minor*, *Chenopodium album*, *Triticum aestivum*, and *Rumex dentatus* [76]. Methanolic needle extract possessed the maximum percentage inhibition effect on germination of *Triticum aestivum*, followed by *Chenopodium album* and *Avena fatua* applied in soil.

3 Application of Allelopathy

Allelopathy has both beneficial and harmful effects. Negative effects of allelopathy include autotoxicity, soil sickness, or biological invasion, while the positive effects are weed control, crop protection, and so on. Currently various investigators are working hard to use allelochemicals as growth regulators, herbicides, insecticides, and antimicrobial crop protection products, and many of them are successful. Following are some of the major application of plant allelopathy.

3.1 Weed Management

3.1.1 Intercropping

Weeds have become one of the major causes for losses in crop production due to various reasons which include cost of weed management, weed crop competition, and intervention of weeds with crop management practices [77–80]. The process of growing crops in the same field at the same time is known as intercropping and can be used for weed management [79]. Research investigation proved that intercropping of allelopathic crops can be useful for weed management, mostly in low absorption agriculture systems [81, 82]. Release of allelochemical, shade effect, and weed-crop competition are the factors by which allelopathic intercrops suppress weed growth [82–84]. Beside this, intercropping has other benefits like enhanced crop yield, great use of resources, and exploitive effects on pests and insect. To increase the crop production, weed management has become very important. A number of weed management approaches are reported by authors to increase crop production [85, 86].

3.1.2 Mulching

In this process plant residue or crop residue is applied to the surface of soil to improve soil fertility and soil moisture and to reduce weed growth. Chemicals released by mulching process inhibit germination and seedling growth of weeds [87–90]. Effect of sorghum mulch was studied by many researchers, for example, 26–37% of weed control was achieved by using sorghum mulch (10–15 t ha^{-1}) in

maize [91], whereas in cotton sorghum mulch (3.5–10.5 t ha^{-1}) decreased the weed by 23–65% [92] and, in aerobic rice, weed density reduced by 50% of total dry biomass [93, 94]. Studies proved that mulching has effect on weed, fruit yield, and economic returns of garden egg (*Solanum melongena*) in Okigwe, Southeastern Nigeria. Different mulching materials, (1) two synthetic materials, polythene and trampoline sheet, and (2) natural or organic materials, 6/ha sawdust and grasses, were used. Results indicated that the plot mulched with sawdust had 7–76% and 6–72% greater fruit yield compared to the other mulching materials in both seasons.

3.2 Allelopathic Activity of Water Extract

Extracted water-soluble allelochemicals are used for controlling the weed [95]. Water extract of sorghum has been effective in suppressing weeds [96–98], for example, sorghum water extract showing allelopathic effect on wheat [98] rice [99], cotton [100], canola [101], mung bean [97], sunflower [102], soybean [103], and maize [91]. Effect of rice straw extract on the growth of the microalga *Chlorella* species and the cyanobacterium *Anabaena* species was evaluated [104]. Different rice straw extract concentrations were prepared in water and methanol, and both extracts show inhibition which was dose dependent. The main phenolic compounds present in water and methanol extract were pyrogallol, gallic acid, and caffeic acid and were considered for its allelopathic effect. This study shows that rice straw extract (aqueous) has a potential to inhibit the growth of *Anabaena* species. Research study showed the allelopathic effects of *Solidago canadensis* on the germination potential of seed and seedling growth of *Lactuca sativa* under the salt stress condition [105]. Leaf extract of goldenrod (*Solidago canadensis*) in high concentration effectively reduces root length, germination percentage, leaf shape index, and germination rate index of lettuce (*Lactuca sativa*). But interestingly at low concentration of leaf extract, there was enhanced leaf width of lettuce (*Lactuca sativa*) and root length. *Chrysanthemum coronarium* aqueous extract shows nematocidal activity against *Meloidogyne incognita* and *Meloidogyne javanica* [106]. In the present scenario, abiotic stresses mainly drought and salinity are one of the major constraints to agricultural productivity and growth of forest tree species at seedling stages [107–114]. It has been suggested that the allelochemicals may improve the resistance against abiotic stresses, for instance. Research studies showed water extract from different plants which have shown the allelopathic potential in stress mitigation. Authors have reported that the exogenous application of allelopathic water extracts improved the stay green, proline accumulation, soluble phenolics, and glycine betaine, which helped to stabilize the biological membranes and improved the tolerance against terminal drought and heat stresses in *Triticum aestivum* [115].

3.3 Allelopathy for the Management of Phytopathogens

Natural compounds obtained from plants are secure and eco-friendly. Research reports show that these natural compounds show allelopathic activity and

can be used for management of phytopathogens. Plants such as Magnoliaceae, Amaranthaceae, Brassicaceae, Acanthaceae, and Chenopodiaceae are well known for their antifungal activities; on the other hand, Papilionaceae, Poaceae, and Asteraceae are known for their nematocidal activity [116]. Allelopathic effect of some essential oils of plants *Eucalyptus tereticornis*, *Callistemon lanceolatus*, *Artemisia vulgaris*, *Ageratum conyzoides*, *Lantana camara*, and *Ocimum kelmandescherium* on ten phytopathogenic fungi *Aspergillus flavus*, *A. niger*, *A. fumigatus*, *A. terreus*, *A. parasiticus*, *Alternaria alternata*, *Fusarium oxysporum*, *Colletotrichum truncatum*, *Trichoderma viride*, and *Helminthosporium tericum* was studied [117]. Four essential oils of *Callistemon lanceolatus*, *Eucalyptus tereticornis*, *Ageratum conyzoides*, and *Ocimum kelmandescherium* inhibited the growth of all the fungi tested. One research study reported that bacterial pathogen can be managed with these secondary metabolites (allelochemicals) [116]. Another research study reported that intercropping of Chinese chive with tomato was able to control soil-borne disease (caused by bacteria *Pseudomonas solanacearum*) by allelopathic approaches which result in safer and higher-quality product at lower cost [118]. Crops showing allelopathic effect can be incorporated into soil (as green manure) to decrease the pathogen population in plants. Crude extract of these crops can be used as spray against pathogen present in air. Research study reported that allelochemicals can also show inhibition of the growth of *Microcystis aeruginosa*, and it was concluded that these allelochemicals can significantly control the toxic cyanobacteria [75]. Other research also supports the plant allelopathic potential in inhibiting the growth of plant pathogens [119–123].

3.4 Development of Herbicides from Allelochemicals

With the increase use of herbicide, weed became resistant against these herbicides. So herbicide with new mode of action is needed today [124]. Various allelochemicals with herbicidal activity have been isolated from different crops [125–127], and allelochemicals with herbicidal activity can be categorized into two major groups: phenolics and terpenoids [128]. One research study reported that phenolic compounds showed their allelopathic potential by inhibiting symbiotic relationship between rhizobium and legume [129]. These natural phytotoxins offer a great opportunity to develop herbicides with a safe mode of action [130]. Another study investigated that *Veronica persica* (Lour.) Merr. had effective herbicidal activity; thus a safer herbicide from allelochemicals can be developed [131]. *Eucalyptus globulus* leaf aqueous extract was tested against the germination potential and early growth of *Lactuca sativa* and *Agrostis stolonifera.* Results showed that this extract has an inhibitory effect on both target species. Also spraying treatment reduced both aerial and root biomass and reduced protein contents and chlorophyll concentrations. HPLC analysis confirmed the presence of eight phenolic

compounds (chlorogenic, two ρ-coumaric derivatives, ellagic, hyperoside, rutin, quercitrin, and kaempferol 3-*O*-glucoside) and other five low weight organic acids [132]. Research study reported bioherbicidal potential of leaf extract of *Delonix regia* on germination and seedling growth of field *Convolvulus arvensis* and *Triticum aestivum* L. [133]. Result shows that lower aqueous concentration (2.5% and 5%) and ethyl acetate (50, 500, 1000 ppm) of leaf extract of *Delonix regia* inhibited germination, root length, shoot length, and seedling dry biomass of *Convolvulus arvensis.*

3.5 Application of Allelopathy in the Agriculture Sectors

Agriculture practices are facing many challenges due to continuous use of chemical in pesticides and insecticides. For sustainable agriculture practices, scientists are exploring the use of natural resources for plant defense. To get rid of environmental pollution caused by chemical pesticide, allelopathy research becomes very important. Use of allelopathy provides a sustainable development in the field of agriculture [134–137]. Utilization of allelopathy effect in agriculture is currently in use. For instance, to cover crop, in crop rotation, intercropping or as green manure [91, 92, 138–148]. In the management of nematode pest, IMP (integrated management practice) is the best method to adopt, and this type of management is followed by Southwestern Nigeria. Recently published research paper described the possible applications of plant volatile organic compounds (VOCs) in agriculture [149] (Fig. 5). Volatile organic compounds are released from leaves of all plants due to abiotic stress [150, 151] or biotic stresses [152]. Isoprenoids are most of the volatile organic compounds [153].

3.6 Effect of Allelopathy in Forestry Sectors

In the present time, management of forest using pesticides and herbicides is a cause of concern. Synthetic herbicide has a toxic effect on wild varieties of animals and plants [154]. Recent studies emphasize on the idea of managing the forest using allelopathy [155, 156]. Allelopathy plays an important role in the establishment of plant community and ecological succession by having an impact on nutrient dynamics, competition between different species, and productivity of desired plant [157]. Research study reported that the rhizosphere which contains signaling compound released by plants involving allelopathy is of utmost use. This study concluded that for the purpose of weed management, signaling compound can be a better option in the future. To study the signaling property, there is a need of better understanding of molecular biology. Mechanism of interaction between plant-plant and plant-pathogen is also directly linked to forest management [158].

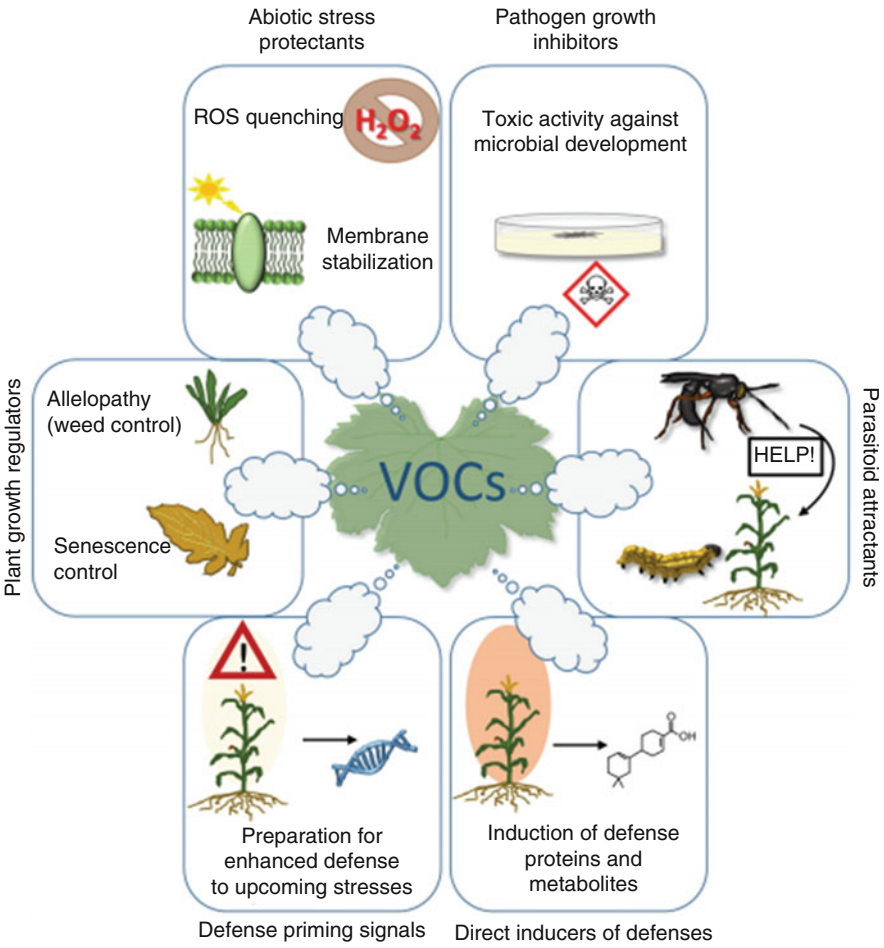


Fig. 5 Possible applications of plant volatile organic compounds (VOCs) in agriculture. (Adapted from Ref. [149])

3.7 Economic Benefits

A lot of compounds are present in plants which are bioactive in nature. A comparison between biological activities of synthetic herbicides to the allelochemicals gives an idea that activity of these secondary metabolites is unquestionable. Use of allelochemical provides an economical way to change agriculture practice. In the recent days, instrumentation is improved which helps in cost-effective and easy identification of biological active compounds as compared to a decade ago. In natural phytotoxins vital cause for scrutiny is that they frequently have new sites of achievement [159]. Besides that preparation of herbicide, pesticide from

natural compound requires less inspection for registration. So, cost of commercialization can be reduced [160].

3.8 Allelopathy and Genetic Manipulation

Allelopathic property can be transferred to different plants by biotechnology or genetic engineering. In China, GM (genetically modified) rice with insect resistance property is at trial stage [161]. Golden rice 2 has been prepared by genetic engineering. Certain enzymes which are obtained from maize are incorporated into rice. This process leads to rise in concentration of beta-carotene (a precursor of vitamin A). This variety of rice can be helpful in a deficiency disease of vitamin A [162]. If a gene transfer for insect resistance is possible, then possibility of transfer of allelopathic gene is also there. It is an area of research that can provide best option for weed control in the future. There are many exciting opportunities in these emerging fields [163].

4 Conclusion and Future Prospective

Allelopathy was considered as evil in previous times, but now it's open for new path for its numerous investigations and applications. There are numbers of phytochemical that are responsible for allelopathy of plant such as phenols, alkaloids, terpenoids, benzoxazinoids, and others. However, phenolic compounds are higher in count responsible for their allelopathic potential. Weeds have become one of the major causes for the losses in crop production, and allelopathy can play an important role in weed management. Various methods such as mulching, intercropping, and use of plant extract are used for weed control. Allelopathic properties have also been used as bioherbicide and abiotic stress mitigation. Overall, in recent years, the phenomenon of allelopathy has shown wider application and future prospective. It will play a great role in a sustainable agriculture with a safe impact on the environment. Allelopathy may facilitate to conserve the available resources and can help to remove the problems raised by use of synthetic chemicals. It can be also used to save the crop in economic and eco-friendly way.

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Biochemical Warfare Between Living Organisms for Survival: Mathematical Modeling

20

S. A. Carvalho and M. L. Martins

Contents

Abstract

Nowadays, evidence is mounting that the race of living organisms for adaptation to the chemicals synthesized by their neighbors may drive competition, coexistence, and community structures. Particularly, some bacterial infections and plant invasions disruptive of the native community rely on the release of allelochemicals that inhibit or kill sensitive strains or individuals from their

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own or other species. In this chapter, we review single and multiscale mathematical models proposed to investigate the dynamics of the biochemical warfare between competing species for survival.

Keywords
allelochemical suppression · population dynamics · multiscale modelling · community assembly

1 Introduction

Microorganisms, such as bacteria [1], yeasts [2], and other fungi [3], frequently secrete antibiotic compounds that kill or inhibit the growth of sensitive strains from their own genotypes or different species. Similarly, plants commonly exude secondary metabolites (phytotoxins) that suppress the germination or growth of neighboring plants [4]. Amazingly, glycolytic carcinoma cells excrete large amounts of lactic acid toxic to the surrounding normal cells. The resulting tissue acidification fosters tumor growth and invasion [5]. In glioblastoma, the most aggressive brain tumor, glioma cells release ATP to the tumor microenvironment. The extracellular ATP itself has a small cytotoxic effect on normal cells, but adenosine formed from ATP degradation by ectonucleotidases overexpressed on the membrane of glioma cells induces significant apoptosis of the adjacent normal cells [6]. These examples suggest that interference competitions mediated by the production of toxic chemical compounds – antibiotic, phytotoxins, lactate, etc. – are ubiquitous in biological communities.

Beyond its paramount relevance for understand microorganism and plant communities, the practical importance of antibiotics and other secondary metabolites is tremendous. Indeed, some exotic invasive plants may use allelopathic suppression to disrupt inherent, coevolved interactions among long-associated native species constituting the communities they invade [7]. This worldwide phenomenon represents a major threat for ecosystems functioning and biodiversity conservation, water availability, and agricultural production [8–11]. Preventing biological invasions and predicting their spreading patterns emerge as imperative tasks in an ecologically sustainable world. Also, a major problem in public health is the invasion of the human organism by some of their symbiont microbes eventually causing serious diseases, as is the case of *Enterococcus faecalis*, a leading cause of hospital-acquired infections [12, 13]. The inter- and intraspecies competition among the Enterobacteriaceae in the inflamed gut is mediated by microcins [14]. Moreover, microcins can act as narrow-spectrum therapeutics to inhibit enteric pathogens and reduce enterobacterial blooms [14].

From the mathematical point of view, patterns of biological invasion are interesting examples of spontaneous symmetry breaking in complex systems. The spreading of an alien species from the place where it has been originally introduced in the habitat (local invasion) can progress in various ways. In a homogeneous environment, invasion frequently generates smooth stationary traveling

population waves [15]. More complicated regimes in which the traveling fronts become transient or oscillatory before the formation of spatial patterns can be observed in a heterogeneous environment, or under the influence of other species [16, 17]. Additionally, the topological and dynamical features of the ecological interacting networks exhibit scaling behaviors highly unlikely to be generated through uncorrelated random processes but instead to be a product of a slow evolution in which stability is a major natural selection mechanism [18].

The race of plants, microbes, and even cells (e.g., within a tumor) for adaptation to the chemicals synthesized by their neighbors may drive species coexistence and community composition. This view contrasts with the conventional explanations of biodiversity as passively shaped by niche differentiation, density-dependent predation pressure, habitat heterogeneity, or fluctuations in the resources required by these communities. However, the astonishing high diversity observed within microorganism communities in seemingly uniform environments – the famous paradox of the plankton [19] – challenges the conventional resource competition framework. Indeed, even a highly structured habitat can hardly maintain such astronomical species numbers.

Nowadays, evidence is mounting that resource competition only is insufficient to explain even the diversity in communities of macrobial organisms such as higher plants and animals. For instance, positive nontrophic interactions between physiologically independent plants also play a major role in plant communities [20, 21]. Also, a theoretical work on the ecosystem stability reveals that mutualism supports biodiversity when the direct competition is weak [22]. An analysis of 59 empirical data sets representing mutualistic plant-pollinator networks provides support to this theoretical work [23].

In this chapter we review single and multiscale mathematical models proposed to investigate the dynamics of competing species in which at least one of them produces secondary metabolites (allelochemicals) affecting the others. The review is focused on models proposed by our group. Models range from deterministic to stochastic, involving either continuous or discrete populations, with or without explicit spatial information, and describing either a single or multiple scales. In this way we hope to provide a wide view of the mathematical tools for modeling the biochemical warfare between living organisms to survive.

2 Mathematical Models for Two Species

As aforementioned, competitions mediated by the production of toxic chemical compounds are ubiquitous in biological communities, and allelopathy is certainly involved in bacterial and plant invasions. Hence, understanding the dynamics of species involving allelochemicals becomes a main issue in both theoretical ecology (emergence of biodiversity in communities) and practical issues (biological invasions and ecosystems conservation). In this section, models for the biochemical warfare involving two species that also compete for common environmental resources are discussed.

2.1 Allelopathically Mediated Invasion

Let us start by considering the population dynamics of two competing species in which one of them (an invader) produces a secondary metabolite that affects the other. We assume that the populations and the released allelochemical are homogeneously mixing as, for instance, when microcin-producing and microcin-sensitive bacteria are grown in liquid cultures. Then spatial fluctuations and correlations can be neglected, and the ecological dynamics can be described by dimensionless ordinary differential equations. Accordingly reference [24], these equations are the following:

$$\begin{aligned} \dot{N} &= N(1 - N - \nu_1 I) - N\Phi(P) \\ \dot{I} &= rI(1 - I - \nu_2 N) \\ \dot{P} &= \beta I - \gamma NP - \delta P \end{aligned} \tag{1}$$

where the dot denotes differentiation with respect to the time t. Here N stands for the native species, I for the invasive species, and P for its allelochemical. The parameters ν_1 and ν_2 are the competition coefficients which measure the extent to which each species presses upon the resources used by the other. Also, r is the reproduction rate of the invasive species; β is the release rate of its allelochemical that naturally degrades at a rate δ. The term $-\gamma NP$ represents toxin consumed by the native species with an absorption rate γP which depends on the toxin level in a linear way. Finally, the term $-N\Phi(P)$ represents native species mortality induced by the uptakes of the allelochemical P. A Holling type II response with a threshold and saturation of the allelopathic suppression is assumed:

$$\Phi(P) = \begin{cases} 0 & , \text{ if } P \leq 1 \\ \mu \dfrac{P-1}{c + (P-1)} & , \text{ otherwise} \end{cases} \tag{2}$$

This functional response was chosen in order to simplify the mathematical analysis. The parameters μ and c control the toxin's efficiency in poisons native species, i.e., the slope μ/c of the response at the threshold concentration $P = 1$.

The mathematical analyses of this system, in which spatial information is lost, proceed by determining their fixed points and linear stability [25]. The fixed points of Eq. (1) and their linear stability are summarized in Table 1. The trivial fixed point $\vec{x}_0^*$ represents the extinction of both species. The fixed points $\vec{x}_1^*$ and $\vec{x}_2^*$ correspond, respectively, to invader extinction (absolutely failed invasion) and the eradication of the native species (completely successful invasion). In addition, depending on the parameter values, at least one, maybe two, new stationary solutions $\vec{x}_3^*$ and $\vec{x}_4^*$, corresponding to coexistence between native and invasive species exist. The details of this analysis can be found in reference [24].

The biochemical warfare taken against the native species markedly alters the scenario of pure intra- and interspecific competition. Indeed, under pure competition, it is well known that the coexistence of native and alien species occurs only under

Table 1 Local linear stability of the fixed points in system (1)

Point	Stability	Condition
$\vec{x}_0^* = (0,0,0)$	Saddle point	Always (eigenvalues $\lambda_1 = 1$, $\lambda_2 = r$, and $\lambda_3 = -\delta$)
$\vec{x}_1^* = (1,0,0)$	Stable	$\nu_2 > 1$. Native species is a strong competitor
	Saddle point	$\nu_2 < 1$. Native species is a weak competitor
$\vec{x}_2^* = \left(0,1, \frac{\beta}{\delta}\right)$	Stable	$\nu_1 + \frac{\mu(\beta-\delta)}{\delta(c-1)+\beta} > 1$. The invader is an effective strong competitor
	Saddle point	$\nu_1 + \frac{\mu(\beta-\delta)}{\delta(c-1)+\beta} < 1$. Invader species is an effective weak competitor
$\vec{x}_3^* = \left(\frac{1-\nu_1}{1-\nu_1\alpha_2}, \frac{1-\nu_2}{1-\nu_1\alpha_2}, \frac{\beta(1-\nu_2)}{\delta(1-\nu_1\nu_2)+\gamma(1-\nu_1)}\right)$	Stable	$\nu_1 < 1$ and $1 - \frac{\gamma+\delta}{\beta} < \nu_2 < 1$. Two weak competitors
	Saddle point	$\nu_1 > 1$ and $\nu_2 > 1$. Two strong competitors
$\vec{x}_4^* = \left(N^*, 1 - \nu_2 N^*, \frac{\beta(1-\nu_2 N^*)}{\gamma N^* + \delta}\right)$	Stable	Sufficient conditions: $\nu_1 + \mu < 1$, $\nu_2\beta < \gamma(c-1)$,$\nu_2 < 1 - \frac{\gamma+\delta}{\beta}$, $\nu_1\nu_2 < 1$, $c > 1$, and $\beta > \gamma + \delta$. Two weak competitors
	Unstable	$\nu_1\nu_2 > 1$. Two strong competitors, but not only

a weak competition regime ($\nu_1, \quad \nu_2 < 1$) [26]. However, even at low stationary toxin concentrations insufficient to provide allelopathic suppression ($P^* \leq 1$), the invader imposes, in addition to the upper bound $\nu_2 = 1$, a lower bound $\nu_2 > 1 - (\gamma + \delta)/\beta$ for its native competitor. Only above this lower bound the coexistence of both species in the regime of weak interspecific competition is possible. Thus, the coexistence imposes a minimum capacity for resource competition to the native species in order to resist to an allelopathic invader. It is worth to notice that such lower bound depends on the characteristics of the allelochemical, namely, its release and degradation rates, β and δ, respectively, as well as its uptake rate γ by the native species. So, the strategy for the invader species is increase the ν_2's lower bound toward the unity, where the fixed point $\vec{x}_3$ no more exists. This can be achieved by secreting a highly stable allelochemical ($\delta \sim 0$) at a high release rate β. In contrast, the strategy for the native species that ensures its coexistence with the invader is counter-intuitive. Indeed, it consists in increasing the allelochemical uptake rate γ in order to decrease the ν_2's lower bound.

In turn, at larger stationary allelochemical concentrations ($P^* > 1$), the mechanism of allelopathic suppression is at work, $\Phi(P^*) = \mu(P^* - 1)/(c + P^* - 1)$, and the coexistence fixed point is $\vec{x}_4^*$. Now the two species coexistence is more constrained since the sufficient conditions demand even smaller competition parameters $\nu_1 < 1 - \mu$ and $\nu_2 < \min\{\gamma(c-1)/\beta, \quad 1 - (\gamma + \delta)/\beta\}$ in order to counterbalance the allelopathic suppression promoted by the invasive species.

2.1.1 Bistability

A basic feature of the spatially homogeneous system – Eq. 1 – is that everywhere in the models' parameter space, two distinct fixed points, either associated with species coexistence or a single species survival, are simultaneously stable. Indeed, according to Table 1, the stability of $\vec{x}_1^*$ implies in the instability of $\vec{x}_3^*$, because $\nu_2 > 1$, and vice versa, because in this case $\nu_2 < 1$. The same holds true for $\vec{x}_2^*$ and $\vec{x}_4^*$. In fact, since $\Phi(P) < \mu, \quad \forall\, P$, then $\nu_1 + \frac{\mu(\beta-\delta)}{\delta(c-1)+\beta} = \nu_1 + \Phi(\beta/\delta)\big) > 1$ implies in $\nu_1 + \mu > 1$, which results in $\vec{x}_2^*$ stable but $\vec{x}_4^*$ unstable. Conversely, if $\nu_1 + \mu < 1$ and $\vec{x}_4^*$ is stable, then $\nu_1 + \Phi(\beta/\delta) < 1$ also and $\vec{x}_2^*$ is unstable.

So, based on the previous analysis, the following scenarios are possible in the models parameter space: (I) only $\vec{x}_1^*$ and $\vec{x}_2^*$ are stable and consequently one species will be extinct; (II) only $\vec{x}_1^*$ and $\vec{x}_4^*$ are stable, leading to either the extinction of the invader or its coexistence with the native species; (III) only $\vec{x}_2^*$ and $\vec{x}_3^*$ are stable, resulting on either the extinction of the native or the coexistence of both species; (IV) only $\vec{x}_3^*$ and $\vec{x}_4^*$ are stable and the coexistence is the rule; (V) only $\vec{x}_1^*$ is stable and the invader species is extinct; and (VI) only $\vec{x}_2^*$ is stable and the native plant is extinct.

Therefore, excepting in the scenarios V and VI, there are always two, and only two, attractors in the phase space, and the system exhibits bistability. The competition outcome will depend on the initial populations N_0 and I_0 and allelochemical concentration P_0, as shown in Fig. 1. In particular, as shown in Fig. 1a, the invasion leads to the extinction of the native plant only if the initial density of the alien species is greater than that of the native one. So, from the ecological viewpoint, if the alien plant is introduced at low level in a native plant community only slightly disturbed, the invasion will fail. Hence, under strong competition, the role of environmental disturbance, here thought as a reduced native population density, is central in determining invasive success, supporting the claim that "there is no invasion of natural communities without disturbance" [27]. However, taking into account the other possible scenarios shown in Fig. 1, coexistence is the rule except in situations where the invader plant is introduced at a large enough number relative to the native species. Again, the main role of environmental disturbance is observed jointly with the chemical stability of the phytotoxin. Under weak competition alien species can invade, but genetic diversity can be sustained.

2.1.2 Invasion from a Single Focus

In reference [24], the invasion spreading starting from a single focus was studied through numerical integration of a spatially explicit version of the model 1. The major prediction of this model version is that an invasion focus spreads as an expanding wave with constant speed. Behind the invasion front, the population densities evolve to stationary and spatially uniform values corresponding to the fixed points of the spatially homogeneous system. These nontrivial fixed points imply on either the extinction of one or the coexistence of both species. Specifically, if the native species is a strong interspecific competitor ($\nu_2 > 1$), it extincts the invader. Instead, if the invader armed with its allelochemical is a strong competitor

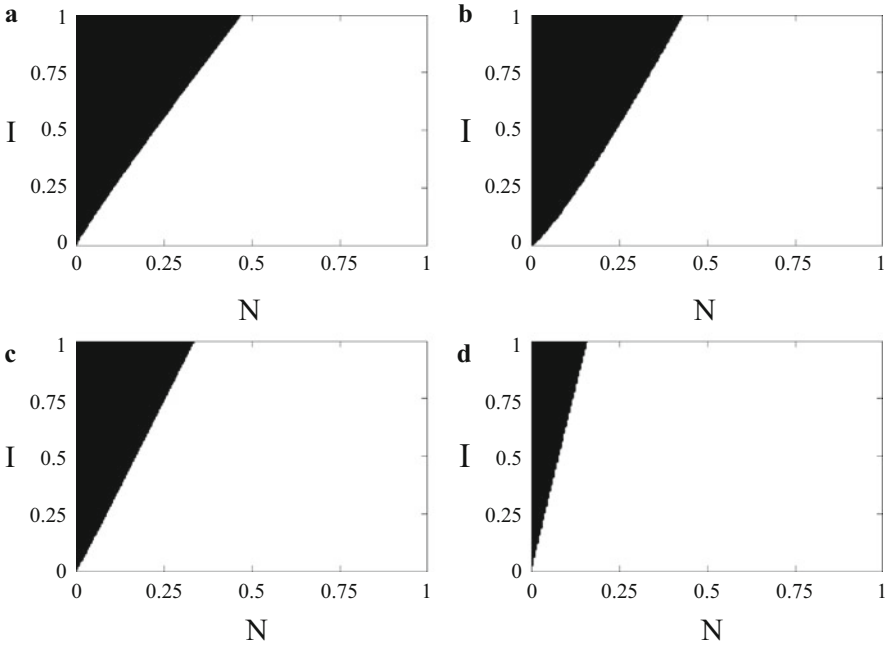

Fig. 1 Basins of attraction in the NI-phase plane ($P = 0$) associated with stable attractors (**a**) $\vec{x}_1^*$ and $\vec{x}_2^*$ (scenario I), (**b**) $\vec{x}_1^*$ and $\vec{x}_4^*$ (scenario II), (**c**) $\vec{x}_2^*$ and $\vec{x}_3^*$ (scenario III), and (**d**) $\vec{x}_3^*$ and $\vec{x}_4^*$ (scenario IV). The region $0 < N \leq 1$ and $0 < I \leq 1$ was partitioned by a uniform grid containing 10,000 sites, each one used as an initial condition. If an initial condition is attracted to $\vec{x}_2^*$ (or $\vec{x}_4^*$), its corresponding site is plotted in black. On the contrary, if the initial condition is attracted to $\vec{x}_1^*$ (or $\vec{x}_3^*$), it is plotted in white. (Figure taken from Ref. [24])

$(\nu_1 + [\mu(\beta - \delta)/\delta(c - 1) + \beta] > 1)$, the native species is extinct. This result neatly demonstrates the advantage of secreting an allelopathic compound. The invader species is able to extinct the native competitor even having a competition coefficient $\nu_1 < 1$. Furthermore, it is worth to notice that the chemical nature of the allelotoxin neatly affects the competition outcome. Indeed, a nonvolatile (small δ), powerful (large μ), and easily released (large β) allelochemical potentially transforms a "weak" species ($\nu_1 < 1$) in a highly successful invader. Figure 2 illustrates typical outcomes for an invasion process starting from a single central focus.

2.2 Allelochemical Warfare

The next natural step is, instead of considering only one species endowed with allelochemical weapons, to study the arms race involving two allelopathic organisms. In reference [28] such an extension was done. There, the following system of dimensionless partial differential equations was proposed:

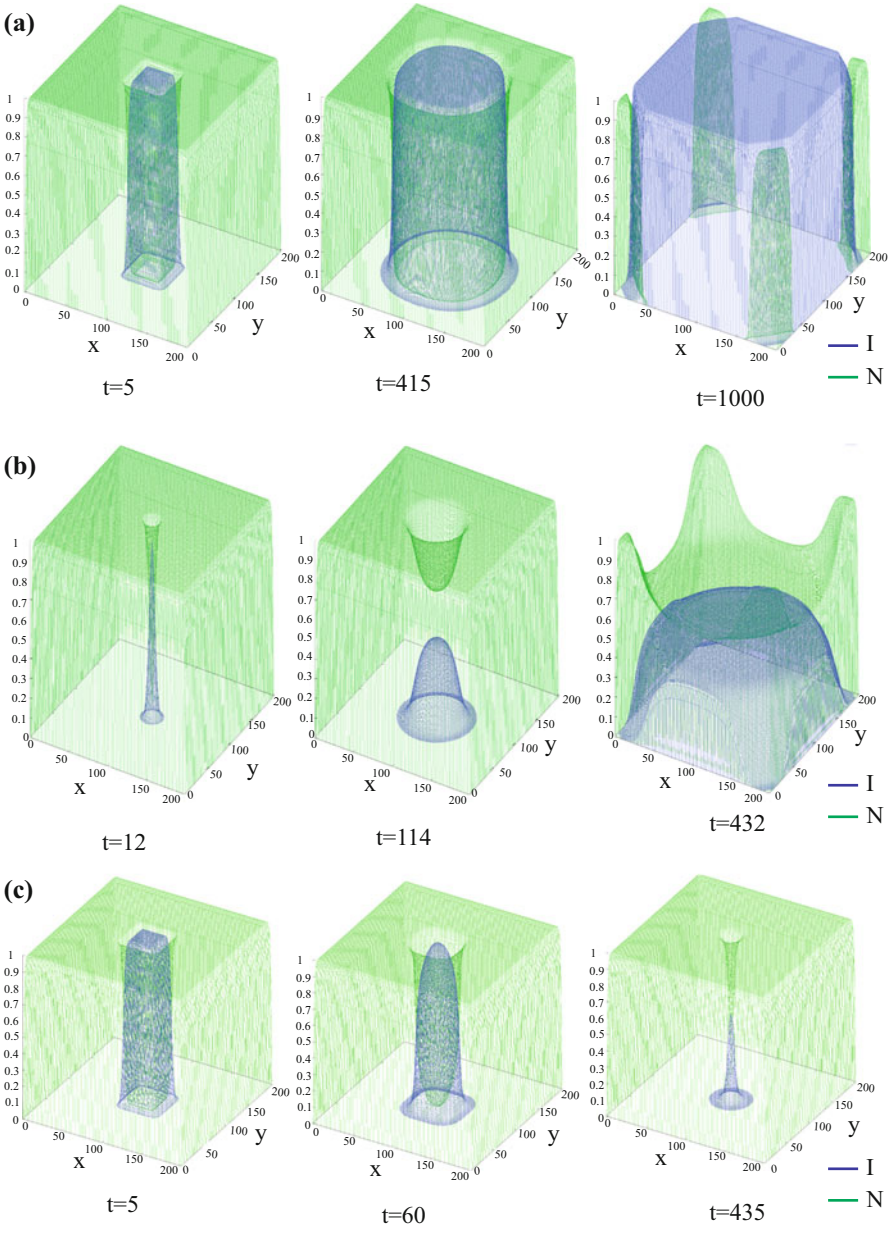

Fig. 2 Invasion spreading from a single focus on a native landscape. Three outcomes are observed: (**a**) the invader plant leads to the extinction of the native species (completely successful invasion); (**b**) both species coexist, but the invader plant spreads throughout the landscape (successful invasion); and (**c**) the alien plant is extinct (unsuccessful invasion). (Figure taken from Ref. [24])

$$\begin{cases} \partial_t N_1 &= \nabla^2 N_1 + (1 - N_1 - \nu_1 N_2) N_1 - \mu_1 \Phi_1(y_2) N_1 \\ \partial_t N_2 &= D_1 \nabla^2 N_2 + r(1 - N_2 - \nu_2 N_1) N_2 - \mu_2 \Phi_2(y_1) N_2 \\ \partial_t B_1 &= D_2 \nabla^2 B_1 + \beta_1 N_1 - \delta_1 B_1 - y_1 \\ \partial_t B_2 &= D_3 \nabla^2 B_2 + \beta_2 N_2 - \delta_2 B_2 - y_2, \end{cases} \tag{3}$$

involving the rescaled response functions $\Phi_i(x)(i = 1, 2)$ given by

$$\Phi_i(x) = \begin{cases} 0, \text{ se } x \leq 1 \\ \dfrac{(x-1)^2}{q + (x-1)^2}, \text{ otherwise.} \end{cases} \tag{4}$$

In this spatially explicit model, it is assumed that both species and their allelochemicals spread in the space through normal diffusion (the terms $\nabla^2 N_1$, $D_1 \nabla^2 N_2$, $D_2 \nabla^2 B_1$, and $D_3 \nabla^2 B_2$). All the remaining terms have the same interpretations as those in Eq. (1). But a key change was introduced: only the quantities y_1 and y_2 of toxins consumed by each species can cause allelopathic suppression. Hence, the functional responses $\Phi_i(x)$ are functions of $y_j = \gamma_i N_i B_j$, $j = 1, 2$, instead of the total amounts B_1 and B_2 of allelochemicals released into the environment, as previously. Indeed, for bacteria, the secreted bacteriocin molecules bind to specific cell receptors on the target bacteria, from which they gain entry into the cell [29]. Also, in contrast to our previous study in which the diffusion coefficient D_N of the native plant was assumed to be a decreasing function of the invader phytotoxin concentration above its threshold, here we do not consider that the species diffusivities are directly affected by the toxins. As a minor change, Holling type III functional responses, instead of type II, are used in the present model.

The spatially homogeneous system associated with Eq. (3) has fixed points similar to those obtained in the previous section, Eq. (1). In Fig. 3, it is shown how the regions of coexistence, bistability, and one species eradication for interspecific competition are changed by endowing only one of the two competing species with allelopathic suppression. Summarizing, the region onto the ν_1, ν_2-plane attracted by the fixed point $\vec{x}_1^* = (1{,}0,\ \beta_1/\delta_1{,}0)$ shrinks, whereas the attraction basin of $\vec{x}_2^* = (0, 1, 0,\ \beta_2/\delta_2)$ enlarges. Similar results are obtained for the converse situation in which $y_1 > 1$, but $y_2 \leq 1$, i.e., the species 2 produces allelochemicals against the species 1.

In Fig. 4 are shown the regions of coexistence, bistability, and one species eradication onto the plane ν_1, ν_2 when each competing species is subjected to the allelopathic suppression induced by the other ($y_1 > 1$ and $y_2 > 1$). Again, a weak competitor can eradicate a strong competitor if endowed with more lethal biochemical weapons. Additionally, the dominance of the stronger allelopathic species is enhanced by the invasion of coexistence and bistability regions.

Figures 5 and 6 partially illustrate the impact of the biochemical warfare on the interspecific competition involving two species in a space-dependent system. The numerical integration of the system 3 was performed in square lattices with length $L = 200$ and null periodic boundary conditions. In both figures, the

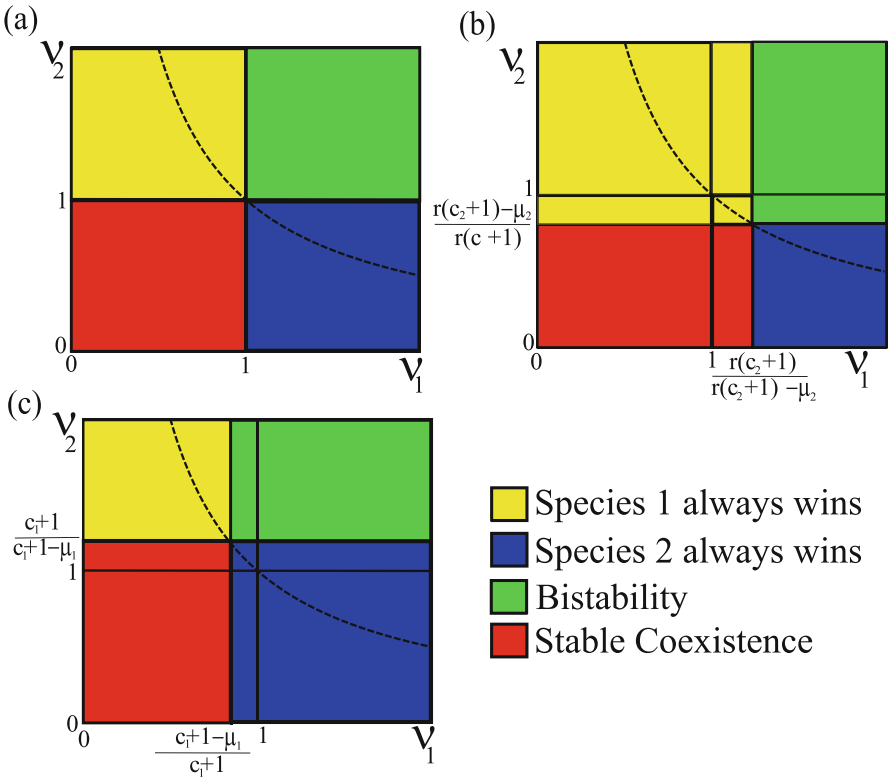

Fig. 3 The effect of allelopathy on the outcomes for two competing populations. In (**b**) the species 1 can allelochemically suppress species 2 ($y_2 \leq 1$ and $y_1 > 1$) and the converse in (**c**) ($y_1 \leq 1$ and $y_2 > 1$). In comparison with pure interspecific competition (**a**), the regions of coexistence, bistability, and one species eradication are changed. The main result is that a weak, but allelopathic, competitor can eradicate or coexist with its stronger competitor

competition coefficients are in the range of values that ensure species coexistence in the homogeneous (space-independent) regime. Figure 5 shows the possibility of a weaker competitor to invade and eradicate a stronger competitor since it is provided with more effective allelochemical weapons. In this example, the wave fronts exhibit constant and isotropic speeds. In turn, Fig. 6 shows an unexpected and very interesting finding for the interaction between two species having equal competition and allelochemical traits. In Fig. 6a an invasion process occurs without species eradication, the predicted outcome at the coexistence regime. However, Fig. 6b, in which only the initial spatial distributions of the interacting species were altered, shows an invasion process with one species eradication. Surprisingly, despite equal competition and allelochemical capacities, the coexistence outcome is replaced by one species eradication. This outcome is impossible in classical competition, whatever the case may be: space-independent or dependent. But it is possible if, in

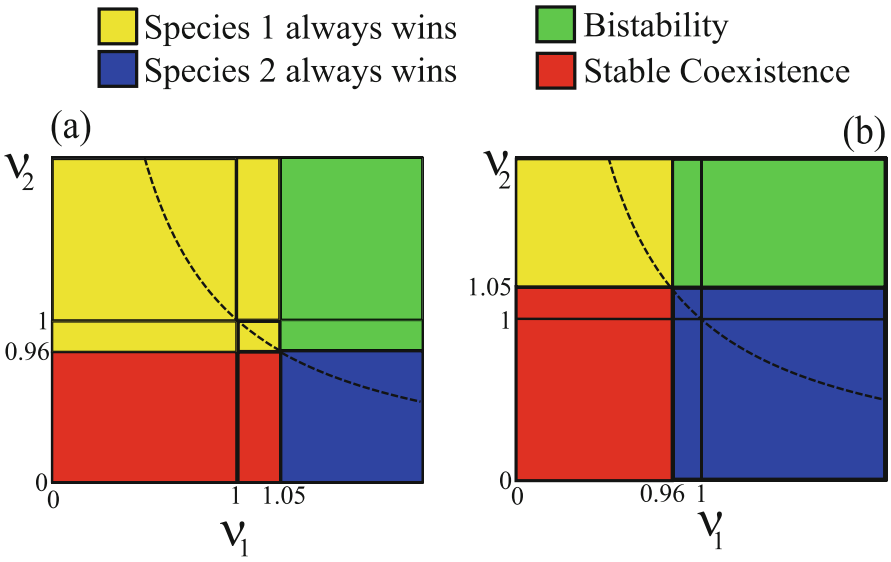

Fig. 4 The outcomes for the allelochemical warfare between two competing species. The value $r = 1$ was used in order to ensure equal species replication rates. Differences in allelopathic suppression relies on toxin release β and degradation rates δ, lethality μ, and efficacy c. In (**a**) the values $\beta_1 = 0.7$, $\beta_2 = 0.3$, $\delta_1 = 0.2$, $\delta_2 = 0.3$, $c_1 = 0.5$, $c_2 = 0.7$, $\mu_1 = 0.3$, and $\mu_2 = 0.4$ were fixed, which provide advantage to species 1 in the allelochemical warfare against species 2. Numerically, the lines $\nu_1 \lesssim 1.05$ and $\nu_2 \lesssim 0.96$ were found to limit the coexistence, bistability, and one species eradication regions. In (**b**) all the parameters fixed in (**a**) are interchanged in order to invert the species allelochemical traits.

addition to competition, allelochemical suppression and spatial heterogeneity are in action. So, our numerical analysis reveals that the present model may exhibit tristability – eradication of either species 1 or 2 and coexistence – ruled by the spatial population distributions.

Thus, depending on the initial population distributions in space, the invasion process can lead to three outcomes, namely, the eradication of the invader organism, the extinction of the native species, and the coexistence of both invader and native organisms. Such unexpected tristability was observed even for two species sharing the same competition coefficients and allelochemical traits. Such major finding is supported by recently studied ecosystems in which interactions involve the coordinate social behavior of the species as, for instance, gather and hunt in herds [30], i.e., nonclassical 1-1 interactions among competing individuals. Instead, since it is assumed that the individuals in both populations stick together, the interactions occur mainly via those individuals living at the perimeter of the territory occupied by the herds.

In system (3) there is no social behavior. Instead, the toxins released by each individual effectively diffuse throughout an area with a characteristic radius fixed by

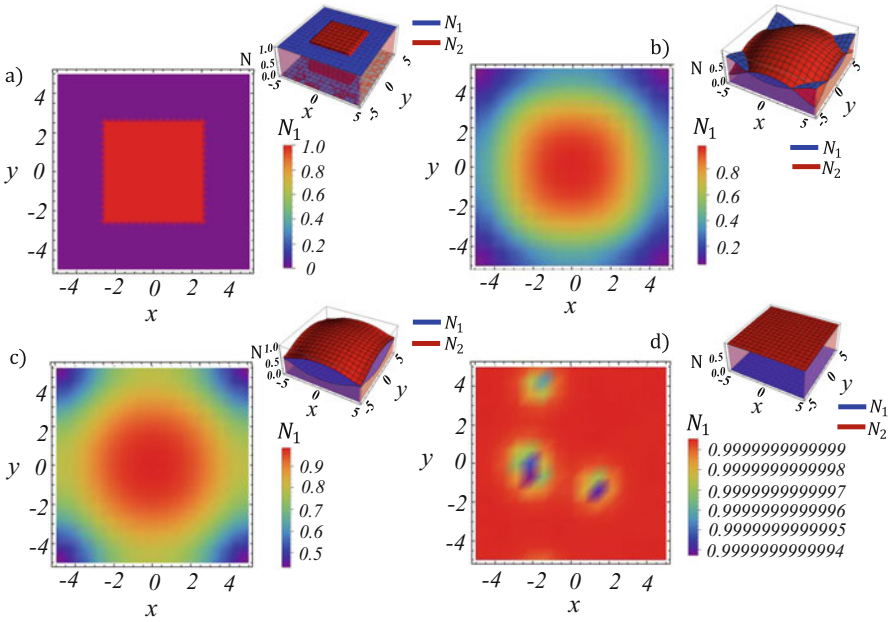

Fig. 5 (**a**–**d**) Evolution in time of a two-dimensional invasion process with allelopathic suppression. The invader species occupies only a 100 × 100 central square patch. The competition coefficients used were $\nu_1 = 0.9$, $\nu_2 = 0.09$ (within the space-independent coexistence range), and the toxins have equal sensitivities ($c_1 = c_2 = 0.1$) but distinct efficacies ($\mu_1 = 0.1$ and $\mu_2 = 1$). All the other parameters are the same for both species ($r = 1$, $D_1 = D_2 = 0.1$, $\beta_1 = \beta_2 = 0.5$, $\delta_1 = \delta_2 = 0.1$, and $\gamma_1 = \gamma_2 = 1.2$). (Figure taken from Ref. [28])

their diffusivities and degradation rates. Only within this area allelopathic suppression can be relevant. So, in the case of an invasion focus, the mutual allelopathic suppressions effectively occur into a rim around the focus. Again, interfacial or peripheral interactions are established, but without socialized behavior. These interfacial interactions emerge from the combination of allelopathy and patchy population distributions in space. It is just this combination that is lost in the regime of pure competition in which allelopathy is absent. Accordingly, the tristability is impossible in classical pure competition. Therefore, our model provides a distinct mechanism for an emergent tristability independent of social behaviors as those discussed in reference [30].

2.3 Spatial Patterns for More Species

It is straightforward to generalize system (3) to take into account any number of species engaged in a biochemical warfare. Such an extension is of paramount relevance for real ecosystems often involving interactions among more than three

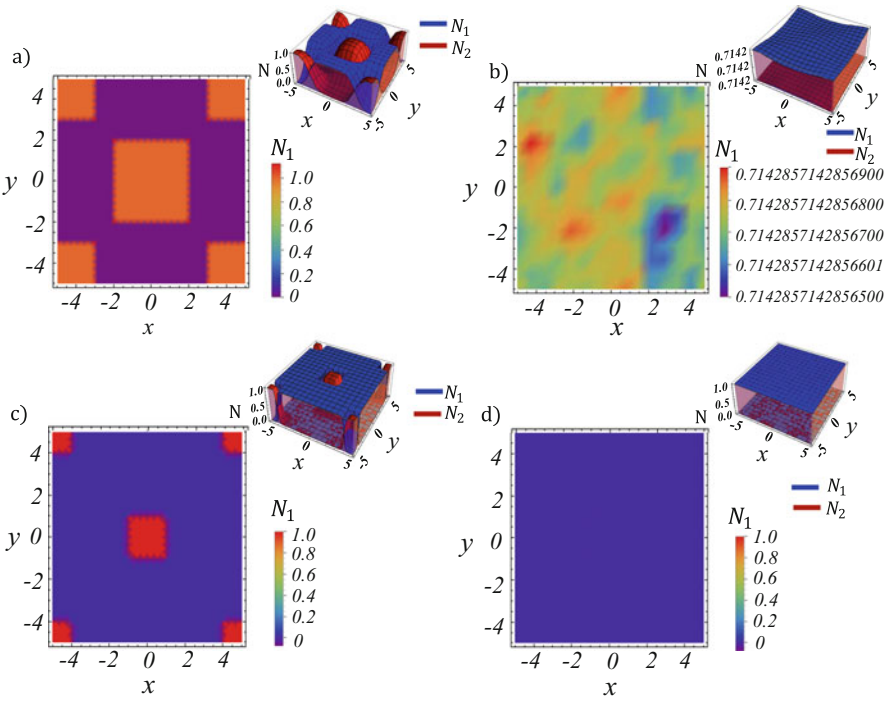

Fig. 6 (**a**) The same as in Fig. 5, but now the invasion leads to species coexistence illustrated in (**b**). In (**c**) the initial spatial area occupied by the species 1 was reduced leading to its eradication as shown in (**d**). The parameters changed were $\nu_1 = \nu_2 = 0.4$ and $\mu_1 = \mu_2 = 0.1$ (equal competition and allelochemical capacities). (Figure taken from Ref. [28])

species. The aim is to understand the self-organized pattern formation processes leading to species coexistence, a fundamental problem in ecology and evolutionary biology [31].

Recently, the paradigm to address the role of population mobility in coexistence and biodiversity is the three-species cyclic game model rock-paper-scissor (RPS) [32]. This model and its whole class of variants are based on individual agents and their rules for dispersion, predation, replication, etc. (the "microscopic" interactions). A hallmark result is that coexistence emerges at small mobilities from the interactions of entangled rotational spiral waves in the landscape. Since then the spiral wave patterns are thought to be the basic dynamical structure supporting coexistence.

In Fig. 7 are shown the spatial patterns and the evolution in time of populations for five species competing for resources and allelopathically suppressing each other. The five species are linked through cyclic suppressions as shown in Fig. 7A1. Our results are qualitatively similar to those obtained for the

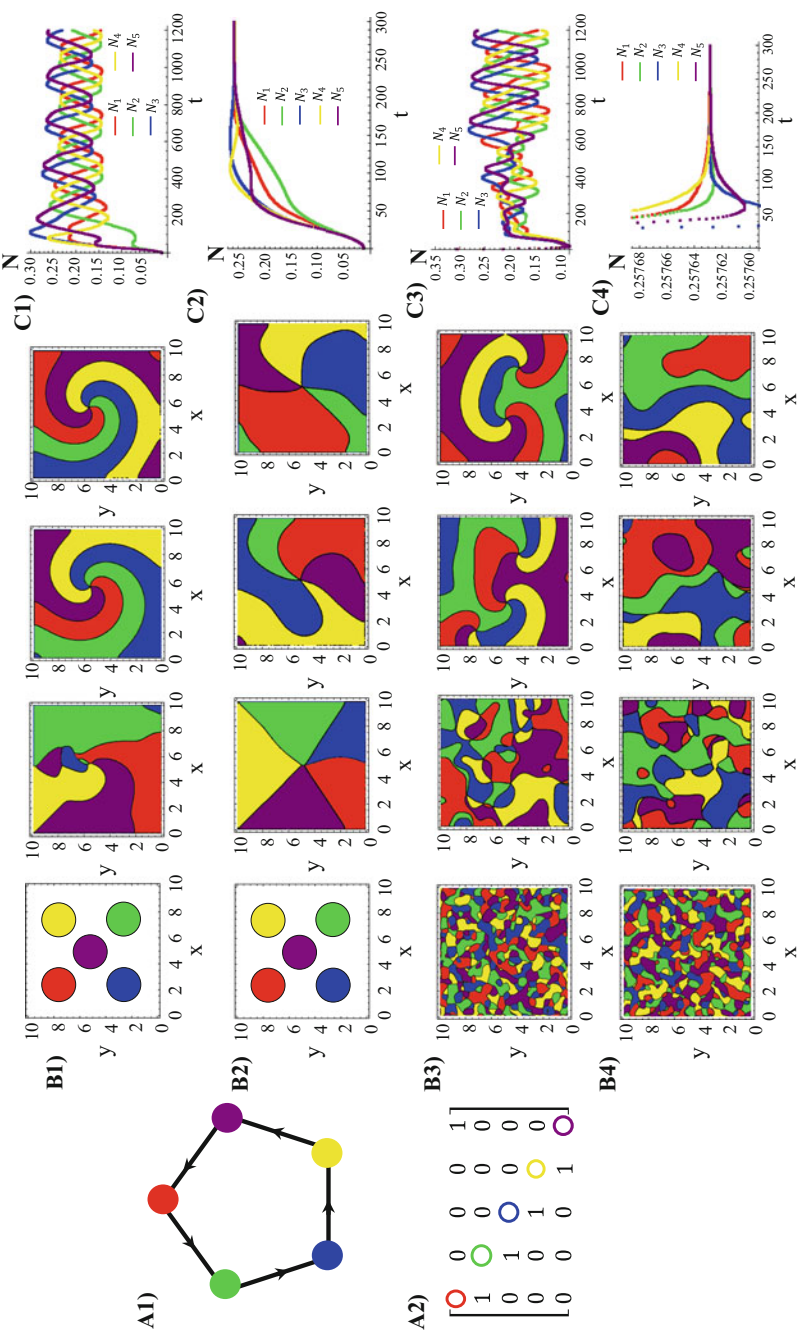

Fig. 7 (continued)

rock-paper-scissor-lizard-Spock (RPSLS) game model to five mobile species [33], although in our graph there are no suppressive interactions connecting each species to its two next nearest neighbors. In particular, our "macroscopic" model, Eq. (3) extended to account for five species, reveals the coexistence of all species even at high allelopathic suppression ($\mu = 0.4$) but small species and toxin diffusivities. For large diffusivities species are led to extinction, as illustrated in Fig. 8, and eventually one species dominates the entire landscape (see Fig. 9). Again, as in the RPSLS model, the coexistence or extinction is generated through the interaction of five distinct local spiral wave patterns. At last, the ecosystem dynamics is attracted (converges) to either a limit cycle, as illustrated in Fig. 7C1, C3 or a fixed point, as seen in Fig. C2, C4. In the case of all the allelochemical parameters maintained fixed, the warfare's outcomes will depend on the way toxins affect the species. If all the local amount of a toxin inhibits its target, the stationary state is either a limit cycle or stable focus. In turn, if only the locally uptaken toxin affects its target, the time evolution is driven to a fixed point.

Nowadays, two distinct mathematical approaches are widely employed to describe the evolution in time and space of biological populations. The first one, called macroscopic models, are based on coupled partial differential equations for continuous time, space, and state variables. The second one relies at the opposite extreme: totally discrete, agent-based models, as the evolutionary game dynamics [32–34] recently proposed. In such microscopic models, species interactions are implemented at the individual level via a set of mechanistic action rules. The key lesson of this subsection is that macroscopic models can provide results qualitatively consistent with those obtained using microscopic models. This is true except nearby the critical values of the model's parameter values as, for instance, the diffusivities on the onset of species extinctions. Inside these critical regions, the nature of spatiotemporal correlations and fluctuations in individual-based models is very distinct from those present in continuous models. Consequently, the correct critical (scaling) behavior is not captured by macroscopic models.

Fig. 7 Five species competing for resources and with cyclic allelochemical suppressions. (A1) Schematic illustration of five-species allelochemical warfare. Arrows point from suppressor to suppressed. (A2) The allelochemical interaction matrix associated with the graph in (A1). The initial populations are spatially distributed either regularly in single, disjunct, isolated, and circular patches (B1,B2) or randomly in adjacent, disjunct but disordered patches (B3,B4). The corresponding spatial distributions of the species at three distinct times are shown for each initial condition. The different colors indicate the locally dominant species. The evolution in time of the population densities are shown in (C1–C4). The results in (B1,C1) and (B3,C3) refer to response functions dependent on the local concentration of allelochemicals, whereas those in (B2,C2) and (B4,C4) are for response only to locally uptaken toxins. The competition and allelochemical traits are the same for all species. Their values were fixed in $D = 0.005$ (small diffusivities), $r = 0.3$, $\nu = 0.5$, $\mu = 0.4$ (high allelopathy), $\beta = 0.5$, $\delta = 0.1$, and $\gamma = 0.1$

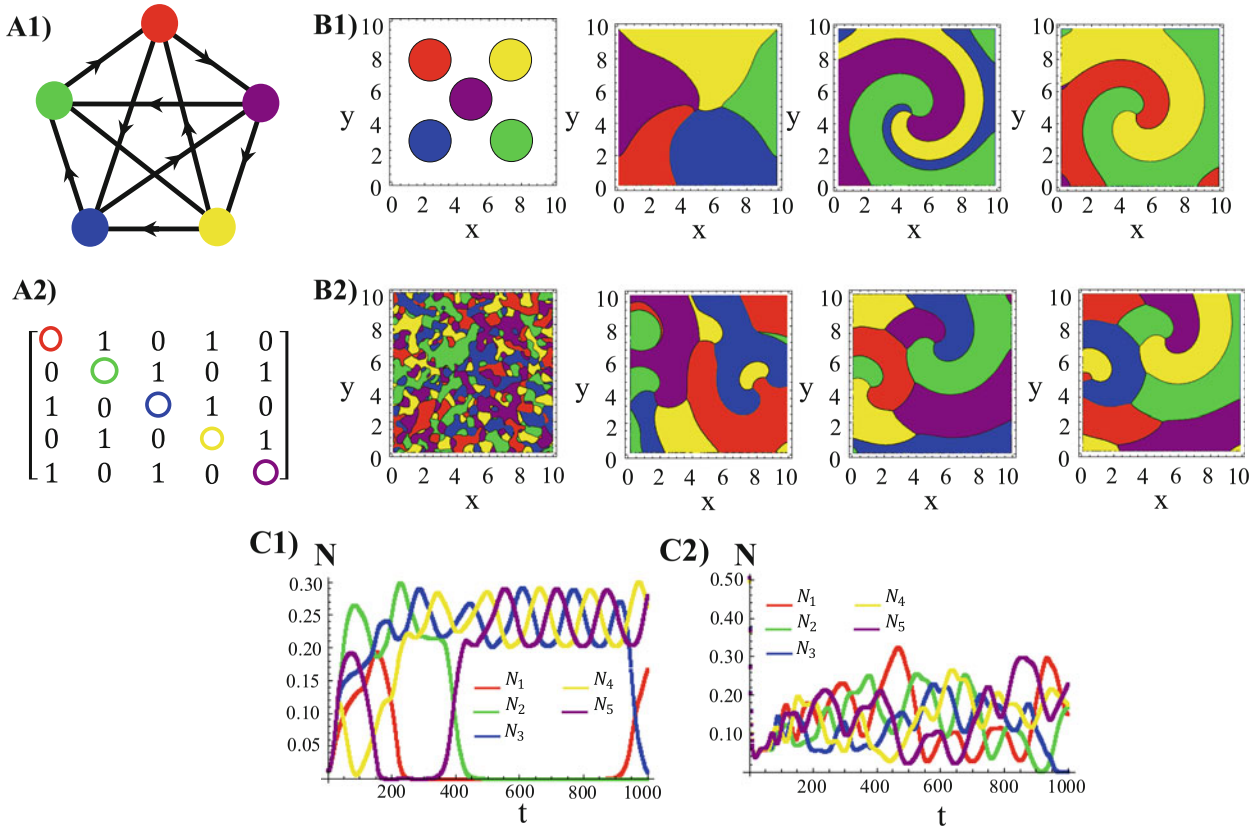

Fig. 8 The same as in Fig. 7, but for five species that, in addition to cyclic allelochemical suppressions, have a suppressor-suppressed relationship with each of its two next nearest neighboring species (A1). In (C1) and (C2) the extinction of two and one species occurs. The data refers to response functions dependent on the total local concentration of allelochemicals

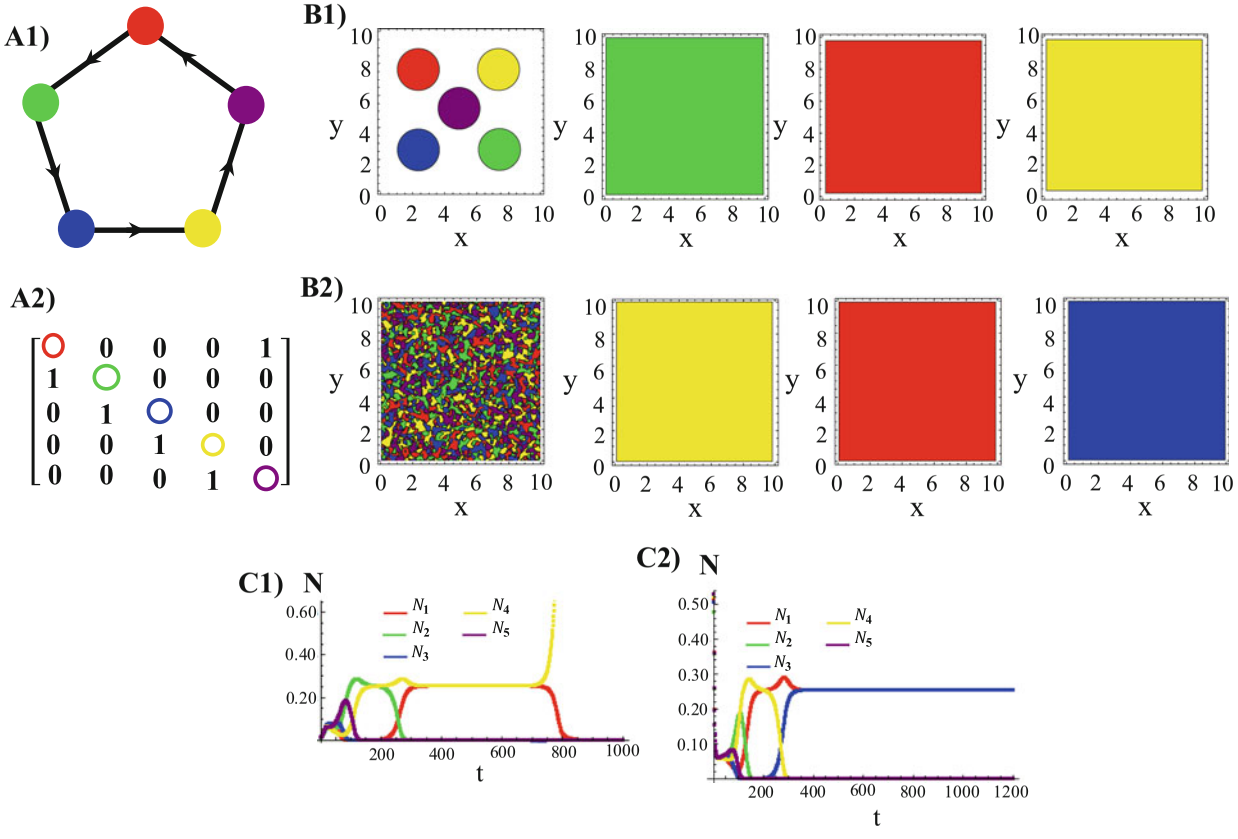

Fig. 9 The same as in Fig. 7, but for large diffusivities $D = 0.5$ and response functions dependent on the total local concentration of allelochemicals

3 Can Allelopathic Interactions Assemble a Community?

In this section, the question if biological communities can emerge from allelopathy, i.e., from competing interactions between their species mediated by toxins, is addressed. Intuitively, this alternative faces a difficulty: multiple toxic environments are the least expected to sustain species diversity.

3.1 An Eco-evolutionary Mathematical Model for Allelochemical Networks

In order to discuss how community structures of populations enforced to adapt and survive to the direct allelochemical suppression of each other are affected by the evolutionary history of the interaction, Eqs. (1) or (3) can be extended to include several species and to integrate ecological and evolutionary processes. In such generalized models, the genetic diversity is generated by mutations that induce changes in the allelochemical traits of the evolving species, and selection is driven by ecological interactions, namely, intra- and interspecific resource competition and allelopathic suppression. These interactions determine how species evolve and enhance or diminish the diversity of communities.

An example of this modeling strategy is the following model [35]. A set S of $l \in \mathbb{N}$ biological species with populations given by $\vec{N} = (N_1,\ N_2,\ .)$ is considered. Every species in S synthesizes and releases toxic secondary chemical compounds (microcins, fitotoxins, etc.) that enhance the mortality of other species. The strengths of such interactions depend on the toxin concentration $\vec{B} = (B_1,\ B_2,\ \ldots)$ and vary in time because B depends on the abundance of species. Furthermore, the community assembly proceeds from an initial subset $S_0 \subseteq S$ by randomly adding new species through mutations-fixed in a fraction of resident species offspring.

Ecological dynamics. The temporal evolution of the biological community in a homogeneous environment is described by the coupled ordinary differential equations

$$\begin{aligned} \dot{N}_i &= r_i\left(1 - N_i - \sum_{j\neq i}^{l}\nu_{ij}N_j\right)N_i - \sum_{j\neq i}^{l}\mu_{ij}\Phi_{ij}^{(k)}\left(y_j\right)N_i \\ \dot{B}_i &= \beta_i N_i - \delta_i B_i - \sum_{j\neq i}^{l}\gamma_{ji}N_j B_i, \end{aligned} \tag{5}$$

the spatially homogeneous version of Eq. (3) in which N_i stands for the population density of the species $i = 1, 2, \ldots$ that produces the allelochemical concentration B_i, respectively. All terms and parameters in these equations have the same interpretation as in Eq. (3). The interacting parameters $\nu_{i,\,j}$, $\gamma_{j,\,i}$ and $\mu_{i,\,j}$ define

ecological networks in which the species are the nodes. Different Holling type *I*, *II*, and *III* functional responses were assumed:

$$\Phi_{ij}^{(k)} = \begin{cases} B_j & (k=1) \\ \gamma_{j,i} N_i B_j & (k=2) \\ \dfrac{B_j}{c_i + B_j} & (k=3) \\ \dfrac{\gamma_{j,i} N_i B_j}{c_i + \gamma_{j,i} N_i B_j} & (k=4) \\ \dfrac{B_j^2}{c_i + B_j^2} & (k=5) \\ \dfrac{\left(\gamma_{j,i} N_i B_j\right)^2}{c_i + \left(\gamma_{j,i} N_i B_j\right)^2} & (k=6), \end{cases} \tag{6}$$

where the parameters c_i control the toxins efficiencies in poison their competing species. All these response functions assume null thresholds for toxin effects, but those with $k \geq 3$ impose saturation to the allelopathic suppression. Also, the response functions indexed by odd *k*s involve the total toxin concentration, in contrast to those indexed by even *k*s for which only the absorbed toxin can induce responses.

The ecological interactions (competition and allelopathy) drive the dynamics (Eq. 5) toward an stationary state $\left(\vec{N}, \vec{B}\right)$ in a short time scale. This stationary state depends on the species initially present and their interaction networks. Eventually, even in the weak interspecific competition (coexistence) regime, some populations are led to extinction by allelopathic suppression, and the community diversity (species richness) decreases.

Evolutionary dynamics. The origin and maintenance of biological communities depends on the interplay between evolutionary processes and ecological interactions that allow species coexistence [36]. Ecological and evolutionary processes are integrated in our model by assuming that mutations in one of the competing species present at the current stationary state of the ecological dynamics generate a new species. This fresh species must survive and evolve in response to novel conditions, and the old species in the community must in turn evolve in response to the new species. Ultimately, the ecological dynamics is driven to another stationary state characterized by distinct populations and interaction networks. After that, additional genetic diversity is generated by adding different species to the current community and so on.

Here, the mechanisms for species introduction is called sequential invasion events (SIE), defined as follows. An alien species, the node $n + 1$, is added to a stationary state currently containing *n* species. It is assumed that the alien species competes for resources with all the *n* pre-existing species. Thus $\nu_{n+1,\,i}$, $\nu_{i,\,n+1} \neq 0$ for $i = 1,\ldots,n$. Concerning allelochemical suppression, the alien species affects k_{n+1}^{out} of the old

ones and is affected by k_{n+1}^{in} of them. So, k_{n+1}^{out} elements $\mu_{n+1,\,i}$ in the line $n+1$ of the enlarged allelochemical interaction matrix are randomly chosen and assigned to non-null values, and the remaining are set to 0. Analogously, k_{n+1}^{in} randomly chosen elements $\mu_{i,\,n+1}$ in the column $n+1$ of the enlarged interaction matrix are assigned to non-null values, and the remaining are fixed in 0. (See reference [35] for details.) Finally, the initial toxin concentration of the alien species is $B_{n+1} = 0$, and its population density is $N_{n+1} = 0.01N_i$, with N_i corresponding to the stationary population density of one species chosen at random between the n current members of the community. Regarding the initial community structure, the SIE evolutionary dynamics starts from a single species.

3.2 Numerical Results

Since our primary interest relied on how allelopathic suppression affects the community structure, $\nu_{i,\,j} = \nu = 0.1$ was fixed in order to ensure equal competition coefficients for every species in a regime of interspecific coexistence.

The scenario of equal (or homogeneous) allelopathic traits was investigated. Thus, each species has fixed toxin sensibility, $c_i = c = 0.1$, release, degradation, and uptaken rates, $\beta_i = \beta = 0.2$, $\delta_i = \delta = 0.2$, and $\gamma_{j,\,i} = \gamma = 0.1$, respectively, $\forall i,\ j$. In turn, two mortality rates induced by allelochemicals were considered, namely, weak ($\mu = 0.1$) and strong ($\mu = 0.5$) $\forall\ i$.

In Fig. 10, the average diversity is shown as a function of the number n_{SIE} of SIE. The diversity or species richness is defined as the fraction of species that survive at the community stationary state. As expected, weak allelopathic suppression allows the assembly of communities exhibiting large diversities. This is true for all response functions tested, and, as expected, the diversity decreases as the response to toxins increases. For instance, in our simulations, $\Phi^{(1)}(x) < \Phi^{(5)}(x) < \Phi^{(3)}(x)$ except for small ($x < 0.11$) or large ($x > 0.89$) toxin concentrations. In contrast, community diversity is drastically reduced at strong allelopathy for all response functions. As an example, the number of surviving species decreases from ~100, at weak, to ~8, at strong allelopathic suppression and response function Φ^1. In this strong regime, diversity seems to decrease slowly after it reaches a maximum as the number of invasion events increases. Also, the effect of toxins uptaken is significant as revealed by the right column in Fig. 10. In these graphs the response functions depend on the absorbed fraction of toxins, not on their total concentration present in the homogeneous environment. So, even the regime of strong allelopathic suppression ($\mu = 0.5$) at low toxins' absorption can become effectively equivalent to the weak ($\mu = 0.1$) regime.

In Fig. 11, the average connectivity of allelochemical networks is illustrated as a function of the number n of surviving species observed at the stationary state reached after a SIE. In a network of size n, the connectivity $C(n)$ is defined as the fraction of non-null elements in its $n \times n$ interaction matrix. Our results indicate that the average connectivity is essentially the same at weak ($\mu = 0.1$) and strong ($\mu = 0.5$)

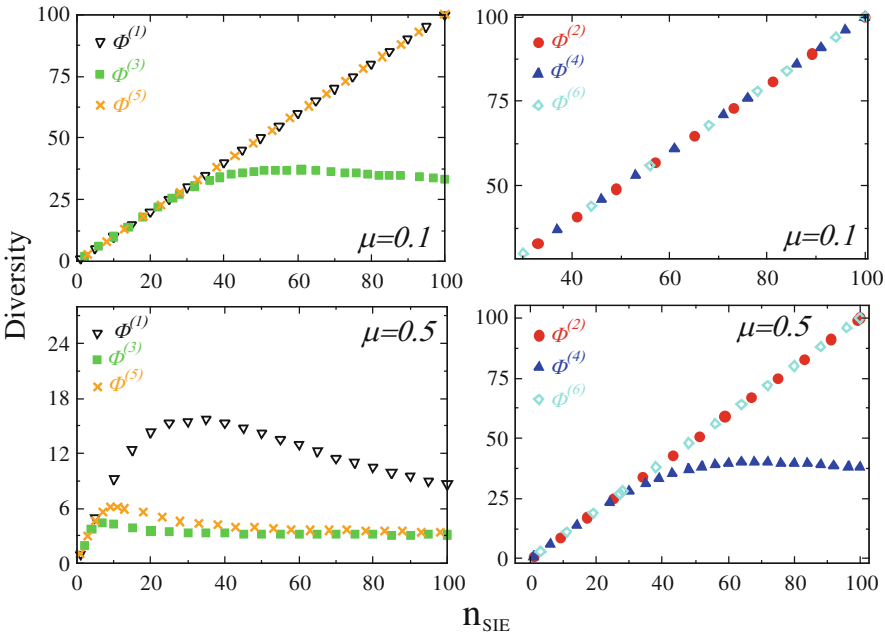

Fig. 10 Average diversity for 200 independent eco-evolutionary dynamics observed after successive invasion events. The initial community is always composed of a single species. The top and bottom plots refer, respectively, to weak ($\mu = 0.1$) and strong ($\mu = 0.5$) allelopathic effects. Homogeneous competition and allelopathy were considered ($\nu_{i,j} = 0$, 1, $\beta_i = 0.1$, and $c_i = 0.1$ were fixed $\forall i, j$)

allelopathy. So, the interaction network is sparsely connected ($C(n) \sim 0.5$) at both weak and strong allelochemical suppression. Furthermore, the connectivity initially increases up to $n \sim 10$ and saturates to a constant value and, for sensitive response functions ($\Phi^{(3,5)}$), exhibits significant fluctuations at strong allelopathic regime. This behavior is very distinct from the power law scaling for large n values observed in random networks, $C(n) \sim n^{-1}$ [31], and a model for growing random networks based on global stability, $C(n) \sim n^{-1.2}$ [18]. Therefore, our results indicate that the communities generated by the SIE dynamics markedly differ from random networks involving positive and negative interactions.

The degree distributions $P(k)$ for allelochemical interaction networks generated by the SIE dynamics are shown in Fig. 12. The distribution $P(k)$ gives the probability that a randomly selected node in a network has k links, i.e., it is connected to k nodes. Normal (Gaussian) and Weibull distributions were observed for in-degree distributions $P(k^{\text{in}})$ depending on the mortality μ and the functional response to allelopathy. For strong allelopathic suppression and functional responses $\Phi^{(1,3,5)}$, $P(k^{\text{in}})$ is a Weibull distribution. In contrast, at weak allelopathic suppression and for the response functions $\Phi^{(4,6)}$ at the strong regime, $P(k^{\text{in}})$ is Gaussian distributed. The apparent anisotropies observed in the insets for $\Phi^{(1,2,4,6)}$ are very weak, as supported

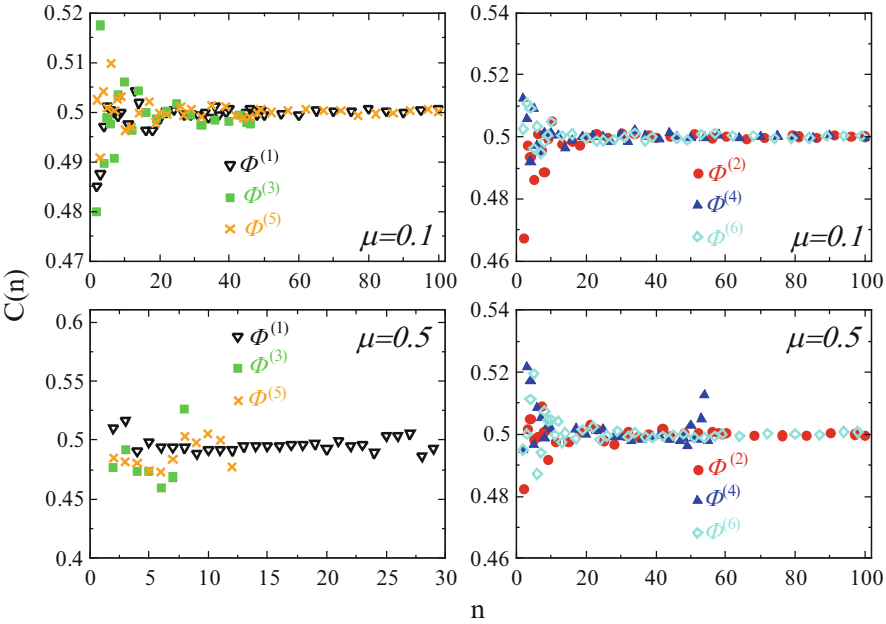

Fig. 11 Average network connectivity $C(n)$ in communities containing n species after a SIE. Again, the initial community is always composed of a single species. (Top) weak and (bottom) strong allelopathic suppression. The parameters $\nu_{i,j} = 0, 1$, $\beta_i = 0.1$, and $c_i = 0.1$ were fixed $\forall i,\ j$, but two distinct $\mu_{i,j} = \mu i \neq j$ were tested

by skewness $S \sim$ O and kurtosis $K \sim 3$ (see reference [35]). However, the ratio $\kappa = \langle k^2 \rangle / \langle k \rangle \sim \langle k \rangle$ is always obtained, indicating that the SIE allelochemical networks are homogeneous [37]. In turn, the degree distributions $P(k^{\text{out}})$ for all scenarios are normal (Gaussian) distributions (data not shown).

Typical allelochemical networks or community structures generated by the SIE dynamics are illustrated in Fig. 13. The nodes in these networks represent species present in the community, and the directed edges between them represent allelopathic interactions. The general rule is that as the allelopathic strength increases, the number of node (surviving species) decreases, but the network topology sustain a highly uniform connectivity pattern for every node. The combined effect of heterogeneous competition and allelopathy dramatically decreases the network size, as shown in Fig. 13c and d.

Lastly, the betweenness centrality measures the extent to which a node lies on paths of minimal length connecting to other nodes [37]. Nodes with high betweenness centrality often have significant influence on the network dynamics. Mathematically, the betweenness centrality x_i of a node i is defined as

$$x_i = \sum_{j,k} n^i_{jk}, \tag{7}$$

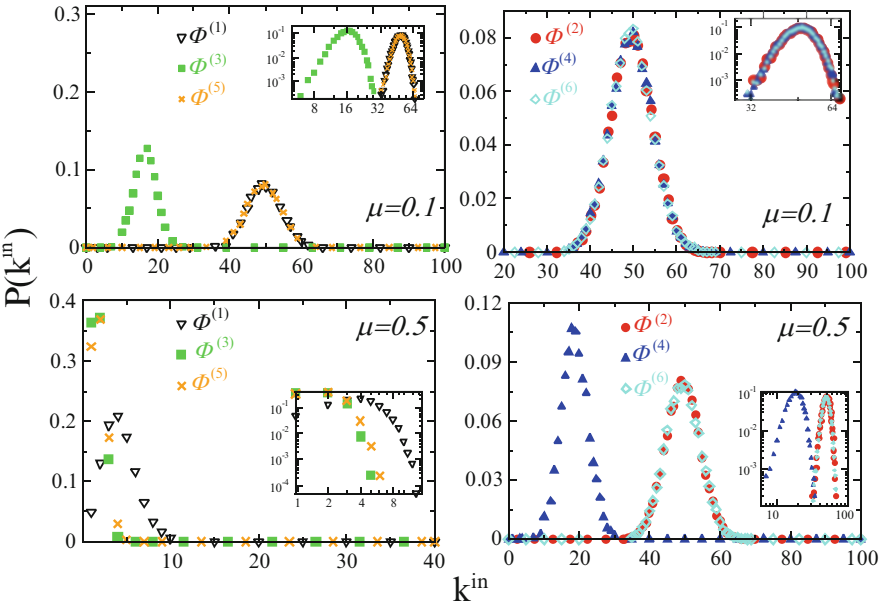

Fig. 12 Degree distribution $P(k^{\text{in}})$ for SIE allelochemical interaction networks in which the competition and allelochemical traits are the same for all species. The top and bottom graphs correspond, respectively, to weak ($\mu = 0.1$) and strong ($\mu = 0.5$) allelopathic suppression

where $n^i_{jk} = 1$ if the node i lies on the path of minimal length from node j to node k and $n^i_{jk} = 0$ if i does not or if there is no such path. In Fig. 14, the average $\langle x_i \rangle$ is plotted for every node i present at the stationary allelochemical network after $l = 100$ SIE. It can be noticed that $\langle x_i \rangle$ decreases dramatically as the strength of allelopathic suppression increases. Indeed, even at weak suppression ($\mu = 0.1$), strong responses to toxins ($\Phi^{(3)}$ lead to small average $\langle x_i \rangle$. Furthermore, despite small fluctuations, the average centrality is practically a constant function indicating a uniform connectivity pattern for every node and the absence of hubs, bridges joining distinct modules, and star graphs in the network.

All these results must be analyzed bearing in mind the scenario for pure intra- and interspecific competition. As the models reported here assume, in the coexistence regime (weak competition, $\nu_{ij} < 1 \ \forall \ i, \ j$), all the surviving species at every stationary state constitute fully connected networks. Since some introduced and/ or resident species are eventually extinct, the community diversity tends to be smaller than the number of invasion or speciation events. Yet, communities with high diversity are the rule. This scenario changes if allelopathic interactions exist.

In the SIE dynamics, ecological networks grow through a succession of species immigration. These alien species allelochemically suppress and are suppressed by resident species at random, eventually leading to the eradication of either the invader or some resident species. Our results, shown in Fig. 10, reveal that communities exhibiting large diversities can be assembled at weak allelopathy, but diversities are

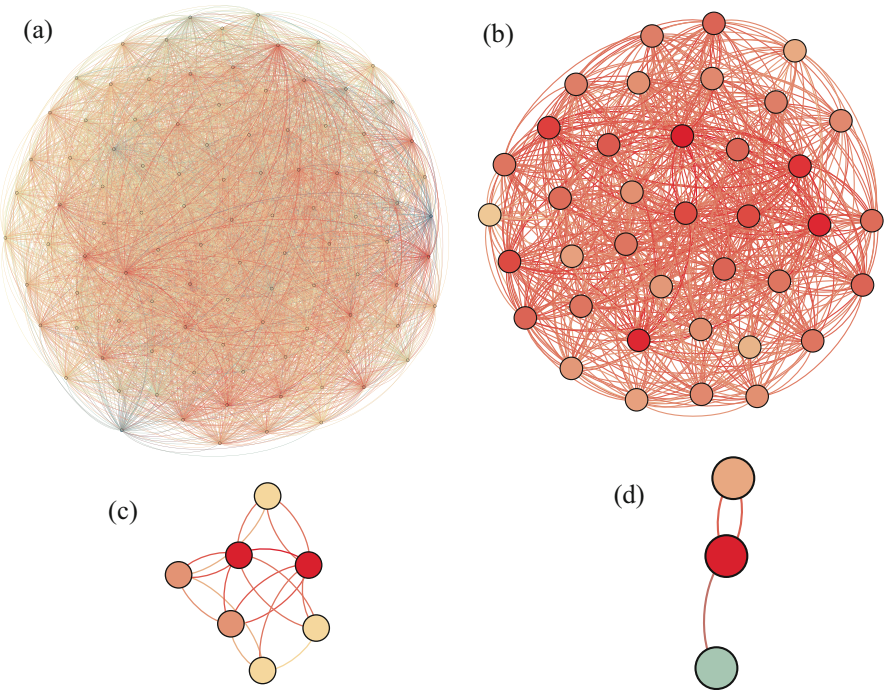

Fig. 13 Typical allelochemical networks generated after $l = 100$ SIEs for (**a**) weak ($\mu = 0.1$) and (**b**) strong allelopathic suppression ($\mu = 0.5$) for homogeneous competition and allelochemical traits ($\nu_{i,j} = 0, 1, \beta_i = 0.1$and $c_i = 0.1, \forall i, \ j$). (**c**) Heterogeneous competition ($\nu_{i,j} \in (0, 1]$ randomly chosen) but weak and homogeneous allelopathy $\mu = 0.1, \beta_i = 0.1$, and $c_i = 0.1, \quad \forall\, i, \ j$). (**d**) Both, competition and allelopathy are heterogeneous ($\nu_{i,j}, \quad \mu_{i,j}, \quad \beta_i, \quad c_i \in (0, 1]$ randomly chosen)

drastically reduced for all response functions effectively leading to strong allelopathy. Furthermore, in the strong suppression regime, species richness either saturates or decreases slowly after reaches a maximum. The maxima occur after ten or more invasion events, depending on the response function to toxins. At the maxima, the average number of species in the communities never exceeds 38. So, the system of interacting species becomes unstable, and the networks stop to grow, consistent with the limit found by May [38]. Beyond these upper bounds, the number of surviving species decreases continuously after each SIE until rest only one (a successful invasion) or very few species, as seen in Fig. 13. Accordingly, the network connectivity distributions change from normal (or Gaussian) to Weibull distributions (Fig. 12). However, the average connectivity of the stationary networks remains constant, as shown in Fig. 11. This suggests network structures with uniform patterns of connectivity for every node, free from hubs, modules, or star graphs, consistently with the structures seen in Fig. 13. Such networks, a subset of almost null measure in a random ensemble, can only be generated through a constrained growth process. Here, the growth process favors the attachment of nodes with few

links, since they modify the interaction matrix stability much less than new nodes with many links.

Summarizing, species-rich communities can be assembled in a homogeneous environment only at weak allelopathy, and even in this regime, species interact with a few others. The plankton paradox stands in the context of a total biochemical warfare between organisms. Maybe, the coexistence of positive (or activatory) and negative (or inhibitory) interactions is necessary to generate stability and diversity. But this will be the focus of future works.

4 Multiscale Modeling for Allelopathy

The complexity and diversity of biological phenomena; the range of spatial and temporal scales over which they act, extending from the molecular to the organism and ecological levels; and the intricate way in which they are interwoven make practically unfeasible the understanding of living systems through intuition alone. Therefore, theoretical multiscale approaches are an essential tool in the quest for a quantitative, "ab initio" ecology. This section will highlight multiscale modeling frameworks to understand the allelochemical warfare between living organisms.

4.1 Multiple Scales in Allelopathy

Plant invasions are intrinsically multiscale in nature. They involve phenomena occurring over a variety of spatial scales ranging from geographic (for instance, regional extinctions of species) to molecular length scales (e.g., use and break of metabolites by microbial communities), while the timescales vary from seconds for signaling events leading to cell death induced by a phytotoxin to tens of years for doubling times of invaded areas. Moreover, all those processes, many of which may still be unknown, are strongly coupled. Indeed, the synthesis of a secondary metabolite may confer a competitive advantage to a given plant, increasing its abundance and altering microbial communities, nutrients, and chemical mosaics in the soil which, in turn, regulate the growth and spatial distribution of plants. To survive in a phytotoxic environment, some plant species may acquire new traits such as metabolic detoxification mechanisms that confer resistance to phytotoxins and promote coexistence. Thus, information flows not only from the finer to coarser scales but between any pair of scales.

The complexity of plant invasion through allelopathy manifests at least in three scales that might be distinguished and described in mathematical models: microscopic, mesoscopic, and macroscopic [4, 7, 39]. Specifically:

- The microscopic scale refers to molecular and subcellular phenomena occurring within the plant cell or at its plasma membrane. Examples are transcriptional events associated with phytotoxic response, dynamics of signaling cascades, and/or metabolic pathways triggered by oxidative stress or defense purposes, fluxes of

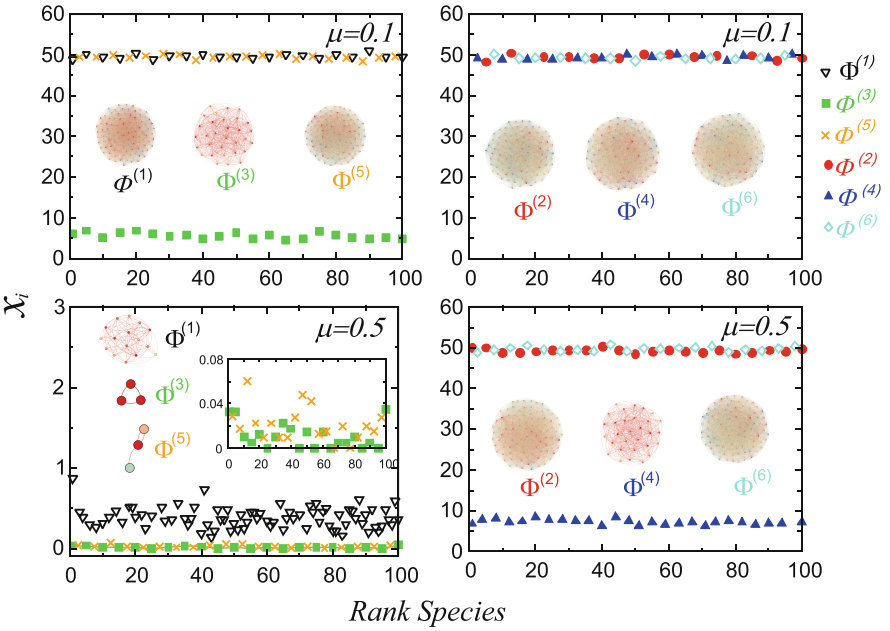

Fig. 14 Average betweenness centrality for each node (surviving species) in communities generated from a single initial species after $l = 100$ SIE. Homogeneous competition and allelopathy, i.e., equal traits for all species, are assumed. (Top) Weak, $\mu = 0.1$, and (bottom) strong, $\mu = 0.5$, allelopathic suppression. Insets: very small but nonvanishing centralities for the response functions $\Phi^{(3)}$ and $\Phi^{(5)}$. Also, typical networks generated in each scenario for distinct response functions are shown

ions, protein secretion, and exudation of micro- and macromolecular metabolites by root border and epidermal cells, etc.

- The mesoscopic scale refers to physiological processes occurring in the rhizosphere or at plant level such as root-root and root-microbe communications, root colonization and growth, seed germination, waves of cell death along the roots, seedling mortality, reduced shoot differentiation, and inhibition of plant growth elicited by phytotoxins, etc.
- The macroscopic scale concerns with processes occurring at the ecosystem level such as invasion fronts, convection and diffusion of nutrients and chemical compounds, seed dispersion, community integration and coevolution, chemical patterning of the soil, etc.

In a multiscale approach, each scale of interest is described in terms of distinct physical models, and all of them are coupled in a single model [40, 41]. Such interwoven levels of description is the main feature typifying multiscale models, neatly evidenced in the general framework expressed through model Eqs. (8, 9, 10, 11, 12, 13, and 14), introduced in the next subsection. Indeed, phenomena at the cellular level (microscopic scale) affect the plant dynamics (mesoscopic scale) and

vice versa, because plant interactions can also alter cell states and consequently the nature and timing of the subcellular processes at the microscopic scale. In turn, plant replication and death and the external introduction of new plants, events associated with the mesoscopic level, lead to new distributions of source/sink of chemical factors and nutrients as well as generate moving boundary conditions that specify the boundary-value problem for these continuous fields at the macroscopic level. Again, in counterpart, the distribution of nutrients and chemical factors in the landscape, components of the macroscopic physical state of the system, clearly affect both the microscopic and mesoscopic scales.

Mathematically, the link between the macroscopic, mesoscopic, and microscopic scales has to be referred to the parameters (growth, death, uptake or absorption, and degradation rates, threshold densities, diffusion coefficients, etc.) characterizing the model. Each parameter refers to a given phenomenon and has a particular effect on a specific plant population, or a chemical substance, or a subcellular process occurring in a plant species. Some of the parameters can be evaluated from biological essays, obtained from generic databases or derived from mathematical models.

So, plant invasion, sometimes called the green cancer, is neatly a multiscale, nonlinear dynamical problem as it is tumor growth in multicellular organisms [42, 43]. The fundamental evolution of these problems cannot be quantitatively described without the help of mathematical models.

4.2 A Multiscale Model for Allelopathic Suppression

The best way to illustrate how multiscale modeling works is to discuss a typical example. Here, an agent-based model for allelochemical suppression in plants, proposed by our group [44], is considered. This model integrates the plant (mesoscopic) and landscape (macroscopic) scales in plant invasion mediated by allelopathy. Specifically, it introduces an effective stochastic kinetics controlled by local probabilities as a strategy to connect the macroscopic diffusion equations for fitotoxins to plant response and interactions at the mesoscopic scale, a central challenge in developing multiscale models.

4.2.1 The Macroscopic Scale: Fitotoxins' Diffusion on the Landscape

The homogeneous environment is represented by a square lattice of $L \times L$ identical patches. Each patch or site has a size scale comparable to those of the plants' rhizosphere. Fixed, null boundary conditions simulating a closed system are used.

The phytotoxins exuded from the roots of invader plants disperse through the soil. It is assumed that the phytotoxin concentration field $F\left(\vec{x},\ t\right)$ is described by the diffusion equation:

$$\partial_t F = D\nabla^2 F + \sum_{\text{invader i}} \beta\delta\left(\vec{x} - \vec{x}_i(t)\right) - \gamma F. \tag{8}$$

This equation includes the simplest diffusive dynamics, the synthesis of the phytotoxin by the alien plants and its natural degradation in time as the only mechanisms involved in the spatiotemporal variation of the phytotoxin concentration. On the right hand side of this equation, the Laplacian term represents the Fick's diffusion which tends to equalize in space the phytotoxin concentration through its flux from regions of high concentrations to regions of low concentrations. In turn, the term containing a sum over Dirac delta functions $\delta\left(\vec{x} - \vec{x}_i(t)\right)$ models the synthesis of phytotoxins by spatially localized sources (invader plants) at sites $\vec{x}_i$ in time t. At last, the third term on the right hand side of the equation represents the degradation of the phytotoxin in proportion to its local, instantaneous concentration. In Eq. (8), D is the diffusion constant of the phytotoxin, and β and γ are its rates of synthesis and degradation, respectively. Thus, the γ term in this equation sets up a characteristic interaction distance between plants.

Dirichlet boundary conditions are imposed to the phytotoxin concentration field. The diffusion constant D and production and degradation rates, β and γ, respectively, are model parameters controlling phytotoxin dynamics. All of them are assumed constant in the model. Eq. (8) is numerically solved through relaxational methods on a square lattice with a lattice unit equals to the radius of the plant rhizosphere.

4.2.2 The Mesoscopic Scale: Plant-Fitotoxins Interactions

Initially, all patches in the landscape are occupied by the native species, except the center of the lattice invaded by an alien plant. Each plant is a cellular automaton (CA) [45, 46] or simply an individual agent. The initial ages of plants are drawn stochastically with uniform probability in the range 1 to $t_{\max}$, the maximum longevity permitted, a parameter model. The effects of competition operate primarily on the individual, eventually affecting its reproduction, survival, and dispersal. The following interaction rules define the CA evolution.

Plant reproduction. The plants can begin to disperse seeds at the age t_m corresponding to the onset of reproductive maturity. Mature plants produce seeds with a probability $p_s = (t_{\max} - t_m)^{-1}$, meaning that in average each one produces a seed crop along their life cycle. Every invader plant produces n_0 seeds, whereas native ones have their seed production affected by the local phytotoxin concentration F according to the expression

$$n_s = \begin{cases} n_0 e^{-a\left(F\left(\vec{x},t\right)-\theta\right)} & , \text{ if } F\left(\vec{x},\ t\right) \geq \theta \\ n_0 & , \text{ otherwise.} \end{cases} \tag{9}$$

where n_s is the number of native seeds produced, a is a parameter measuring the phytotoxin inhibition of seed production, and θ is the concentration threshold for phytotoxicity.

Growth (aging) and death. At any time step, an alien plant can die with a probability $p_d = 1 - q$, in which q is the adult survival probability. For native plants the death probability is affected by the local phytotoxin concentration F as follows:

$$p_d = \begin{cases} 1 - qe^{-b\left[F\left(\vec{x},t\right)-\theta\right]} & \text{, if plant's age } \leq t_e \text{ and } F\left(\vec{x},\ t\right) \geq \theta \\ 1 - q & \text{, otherwise.} \end{cases} \tag{10}$$

Here, the parameter b is a measure of the phytotoxin's efficiency in kill native plants. So, the model assumes that the mortality of young native plants (age $\leq t_e$, where t_e is the establishment time) is an increasing function of the local phytotoxin concentration, but it is unaffected for established plants (age $> t_e$). In the case of plant death, the corresponding site becomes empty and available for future colonization.

In turn, the value of q is calculated so that the probability of a plant to survive over $t_{\max}$,

$$P(t > t_{\max}) = 1 - \sum_{t=1}^{t_{\max}} (1-q)q^{t-1} = 1 - \frac{1 - q^{t_{\max}+1}}{q}, \tag{11}$$

is smaller than 0.05 [47].

The age of every surviving plant is increased by a unit at each time step. Therefore, the model assumes that the natural survival probability q of native and invader species does not vary with age.

Dispersal and colonization. Mature plants (age $\geq t_m$) can produce n_s seeds at each time step with a probability p_s. These seeds are dispersed through a neighborhood of radius $r_{\max}$ of the site of the parental plant accordingly an exponential distribution:

$$n(r) = \frac{n_s}{1 + \sum_{r=1}^{R_{\max}} e^{-r/r_c}} e^{-r/r_c}. \tag{12}$$

Here, r_c is the characteristic length of seed dispersion, and distances are determined using the Manhattan metric ($d_{ij} = |x_i - x_j| + |y_i - y_j|$). So, the model assumes that seed dispersion is spatially isotropic in a two-dimensional lattice and uses a cutoff length $r_{\max}$ in order to truncate the exponential distribution for seed dispersion. The number $n\left(\vec{x},\ t\right)$ of seeds present in every site decays at a rate γ_s after each time step. Thus, the parameter γ_s determines the seed viability in time. Finally, open boundary conditions, for which any site do not receive seeds from outside the lattice, were used.

Seed germination occurs only in empty sites with a fixed probability $p_g = p_0$ for the seeds of the alien plant, whereas their native counterparts germinate with a probability that decreases as the local phytotoxin concentration increases. Specifically, the probability of native seed germination is given by

$$p_g = \begin{cases} p_0 e^{-c\left[F\left(\vec{x},t\right)-\theta\right]} & \text{, if } F\left(\vec{x},\ t\right) \geq \theta \\ p_0 & \text{, otherwise.} \end{cases} \tag{13}$$

Once germinated, native and/ or alien species can colonize the empty site with a probability

$$p_c = 1 - \left(1 - p_g\right)^{n_{\text{seeds}}}, \tag{14}$$

where n_{seeds} is the total number of seeds of the corresponding species (native or invader) present in the site. The empty site will be colonized by the plant species having the greatest p_c value. If eventually both native and alien plants have the same value of p_c, then one of them is selected with equal chance and wins the competition.

CA Simulation protocol. The CA simulations were implemented through the following procedure. Initially, a single invader plant with an age randomly chosen between t_m and $t_{\max}$ was introduced in the center of the lattice. At each time step, (i) plants can die with probability p_d; (ii) any survival plant ages, and the mature ones can produce and disperse seeds with probability p_s; (iii) the empty sites can be colonized, with probability p_c, by native or alien plants; (iv) the non-stationary amount of phytotoxin is determined according to Eq. (8) for each lattice site; (v) the quantities of interest, namely, evolution in time of plant populations, gyration radius, and roughness of the border of the invaded region, are determined. At the end of this sequence of actions, a new time step (Monte Carlo step-MCS) begins, and the entire procedure is iterated. Additional details can be found in reference [44].

4.3 Simulational Results

In order to investigate the controversial role of allelopathy in plant invasion, simulations were performed using basically the same values of the parameters for native and invader species. So, both native and alien plants have exactly the same skews in the competition for resources if allelopathic suppression is neglected. These values, listed in Table 1 of reference [44], were inspired in typical herb species with annual life cycle such as *Euphorbia heterophylla* L. It must be noticed that the model parameters are rather arbitrary since the details of most invasion processes are largely imprecise. This apparent handicap really represents the strength of the model able to investigate the dynamics of invasion in a broad range of parameter values.

Figure 15a shows the invasion probability as a function of the ratio between the seed productions of invasive and native plants. In turn, the invasion probability as a function of the initial fraction of resistant plants in the native community is shown in Fig. 15b. Here, resistant plants have a phytotoxic threshold 100 times greater than the “normal” native plants. As expected, the chance of a successful invasion increases if the alien plants produce more seeds than the native species. Also, the invasion probability decreases as the fraction of resistant plants increases in the native community. Finally, Fig. 15c and d show the invasion probability as a function of the phytotoxic threshold and the maximum dispersion radius of the alien seeds, respectively.

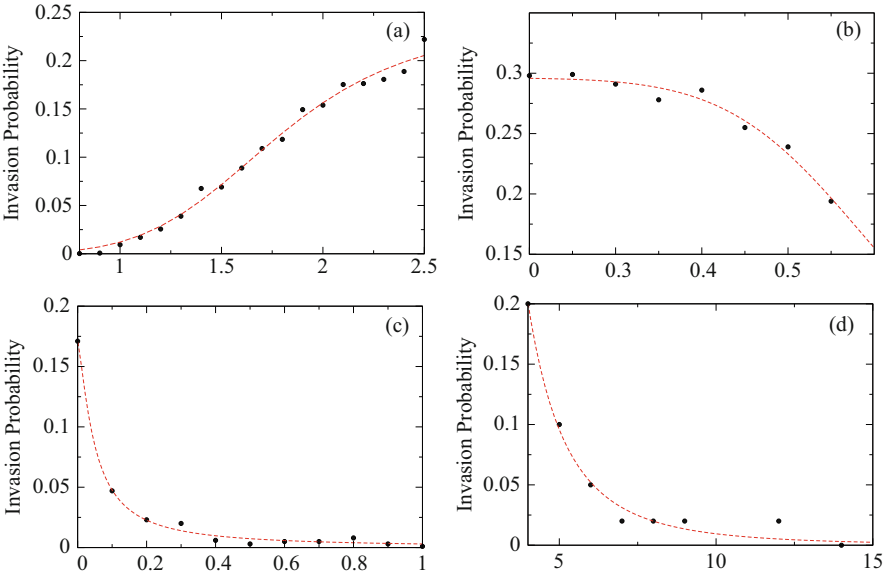

Fig. 15 Invasion probability as a function of (**a**) the ratio between the numbers of seeds produced by the invasive and the native plants, (**b**) the initial fraction of resistant plants, (**c**) the phytotoxic threshold, and (**d**) the maximum dispersion radius of the alien seeds. Here, the linear length of lattices used is $L = 256$; the data correspond to averages over 5000 independent samples and to a total evolution time of 6×10^3 MCS. (Figure taken from Ref. [44])

Typical evolutions in time of native plant populations in successful invasions are shown in Fig. 16. The fastest decrease of this population occurs when the native community is homogeneous and constituted entirely of plants with a low resistance to the alien phytotoxin. The slowest decay of the native population occurs in heterogeneous communities with resistant plants distributed at patches in the habitat.

In Fig. 17 are shown spatial patterns of invasion at different time steps corresponding to a homogeneous native plant community with low resistance to the phytotoxin secreted by the alien species. These invasion patterns are circular with gyration radii that scale as the square root of the numbers of invader plants N_{inv} and smooth surfaces (Hurst exponent $H = 1$) at the asymptotic limit. In turn, the morphology of the invasion patterns changes if a fraction of the native community exhibits a high resistance to the phytotoxin. Typical invasion patterns for resistant plants initially distributed in patches on the native habitat are shown in Fig. 18. It can be noticed that the invasion patterns lost the radial symmetry, yet their gyration radii scale again as $N_{\text{inv}}^{1/2}$. However, the border of the invaded region is rough and characterized by a Hurst exponent $H < 0.5$. In addition, spatial patterns of resistant plants also change with time, exhibiting large patches nearby the invasion front and an exponential decay of their cluster size distributions (see reference [44]).

In all the simulations, the gyration radius of the invasion pattern increased linearly in time, and thus the invasion speed is constant. The speed of invasion is

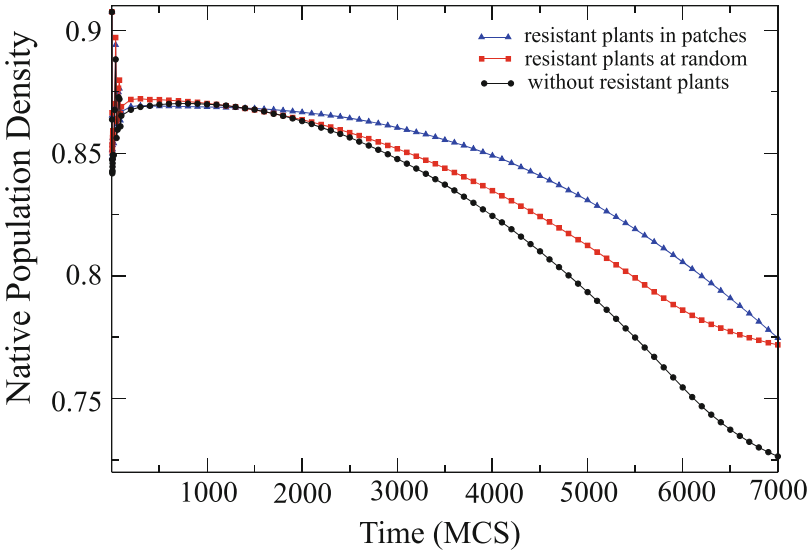

Fig. 16 Evolution in time of native plant populations in successful invasions. The data correspond to averages over 200 independent samples. (Figure taken from Ref. [44])

significantly lower only in the case of resistant plants initially distributed in patches. Furthermore, adjacent to the invasion front, a rim of empty sites is established, as seen in Figs. 17 and 18. So, the invasion progresses by suppressing the native plants nearby its expanding border.

This model demonstrates that seed production and dispersal distance, both traits associated with the invader species, as well as the native susceptibility to the alien phytotoxin determine the success probability and the speed of invasion. A greater seed production by the alien species is necessary, and a higher native sensitivity to its phytotoxin enhances the invasion success. Indeed, the chance of the invader plant to colonize new sites increases due to a larger alien seed bank present at this site coupled with a decreased germination probability of the local native seeds. Also, the greater death rate of the native juveniles affected by the invader phytotoxin further enhances the displacement of the native plants. However, a long-range dispersal of the invasive seeds has an opposite effect, since their seed banks nearby the already invaded area decrease. But it is just on the edge of the invaded region, where a greater phytotoxin concentration strongly impairs native seed germination and seedling establishment, that the colonization opportunities occur.

In addition, invasion occurs even in natural communities without disturbance, at least for allelopathic invaders (i) exuding phytotoxins with large and multiple effects on the native plants and (ii) having a higher rate of seed production. So, the model demonstrates that these two conditions are sufficient for a successful allelopathic invasion. The patterns of invasion shown in Figs. 17 and 18 range from circular with smooth fronts, in a homogeneous native community, to irregular shapes with rough fronts, in a heterogeneous native community containing resistant plants.

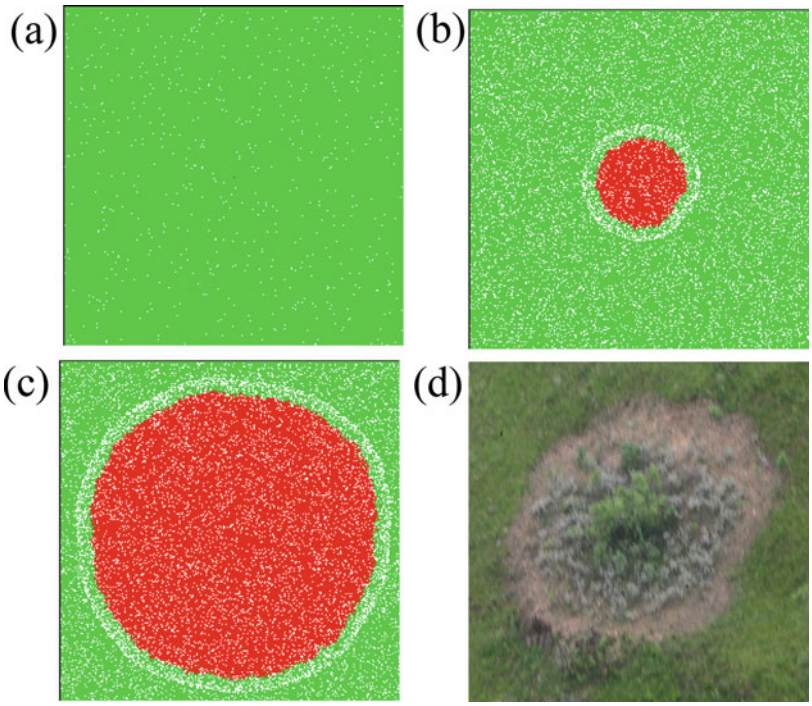

Fig. 17 Spatial patterns of a typical invasion in a homogeneous native plant community with low resistance to the phytotoxin secreted by the alien plant. Three different time steps (**a**) $t = 1$, (**b**) $t = 1000$, and (**c**) $t = 3000$ are shown. Green, red, and white pixels correspond to native plants, invader plants, and empty sites, respectively. Again, $L = 256$ was used. The invasion patterns should be compared with the one shown in (**d**) for the *Vochysia* sp. (*Vochysiaceae*), in Brazil. (Figure taken from Ref. [44])

Such patterns are, for instance, qualitatively very similar to the invasion patterns of *Vochysia* sp. (*Vochysiaceae*) (Fig. 17d) and *Myracrodruon urundeuva* (*Anacardiaceae*) (Fig. 18d), in Brazil. This naive comparison suggests that the variety of patterns generated by this model is able to represent the range of shapes associated with the allelopathic spreading of invasive plants with short-distance seed dispersal. Indeed, initial patterns distinct from the single seed used in simulations will certainly lead to spatial structures more similar to the patterns of allelopathic invasion observed in nature. Even branched or dendritic invasion patterns are possible in a heterogeneous environment in which, for example, the phytotoxin's diffusivity exhibits a spatial variation.

Concerning population heterogeneity, the inclusion of naturally more resistant native plants to the invader phytotoxins did not lead to a failure of the invasion processes but decreased their probabilities of success and lowered the invasion velocities. Moreover, the invasion front can be locally pinned by the resistant native plants, but eventually advances in between the blocked areas as shown in Fig. 19. From a biological point of view, this is an interesting concept that brings to the focus

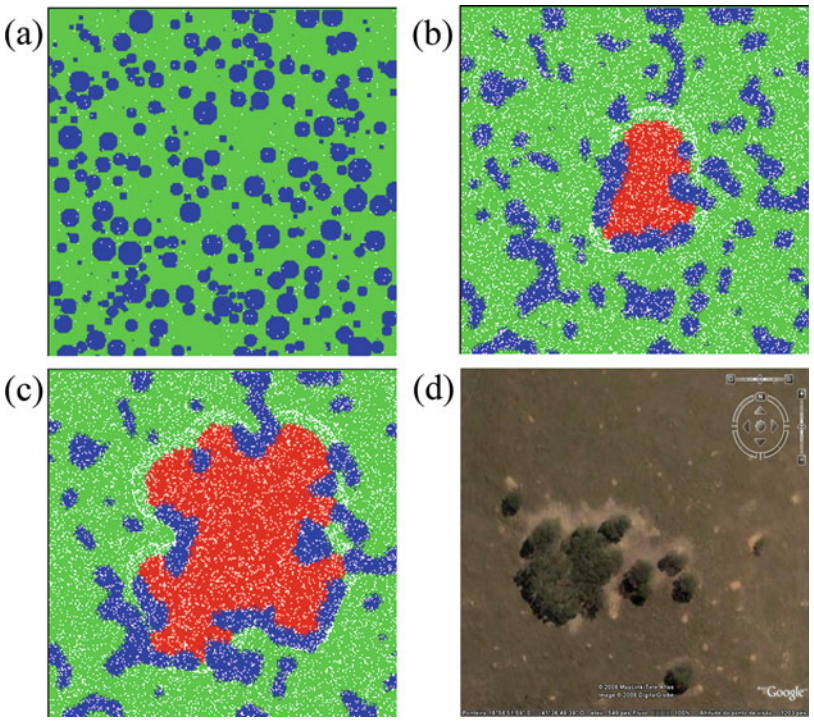

Fig. 18 Spatial patterns of a typical invasion in a heterogeneous native community with high-resistant plants distributed in patches. Three different time steps (**a**) $t = 1$, (**b**) $t = 1500$, and (**c**) $t = 3000$ are shown. For comparison, an invasion pattern of *Myracrodruon urundeuva* (*Anacardiaceae*) in Brazil is shown in (**d**). Green, red, and white pixels correspond to native plants, invader plants, and empty sites, respectively. Again, $L = 256$ was used. (Figure taken from Ref. [44])

the ecological factors that possibly determine the pinning-depinning threshold. Interfering in such factors in order to pinning the invasion front might act as an effective control strategy of biological invasions.

5 The Future of Theoretical Allelopathy

The complexity and diversity of phenomena underlying species competition, coexistence, and invasions; the range of spatial and temporal scales over which they act, extending from the molecular to the ecological levels; and the intricate way in which they are interwoven make practically unfeasible the understanding of the biochemical warfare between living organisms through intuition alone. The development of quantitative theoretical models for these phenomena, such as the one presented here, might be a very useful approach to deduce how distinct mechanisms interact to generate community stability and biodiversity, to integrate the rapidly

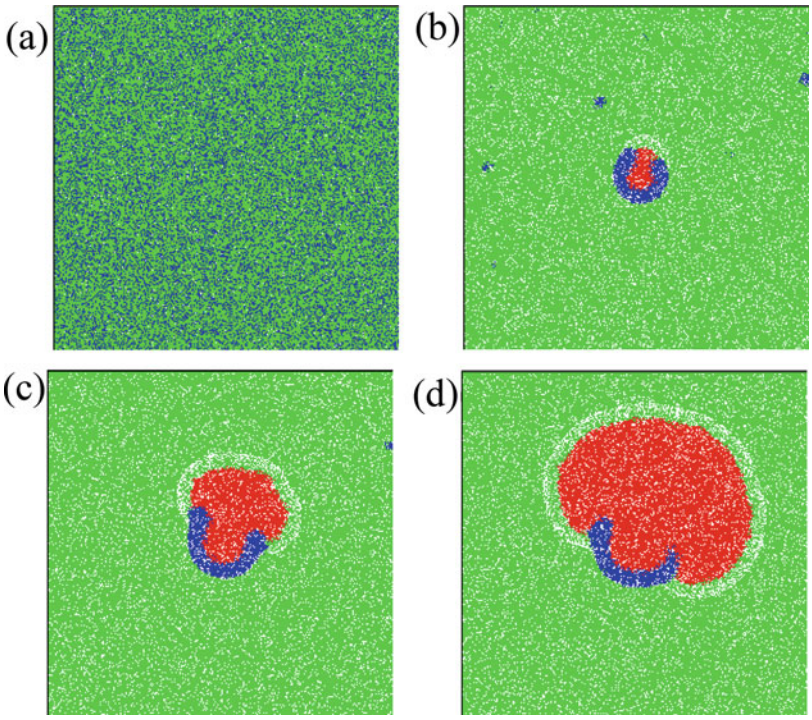

Fig. 19 Spatial patterns of an invasion process locally pinned by the presence of native plants resistant to the phytotoxin secreted by the alien plant. Four different time steps (**a**) $t = 0$, (**b**) $t = 1000$, (**c**) $t = 2000$, and (**d**) $t = 3000$ are shown. Green, red, and white pixels correspond to native plants, invader plants, and empty sites, respectively. Again, $L = 256$ was used. (Figure taken from Ref. [44])

increasing amount of information obtained at the various scales in accurate models, and to predict the macroscopic response of the system to control interventions. Such mechanistic models can provide real insights into critical traits that regulate invasion success, coexistence, and diversity in eco-evolutionary systems. They can also guide the design of new assays by indicating relevant processes for further investigation and prevent excessive experimentation needed to develop effective control strategies.

In particular, the future of multiscale modeling in biology seems to be promising in an age, the era of "omics," in which unprecedented views of organisms at work are being produced. Integrating all the facets of ecology and evolution beginning at the level of genes cannot rely on intuition alone. Theoretical multiscale approaches might be essential tools to quantitatively describe the complexity of biological communities and to predict its response to endogenous or exogenous imbalances. Clearly, computer simulations will be essential for the extensive investigation of detailed and realistic multiscale models. But, at least in the near future, in silico ecology will not be able to replace entirely field observations and

in vivo experimentation. Indeed, the inherent nonlinearity of biological systems imply in sensitivity to the initial conditions, even if their dynamics were completely known. Furthermore, they are probably algorithmically incompressible, and their simulation through any algorithm simpler than themselves can only be approximated.

Today, the quantitative success of multiscale modeling is limited, whereas the unresolved scientific problems are widespread. However, the increasing computer power, the development of inherently multiscale modeling and theoretical ideas, and the growing interest from physicists, mathematicians, and biologists on this type of multidisciplinary approach will certainly accelerate the progress and the broad applicability of the multiscale program in biological sciences. Hence, we can confidently predict a major role for multiscale models in future ecological and evolutionary research, transforming biology in a highly computer-intensive science. The hard calculations and extensive simulations necessary for the solution of multiscale mathematical models can be so vital to the rise of quantitative and predictive systems biology as biologists never dreamed before. As Cohen said: "Mathematics is biology's next microscope, only better" [48].

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Allelopathy for Weed Management

21

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Abstract

A large number of plant and weed species produce secondary metabolites known as allelochemicals, and the process is known as allelopathy. Allelochemicals can be used to control weeds in agricultural systems by using allelopathic crops for intercropping, crop rotation, or mulching. A few important examples of crop species with high allelopathic potential may include (but not limited to) wheat, rice, sorghum, rye, barley, and sunflower. The naturally produced allelochemicals in these crops could be manipulated to suppress weeds and witness an environment-friendly and sustainable agricultural production system.

Keywords

Allelopathy · Weed control · Allelopathic crops · Crop rotation · Intercropping · Cover crops

1 Introduction/Importance of Allelopathic Weed Control

Sustainable weed control is critical to ensure food security for future generations. Chemical weed control has been the most effective among the various weed management methods that have been used to control weeds in different crops and ecological conditions. However, the sustainability of chemical weed control is at stake due to evolution of herbicide resistance in weeds, environmental concerns, and damages to human health.

Allelopathy, a biochemical interaction among living organisms, can provide an effective environment-friendly alternative to chemical weed control [1, 2]. Various natural herbicidal compounds have been identified from different microbes and crop species [1, 3, 4]. These natural phytotoxins could be applied directly as natural herbicides or could be used to develop novel herbicide mode of actions [5]. Allelopathy can be used to manage weeds in field crops through intercropping, cover cropping, crop rotation, mulching and residue incorporation, and allelopathic extract and by utilizing hormetic potential of allelochemicals [2, 6].

In this chapter, we have discussed the potential allelopathic crop and weed species and the possible ways to utilize allelopathic potential for weed management in field crops. Moreover, challenges to allelopathic weed control and future directions are also discussed.

2 Rich Sources of Allelochemicals

2.1 Allelopathic Crops

Various crop species have shown allelopathic potential that can be used to manage weeds in field crops by using them as cover crops, surface mulch and/or residue incorporation, intercropping, and rotation and using crop extract with reduced dose of herbicides [7]. Researchers have screened various crop cultivars with strong allelopathic traits. Common field crops including rice (*Oryza sativa* L.), wheat (*Triticum aestivum* L.), sunflower (*Helianthus annuus* L.), maize (*Zea mays* L.), canola (*Brassica napus* L.), sorghum (*Sorghum bicolor* L.), millet (*Pennisetum glaucum* (L.) R.Br.), and buckwheat (*Fagopyrum esculentum* Moench) possess variety of allelochemicals that can be used to suppress weeds [1, 7]. Alfalfa (*Medicago sativa* L.) a common fodder crop cultivated worldwide provided significant control (up to 80%) to various weeds of rice ecosystem [8]. Rice a major cereal also possess various herbicidal compounds; Xuan et al. [9] reported that rice allelopathy can provide up to 88% control of weeds. Similarly, buckwheat residues caused up to 80% weed control in rice field [10]. Furthermore, allelochemicals released from sunflower, rye (*Secale cereale* L.), wheat, and sorghum could be utilized to provide effective weed control in various crops [1, 11–19]. Allelopathic potential of crops against weeds varies among crop species. For example, Batish et al. [91] tested the weed control potential of 35 crops; all tested crops showed weed suppression potential; some common crops including rice, wheat, maize, sugarcane, alfalfa, and vegetable crops (cucumber (*Cucumis sativus* L.), soybean (*Glycine max* [L.] Merr.), fennel (*Foeniculum vulgare* Mill.), and carrot (*Daucus carota* L.)) showed strong allelopathy and even caused autotoxicity under some conditions. Various allelochemicals having inhibitory effects against the weeds have also been identified from different common crop species [7, 20].

Different genotypes of the same crop may have different allelopathic potential. Thirty eight wheat cultivars were tested for their allelopathic effect on *Lolium rigidum* Gaud. Wheat cultivars showed differential allelopathic potential [84]. The above discussion indicates that the allelopathic potential of a number of field crops has been established in the last decades. These crops can be potentially used to manage weeds in agroecosystems in different ways to ensure sustainable weed control.

2.2 Allelopathic Weeds

A large number of allelochemicals that can suppress the growth of other plant species have been identified in various weeds. Similar to crops, weed species also produce allelochemicals; these allelochemicals are supposed to be more toxic because weeds normally grow under stress conditions. Different weed species including *Chenopodium album* L., *Medicago denticulata* L., *Melilotus indica* L., *Convolvulus arvensis* L., *Vicia hirsute* L., *Lathyrus aphaca* L., and *Rumex acetosella* L.

showed strong herbicidal potential to control *Phalaris minor* Retz. [21]. *Acroptilon repens* L., a commonly found weed in the western United States, showed herbicidal potential against *Echinochloa crus-galli* (L.) P. Beauv., *Agropyron smithii* Rydb., and *Bromus marginatus* Steud. [22]. Aqueous extract of different plant parts of *Croton bonplandianum* Baill. exhibited herbicidal potential against the weeds including *Melilotus alba* L., *Vicia sativa* L., and *Medicago hispida* Gaertn. [23]. Two weed species, i.e., *E. crus-galli* and winter cherry (*Withania somnifera* (L.) Dunal), were tested for their potential to control *Avena fatua* L., and allelopathic extracts from both the weeds inhibited the germination and seedling growth of *A. fatua* [24]. However, rare studies are available on identification and extraction of herbicidal compounds from weed species.

D'Abrosca et al. [25] identified 24 different phytotoxic compounds in *Sambucus nigra* L. belonging to various groups including lignans, cyanogenins, phenolic glycosides, and flavonoids. These phytotoxic compounds showed strong inhibitory effects on germination and growth of lettuce (*Lactuca sativa* L.), onion (*Allium cepa* L.), and radish (*Raphanus sativus* L.) [25]. Honeyweed (*Leonurus sibiricus* L.) contained various phytotoxic compounds that showed an inhibitory effect on rice, wheat, and mustard [26]. Aqueous extract of *Conyza canadensis* L. showed a strong inhibitory effect on various crops due to the presence of different phenolics, including gallic acid, syringic acid, catechol, and vanillic acid [27]. Sasikumar et al. [28] stated that the strong inhibitory effect of different plant parts of *Parthenium hysterophorus* L. on the germination and growth of various crops was due to the presence of phenolic acids identified in this weed. Similarly, *Chenopodium ambrosioides* L. and *E. crus-galli* also contain various phytotoxic compounds that were found to inhibit the germination and growth of different crop species [29, 97]. Zohaib et al. [30] reviewed more than 30 weed species containing phytotoxic compounds that showed strong inhibition against various crops and weeds; the phytotoxic potential of these weeds can be explored to manage weeds. The most commonly found phytotoxic compounds in weeds were alkaloids, fatty acids, phenolics, terpenoids, indoles, lignans, cyanogenins, flavonoids, and coumarins [30]. Furthermore, allelopathic compounds released from aquatic weeds showed more phytotoxic activity against various terrestrial weeds and crop plants [31], because plants of a certain ecosystem might be well adapted to the allelochemicals compared to the ones of any other ecosystem [31, 32]. Thus, phytotoxic compounds released from aquatic weeds can be identified and used as potential bio-herbicides. In crux, the use of weeds to make herbicides can be an environment-friendly option to control weeds in crops for sustainable crop production.

3 Ways to Use Allelopathic Potential for Weed Management

3.1 Intercropping

Growing of crops together at the same time in the same field is an important strategy to increase input (land, fertilizer, and water) use efficiency and to enhance crop yield and economic returns [33]. In addition, intercropping especially with

allelopathic crops can provide eco-friendly alternative to chemical weed control [34]. Recent studies have explored the effectiveness of intercropping with allelopathic crops as a good alternative to chemical weed control [6]. Intercropping of fodder legumes in maize helped to control the giant witchweed (*Striga hermonthica* [Del.] Benth) invasion than the sole maize crop [99]. Intercropping of various allelopathic crop species in maize was effective to control different narrow- and broad-leaved weed species [35]. Infestation of purple nutsedge (*Cyperus rotundus* L.) in cotton crop was significantly reduced with intercropping of sesame (*Sesamum indicum* L.), soybean, and sorghum on alternate rows [100]. In another field trail, the intercropping of white clover (*Trifolium repens* L.), black medic (*Medicago lupulina* L.), alfalfa, and red clover (*Trifolium pratense* L.) in wheat crop was effective to control various weed species and to enhance wheat yield [101]. Similarly, intercropping of pea (*Pisum sativum* L.) with barley (*Hordeum vulgare* L.) [102], sorghum with cowpea (*Vigna unguiculata* (L.) Walp.) [36], wheat with canola [37], and wheat with chickpea (*Cicer arietinum* L.) [38], reduced the weed infestation as compared to sole crop and enhanced farm income. Therefore, intercropping of allelopathic crops with the main crop has potential to control weeds through release of allelochemicals.

3.2 Cover Crops

Cover crops with allelopathic properties can provide effective weed control in addition to their other benefits including protection from soil erosion, snow trapping, nitrogen fixations, and improvement of soil structure and fertility [39]. The weed suppression potentials of cover crops including physical suppression, shade effect, decrease in temperature, and competition with weeds for inputs can be further increased through selection of strong allelopathic crops as cover crops. Furthermore, the release of allelochemicals from cover crops through root exudates, leaf shading, and washing by rain will help to decay the weed seedbank. The weed control efficiency of cover crops depends on its allelopathic potential and duration in the field; strong allelopathic crop for long duration in the field will provide more efficient weed control [40]. The weed control efficiency of cover crops also depends on weed species, e.g., sorghum as cover crop provides effective control of broad-leaved weeds; however narrow-leaved weeds were not controlled [41]. Environmental factors also influence weed control potential of cover crops by changing allelopathic potential, e.g., rye grown under nutrient stress conditions was more phytotoxic as compared to rye grown under high fertility [42]. Herbicide-resistant weeds, which are a major problem for sustainable weed management, may be controlled with allelopathic cover crops. The allelopathic crops that can be used as cover crops include rye, barley, sorghum, oat, wheat, canola, black mustard, buckwheat, clover species, and hairy vetch [39, 2]. In a recent study, allelopathic cover crops such as buckwheat and hairy vetch were effective in controlling apricot weeds [39].

3.3 Crop Rotation

Crop rotation is system in which different plants are grown in a sequence in a specific field for definite time period. It is important to reduce pest (weeds, pathogens, and insects) pressure, to overcome autotoxicity, and to sustain soil fertility [6, 43, 92]. Diversified rotation is key for sustainable weed control as it creates unstable conditions for weeds and helps to reduce weed seedbank [44]. Allelopathic crop in a rotation can potentially suppress its associated weeds and reduce weed infestation in the crop following in the rotation [45]. Both root exudates and decomposing crop residues an allelopathic crop in the rotation add allelochemicals to the soil that help to reduce weed pressure [46]. For example, weed infestation is reduced in wheat crop if grown following the sorghum crop due to release of allelochemicals from sorghum [47]. For instance, in sunflower-wheat rotation, the weed infestation in wheat crop grown after sunflower was considerably reduced [6]. Inclusion of rapeseed in rotation caused about 40% reduction in weed density in the subsequent crop in rotation [46].

Weed seed germination inhibition potential of allelopathic crop in rotation can also negatively affect the seed germination of subsequent crop in rotation. For example, wheat germination was delayed when it was grown in rotation with sorghum [48]. However, wise use of allelopathic crops in rotation and tillage timing can help to reduce the inhibitory effect on crop [49]. Therefore, good crop rotation with inclusion of allelopathic crop can help to avoid autotoxicity and to reduce weed problem with minimum dependence on chemical weed control method.

3.4 Mulching and Residue Incorporation

In allelopathic mulching, the crop or weed residues are applied on soil surface or incorporated in the soil. Mulching with allelopathic crop/weed residues inhibits weed germination and growth due to release of allelochemicals in the rhizosphere, physical suppressing and depriving weed seeds from light [50–53]. In addition to weed control, mulching increases water holding capacity, increases soil fertility, enhances organic matter, and works as buffer to maintain soil temperature [54–56]. Commonly, farmers use economic parts of the crop while incorporating the remaining crop parts in the field as organic matter. The allelopathic plant parts left in the field inhibit the weeds. Recently, many studies have been done to explore the weed control potential of allelopathic mulches and residue incorporation in field crops. For instance, application of sorghum crop straw as surface mulch in maize provided up to 37% weed control [57], while in cotton and rice, about 60% and 50% weed control, respectively, was achieved with sorghum surface mulch [58] Sorghum residue incorporation or surface mulches provided effective control of various noxious weed species including *C. rotundus*, broad-leaved dock (*Rumex obtusifolius* L.), *P. minor*, *C. arvensis*, *C. album*, and scarlet pimpernel (*Anagallis arvensis* L.) [59, 60]. In another field study, it was observed that maize residues added in the field after maize harvest caused significant reduction in weed infestation in the succeeding

broccoli (*Brassica oleracea* L.) crop [61]. Similarly, sunflower residues and surface mulches have potential to control various weed species in the field crops [62]. In another study, application of barely mulch in maize provided up to 80% weed control and 45% increase in maize grain yield over control [63]. Abbas et al. [50] reported that the mulches of allelopathic crops including rice, maize, sorghum, and sunflower at 12 t ha^{-1} provided effective control of herbicide-resistant *P. minor* in wheat. Mulches and residues of various crops including rye, clover, rice, maize, and canola have been reported for their potential as weed control [1, 7, 11–18].

Combined use of different allelopathic mulches can enhance their weed control potential due to the availability of diverse allelochemicals. Furthermore, allelochemicals have been known for their synergistic effect [6]. For example, combined use of canola, sunflower, and sorghum mulches provided more efficient weed control in maize as compared to the sole use of individual mulch material [64]. Therefore, residues of allelopathic crops can be used either as surface mulch or soil residue incorporation to control weeds in different crops.

3.5 Development of Herbicides from Allelochemicals and Their Derivatives

Herbicides with new modes of actions are badly needed due to fast-increasing herbicide resistance in weeds against all the major herbicide groups [65]. In addition, weed management in organic production systems is a great challenge [66]. Various natural herbicidal compounds have been identified from different microbes and crop species [1, 3, 4, 11]. These herbicidal compounds can be categorized in two major groups: phenolics and terpenoids [67]. These natural phytotoxins offer a great opportunity to be directly used as natural herbicides and to develop novel herbicide mode of actions [5]. The toxicity of allelopathic compounds depends on various factors including cultivar, plant part, concentration of extract, donor plant growth stage, and environmental conditions [68]. In this regard, several crop and weed species are now getting importance as a potential weed-controlling agent because of having various allelochemicals [1, 11–18].

In conclusion, allelochemicals from various crop and weed species can be directly used as herbicides or can provide basis for development of herbicides with new modes of actions.

3.6 Utilizing Hormetic Potential of Allelochemicals to Enhance Crop Competitiveness

The phytotoxic response of allelochemicals is dose dependent; allelochemicals cause growth enhancement (hormesis) at their low concentrations. The growth-promoting response of allelochemicals can be used to enhance crop growth. It will provide crop plants a competitive advantage over weeds. Allelochemicals can cause up to 50% and 42% increase crop growth under laboratory and

field conditions, respectively [69, 90]. The hormetic response of allelochemicals varied depending on the type of allelochemicals, source of allelochemicals, time of application, and crop trait [90]. For example, aqueous extracts of sorghum, maize, and rice at low concentrations caused up to 35% increase in maize grain yield; each extract caused different levels of enhancement [70]. Similarly, the sorghum extract at 3% w/v concentration caused up to 42% increase in canola and maize yield, respectively [71]. Allelochemicals from various sources have been reported for their hormetic effect on different crop species in field conditions both under normal and stress environments [90].

In addition to growth enhancement of crop plants, allelochemicals can suppress the weed growth directly by acting as herbicide. The selectivity can be achieved by applying allelochemicals at crop tolerant stage and weed sensitive stage (early growth stage) [90]. Therefore, hormetic potential of allelochemicals can be used to suppress weeds by providing crop plants competitive advantage over the weeds.

4 Challenges in Implementing Allelopathic Weed Control

Establishing allelopathic weed control as tool for weed management in field crops might be a difficult task as other interference appliances (competition for inputs, soil microbial impact, and nutrient immobilization) work in parallel [72]. Estimation of herbicidal potential of allelochemicals after their entry in soil is an important task because various allelochemicals only showed inhibitory effect in bioassays, but no inhibition occurs when applied with soil [72]. Moreover, various types of stresses in the ecosystem also influence the allelopathic effect [73]. Hence, it is difficult to prove the mechanism of allelopathy [74]. Type and concentration of allelochemicals released by any specific plant species depend both on plant factors (species, growth stage, and plant part) and environmental factors (soil fertility, moisture level, temperature, climatic conditions, etc.) [75]. Furthermore, fortune of allelopathic effect in soil is not well known [76]. Soil environment affects the activity of allelochemicals due to various physical, chemical, and biological interactions [77, 78]. Furthermore, in the complex agroecosystem, allelochemicals do not reproduce and are susceptible to chemical and microbial degradation. Herbicidal potential of allelochemicals is a collective/synergistic response of various chemicals in the mixture and not due to any particular chemical [75]. Thus, type of allelochemicals and their integrated effects in the mixtures is important to be considered.

High production cost (e.g., tentoxin), low efficacy, and poor selectivity are also major limitations in using allelochemicals as potential weed control agents [4]. These herbicides might be toxic to nontarget crop species, for example, a natural plant-released phytotoxin alpha-terthienyl extracted and isolated from common marigold (*Tagetes erecta* L.) roots for use as a herbicide was equally toxic to crop plants in addition to weeds [77]. Generally, allelochemicals have short half-

lives [79], and additionally the nature of allelochemicals, soil type, allelochemical concentration, and soil enzymatic and microbial community are also important [72]. Moreover, allelochemicals can be toxic to animals, e.g., fumonisin is toxic to animals and sorgoleone causes dermatitis [77]. Furthermore, allelochemical concentration (10^{-2}–10^{-5} M) that causes herbicidal effect is higher than the ideal concentration (10^{-5}–10^{-7} M) of natural herbicidal compounds according to environment safety standards [80]. In simple, issues regarding development of natural herbicides that form allelochemicals are much complicated and uneconomical as compared to synthetic herbicides. Additionally, less stability, low weed control efficacy, poor selectivity, and high cost are major limitations for development of natural herbicides by industries. However, the artificial modifications in the structure of plant-released herbicidal compounds may help to increase their selectivity and weed control efficacy. In addition, experiments considering the change in allelopathic effects with application of nitrogen fertilizers, activated charcoal, and environmental stresses may help to understand the fate of allelochemicals in soil.

5 Future Directions

5.1 Germplasm Selection to Enhance Allelopathic Potential

Importance of crop cultivars with improved weed suppressive ability has highly increased due to fast-increasing herbicide resistance in weeds and environmental concerns of herbicide use. In this scenario, it is important to breed crop cultivars with high allelopathic potential to suppress weeds and reduce herbicide usage. Different crop species and even the cultivars within same crop species vary in their allelopathic potential [81]; this weed-suppressing potential can be used as an alternative to herbicides. Studies on genetics of allelochemicals have not gained much attention in the past. The variability in allelopathic traits can be used to breed crop cultivars with more weed suppressive ability, e.g., rice produced from two inbred lines one with allelopathic gene and second with restorative gene had strong suppressive ability against *E. crus-galli* [82]. Crossing between old cultivars (having high allelopathic potential) with new crop cultivars can also help to enhance allelopathic potential [83]. Marker-assisted selection can help to develop crop cultivars with enhanced allelopathic potential. For instance, two major QTLs linked with allelochemical production were identified on 2B wheat chromosome [84]; thus allelopathic potential of wheat can be increased with the discovery of markers linked with the genes that control allelopathic traits. Recently successful attempts have been made to produce rice cultivars with high allelopathic potential in the United States (Arkansas), Asia, and Africa [85]. Bertholdsson [83, 86] also improved the weed suppressive potential of wheat and barley using breeding techniques; however the developed cultivars showed low grain yield potential. Further studies are required to breed crop cultivars with enhanced allelopathic potential and good yield potential for sustainable weed management.

5.2 Exploring Hormesis to Suppress Weeds

Hormesis of allelochemicals can play an important role for sustainable weed management. The dose-response effect of allelochemicals (inhibition at higher concentrations and growth enhancement at low concentrations) can be used in crop production to enhance crop growth with inhibition in weeds [90]. For example, application of hormetic dose to crop plants can produce herbicidal effect to weeds at their growth sensitive stage (early seedling stage). Optimization of dose, source, and application time of allelochemicals to produce hormesis in crop plants and inhibition in weeds might be an effective way to control weeds on a sustainable basis. Abbas et al. [90] reviewed that the various types of allelochemicals released from different crop and weed species produced hormesis (growth enhancement) in range of crop species at low doses, the same hormetic doses produced herbicidal effect when applied to weeds at their early seedling stage. Thus hormesis of allelochemicals will increase weed suppressive ability of crop by enhancing crop growth and by inhibiting weed growth due to their herbicidal effect. Therefore, future research in this regard will open new horizons for sustainable weed management.

5.3 Allelopathy of Unexplored Fields

The allelopathy of unexplored field can help to provide novel allelochemicals with more herbicidal potential. For example, allelopathic effect of aquatic weeds was more suppressive against various types of terrestrial weed species (Abbas et al. 2017). The susceptibility of crop weeds against allelochemicals from different ecosystem (aquatic in this case) was due to low adaptability of terrestrial weeds. Thus, more studies about determination and identification of allelochemicals of aquatic weeds can help to evaluate strong natural herbicide candidates. Furthermore, secondary metabolites released from lichen showed phytotoxicity to lichen photosynthetic process both when used alone and in the form of naturally occurring mixtures [87]. Similarity, allelochemicals released from fungi showed herbicidal effect against some weed species, e.g., *P. hysterophorus* L. [88]. Further studies in these ignored fields might identify novel strong herbicidal candidates.

5.4 Understanding About Mode of Action of Allelochemicals

Studies about how allelochemicals work have prime importance in allelopathic studies and require understanding at the molecular level, e.g., the structure of binding sites of protein or DNA. The knowledge about mode of action of natural phytotoxins can help to fasten the further industrial level consideration to produce natural herbicides. Recently few research attempts have been made to understand the mode of action of allelochemicals, e.g., Ren et al. [89] revealed that β-cembrenediol an important allelochemical inhibits the receptor plants through oxidative damage due to increased production of reactive oxygen species.

6 Conclusion

Allelopathic weed control can provide environmentally friendly tool to control weeds in cropping systems without dependence on chemical herbicides, as chemical weed control causes various hazards to environment, biodiversity, and human health. The use of allelopathic weed control through intercropping, crop rotation, cover cropping, mulches, residues, and water extract alone or in combination with synthetic herbicides will not only provide sustainable weed control but also sustainable crop production due to positive effects of these strategies on soil fertility, organic matter contents, and ecosystem biodiversity. Furthermore, efforts to motivate industries to produce allelochemical-based herbicides, breeding of more weed suppressive crop cultivars, exploring the allelopathy of unexplored fields, the use of allelochemical hormesis, and understanding about mode of action of allelochemicals will enhance the efficacy of allelopathic weed control.

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Prosopis juliflora: Phytochemical, Toxicological, and Allelochemicals

22

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Contents

Abstract

Prosopis juliflora (Fabaceae), which is also known as mesquite, is particularly invasive in exotic environments and has become one of the world's 100 most invasive species that is globally distributed. This scenario is mainly due to the allelochemicals released by its roots, leaves, and fruits that inhibit seed germination of neighboring species. Therefore, ecosystem-level changes create monospecific stands and impair the chemistry and biophysical properties of soil. The metabolites from *Prosopis juliflora* with allelopathic properties result from two major biosynthetic pathways: shikimic acid metabolites and piperidine alkaloids. Several *Prosopis* species have substantial impacts on biodiversity, ecosystem services, and local and regional economies in their native terrain; others provide multiple benefits to local communities. Overall, *P. juliflora* has demonstrated to

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be a versatile raw material, widely applicable in the food, cosmetic, pharmaceutical, agricultural, and renewable energy industries, providing progress in several fields of science and technology.

Keywords
Prosopis juliflora · Fabaceae · Mesquite · Alkaloids · Shikimic acid

1 Introduction

A plant's natural habitat offers several biotic and abiotic challenges, which, over time, stimulates the development of defense mechanisms in order to survive in such hostile environments [1]. Natural products that have toxic, repellant, or antidigestive effects, such as cyanogenic glucosides, glucosinolates, alkaloids, terpenoids, phenolics, and proteinase inhibitor, represent direct defenses against herbivores. In the same way, it can be highlighted that there are compounds released by plants that impair plant-plant interactions by inhibiting germination and seedling growth of neighboring species, providing more energy resources to the emitting plant and thus enabling better chances of survival [2]. Physical barriers, such as cuticles, trichomes, suberin, callose, and thorns that impede, for instance, pathogen ingression and arthropod access to plant tissues are also considered direct defense mechanisms [3, 4]. Therefore, this co-evolutionary struggle between plants, environment, and other organisms leads to an improvement in a huge variety of specialized compounds that may act as a direct or indirect guard against what is known as allelochemicals [5].

Allelochemicals are nonnutritional secondary metabolites produced by plants and other living organisms that display positive or negative effects upon the growth, health, behavior, or population biology of neighboring organisms [6]. In plant–plant allelopathy, the more common impaired functions are respiration; photosynthesis; water balance and stomatal function; stem conductance of water; xylem element flux; membrane permeability; cell division and development; protein synthesis; and enzyme activity alteration [7]. Allelochemicals are widespread throughout different plant structures such as seeds, flowers, pollen, leaves, stems, and roots, or sometimes can be found in just one or two such locations [2].

Strictly, allelochemicals should really only be applied to those substances established by quality experimental evidence (especially in genuine field circumstances) and not simply to a plant-derived compound which shows toxicity toward some (perhaps irrelevant) other plant during in vitro bioassay [2]. Studies have been reported in allelopathy showing the ecological mechanisms of exotic plant invasion as well as demonstrating ecosystem-level vegetation change following exotic invasion [8, 9]. Not only do these exotic plants bring with them novel allelochemicals that adversely affect germination and growth of native plants, but the chemicals stimulate the synthesis of allelochemicals by their rhizospheric biota [10, 11].

In this chapter, we attempt to discuss certain features of *Prosopis juliflora*, its most important allelochemicals, and its future prospects in order to make a rational use of this invasive species.

2 *Prosopis juliflora*

The *Prosopis* genus belongs to Fabaceae (Leguminosae) and is comprised of 44 species, in which 40 are native to the Americas [12, 13]. *Prosopis juliflora* is an invasive tree species present in arid and semi-arid zones, which is native to the rangelands in South America, Central America, and the Caribbean. This species has easy dispersion, grows fast, and is adapted to survive and grow in harsh desert environments [14].

In this context, *Prosopis* seedlings were deliberately distributed into myriad potentially suitable habitats across the Caatinga and the initial introduction of *Prosopis juliflora* was in the 1940s [15]. Despite its recent invasion history, *Prosopis* are the most widespread invasive plants in the Caatinga [16, 17], where they transform the native ecosystem, causing impacts on community structure, hydrology and soil properties [14]. This species is used by local populations as feed and as fodder, in the production of poles, cuttings, and fences or as fuel, in the form of firewood and charcoal [17, 18]. Some rural communities use this species as a medicinal plant, recommending its use for skin problems [19]. It is also important in honey production because its flowers are highly sought after by bees [20].

It can be easily identified as being a tree that reaches about 10 meters in height; branches with bipinous leaves and solitary spines at each node, reaching ca. 30 cm in length; flowers arranged in spikes, pedicelate, with light yellow coloration; indehiscent fruit, coriaceous, and segmented endocarp, fleshy and sweet mesocarp and yellowish coloration [20–22].

2.1 Allelochemicals from *Prosopis juliflora*

Prosopis juliflora, which is also known as mesquite, is particularly invasive in exotic environments and has become one of the world's 100 most invasive species that is globally distributed [23]. This scenario is mainly due to the allelochemicals released by its roots, leaves, and fruits that inhibit seed germination of neighboring species; therefore, the growth of neighboring plants is impaired leading to an increased availability of nutrients and water to ensure its survival [24]. In addition to the extensive use of allelochemicals, in conjunction with its unique seed regenerating strategy and perennial habit of the invasive plants, we can observe ecosystem-level changes creating monospecific stands and changes in the chemistry and biophysical properties of soil [8].

Regarding the production of allelochemicals, the defenses may be categorized as constitutive (or static) and induced (or active), when the defensive traits are only expressed in response to an environmental change or biological threat [25, 26]. Nevertheless, both defense kinetics are costly to plants, compromising its growth and reproduction; therefore, plants use sophisticated regulatory networks to maintain a balance between growth and defense response in order to ensure its survival [27, 28]. Advanced studies remain necessary to discover how exactly the plants manage this sophisticated network and its protective mechanisms. However, it is

already known that they are regulated by phytohormones (i.e., jasmonic acid) and triggered by specific signals which are further converted to large-scale biochemical and physiological changes, promoted by downstream signal events. Some mechanisms previously reported involve membrane depolarization, ion flux, activation of mitogen-activated protein kinase (MAPK), changes in gene expression, alteration of the plant proteome, and finally, production of specific specialized metabolites [5, 29].

Dry leaves of *Prosopis juliflora* mainly present phenolic-derivatives [30] and alkaloids, which were reported for the first time in the literature, being named after the species, and are known as: juliflorine [31], julifloricine [31], julifloridine [32], juliprosinene [33], juliprosine [34], juliprosopine [32], and mesquitol [35].

Previous research have attributed the allelopathy of *P. juliflora* to phenolic compounds [36] as well as to aqueous extracts of *P. juliflora* leaves, suggesting that the foliage species may contain water-soluble allelochemicals. These metabolites may be released into its vicinity by rain water from the leaves even before hitting the ground [37]. These lixiviated chemicals present an inhibitory growth effect on many plant parts and were isolated, evaluated, and identified as syringin, (-)-lariciresinol, L-tryptophan, juliprosopine, juliprosine, and juliprosopinal, in which, juliprosine and its derivatives demonstrated the most pronounced allelopathic activity (Fig. 1) [34, 38–40].

The metabolites from *Prosopis juliflora* with allelopathic properties result from two major biosynthetic pathways (Fig. 2): shikimic acid metabolites [(-)-lariciresinol for instance, is a lignoid (i.e., phenylpropanoids)] and piperidine alkaloids [secojuliprosopinal, derived from the acetic acid or polyketide metabolic pathway through the lysine amino acid pathway] [41].

The composition of individual metabolites and their concentration are not static but differs from organ to organ, not only within the developmental cycle of a plant but also within and between populations [42]. This variation, which leads to complex mixtures of secondary metabolites, is probably a strategy against the selection of specialized herbivores or pathogens as well as other plants [43].

2.1.1 *Prosopis juliflora* Alkaloids

In Brazil, *P. juliflora* is widespread in the Caatinga biome, providing shelter, helping to reduce soil erosion and improving the microclimate of these regions. In addition, this plant has an important use for humans and animals, as a source of food, feed, fuel, medicines, and cosmetics [24, 44]. However, the allelopathic potential of this species is shown by inhibiting the germination or growth of other plants around it, monopolizing spaces and nutrients [24]. This allelopathic potential might be caused either by fallen leaves or plant leachates or root exudates, through the release of allelochemicals into the environment [45, 46]. It is known that these allelochemicals can induce toxicity to other biotas [47–49]. These toxic effects are commonly reported in animals with free access to *P. juliflora* as a food source, and this toxicity has been attributed to the presence of alkaloids in the algaroba pods ingested by animals [50–52].

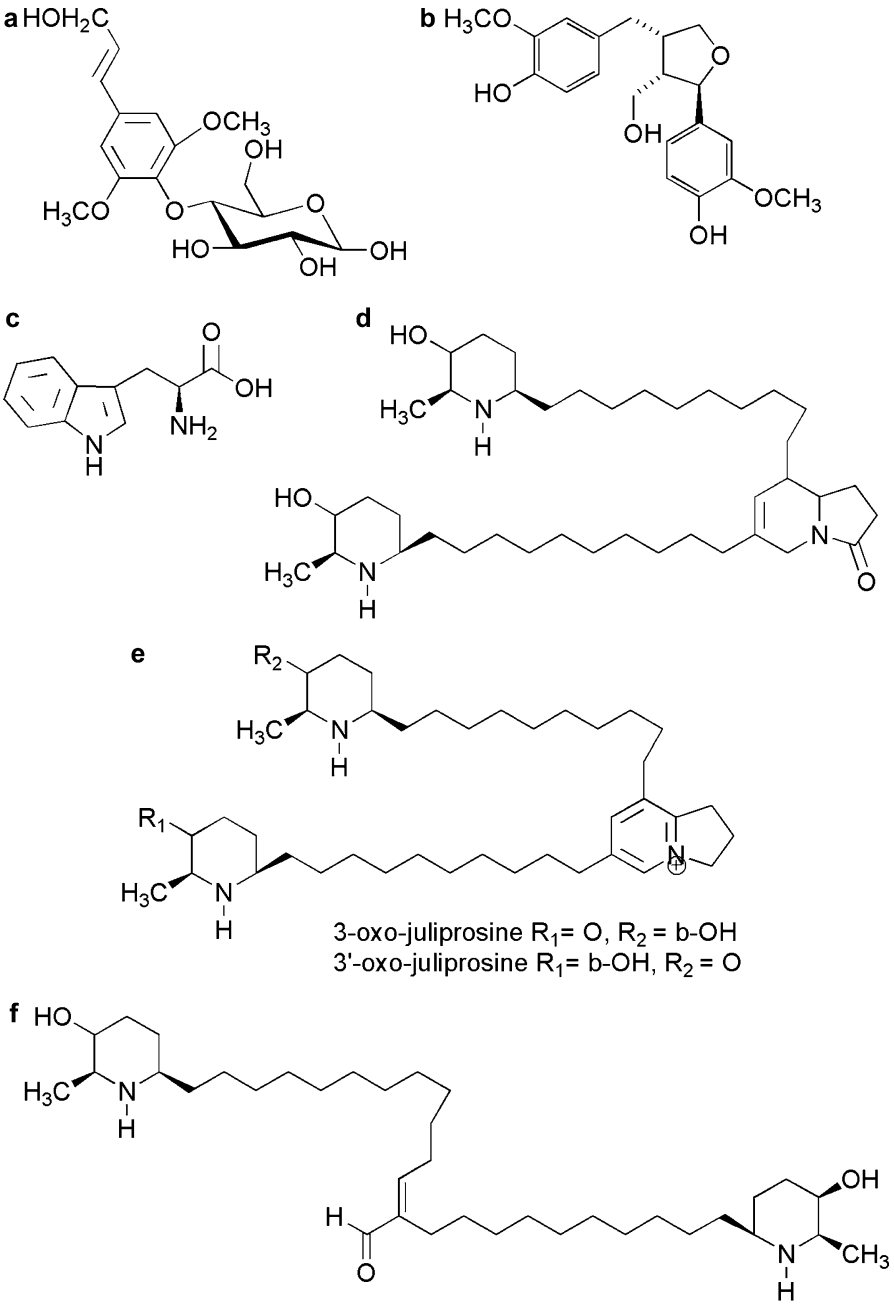


Fig. 1 *Prosopis juliflora* chemical compounds with growth inhibitory effect. (**a**) syringin, (**b**) (-)-lariciresinol, (**c**), L-tryptophan, (**d**) juliprosopine, (**e**) juliprosine, and (**f**) juliprosopinal

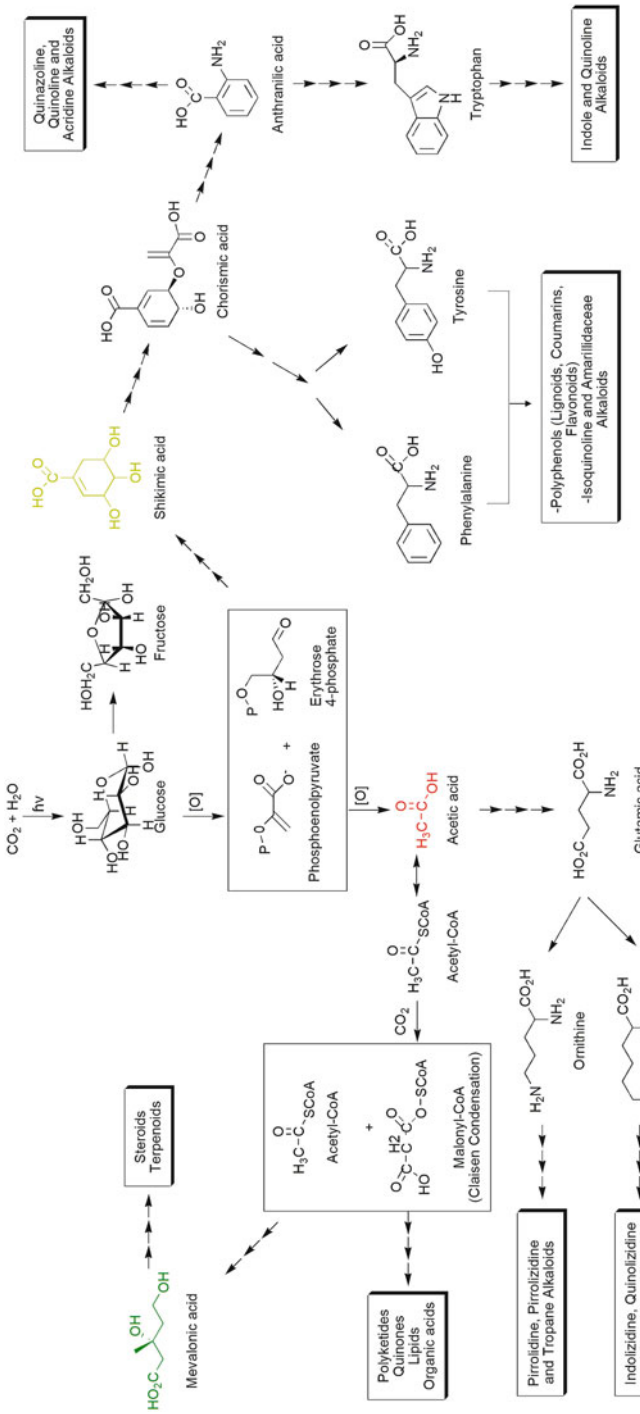


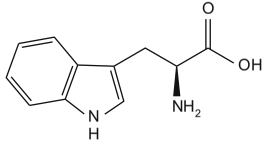
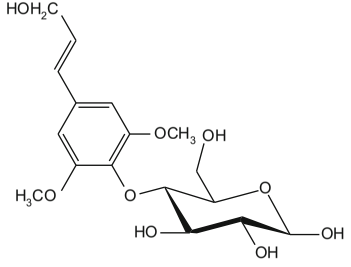
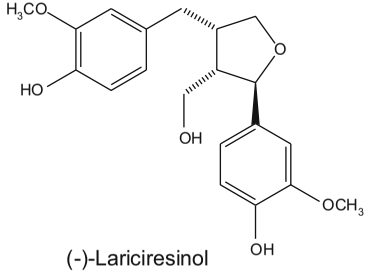
Fig. 2 General overview of the major metabolic pathways of plants

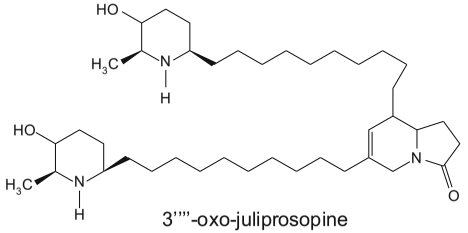
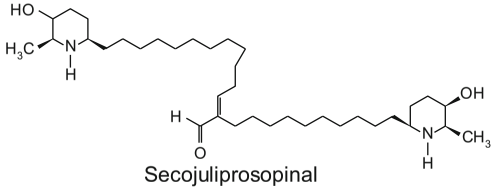
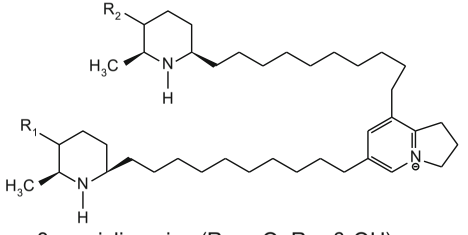
Several studies have been carried out on the isolation, structural elucidation, and evaluation of pharmacological and toxicological activities of alkaloids from *Prosopis juliflora* [24, 30, 53]. Among the activities already described for the mesquite, alkaloids could be highlighted the likely function of allelochemicals. *Prosopis* are rich source of piperidine alkaloids [54], such as juliflorine, julifloricine and julifloridine, juliprosine, juliprosinene and juliflorinine, 3′-oxojuliprosopine, secojuliprosopinol, 3-oxojuliprosine, and 3′-oxo-juliprosine and julifloravizole [14]. These alkaloids are complex structures derived from L-lisine and have been isolated from leaves, stem, seeds, pods, roots, and flowers [54, 55]. Among these, juliprosopine was found to be the major alkaloid in leaves; the abundance and diversity of alkaloids in aerial parts of the plant may be attributed to the protective mechanism of the plant against animals and pathogens [54].

The allelopathic potential of *P. juliflora* alkaloids, and phenylpropanoids, has been demonstrated through the use of extracts from their tissues and the evaluation of their effects against germination and plant growth (Table 1), as well as its action against fungi responsible for the infection and loss of other plant species. One of the pioneering studies of this issue was accomplished by Nakano, Nakajima, Fujii, Shigemori, Hasegawa [56] who isolated alkaloids from *Prosopis* leaves and demonstrated their effect on the growth of cress seedlings. The alkaloids 3′-oxo-juliprosopine and secojuliprosopinal showed inhibitory effects on root and shoot growth of cress in a dose-dependent manner and a mixture (1:1, w/w) of 3-oxo-juliprosine and 3′-oxo-juliprosine had a much stronger inhibition on both the root and shoot growth. Furthermore, the alkaloids from *P. juliflora* inhibited shoot and root growth of monocotyledonous plants, barnyard grass, rice and timothy grass, dicotyledonous plants, amaranth, lettuce, and cress. All alkaloids tested showed inhibitory growth against both monocotyledonous and dicotyledonous plants [34].

Aqueous extracts of *Prosopis juliflora* leaves have also showed insecticide potential against whiteflies from *Bemisia tabaci* (Hemiptera: Aleyrodidae). Their activity was attributed to alkaloids detected on extract that promoted 43.6% of mortality in eggs and 75.1% in nymphs in relation to control [47]. Alkaloids from *P. juliflora* displayed antifungal activity against several species, such as those of genus *Fusarium*, *Drechslera*, *Alternaria*, and *Colletotrichum* [48, 57]. Among different extracts from *Prosopis* leaves tested against the fungi *Alternaria alternata*, methanol and ethanol extracts demonstrated highly significant antifungal activity by inhibition of the mycelial growth [48]. Fungus species such as genus of *Alternaria*, *Curvularia*, *Fusarium*, *Drechslera*, and *Phoma* cause grain mold disease in sorghum (*Sorghum bicolor* L.), so they destroy grains during storage thereby rendering this Poaceae species unfit for human consumption. Alkaloid-enriched extracts from *Prosopis* showed a function of pesticide and showed a significant percentage of reduced incidence of biodeterioration, with an increase in seed germination and seedling vigor [48]. In the same way, *Prosopis juliflora* exhibited superior inhibitory effect against the fungi *Colletotrichum musae,* which causes anthracnose disease in bananas; however, further studies need to be realized to identify the active compounds responsible from the extracts with fungicidal potential [57].

Table 1 Allelopathic activity of isolated metabolites from *Prosopis juliflora*

Compounds from *P. juliflora*	Allelopathic activity	Reference
 L-tryptophan	Inhibitory effect on radicle growth of lettuce and barnyard grass at concentrations greater than 1.5 μM	(Nakano et al. 2001)
 Syringin	Inhibitory effect on root growth of lettuce seedlings (I_{40} = 350 μM) Inhibitory effect on shoot growth of lettuce seedlings (I_{40} = 600 μM) Inhibitory effect on root growth of Barnyard grass seedlings (I_{40} = 400 μM) Inhibitory effect on shoot growth of Barnyard grass seedlings (I_{40} = 400 μM)	(Nakano et al. 2002)
 (-)-Lariciresinol	Inhibitory effect on root growth of lettuce seedlings (I_{40} = 600 μM) Inhibitory effect on shoot growth of lettuce seedlings (I_{40} = 600 μM) Inhibitory effect on root growth of Barnyard grass seedlings (I_{40} = 30 μM) Inhibitory effect on shoot growth of Barnyard grass seedlings (I_{40} = 100 μM)	(Nakano et al. 2002)

 3’’’’-oxo-juliprosopine	Inhibitory effect on root growth of cress (*Lepidium sativum* L.) seedlings (I_{50} = 400 μM) Inhibitory effect on shoot growth of cress (*Lepidium sativum* L.) seedlings (I_{50} = 10 μM)	(Nakano et al. 2004b)
 Secojuliprosopinal	Inhibitory effect on root growth of cress (*Lepidium sativum* L.) seedlings (I_{50} = 500 μM) Inhibitory effect on shoot growth of cress (*Lepidium sativum* L.) seedlings (I_{50} = 100 μM)	(Nakano et al. 2004b)
 3-oxo-juliprosine (R_1= =O, R_2= β-OH) 3’-oxo-juliprosine (R_1= β-OH, R_2= =O)	Inhibitory effect of a 50:50 mixture of 3-oxo-juliprosine and 3′-oxo-juliprosine on root growth (I_{50} = 100 μM) and shoot growth (I_{50} = 1 μM) of cress seedlings	(Nakano et al. 2004b)

2.1.2 Allelophatic Effects of *P. juliflora* Carbohydrates and Phenolic Compounds

In addition to the presence of alkaloids in its chemical constitution, polysaccharides and several secondary metabolites such as flavonoids, phenolic derivatives, tannins, saponins, terpenoids, and coumarin were detected from different parts of *Prosopis juliflora* [30, 58, 59]. Many secondary metabolites produced by plants and also microorganism can act as a potential allelochemicals, but just having the presence of these compounds does not establish the allelopathic process [60, 61].

Regarding the *P. juliflora* polysaccharides, the presence mainly of galactomannans with different proportions of mannose and galactose has been reported, as well as compounds formed by arabinose and glucose [58, 62–68]. However, although the presence of carbohydrates in *P. juliflora* is well reported, along with its many activities, probably the allelopathic activity reported in the species cannot be attributed to these primary metabolites. Therefore, additional efforts to better comprehend this issue are necessary.

Only one paper that related the presence of carbohydrates in vegetal growth has been found so far. Hedin, McCarty, and Dollar [69] evaluated the effect of commercial formulations on the yield of cotton (*Gossypium hirsutum*) lint. According to the authors, bioregulators have an important role in plant growth and may induce the biosynthesis of allelochemicals. This way, the commercial product tested presents a carbohydrate fraction composed of xylose, mannose, galactose, and glucose that when applied as a foliar spray tends to increase the yield of cotton lint. Although the results were not statistically significant, they did show consistently small increases which have not been reported so far for carbohydrates [69].

Historically, the literature reports several studies using different concentrations of aqueous extracts of the leaves, barks, and roots of *P. juliflora* in the search for allelochemical compounds. Thus, it is well reported that in areas adjacent to species and especially beneath the tree canopy, there is a decrease in seed germination and growth of other competing species caused by the action of allelochemical compounds of *P. juliflora* [70–73].

This way, the allelopathy exercised by *P. juliflora* described in the literature is related to the secondary metabolites of the species, which may act as the main allelochemical compounds [70–73]. According to Getachew, Demissew, and Woldemariam [73], *P. juliflora* presents its allelochemical compounds in different parts of the plant and in different amounts and the leaves are responsible for the greater inhibitory effects, whereas the barks and roots present less activity.

Phenolics are a common class of secondary metabolites which depending on the nature and availability in the soil and could play an important role in allelopathy. The term “phenolic compounds” also could be related to aromatic phenols, benzoic acids and aldehyde, cinnamic acids, coumarins, tannins, and flavonoids [2, 60, 61, 74].

The phenolic compounds can act through several mechanisms such as (1) changing the membrane permeability leading to increased permeability, contents spill and increased lipid peroxidation and inhibiting plants from absorbing nutrients; (2) inhibiting root elongation, cell division and changes in cell structure; (3) weakening oxygen absorption capacity and reducing chlorophyll content and photosynthetic

rate; (4) changing the activity of many enzymes like phosphorylase, peroxidase, catalase and ATPase; (5) reducing or inactivating the activity of plant hormones; and (6) inhibiting protein synthesis and reducing DNA and RNA integrity [61].

Low-molecular-weight organic compounds such as phenolic acids are present in root exudates and decomposition residues, among them acids trans-cinnamic acid, vanillic, p-coumaric, ferulic, and salicylic acids have been reported, as summarized by Tian, Bi, Sun, Zhang [75]. Using as a background the knowledge that the effects of allelochemicals are dose-dependents [76], it is known that in low concentrations they can boost plant growth and in high concentrations they inhibit, promote, or have no effect on plant growth [77]. Tian, Bi, Sun, and Zhang [75] studied the allelopathic effects of phenolic acids on *Colletotrichum gloeosporioides,* a species of fungus responsible for damage to strawberry crops. The authors detected the presence of ten phenolic acids in soils: gallic acid, protocatechuic acid, chlorogenic acid, p-hydroxybenzoic acid, caffeic acid, syringic acid, vanillin, p-coumaric acid, ferulic acid, and trans-cinnamic acid and observed that in the lowest concentration tested, the germination and growth could increase whereas in higher concentrations, they could reduce the disease in strawberry crops, demonstrating the potential beneficial allelophatic use.

In order to study the chemical composition and the allelophatic potential of *Nigella sativa,* Zribi, Omezzine, and Haouala [78] used aerial parts and seeds aqueous extracts from different developmental stages (vegetative, flowering, and fruiting). According to the authors, the phytochemical composition formed by phenolics, flavonoids, flavonols, flavones, and alkaloids were high in the vegetative stage and they contributed significantly to the allelophatic activity of the species; some of them are also present in the *P. juliflora* chemical composition [78]. The aqueous extracts delayed germination and seedling growth depending on the stage of development, showing that this is an important parameter related to allelochemical compounds [78, 79].

Specifically related to *P. juliflora,* Kaur, Callaway, and Inderjit [74] evaluated the production, accumulation, and inhibitory effects of phenolic in soils found under *P. juliflora* compared to the seed growth of *Brassica campestris*. The authors found out that the soils beneath *P. juliflora* present higher levels of total phenolics than the soil from open areas and suppression in *Brassica campestris* growth. These results were similar to those presented by Kaur, Gonzáles, Llambi, Soriano, Callaway, Rout, Gallaher, and Inderjit [80], where the soil beneath the *P. juliflora* canopy showed higher levels of total phenolics and those one published by Inderjit, Seastedt, Callaway, Pollock, and Kaur [45], which presented a phenolic-rich soil under *P. juliflora* causing suppression in *Bambusa arundinacea* growth.

Recently, a study evaluated phytotoxicity of *P. juliflora* and its potential on weed control and yield of wheat through a two-year experimental design. The author used *P. juliflora* extracts from leaves, bark, and root and used as parameters the following: weed density, fresh weed biomass, dry weed biomass, chlorophyll content, leaf area index, leaf area duration, crop growth rate, net assimilation rate, plant height, and number of tillers. After application of the extracts, all parameters were significantly reduced, showing that the phytotoxic compounds of *P. juliflora* can also cause negative effects on agricultural crops [81].

3 Future Directions and Prospect

The genus *Prosopis* are among the world's most damaging invasive species. Invasive species can cause ecological, economic, and social impacts and are responsible for global change. The highly invasive nature of *Prosopis* includes different factors: the production of large numbers of seeds; rapid growth; the species can grow in a wide range of conditions from 50 to 1500 mm mean annual rainfall and in temperatures as high as 50 °C (air temperature) and 70 °C (soil temperature); and they are not limited by alkaline, saline, or infertile soils and by allelopathic and allelochemical effects from other plant species [23, 82].

Prosopis species, such as *Prosopis juliflora,* have colonized several countries from America, Asia, and Australia. Due to their invasive character, each country has used different methods and strategies to eradicate it. The control of *Prosopis* species includes different management techniques globally, presenting advantages and disadvantages: (i) biological control, (ii) mechanical control, (iii) chemical control, (iv) utilization, and (v) cultural control/other control [23, 82]. However, these techniques have been expensive and ineffective.

What is more, it is important to understand the dynamics of invasive species to reduce their negative impacts and maximize their benefits. Several *Prosopis* species have substantial impacts on biodiversity, ecosystem services, and local and regional economies in their native terrain; others provide multiple benefits to local communities [82].

Prosopis juliflora can be used to make stock-proof living fences, and charcoal and biochar in India Bartlett, Milliken, and Parmar [83]. The authors highlighted it as a potential solution to decrease the impact on rural livelihood and to reduce its prevalence and further spread. These actions can also provide additional local employment opportunities. In Kenya, considerable effort was taken to improve the policies and educate the population to use *Prosopis* species. They created a cookbook to teach the communities how to adapt its use as a flour [23]. Communities in Kenya have benefited from the sale of charcoal and the use of *Prosopis* pods for fodder, improving the local economy by US$1.5 million per year [84]. In South Africa, one company uses the pods to make medicines able to control the blood sugar levels in humans. The company's annual profit generated from local sales is US$ 106,000 [85].

Several applications concerning *Prosopis juliflora* have been previously reported in the technology fields of energy, agriculture, biotechnology, pharmacology, food, cosmetology, and pharmaceutical [24, 52]. The species provides several properties and services, from primary consumption to specific applications; for instance, its wood has been used as a primary source of fuel for subsistence, due to its high calorific value, low ash production, and its ability to burn well even when it is still fresh [13, 86].

The potential of *P. juliflora* as a source of animal feed has also been explored; however, caution should be used concerning its constitution, since feed comprised exclusively or mostly of *P. juliflora* parts are not safe [87], due to its neurotoxic effects, which are related to its piperidine alkaloids: juliprosopine and juliprosine,

the main constituents of the species [88]. Therefore, the authors recommend that *P. juliflora* must constitute no more than 20% of any feed in order to avoid animal health risks [87].

Regarding specific applications, which require complex analyzes and further scientific research, several synthetic insecticides have been employed in order to control agricultural pests; however, their constant use has led to the development of insecticide resistance, promotion of harmful effects on humans, and other nontarget organisms, along with severe environmental damage. In order to overcome these events, the search for alternative pest control methods involving natural plant products has increased substantially. In this context, previous studies have demonstrated larvicidal activity of *P. juliflora* seed pod hexane extract against *Spodoptera litura*, with an LC_{50} of 200.40 ppm, which, according to the authors, may be due to the presence of 9-Octadecyne, the main compound of the afore mentioned extract [89]. Other studies regarding *P. juliflora* leaf aqueous extracts have also demonstrated its mortality against whitefly eggs (43.6%) and nymphs (75.1%) at a concentration of 10% [47], taken together with the insecticidal activity towards mosquito vectors of malaria, dengue, Chikungunya, and filariasis [90, 91]; this suggests that *P. julifora* derivatives may act not only as biopesticides but also in disease control.

According to the literature, all parts of *P. julifora* have been explored in folk medicine [13, 52, 92]. The tree's flour is used in syrup as an expectorant, and its tea infusion, to treat skin lesions and improve digestion [92]. Additionally, its bark extract is used as an antiseptic, the decoction of its wood chips is applied for skin tonification, and its gum is used to treat eye infection [93].

Besides folk medicine application involving *P. juliflora*, several studies have demonstrated the potential of the species regarding pharmaceutical technology [52]. The gum from the species is very similar to Arabic gum and has been described as an emulsifying agent for oil-water (O/W) emulsions [94–96] and as a good encapsulating agent for essential oils and natural dyes [97–99]. These properties of *P. juliflora* gum can be easily employed by the cosmetic industry, in regard to, for instance, the development of emulsion cosmetic formulations and encapsulated aromatic oils. Due to this property, the *P. juliflora* gum can be used in food, cosmetology, and pharmaceutical industries as natural viscosity modifier to improve the formulation stability. Rincón, Muñoz, Ramírez, Galán, and Alfaro [58] studied the rheology of aqueous dispersion of galactomannan (to 0.6 from 1.4%) from *P. juliflora* and related its use as a natural thickener with different applications.

Polysaccharides present in *P. juliflora*, such as galactomannan, have demonstrated a good disintegrating and binding efficiency, when applied to the pharmaceutical industry as plant excipients, and have shown importance over the years, due to their abundance in nature, their safety, and economic viability [100]. Previously, Reis, Cavalcanti, Rubira, and Muniz [101] have chemically modified galactomannan obtained from *P. juliflora*, with glycidil methacrylate, suggesting that this new component may be used by the pharmaceutical industry as an alternative to develop controlled release systems. In the cosmetology field, the polysaccharides have been used as a moisturizing agent. Barreto et al. [102] evaluated the skin moisturizing

effect of nanoemulsions containing polysaccharide-enriched fraction obtained from the crude extract of the by-product from the processing of the leaves of *A. sisalana*. According to the authors, nanoemulsions showed moisturizing properties, improving the increase in the water content of the stratum corneum while maintaining the skin barrier function. Ribeiro, Barreto, Ostrosky, da Rocha, Verissimo, and Ferrari [103] related the skin moisturizing activity for O/W nanoemulsion containing 1% of *Opuntia ficus-indica* (L.) Mill hydroglycolic extract. They attributed its properties to the chemical composition of *Opuntia ficus-indica* (L.) Mill which is rich in carbohydrates.

As previously discussed, several studies reported the presence of tannins, phenolics, flavonoids, alkaloids, terpenes, and steroids in extracts of certain parts of *Prosopis*. This chemical composition demonstrates *P. juliflora* as a potential candidate for the food, cosmetic, and pharmaceutical industries [82]. Over time, the search for phenolic compounds has increased substantially by the industries [104], due to their ability to scavenge reactive oxygen species (ROS) generated in numerous oxidative mechanisms, which reduce the cell aging process, as well as having anti-inflammatory, antiallergic, antiviral, anticarcinogenic properties [105] and antimalarial properties [106]. The antioxidant activity of *P. juliflora* extracts was evaluated and showed the presence of flavanols, attributing the antioxidant activity to its main compound: (-)-mesquitol, suggesting that *P. juliflora* could be an important natural source of antioxidants for such industries Sirmah, Mburu, Iaych, Dumarçay, and Gérardin [104].

Other pharmacological properties have been reported. The antiemetic activity of *P. juliflora* shows methanol extract, which demonstrated that a dose of 150 mg/kg could reduce the retching in chicks by 73.64% when compared to the standard drug (chlorpromazine) [107]. Scientific studies have also discovered that juliflorine, a specific alkaloid, only found in *P. juliflora*, can suppress acetylcholinesterase and butyrylcholinesterase enzymes in a concentration dependent manner, leading to a potential drug for Alzheimer's disease [108, 109]. Finally, Malik, Ahmed, and Khan [110] reported that the *P. juliflora* pod extract is a novel source of anticancer, antitumor, and chemoprotective activity, especially due to the presence of their terpenoids.

Beyond all the applications already mentioned, in regard to *Prosopis juliflora*, the species has other potential, in the field of biotechnology. For instance, wood is considered a lignocellulosic biomass and may be used as an important substrate for the extraction of sugars, enabling the production of second generation (2G) ethanol [111]. However, in order to produce 2G ethanol, commercial viability must be achieved by process optimization. In this context, *P. juliflora* is very suitable as a lignocellulosic substrate, since the species is a nonfood plant, has a cosmopolitan distribution, grows in a wide range of soils, and has a very high content of carbohydrates. Recent studies have reported the production of ethanol at 10.85 g/L, with a fermentation efficiency of 87.34%, using *P. juliflora* lignocellulosic biomass as substrate, and a co-culture of *Saccharomyces cerevisiae* VS3 and *Pichia stipitis* NCIM 3498 within 36 h in a sequential manner [112, 113]. Seeds from *P. juliflora* may also be considered an important source of fuel, since the constituents of its oil

can be easily transformed into its alkyl esters derivatives (biodiesel) by NaOH-Catalyzed transesterification reaction [114]. This represents as an important renewable energy source and a possible substitute to fossil fuels.

Additionally Kailappan, Gothandapani, and Viswanathan [115] have produced activated carbon with 56.9% yield, submitting *P. julflora* wood powder to temperatures of 600 °C for 1 h followed by activation with zinc chloride for 30 min, demonstrating that with applied scientific research, low value raw material such as lignocellulosic feedstock can be transformed into high value additional products.

It is known that *P. juliflora* has natural coagulants capable of removing turbidity from water [116] and that its ethanolic extracts can remove 78% of total dissolved solids in tannery and paint industry effluents [117, 118]. Previous studies have reported steel corrosion inhibition from its methanolic extracts from leaves [119].

The capacity of the species to accumulate fluoride was also reported by Saini, Khan, Baunthiyal, and Sharma [120], thus providing to be a suitable candidate to decontaminate fluoride loaded soils. Moreover, the tree rings of *P. juliflora* can hyper accumulate and immobilize heavy metal such as nickel (Ni), copper (Cu), cadmium (Cd), and chrome (Cr), making it a good bioindicator of heavy metal contamination in ecosystems and an alternative to bioremediate heavy metals-polluted sites at the same time [121–123]. Several other applications of *P. juliflora* regarding its biotechnological potential are reported by [52]; these include: synthesis of bio-based polyurethane, production of high-energy Li-ion hybrid electrochemical capacitors, and so on.

4 Conclusions

Prosopis juliflora is among the world's most damaging invasive species that can cause ecological, economic, and social impacts and are responsible for global change. However, mesquite has demonstrated to be a versatile raw material, widely applicable in the food, cosmetic, pharmaceutical, agricultural, and renewable energy industries, providing progress in several fields of science and technology. Therefore, the strategies to control the invasive character of *Prosopis juliflora* using transdisciplinary approaches would also improve feasible solutions.

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Ecological Management of Agricultural Pests Through Allelopathy

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Abstract

Allelopathy is a naturally occurring ecological phenomenon in which the living organisms produce and release the biochemicals (allelochemicals) in the environment that affect the growth, development, reproduction, and survival of other living organisms in the surrounding environment. For field crops, the phenomenon of allelopathy can be exploited in the form of intercropping, use of cover crops, mulching, crop rotations, and use of plant water extracts alone or in combination with reduced doses of herbicides to provide effective control of the agricultural pests and diseases. For the control of insect pests (field and storage insect pests), the use of allelopathic plant water extracts and the powder of allelopathic plants may be quite useful. The allelochemicals affect the growth of unwanted plants (e.g., weeds) through changes in the cell structure, inhibition of cell elongation/division, disruption of membrane structures, and disruption of water and nutrient uptake and the process of photosynthesis. The phenomenon of allelopathy is ecofriendly, and it may help significantly reduce the usage of pesticides. Thus, the phenomenon of allelopathy provides an attractive ecological alternative to pesticides for controlling the pests and diseases of the agricultural crops. In this chapter, we have discussed the mechanism of allelochemicals for growth inhibition in plants and the role of crop rotation, allelopathic mulches, allelopathic cover crops, intercrops, and allelopathic water extracts (alone or with reduced doses of herbicides) in weed management. The role of allopathic water extracts and allelopathic powders for managing the insect pests and diseases has also been described.

Keywords

Allelopathy · Weeds · Crop rotation · Intercropping · Cover crops · Mulching · Insect pests · Diseases

1 Introduction

There is continuous increase in the food demands owing to continuously increasing global population. The fast growth of world population calls for producing foods in bulk quantities to ensure food security [1]. Although, the production of field crops has increased several folds, the pressure of agricultural pests (weeds and insects) and diseases is also increasing. These demands develop strategies to control the pests and diseases with no or low dependence of synthetic pesticides, to save the ecosystem and ensure the production of good quality food [2]. In the past, the increase in herbicide residue in food and groundwater has threatened the long-term usage of herbicides in the diverse agroecosystems for controlling weeds. The herbicide and insecticide resistance has also emerged as a serious issue which may threaten the future food security [3, 4].

In this scenario, the effective use of phenomenon of allelopathy in agroecosystems may offer pragmatic option to reduce the pests and diseases pressure with low reliance on synthetic chemicals. According to Rice [5], allelopathy is

"the effect of one plant on the growth and development of other plant, comprising microorganisms, through the release of the chemical substances in the neighboring environment." Farooq et al. [6] defined allelopathy as "the phenomenon in which fungi, viruses, other microorganisms and plants produce secondary metabolites that affect the biological and agricultural systems."

Indeed, the allelochemicals are the byproduct of the primary metabolism and are thus called as secondary metabolites. The allelochemicals are present in different plant parts (e.g., seed, leaves, stem, flowers, rhizomes, pollen, and roots pollen) which enter to the agroecosystems through the exudation from roots, residue decomposition, and volatilization from the aerial plant parts. The microorganisms also modulate the production and transformation of secondary allelochemicals [5, 7].

The important allelochemicals are categorized into phenolics, steroids, amino acids, alkaloids, terpenoids, flavonoids, carbohydrates, etc. [8]. Among plant secondary metabolites, phenolic compounds are the most prevalent and are involved in plant developmental cascades under optimal and suboptimal conditions. The phenolics also act as defense agent against the attacking organisms and work as a signal molecule thus impacting the plant/cell growth and development [9]. The process of germination, growth, and development may be affected when a susceptible plant is exposed to allelochemicals of some other plants. Inhibition of the germination of seeds, elongation of coleoptile, and development of root/shoot are the most evident effects of allelochemicals on other plants [8].

For field crops, the phenomenon of allelopathy may be exploited through crop rotations, as green manure crops, as cover crops, intercrops, and the use of allelopathic water extracts and allelopathic powders (either alone or in combination with reduced doses of pesticides) [7, 10–18].

In this chapter, the role of crop rotation, allelopathic mulches, allelopathic cover crops, intercrops, and allelopathic water extracts (alone or with reduced doses of herbicides) in weed management has been described. The role of allelopathic water extracts and powders for the management of crop insect pests and diseases in agronomic and horticultural crops has also been discussed.

2 Mechanism of Action of Allelochemicals for Agricultural Pest Management

Upon release of allelochemicals in the environment, they may affect the physiological and biochemical processes in other plants and organisms present in the vicinity [19, 20]. In the following lines, mechanism of action of allelochemicals for agricultural pest management has been described.

2.1 Changes in the Micro- and Ultrastructure of Cell

The allelochemical present in different plant parts affects the cell structure and its shape. The root cells can widen or shorten by the volatile allelochemical (e.g.,

monoterpenes, eucalyptol, and camphor), in addition to the nuclear abnormalities within the vacuole [21, 22]. For instance, the activity of mitotic cells was reduced by more than 50% in watermelon (*Citrullus lanatus* (Thumb.) Matsum. & Nakai.) by the application of maize (*Zea mays* L.) pollen extract. It also increased the irregularities of nuclear and pyknotic nuclei and reduced the growth of radicle and hypocotyl [23]. The allelochemicals from barley (*Hordeum vulgare* L.) roots (i.e., hordenine and gramine) caused damage in the tips of cell walls of radical in white mustard (*Sinapis alba* L.) and enhanced the organelle disorganization and autophagy of cell [24]. Similarly, the ultrastructure of chloroplasts and mitochondria in cucumber (*Cucumis sativus* L.) was distorted by an allelochemical "cinnamic acid" [25]. The random amplification of the polymorphic DNA profiles of various plants has also been reported to be affected through the allelochemicals released from the catmint (*Nepeta meyeri* Benth.) and field bindweed (*Convolvulus arvensis* L.) [26, 27]. The disturbance of microtubules in the roots of wheat (*Triticum aestivum* L.) and *Arabidopsis* (*Arabidopsis thaliana* L.) is strongly influenced by an allelochemical citral [28, 29]. The citral cell caused ultrastructure changes and cell-wall condensation and decreased the intercellular communication and root hair development in *Arabidopsis* [30].

2.2 Inhibition of Cell Division and Cell Elongation

In plant meristems, DNA synthesis and cell proliferation are affected by allelochemicals, e.g., monoterpenoids (i.e., camphene, beta-pinene, camphor, alpha-pinene, and 1,8-cineole) [31]. Reduction in the process of mitosis particularly in lettuce (*Lactuca sativa* L.) has also been reported in response to exposure to allelochemicals [32]. In a study, the cell number in each period of cell division was decreased by sorgoleone [an allelochemical in sorghum (*Sorghum bicolor* (L.) Moench)] with subsequent damage in tubulins and polyploidy nuclei in bean plant (*Phaseolus vulgaris* L.) [33]. A rye (*Lolium rigidum* Gaud.) allelochemical, i.e., DIBOA (2, 4 dihydroxy-1,4 (2H)-benzoxazin-3-one), has been reported to reduce the renewal of root cap cells of cucumber with simultaneous reduction in growth [34]. In soybean (*Glycine max* (L.) Merr.), the application of allelopathic water extracts of jimson weed (*Datura stramonium* L.) decreased the primary/lateral root elongation, inhibited the length and density of root hairs, suppressed the root tip cell division, and increased the chromosomal aberration in micronucleus index especially at higher rates of extract application [35].

2.3 Increase in the Cell Membrane Permeability

The allelochemicals increase the production of free radicals, which results in higher lipid peroxidation and alteration in the membrane permeability, which destruct the biological membranes in the plant system [19, 36–39]. For example, the application of the allelopathic water extracts, prepared from the aerial plant parts of

barley, decreases the growth of wild mustard (*Sinapis arvensis* L.) and wild barely (*Hordeum spontaneum* L.) saplings through increase in the lipid peroxidation [40, 41]. The application of non-sterile aerial parts of wheat and water foxtail (*Alopecurus aequalis* L.) increases the ROS (reactive oxygen species) activity and leaf malondialdehyde contents in the seedlings of non-transgenic and transgenic potato (*Solanum tuberosum* L.) owing to leakage of biological membranes [42]. The use of lemongrass (*Cymbopogon citratus* L. (DC) Stapf) essential oil enhanced the lipid peroxidation and thus caused damage to the biological membranes in barnyard grass (*Echinochloa crus-galli* (L.) P. Beauv) [43]. The exposure of cucumber seedlings to cinnamic acid enhanced the ROS production, thus enhancing the lipid peroxidation and reducing the membrane H^+-ATPase activity [44].

2.4 Impact on the Plant Growth Regulatory System

Allelochemicals may cause imbalances to the several phytohormones or change the plant growth regulator contents, which reduce the plant growth and development including the germination of seed and growth of seedling. The phenolic allelochemicals may cause increase in the activity of indole acetic acid (IAA) oxidase and may decrease the reaction among peroxidase and IAA [45]. In barnyard grass, application of aqueous extracts of rice (*Oryza sativa* L.) negatively affected the growth of seedling through reducing the IAA levels with immediate increase in IAA oxidase activity [46]. In tomato (*Lycopersicon esculentum* L.), the application of cyanamide (1.2 mM) caused an imbalance in two plant hormones, viz., auxin and ethylene [47]. In another study, the exposure of seedlings of wheat to ferulic acid (1.50 mM) decreased the seedling growth through accumulation of cytokinins, IAA, and gibberellic acid [48].

In rice seedlings, Yang et al. [49] investigated the impact of two different allelochemicals (i.e., DTD and HHO) on the abscisic acid, ZR contents and IAA. Both of these allelochemicals were extracted from the *Ageratina adenophora* Sprengel weeds. The application of the DTD allelochemical, at higher concentrations (i.e., 1.5 mM), increased the abscisic acid contents in rice root which was followed by a sharp decline in its contents after 96 h of the application of DTD allelochemical. The application of HHO allelochemical enhanced the abscisic acid contents for 48/96 h. Nonetheless, the HHO and DTD application reduced the ZR and IAA contents in rice roots.

2.5 Influence on Photosynthesis Process

The main effect of allelochemicals on plant photosynthesis process can be visualized through decrease in photosynthesis or destruction of the photosynthetic machinery. Subsequently, the pigment contents of photosynthetic apparatus are reduced, which lumps the power and transfer of electron, and reduce the ATP synthesis, enzyme activity, and disturbs the stomata which eventually reduce the process of

photosynthesis [25, 50, 51]. In the process of photosynthesis, the allelochemicals mainly disturb the function of photosystem II [52, 53], followed by damage of D1 proteins[54]. It has been reported that the F_v/F_m and growth of the weeds [55] as well as the number of active reaction center in the electron transport chain [56] are decreased by the sorgoleone. In barnyard grass, the green pigments (i.e., chlorophyll a and b, carotenoids) in leaves, the alpha-amylase activity in seeds, and the metabolism of photosynthetic process were significantly inhibited by a higher application of essential oil from leaves of lemongrass [43]. In cucumber, application of root extracts and root exudates of cucumber and derivatives of cinnamic acid and benzoic acid reduced the net photosynthetic rate, transpiration, intercellular CO_2 concentration, and stomatal conductance [50].

Sorgoleone, an allelochemical found in sorghum, is inhibitor of PSII [57–59] which interferes the binding of plastoquinone at the DI protein [59]. Some other allelochemicals (e.g., 5-ethoxysorgoleone) found in sorghum have also been reported to inhibit the activity of PSII [60]. Some allelochemicals produced by cyanobacteria inhibit the electron transport after binding to PSII sites [61], e.g., fischerellin from the *Fischerella muscicola* [62]. The *Myriophyllum spicatum* (an aquatic angiosperm) produce an allelochemical, i.e., tellimagrandin II, which disturbs the PSII mechanism by affecting the electron transport at non-heme iron [63].

2.6 Effect on Nutrient and Water Uptake

Nutrient and water uptake in the plant roots is also affected by the allelochemicals. The functions of Na^+/K^+-ATPase is well known in the uptake and transportation of ion at the plasma membrane which is reduced by the allelochemicals. In a study, the allelochemicals (cinnamic acid and p-hydroxybenzoic acid) highly reduced the root dehydrogenase capacity and ATPase activity and thus decreased the uptake of potassium, nitrate, and phosphorus [64]. H^+-ATPase action across the root cell plasma lemma is decreased by sorgoleone and juglone, which influence the uptake of water and solutes in the soybean, maize, and peas (*Pisum sativum* L.) [65, 66]. In maize seedlings, different allelochemicals such as *p*-coumaric acid, ferulic acid, and trans-cinnamic acid have been reported to affect the uptake of nitrate and H^+-ATPase function in the plasma membrane [67]. The growth, uptake, and translocation of nutrients in radish (*Raphanus raphanistrum* L.) plants are affected by the residues of sunflower (*Helianthus annuus* L.) [68].

In a study on wheat, Yuan et al. [69] correlated the allelochemical (e.g., 4-tert-butyl benzoic acid, ferulic acid, and benzaldehyde) application with the nitrogen absorption. However, the correlation was more negative for ammonical form of nitrogen than the nitrate form of nitrogen. Yu and Matsui [70] also reported reduction in the uptake of nitrate, sulfate, potassium, calcium, magnesium, and iron due to application of cinnamic acid and the root exudates of cucumber in cucumber seedlings. It is interesting to note that the impact of allelochemicals on the ion uptake is linked with the concentration and type of allelochemical. For example, low concentration of an allelochemical (i.e., dibutyl phthalate) may enhance the

nitrogen absorption and may reduce the potassium and phosphorus uptake. However, a higher concentration of this allelochemical reduces the absorption of potassium, phosphorus, and nitrogen. Likewise, low concentration of an allelochemical "diphenylamine" encourages the nitrogen absorption and discourages the phosphorus absorption of phosphorus in tomato roots [71].

3 Role of Allelopathy in Weed Management

The weeds are believed to be the most important competitors of the crop plants causing substantial yield reduction through competition for nutrients, space, light, and water. In different cropping systems, allelopathic weed management may provide an effective strategy with no or less resilience on synthetic herbicides. The phenomenon of allelopathy may be used in weed management through crop rotation [72], use of smother or cover crops [73, 74], intercropping, crop residue incorporation, [10, 73, 75], mulching [10, 76], and use of allelopathic aqueous extracts alone [7, 77] or in combination with reduced doses of herbicides [78–82]. As described above, the allelochemicals affect cell division, biosynthesis of hormones, uptake/transportation of nutrients [83], permeability of membrane [84], oscillations of stomata, photosynthetic pigments [85], respiration process, protein metabolisms [8], and plant water relation [5], when being applied at higher concentration, which may cause significant growth decline in weed plants. The application of allelopathy for weed control in field crops has been discussed in the following section.

3.1 Intercropping

In intercropping, the compatible crops are grown together in order to get high yield and economic profits. Further, intercropping increases the resource use efficiency and helps controlling the weeds (Table 1; [99]). In particular, weed population can be reduced, and the crop yield may be increased if the allelopathic crops are used in intercropping. For instance, weed density of jungle rice (*Echinochloa colona* (L.) Link.), jute mallow (*Corchorus olitorius* L.), common purslane (*Portulaca oleracea* L.), and crowfoot grass (*Dactyloctenium aegyptium* (L.) Willd.) was decreased when maize and cowpea (*Vigna unguiculata* [L.] Walp.) were intercropped [100]. The weed infestation in wheat crop can be reduced by intercropping legumes in wheat as compared to sole wheat crop [101]. In a study, the growth of *Orobanche* spp. was decreased by introducing berseem (*Trifolium alexandrinum* L.) as intercrop in legumes [102].

In cotton (*Gossypium hirsutum* L.), intercropping of sorghum and sunflower crops reduced the weed density by 60–62% and enhanced the cotton production by 17–22% [103]. In another study, the intercropping of pea with barley decreased the population of wild mustard and common lamb's quarters (*Chenopodium album* L.) than the sole crops. Furthermore, the weeds also extracted

Table 1 Weed suppression through intercropping of allelopathic crops in main crops

Weed species controlled	Intercropping system	References
Horse purslane (*Trianthema portulacastrum* L.), crowfoot grass, purple nutsedge (*Cyperus rotundus* L.)	*Sesbania* (*Sesbania sesban* (L.) Merr.) intercropping in direct seeded rice	[76]
Swine cress [*Coronopus didymus* (L.) Sm.], honey clover (*Melilotus albus* Medik.), common lamb's quarters, purple nutsedge, wild oat (*Avena fatua* L.), sweet clover (*Melilotus indica* (L.), scarlet pimpernel (*Anagallis arvensis* L.), Bermuda grass (*Cynodon dactylon* (L.) Pers.), Pall.)	Chickpea (*Cicer arietinum* L.) intercropped in wheat	[86]
Jungle rice, yellow foxtail [*Setaria glauca* (L.) Beauv.], large crabgrass [*Digitaria sanguinalis* (L.) Scop.]	Black gram (*Phaseolus mungo* L.) intercropped in rice	[87]
Littleseed canarygrass (*Phalaris minor* Retz.), broad-leaved dock (*Rumex obtusifolius* L.), swine cress, common lamb's quarter	Canola (*Brassica napus* L.) intercropped in maize	[88]
Purple nutsedge, field bindweed, horse purslane	Sorghum, sunflower, and mung bean (*Vigna radiata* [L.] R. Wilczek) intercropped in maize	[89]
Annual ryegrass, common lamb's quarter	Canola intercropped in maize	[90]
Giant witchweed [*Striga hermonthica* (Del.) Benth]	Green leaf *Desmodium* (*Desmodium intortum* (Mill.) Urb.) intercropped in finger millet (*Eleusine coracana* L.)	[91]
Purple nutsedge	Sesame, soybean, and sorghum intercropped in cotton	[12]
Black bindweed [*Fallopia convolvulus* (L.) Á. Löve], German chamomile (*Matricaria chamomilla* L.), common sowthistle (*Sonchus oleraceus* L.)	False flax (*Camelina sativa* L. Crantz) intercropped in pea	[92]
Common groundsel (*Senecio vulgaris* L.)	Leek (*Allium porrum* L.) intercropping with celery (*Apium graveolens* L.)	[93]
Redroot pigweed (*Amaranthus retroflexus* L.), field bindweed	Bitter bottle gourd (*Lagenaria siceraria* L.) intercropped in maize	[94, 95]
Itchgrass [*Rottboellia cochinchinensis* (Lour.) W.D. Clayton]	Hyacinth bean (*Lablab purpureus* L.), jack bean (*Canavalia ensiformis* L.), butterfly pea (*Clitoria ternatea* L.) intercropped in maize	[96]
Tridax daisy (*Tridax procumbens* L.), *Amaranthus* species, large crabgrass, waterleaf (*Talinum triangulare* (Jacq.) Willd), chickweed (*Ageratum conyzoides* L.), slender cyperus (*Cyperus distans* L.f), ditch grass (*Paspalum orbiculare* G. Forst.), Indian	Cassava (*Manihot esculenta* Crantz) intercropping with maize	[97]

(*continued*)

Table 1 (continued)

Weed species controlled	Intercropping system	References
goosegrass (*Eleusine indica* (L.) Gaertner), Bermuda grass and morning glory (*Ipomoea involucrata* P. Beauv.)		
Corn buttercup (*Ranunculus arvensis* L.)	Wheat intercropped in linseed (*Linum usitatissimum* L.)	[98]

a high quantity of nitrogen (30%) in the sole crop of pea than pea-barley intercrop [104]. In conclusion, weed density and biomass can be reduced with substantial improvement in crop yield due to intercropping of allelopathic crops (Table 1).

3.2 Crop Rotation

Crop rotation is the consecutive planting of different crops in a specific field over a certain period of time. In crop rotation, the allelochemicals are exuded through roots of allelopathic crops and are also released by crop residue decomposition which help to control weeds (Table 2; [117, 118]) following the allelopathic crops. The crops succeeding sorghum face less weed competition due to decline in weed densities owing to allelochemicals release into the soil from the sorghum crop [105, 119].

In several Asian countries, rice-wheat system is practiced in large area. This system mostly depends on herbicides to control weeds. In this system, the growing of allelopathic crops such as sorghum, maize, and pearl millet (*Pennisetum glaucum* L.) after wheat harvest and prior to rice plantation provides effective control of rice weeds for initial 45 days of crop cycle [6]. In case of weed-infested field of wheat, fodder crops such as Egyptian clover (*Trifolium alexandrinum* L.) or oat (*Avena sativa* L.) can be useful for natural control of weeds for one season [120]. In red clover (*Trifolium pratense* L.), a parasitic weed, i.e., *Orobanche minor* (JE Smith), can be controlled if planted in wheat fields. Wheat has the capacity to encourage seed germination of parasitic weeds without attachment, and thus it can be useful to control the parasitic weeds [121].

3.3 Cover Crops

Weeds suppression, soil conservation, suppression of weeds, and increased recycling of nutrient and fodder supply are the benefits of cover crops (Table 2; [122, 123]). Sunn hemp (*Crotalaria juncea* L.), sweet clover, yellow sorghum, alfalfa (*Medicago sativa* L.), cowpea, red clover (*Trifolium pratense* L.), and ryegrass are the main cover crops grown across the globe. The investigations from the farmers' field and from the long-term experiments have shown that weed population and dry biomass are reduced with allelopathic cover crops due to release of allelochemicals in the rhizosphere [124]. For instance, the barnyard grass density in maize can be reduced with legume cover crops such as jumbie bean (*Leucaena leucocephala* L.), velvet bean (*Mucuna*

Table 2 Weed control through crop rotation, allelopathic mulches, crop residue incorporation, and cover crops

Weed species	Allelopathic source	Application mode	Weed dry weight reduction (%)	References
Horse purslane	Sorghum crop	Wheat-sorghum-rice rotation	13.25–25% reduction in weed density in rice	[105]
Purple nutsedge	Sorghum crop	Wheat-sorghum-rice rotation	5.0–32.1% reduction in weed density in rice	
Jungle rice	Sorghum crop	Wheat-sorghum-rice rotation	10.4–32.2% reduction in weed density in rice	
Crowfoot grass	Sorghum crop	Wheat-sorghum-rice rotation	9.4–23.2% reduction in weed density in rice	
Horse purslane	Sunflower + rice + brassica	Soil incorporation	60.1	[106]
Littleseed canarygrass	Kabling-parang (*Anisomeles indica* L.)	Dried leaf and root powder mulch	33.9–72.9	[107]
Hoorah grass (*Fimbristylis miliacea* (L.) Vahl), common water clover (*Marsilea quadrifolia* L.), Chinese sprangletop (*Leptochloa chinensis* L.), Indian toothcup (*Rotala indica* L.), smallflower umbrella sedge (*Cyperus difformis* L.), goose weed (*Sphenoclea zeylanica* Gaertn), Asian spiderwort (*Murdannia keisak* Hassk.), climbing dayflower (*Commelina diffusa* Burm. f.), buffalo grass (*Brachiaria mutica* Forssk.), Egyptian grass (*Dactyloctenium aegyptium* (L.) Willd.), pickerel weed	Hairy beggarticks (*Bidens pilosa* L.)	Dried material applied as mulch	84.9	[108]
Horse purslane	Sorghum crop	Applied as surface mulch	42.2–48.7% reduction in weed density in rice	[105]

(*continued*)

Table 2 (continued)

Weed species	Allelopathic source	Application mode	Weed dry weight reduction (%)	References
Purple nutsedge	Sorghum crop	Applied as surface mulch	22.2–59.9% reduction in weed density in rice	
Jungle rice	Sorghum crop	Applied as surface mulch	16.6–35.4% reduction in weed density in rice	
Crowfoot grass	Sorghum crop	Applied as surface mulch	33.3–63.3% reduction in weed density in rice	
Swine cress	Rice crop	Applied as surface mulch	65.2% reduction in weed density in wheat	[76]
Toothed dock (*Rumex dentatus* L.)	Rice crop	Applied as surface mulch	76.9% reduction in weed density in wheat	
Common lamb's quarter	Rice crop	Applied as surface mulch	62.5% reduction in weed density in wheat	
Littleseed canarygrass	Rice crop	Applied as surface mulch	46.2% reduction in weed density in wheat	
Barnyard grass, pickerel weed, Indian jointvetch (*Aeschynomene indica* L.)	Billy-goat weed (*Ageratum conyzoides* L.)	Mixed in soil as powder	70–100	[109]
Wild oat	Black mustard (*Brassica nigra* L.) crop	Soil incorporation	68	[110]
Pickerel weed, smallflower umbrella sedge, hemlock beggarticks (*Bidens biternata* L.)	Lilyturf (*Ophiopogon japonicus* K.)	Mixed in soil as powder	82.6–100	[111]
Cuban jute (*Sida rhombifolia* L.), common purslane, common cocklebur (*Xanthium strumarium* L.), *Ipomoea* spp., wild senna (*Cassia obtusifolia* L.)	Rye crop	Cover crop	80–90	[112]

(*continued*)

Table 2 (continued)

Weed species	Allelopathic source	Application mode	Weed dry weight reduction (%)	References
Common lamb's quarter, velvetleaf (*Abutilon theophrasti* Medik)	Rye crop	Cover crop	–	[113]
Goosegrass, Palmer amaranth (*Amaranthus palmeri* S. Wats), pitted white morning glory	Wheat crop	Cover crop	–	[114]
Littleseed canarygrass, common lamb's quarter, toothed dock, fumitory	Sorghum crop	Soil incorporation	42–56	[115]
Horse purslane, field bindweed, Bermuda grass, purple nutsedge	Sorghum crop	Surface mulch	50–96.6	[116]

pruriens [L.] DC.), jack bean, and wild tamarind (*Lysiloma latisiliquum* L.). Similarly, crabgrass and barnyard grass population in soybean was reduced when barley was grown as a cover crop [125]. Hyacinth bean and jack bean as cover crop can efficiently control the mission grass (*Pennisetum polystachion* L.), a noxious weed in rubber (*Hevea brasiliensis* Mull. Arg.) plantations [126]. Red spider lily (*Lycoris radiate* L.) can be grown as a cover crop as its dead leaves have lycorine (0.08%) allelochemical, which can reduce the germination and root/shoot growth of rice weeds [127]. Leaf water extracts of the cover crops [e.g., trefoil (*Oxalis brasiliensis* L.)] have been reported to decrease the growth of lettuce, star-of-Bethlehem (*Ornithogalum umbellatum* L.), red spider lily, European pennyroyal (*Mentha pulegium* L.), creeping thyme (*Thymus serpyllum* L.), moss pink (*Phlox subulata* L.), and chamomile (*Chamaemelum nobile* L.) [128, 129].

Cover crops also suppress weed in conservation tillage systems. For example, rye as a cover crop can control the weed population in soybean crop in no-till system [113]. Use of rye and wheat as cover crop in cotton also helps to control diverse weeds [114]. Cover crops have also been reported to decrease the biomass of goosegrass, pitted white morning glory (*Ipomoea lacunosa* L.), and palmer amaranth (*Amaranthus palmeri* S. Watson) with decreased seed bank in the soil [74]. The cover crop residues enhance the nutrient level and allelochemicals in the soil that discourage the plant pests, mainly the diseases caused by the soilborne pathogens [130, 131].

3.4 Mulching and Soil Incorporation of the Allelopathic Crop Residues

Use of allelopathic mulches is also a pragmatic way to control weeds in field crops (Table 2; [17, 105, 76, 132]). The allelopathic mulches decrease the seed

germination and seedling growth through the release of allelochemicals in the rhizosphere [133, 134]. In addition, the use of allelopathic mulch can increase the soil fertility and the soil organic matter, soil moisture, and soil water infiltration and can influence the effects of raindrops on soil, thus altering the soil temperature, activities of soil microbes with simultaneous reduction in soil erosion [135, 136].

Different rice weeds such as barnyard grass, flat sedge (*Cyperus difformis* L.) purple nutsedge, and jungle rice were reduced by 70%, and paddy yield was enhanced by 20% when the allelopathic plant mulch (1–2 t ha^{-1}) was applied [137]. The annual weeds such as common chickweed (*Stellaria media* L.), shepherd's purse (*Capsella bursa-pastoris* L.), meadow grass (*Poa annua* L.), German chamomile, and henbit deadnettle (*Lamium amplexicaule* L.) in wheat, sunflower, and maize were decreased when the soil was amended with olive (*Olea europaea* L.) waste [138]. In addition, when purple passion fruit (*Passiflora edulis* L.) was applied at 2 t ha^{-1} as surface mulch in paddy field, it enhanced the rice yield by 35% and decreased the density of monochoria (*Monochoria vaginalis* L.) and barnyard grass than control treatment [139]. Similarly, weeds such as barnyard grass and pickerel weed (*Monochoria vaginalis* (Burm. f.) C. Presl.) were controlled by mulch of alfalfa (*Medicago sativa* L.) crop [137]. Use of wheat residue as soil cover conserved the soil moisture and reduced the weed population and biomass in broad bean (*Vicia faba* L.) [134]. In another study, mulching with wood chip material efficiently inhibited the weed density and also increased the soil organic matter and water-holding capacity of soil [140].

3.5 Use of Allelopathic Water Extracts

Allelochemicals when extracted in water from different parts of plant are also good alternatives of herbicides for weed control (Table 3; [14, 145]). Several studies conducted under the laboratory and field conditions revealed that weed density and dry biomass were reduced by the use of allelopathic water extracts [77, 142, 145], especially the sorghum crop water extract [146].

As natural herbicide, sorghum is considered as one of the very commonly used crops for water extract preparation. The horse purslane, field bindweed, Bermuda grass, and purple nutsedge were reduced by 39.7, 58.4, 26.5, and 11.4 due to sorgaab (sorghum water extract) application, respectively, in cotton [143]. Weeds of cotton, sunflower, and mung bean are also controlled by sorgaab [147]. In wheat, weed population is decreased, and grain yield is increased by the application of sorghum extract extracts at various rates [146]. For example, weeds such as wild oat, littleseed canarygrass, common lamb's quarter, and field bindweed were controlled by application of sorghum water extract [115].

Various other studies have reported that weed population in wheat [115], rice [148], cotton [12], maize [132], canola [77], and mung bean [116] was reduced by 18–44% by the application of sorghum water extract. In comparison to sole application of sorghum water extract, the combined application of sunflower, eucalyptus (*Eucalyptus camaldulensis* Dehnh.), and sorghum water extracts was found to be more efficient for the control of weeds in wheat fields [149]. Likewise, the

Table 3 Weed management through allelopathic crop water extracts

Weed species	Crop	Allelopathic water extract	Rate and timing of application	Reduction (%)		References
				Weed density	Weed dry mass	
Horse purslane	Direct seeded rice	Sorghum	18 L ha^{-1} at 20 days after sowing (DAS)	26.7–48.7	–	[105]
Purple nutsedge	Direct seeded rice	Sorghum	18 L ha^{-1} at 20 DAS	19.6–34.0	–	
Jungle rice	Direct seeded rice	Sorghum	18 L ha^{-1} at 20 DAS	10.7–35.4	–	
Crowfoot grass	Direct seeded rice	Sorghum	18 L ha^{-1} at 20 DAS	19.6–23.2	–	
Horse purslane	Transplanted flooded rice	Sorghum	18 L ha^{-1} at 20 DAS	23.0–27.8	–	
Purple nutsedge	Transplanted flooded rice	Sorghum	18 L ha^{-1} at 20 DAS	11.1–31.2	–	
Jungle rice	Transplanted flooded rice	Sorghum	18 L ha^{-1} at 20 DAS	12.5–26.1	–	
Crowfoot grass	Transplanted flooded rice	Sorghum	18 L ha^{-1} at 20 DAS	24.5–33.3	–	
Jungle rice, purple nutsedge, rice flat sedge (*Cyperus iria* L.)	Rice	Sorghum	15 L ha^{-1}	–	40.4	[141]
Little seed canarygrass, wild oat	Wheat	Sorghum + brassica	12 L ha^{-1} (each) at 30 and 40 DAS	–	18–27	[142]
Little seed canarygrass, wild oat	Wheat	Sorghum + sunflower	12 L ha^{-1} (each) at 30 and 40 DAS	–	24–39	
Horse purslane	Cotton	Sorghum	One spray at 20 DAS	–	39.7	[143]
Field bindweed	Cotton	Sorghum	Two sprays at 20 and 40 DAS	–	58.4	
Bermuda grass	Cotton	Sorghum	Three sprays at 20, 40, and 60 DAS	–	26.5	
Purple nutsedge	Cotton	Sorghum	–	–	11.4	
Littleseed canarygrass, wild oat	Wheat	Sorghum + eucalyptus	12 L ha^{-1} (each) at 30 and 40 DAS	–	13–32	[142]
Common lamb's quarter,	Mung bean	Sorghum	One spray at 20 DAS at	11.4	13.85	[116]

(*continued*)

Table 3 (continued)

Weed species	Crop	Allelopathic water extract	Rate and timing of application	Reduction (%)		References
				Weed density	Weed dry mass	
purple nutsedge, field bindweed			300 L ha^{-1} each			
Common lamb's quarter, purple nutsedge, field bindweed	Mung bean	Sorghum	Two sprays at 20 and 30 DAS at 300 L ha^{-1} each	17.54	23.73	
Common lamb'squarter, purple nutsedge, field bindweed	Mung bean	Sorghum	Three sprays at 20, 30, and 40 DAS at 300 L ha^{-1} each	31.58	44.11	
Common lamb's quarter, purple nutsedge, field bindweed	Mung bean	Sorghum	Four sprays at 20, 30, 40, and 50 DAS at 300 L ha^{-1} each	38.60	47.59	
Purple nutsedge, horse purslane	Sunflower	Sorghum	One spray at 20 or 40 DAS	15.8–19.3	19.1–27.2	[144]

applications of sorghum water extract with eucalyptus, sesame (*Sesamum indicum* L.), sunflower, tobacco (*Nicotiana tabacum* L.), and *Brassica* sp. efficiently controlled the weeds such as littleseed canarygrass and wild oat in wheat [142].

3.6 Use of Allelopathic Water Extracts with Reduced Doses of Herbicides

Although, sole application of allelopathic water extracts is beneficial and eco-friendly for weed control, nonetheless fair weed control is not achieved with this. The combined use of allelopathic water extracts with reduced doses of herbicides may be more helpful to control different weed biotypes (Table 4; [6, 79, 157]). For example, Cheema et al. [158] compared the standard dose of atrazine (300 g a.i. ha^{-1}) with the combined application of sorghum water extract (12 L ha^{-1}) and reduced dose of atrazine (50, 100, and 150 g a.i. ha^{-1}). They found that the combined application of sorghum water extract (12 L ha^{-1}) and reduced dose of atrazine (150 g a.i. ha^{-1}) was as useful as the standard dose of herbicide for controlling different weeds such as field bindweed, purple nutsedge, and horse purslane. In a similar study, the combined application of sorghum water extract

(12 L ha^{-1}) with pendimethalin (0.5 g a.i. ha^{-1}) and S-metolachlor (1.0 kg a.i. ha^{-1}) reduced the density and dry biomass of horse purslane [159]. A reduction of 53–95% was recorded in the weeds' dry weight when sorghum water extract (10 L ha^{-1}) was sprayed in combination with reduced doses of herbicides [160]. Various studies have reported that the combined application of allelopathic water extracts in combination with reduced dose of pre- and postemergence herbicides reduced the weed density and dry biomass (Table 4).

4 Allelopathy for Insect Pest Management

Insects cause considerable damages to grain crops, fiber crop, legumes, and vegetable crops. Insecticides have harmful effects on the environment and affect the health and cause hygienic problems. Due to frequent and irrational application of insecticides, the insects have evolved resistance against insecticides. In this scenario, the use of secondary metabolites (i.e., allelochemicals) is the effective method to control the insect pests (Table 5; [6]). There have been several advantages of these metabolites such as easy handling, easy biodegradation, economical affordability, and the environmental safety.

The chickpea beetle (*Callosobruchus chinensis*) is efficiently controlled by application of secondary metabolites derived from olive, bhang (*Cannabis sativa* L.), tea (*Thea chinensis* Sims), garlic (*Allium sativum* L.), black pepper (*Piper nigrum* L.), and red chillies (*Capsicum annum* L.) [169]. The eucalyptus volatile oil was useful for the control of rice moth (*Corcyra cephalonica* St.). Aphids and sucking insects of *Brassica* spp. can be controlled by allelopathic water extracts from mulberry (*Morus alba* L.), sorghum, sunflower, and mustard species [6]. The strawberry aphids (*Chaetosiphon fragaefolii*; nymphs and adults) are controlled by use of neem (*Azadirachta indica* A. Juss) seed oil [170]. The green cicadellid (*Jacobiasca lybica*), whitefly (*Bemisia tabaci*), and *Ashbya gossypii* were controlled by azadirachtin, an allelochemical extract from different parts of neem plant [168]. The conifer plantations provide shelter for large pine weevil (*Hylobius abietis* L.) which can be managed by treating with neem oil. Azadirachtin, salannin, and nimbin are the allelochemicals found in neem oil which help to control feeding weevil of Sitka spruce (*Picea sitchensis* B.) [171].

The phenolics such as ferulic acid and *p*-coumaric acid can be used to control wheat midge (*Sitodiplosis mosellana* G.) [172]. The mosquito (*Culex pipiens* L.) and Mediterranean fruit fly [*Ceratitis capitata* (Wiedemann)] larvae are controlled by common rue (*Ruta graveolens* L.), a fragrant plant that has coumarins and flavonoid compounds. Cover crop residues enhance the nutrient level and allelochemical in the soil that discourage plant pests, mainly diseases of soilborne pathogens [130, 131]. In a study, the larva of rice moth (*Corcyra cephalonica* St.) was controlled by the application of eucalyptus volatile oils [166]. The allelopathic water extracts of mustard, sunflower, and sorghum in combination with the mulberry extracts were effective for managing the different sucking insects (e.g., aphid) in *Brassica* spp. Sorghum and sunflower water extract were found to be more effective for aphid mortality [77].

Table 4 Weed management in field crops through combined application of allelopathic water extracts and reduced herbicides doses

Weeds controlled	Allelopathic extract + herbicide	Crop	Reduction (%)		References
			Weed density	Weed dry weight	
Swine cress, littleseed canarygrass	Sorghum + sunflower (18 L ha^{-1} each) + metribuzin (Sencor 70 WP) at 52.5 g a.i. ha^{-1})	Wheat	83.3	77.9	[82]
Swine cress, littleseed canarygrass	Sorghum + sunflower (18 L ha^{-1} each) + bensulfuron + isoproturon (Cleaner 70 WP) at 52.5 g a.i. ha^{-1}	Wheat	88.2	88.5	[82]
Swine cress, littleseed canarygrass	Sorghum + sunflower (18 L ha^{-1} each) + metribuzin + phenoxaprop (Bullet 38 SC) at 57 g a.i. ha^{-1}	Wheat	87.3	91.6	[82]
Swine cress, littleseed canarygrass	Sorghum + sunflower (18 L ha^{-1} each) + mesosulfuron + idosulfuron (Atlantis 12 EC) at 36 g a.s. ha^{-1}	Wheat	87.3	92.9	[82]
Swine cress, littleseed canarygrass	Sorghum + sunflower (18 L ha^{-1} each) + mesosulfuron + idosulfuron (Atlantis 3.6 WG) at 4.32 g a.i. ha^{-1}	Wheat	87.3	90.3	[82]
Horse purslane	Atrazine (125–250 g a.i. ha^{-1}) + sorghum + brassica + sunflower + mulberry water extracts (20 L ha^{-1} each)	Maize	15.6	85–90	[150]
Barnyard grass, rice flat edge, jungle rice, purple nutsedge, crowfoot grass	Sorghum water extract (7.5 L ha^{-1}) + ryzelan (15 mL ha^{-1})	Rice	38.4	35	[141]

(*continued*)

Table 4 (continued)

Weeds controlled	Allelopathic extract + herbicide	Crop	Reduction (%)		References
			Weed density	Weed dry weight	
Field bindweed, redroot pigweed	Furamsulfuron (half dose) + sorgaab	Maize	–	57.3	[151]
Barnyard grass	Sorghum/ sunflower/rice water extract (15 L ha^{-1}) + butachlor (400–600 g a.i. ha^{-1})	Rice	65–75	63–79	[152]
Barnyard grass	Sorghum/ sunflower/rice water extract (15 L ha^{-1}) + pretilachlor (208–313 g a.i. ha^{-1})	Rice	64–76	57–79	[152]
Barnyard grass	Sorghum/ sunflower/rice water extract (15 L ha^{-1}) + ethoxysulfuron (10–15 g a.i. ha^{-1})	Rice	65–72	58–72	[152]
Rice flat sedge	Sorghum/ sunflower/rice water extract (15 L ha^{-1}) + butachlor (400–600 g a.i. ha^{-1})	Rice	60–72	49–71	[152]
Rice flat edge	Sorghum/ sunflower/rice water extract (15 L ha^{-1}) + pretilachlor (208–313 g a.i. ha^{-1})	Rice	61–72	36–60	[152]
Rice flat edge	Sorghum/ sunflower/rice water extract (15 L ha^{-1}) + ethoxysulfuron (10–15 g a.i. ha^{-1})	Rice	61–69	26–74	[152]
Crowfoot grass	Sorghum/ sunflower/rice water extract (15 L ha^{-1}) +	Rice	63–74	62–76	[152]

(*continued*)

Table 4 (continued)

Weeds controlled	Allelopathic extract + herbicide	Crop	Reduction (%)		References
			Weed density	Weed dry weight	
	butachlor (400–600 g a.i. ha^{-1})				
Crowfoot grass	Sorghum/ sunflower/rice water extract (15 L ha^{-1}) + pretilachlor (208–313 g a.i. ha^{-1})	Rice	62–74	50–81	[152]
Crowfoot grass	Sorghum/ sunflower/rice water extract (15 L ha^{-1}) + butachlor (400–600 g a.i. ha^{-1})	Rice	67–75	31–69	[152]
Purple nutsedge, horse purslane	Sorghum water extract at 10 L ha^{-1} + pendimethalin (1.0 kg a.i. ha^{-1})	Cotton	39.1–51.9	37.2–50.3	[153]
Purple nutsedge, horse purslane	Sorghum water extract at 10 L ha^{-1} + S-metolachlor (2.15 kg a.i. ha^{-1})	Cotton	47.8–53.9	56.6–62.8	[153]
Common lamb's quarter, swine cress	Sunflower + sorghum water extract each at 15 L ha^{-1} + pendimethalin (825 ml ha^{-1})	Sunflower	84	67.3	[154]
Purple nutsedge, annual yellow sweet clover	Sorghum water extract at 12 L ha^{-1} + S-metolachlor (2.15 kg a.i. ha^{-1})	Cotton	77	77	[80]
Horse purslane, purple nutsedge, common lamb's quarter, swine cress	Brassica + sorghum water extract each at 15 L ha^{-1} + pendimethalin (1.2 kg a.i. ha^{-1})	Canola	42.8–94.2	37.46–94.18	[155]
Littleseed canarygrass	Sorghum water extract at 12 L ha^{-1} + isoproturon (1.0 kg a.i. ha^{-1})	Wheat	94.2	64.8	[156]

Table 5 Suppression of insect pests through allelopathy

Allelopathic source and application rate	Insect pests suppressed	Mortality (%)	References
Kalonji (*Nigella sativa* L.) oil extract (10% concentration)	Red flour beetle (*Tribolium castaneum*; Coleoptera: Tenebrionidae)	48.0 repellency against grubs	[161]
Clove [*Syzygium aromaticum* (L.) Merr. et Perry)] oil extract (10% concentration)	Red flour beetle	47.5 repellency against grubs	
Olive oil extract (10% concentration)	Red flour beetle	46.0 repellency against grubs	
Neem leaf powder (100 g in 1 L water)	Asian citrus psyllid (*Diaphorina citri* Kuwayama) nymphs	31.5	[162]
Neem leaf powder (100 g in 1 L water)	Asian citrus psyllid adults	26.1	
Datura (*Datura alba* Nees) leaf powder (100 g in 1 L water)	Asian citrus psyllid nymphs	31.5	
Datura leaf powder (100 g in 1 L water)	Asian citrus psyllid adults	20.8	
Fig-leaf goosefoot (*Chenopodium ficifolium* Sm.) ethanol extract (5000 mg mL^{-1})	Aphid (*Aphis gossypii* Glover)	86	[163]
California pepper tree (*Schinus molle* Rev L.) ethanol extract (4.7% w/v)	Elm leaf beetle (*Xanthogaleruca luteola* Muller)	92	[164]
Birbira (*Millettia ferruginea* Hochst.) (seed crude extract)	*Macrotermes* termites	93–100	[165]
Neem oil volatiles	*Corcyra cephalonica* St.	26	[166]
Neem seed kernels water extract (2%)	Flower thrip (*Taeniothrips sjostedti* Trybom)	54	[167]
Fig-leaf goosefoot methanol extract (5000 mg mL^{-1})	Aphid	83	[163]
Fig-leaf goosefoot n-Hexane extract (5000 mg mL^{-1})	Aphid	54	
Birbira seed crude extract	Sorghum chaffer (*Pachnoda interrupta* Oliver)	45–60	[165]
Eucalyptus (*Eucalyptus camaldulensis* L.) oil volatiles	*Corcyra cephalonica* St.	67–78	[166]
Hot pepper (*Capsicum annuum* L.) fruit water extract (2%)	Flower thrip	54	[167]
Hot pepper fruit water extract (2%)	Pod borer (*Heliothis armigera* Hb.)	31	
California pepper tree water extract (5.6% w/v)	Elm leaf beetle	28	[164]

(*continued*)

Table 5 (continued)

Allelopathic source and application rate	Insect pests suppressed	Mortality (%)	References
Neem seed kernel water extract (2%)	Pod borer	32	[167]
Fig-leaf goosefoot [acetone extract (5000 mg mL^{-1})]	Aphid	47	[163]
Tomato [leaf water extract (4%)]	Flower thrip	32	[167]
NeemAzal-T/S® (20 g a.i. ha^{-1})	*Jacobiasca lybica* (Berg. and Zanon)	92	[168]
Tomato leaf water extract (4%)	Pod borer	12	[167]

In a study, the application of ethanol leaf water extracts of California pepper tree (*Schinus molle* L., 4.3–4.7% w/v) reduced the population of elm leaf beetle (*Xanthogaleruca luteola* M**ü**ller.) by 97% [164]. Some weeds such as ragweed (*Ambrosia trifida* L.), chickweed, and Spanish flag (*Lantana camara* L.) have a great allelopathic activity against insect pests [173].

5 Allelopathy for Disease Management

Several crops including oilseed, cereal, sugar crops, and especially vegetables are seriously affected by the plant diseases. The main causing agents of soilborne and seed-borne diseases are fungi, bacteria, viruses, and certain nematode pathogen. A decrease in crop stand and production quality has been reported due to incidence of soilborne diseases with simultaneous reduction in the final yield. Although cultural operations like setting of infested plant residues to fire and adopting resistant genotypes against disease may provide some control, still plant diseases may cause considerable yield losses. The control of diseases through chemical is non-ecological and may deteriorate the environment [6]. This indicates demand for the control of pathogens and diseases and through ecological approaches like allelopathy. A fungus *Sclerotinia sclerotiorum* in beans was controlled by the application of water extracts of various cereals, sweet clover, canola, and lentil (*Lens culinaris* Medik.) at low concentrations [174].

The bark of sugi (*Cryptomeria japonica* D.) has been used to control the root infection diseases in tomato [70]. The growth of bacterial wilt (*Pseudomonas solanacearum* Smith) was inhibited by the application of root exudates of the Chinese chive (*Allium tuberosum* L.) without adverse effect on tomato [175]. Likewise, the aerial parts of some marigold (*Tagetes erecta* L.) species produce the volatile allelochemicals which are useful to control tomato early blight disease (90% reduction), caused by *Alternaria solani* [70]. In tomato intercropped with cowpea, the tomato bacterial wilt, caused by *Ralstonia solanacearum*, was controlled to a great extent [175]. The growth of root-knot nematode (*Meloidogyne javanica* T.) can be controlled by using the neem leaves/cakes [176]. Volatile sulfur compounds

(glucosinolates) are produced by *Brassica* spp. in soil microenvironment of soil which can be useful for the reduction of fungal pathogens [177] and soil nematodes.

Application of canola (1%), barley, oat, and lentil extracts (each 2% and 4%) significantly decreased the germination of sclerotia than control. However, ascospore germination was controlled by the application of 2 and 4% barley and lentil extracts, respectively, while ascospore germination was encouraged by these similar applications of wheat, canola, sweet clover, lentil, and rye extracts [178]. The root rot disease in cumin (*Cuminum cyminum* L.), caused by *Fusarium oxysporum* and other *Fusarium* spp. (e.g., *F. moniliforme*, *F. lateritium*, *F. equiseti*, *F. solani*, and *F. dimerum*), was controlled by oil extract from cumin (*Cuminum cyminum* L.), basil (*Ocimum basilicium* L.), and the rose geranium (*Pelargonium graveolens* L [179]). Similarly, plant pathogens, such as *Fusarium verticillioides*, *Bipolaris sorghicola*, *Trichothecium roseum*, *F. solani*, *Alternaria alternata*, *Curvularia lunata*, *Cladosporium cladosporioides*, *F. oxysporum*, *A. strictum*, *Aspergillus flavipes*, etc., were controlled by seaweed extract (0.3%) in sorghum seeds. They also increased the activities of defensive enzymes such as ammonia lyase, peroxidase, phenylalanine, β-1, 3-glucanase, and chitinase [180]. In another study, the application of seed meal of Ethiopian mustard (*Brassica carinata* L.) at 2.5 t ha^{-1} enhanced the yield of tomato and reduced the root knot [181]. Two fungal pathogens including *Rhizoctonia solani* Kühn and *Pyricularia oryzae* (Cavara) were controlled, through inhibition of spore germination. There had been proposed another important component of the defensive system of rice against diseases and weeds by two allelochemicals present in rice, i.e., the 3-isopropy l-5-acetoxycyclohexene-2-one-1 and 5,7,40-trihydroxy-30,50-dimethoxyflavone [182]. The use of crop allelochemicals is, thus, a cheap and environment-friendly way for managing crop diseases (Table 6).

6 Conclusion

Various plants such as rice, sorghum, wheat, sunflower, eucalyptus, mulberry, and neem have great allelopathic potential which can be exploited to reduce weed pressure and control the insects and diseases in field and horticultural crops. For weed control, the allelopathy can be exploited through crop rotation, use of allelopathic water extracts alone or in combination, intercropping and use of cover crops. Nevertheless, special care is required to avoid any damaging influence of the allelopathic phenomenon on the agricultural systems. The future research strategies should include the breeding of crop cultivars with higher allelopathic potential to cope with biotic and abiotic stresses. Although the allelochemicals extracted from crop plants have been used for decades as eco-friendly pesticides, there have been very few allelochemical-based pesticides available in the market across the globe. Thus, due to increase in the area under organic crops, and to protect the environment from the hazards of pesticides, the ecological and physiological mechanisms of the allelopathy need greater attention in future studies with great emphasis on development of allelochemical-based pesticides. Although, the molecular mechanism of

Table 6 Allelopathic suppression of diseases and nematodes

Allelopathic source	Application mode/rate	Pathogen/disease suppression	References
Neem cake	1% (mass/mass soil)	67–90% decrease in lesion (*Pratylenchus penetrans*) number and root-knot nematodes in the tomato crop	[183]
Neem cake	1% (mass/mass soil)	23% decrease in nematodes in maize roots and 70% decrease in root-knot nematodes in the soil around roots	
Rice	Root exudates (1.5 mL)	37% decrease in the spore germination of *Fusarium rum* f. sp. *Niveum*	[184]
Rice	Root exudates (20 mL)	71.9% decrease in the spore germination of *Fusarium rum* f. sp. Niveum	
Neem	Leaf water extract (20% w/v)	53.22% decrease in the growth of *Fusarium solani* f. sp. *Melongenae*	[185]
Sweetworm wood (*Artemisia annua*)	Leaf water extract (20% w/v)	42.2% decrease in the growth of *Fusarium solani* f. sp. *Melongenae*	
Neem cake	3% (w/w)	61.03% decrease in the number of female root-knot nematode (*Meloidogyne javanica*) in roots	[176]
Barley + potato	Grown in rotation	55.1% decrease in the inoculum intensity of *Rhizoctonia solani* (JG Kühn)	[186]
Rice	Root exudates (20 L)	71.88% decrease in the spore production of *Fusarium oxysporum* f. sp. Niveum	[184]
Eucalyptus	Leaf water extract (20% w/v)	46.76% decrease in the growth of *Fusarium solani* f. sp. Melongenae	[185]
Neem cake	3% (w/w)	63.7% decrease in egg masses of root-knot nematodes in roots	[176]
Neem leaves	3% (w/w)	38.3% decrease in the number of female root-knot nematode in roots	[176]
Indian mustard (*Brassica juncea* L.) + potato	Grown in rotation	45.5% decrease in the inoculum intensity of *Rhizoctonia solani* (JG Kühn)	[186]
Neem leaves	3% (w/w)	60.34% decrease in the egg masses of root-knot nematodes in the roots	[176]
Rhubarb (*Rheum emodi* L.)	Leaf water extract (20% w/v)	37.2% decrease in the growth of *Fusarium solani* f. sp. Melongenae	[185]
Tulsi (*Ocimum sanctum* Linn)	Leaf water extract (20% w/v)	44% decrease in the growth of *Fusarium solani* f. sp. Melongenae	
Turnip (*Brassica rapa* L.) + potato	Grown in rotation	56.2% decrease in the inoculum intensity of *Rhizoctonia solani* (JG Kühn)	[186]

allelopathy has been explored, further research is needed for widespread application of allelopathy in agricultural production worldwide. The phenomenon of allelopathy, thus, offers an attractive way to attain the sustainability of agriculture, environmental safety, food safety, conservation of resources, and economic constancy.

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Part IV

Biotic/Abiotic Stress and Secondary Metabolites in Plants

Decrypting Early Perception of Biotic Stress on Plants

24

Simon A. Zebelo

Contents

Abstract

Plant response to biotic stress induced by various herbivores and pathogens involves different defense mechanisms. Plant defense strategies against biotic stressors start in the plasma membrane, where the biotic stressors interact physically by mechanical damage and chemically by introducing elicitors or triggering plant-derived signaling molecules. The concept of "early" is relative and depends on the dynamics of plant cells responding to stimuli. The stimuli triggered by different biotic stressors result in different rates of plant responses, which often depend on the intensity and the rate of the stimulus. In plant responses to stimuli, the term "early" is often used to indicate the first visible or detectable plant response. Plant early biotic stress responses vary based on the type of the stressors. Based on the type of stressors, the rate of early responses is classified

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as (1) early responses to microbes, (2) early responses to herbivores, and (3) early response to nearby plants. This chapter discusses the variability in early plant responses to stimuli caused by biotic stressors and the importance of understanding the timing of plant responses to changing biotic stimuli.

Keywords
Biotic stimuli · Early signaling · Signal transduction · Molecular patterns

Abbreviations

AM	Arbuscular mycorrhiza
BAK1	Brassinosteroid-insensitive 1 (BRI1)-associated kinase 1
BIK1	Botrytis-induced kinase 1
BRI1	Brassinosteroid-insensitive 1
CCaMK	Calcium−/calmodulin-dependent protein kinase
CDPK	Ca^{2+}-dependent protein kinases
CERK1	Chitin elicitor receptor kinase 1
CSSP	Common symbiotic signaling pathway
DAMPs	Damage-associated molecular patterns
EF-Tu	Elongation factor-Tu
ETI	Effector-triggered immunity
FACs	Fatty acid amino acid conjugates
FLS2	Flagellin-sensitive 2
GA	Gibberellic acid
GLV	Green leaf volatile
HAMPs	Herbivore-associated molecular patterns
IAA	Indole acetic acid
LCO	Lipochitooligosaccharidic
LRR	Leucine-rich repeat
LysM	Lysine motifs
MAMPs	Microbe-associated molecular patterns
MeSA	Methyl salicylate
MTI	MAMP-triggered immunity
NF	Nodulation (Nod) factors
OS	Oral secretions
PAMPs	Pathogen-associated molecular patterns
PGPR	Plant growth-promoting rhizobacteria
PNG	Peptidoglycan
PRRs	Pattern recognition receptors
PTI	PAMP-triggered immunity
RLKs	Receptor-like kinases
RLPs	Receptor-like proteins
ROS	Reactive oxygen species
Vm	Transmembrane potential
WIPK	Wound-induced protein kinase

1 Introduction

In plant cells, the perception of stimuli triggered by small perturbations in their surrounding environment, and a prompt response, is mounted to prevent severe and perhaps irreversible damage. An early response to external stimuli allows plants to cope with stress, and the perception time ranges from seconds to minutes or hours of exposure. Failure to rapidly perceive stimuli, transduce the signal, and regulate the relevant genes at the appropriate time ultimately results in the inability of plant growth and development, which eventually leads to death [1].

In plants, the concept of "early response" is related to the type and intensity of response to external stimuli. Biotic stimuli caused by viruses, bacteria, fungi, herbivores, and plants have different levels of intensity that correspond to different response rates. A few hours is considered an early response time when some responses to stimuli take days to become apparent. However, the extremely rapid response to the rapid and intense cell and tissue damage caused by herbivores requires response times that range from the fraction of seconds to minutes. Therefore, when defining the term "early," we must always consider the intensity and duration of the external stimulus.

Regardless of the type of biotic stimulus, the first general sensor is the plasma membrane, which is considered to be the first barrier between a living cell and the outside environment. As a dynamic system, the plasma membrane contains receptors, proteins, enzymes, transporters, and channels that all contribute to the stimulus perception and subsequent signal transduction [1]. Second messengers have a basic role in signal transduction, and intracellular calcium variations are involved in the early signals of many biotic stimuli perceptions, representing a common mechanism of the cellular response to the external environment. Tissues and organs exposed to the environment respond specifically and sometimes differently to stimuli, allowing the plant to make an integrated response. The timing of detection of plant responses is important to better assess productivity, stress adaptation, natural variation, population dynamics, and plant-plant interactions in changing environmental conditions. From this perspective, we will examine the different timing of plant responses to biotic environmental stimuli by exploring the variability of early responses caused by viruses, bacteria, fungi, herbivores, and nearby plants.

2 Early Perception of Stress Caused by Microorganisms

Pathogenic and beneficial microbes are initially perceived as harmful aggressors in order to limit their invasion [2]. Early perception of pathogenic and beneficial microbes takes place by pattern recognition receptors (PRRs) in the plant plasma membrane. PRRs play a key role in recognizing microbe- or pathogen-associated molecular patterns (MAMPs/PAMPs) which are microorganism molecular signatures. These molecular signatures include microbial structures, such as cell wall components (chitin, chitin fragments (chitooligosaccharides), peptidoglycan (PNG)) and motility organs (flagellin) [3–5]. The PRRs are generally receptor-like kinases

(RLKs) or receptor-like proteins (RLPs) [3, 5]. Lysine motifs (LysM) or lectin motifs are common extracellular domains in RLKs and RLPs; the extracellular motif is the most characterized domain in plants [6, 7]. PRRs can perceive also self-molecular signatures termed as damage-associated molecular patterns (DAMPs), which are plant cell wall fragments produced as a result of mechanical damage or enzymatic degradation by microbial activities [4, 5]. PRR-mediated microbe perception induces a comprehensive defense responses in plants called MAMP- or PAMP-triggered immunity (MTI/PTI) [5]. MTI/PTI leads to early plant defense signaling which is initiated with variations in the plasma transmembrane potential (Vm), promoting intracellular Ca^{2+} influx; these events eventually lead to the massive transcriptional reprogramming that initiates defense responses such as production of antimicrobial secondary metabolite, stomatal closure, and thickening of the cell wall [8–10]. MTI/PTI prevents non-adapted microbes from infecting and is therefore an important barrier against disease. The adopted pathogens overcome the MTI/PTI responses by batteries of virulence factors known as effectors [9]. Host plants evolved to win back the arm race with the adopted microbes by host-specific intracellular receptor (R) proteins that sense effectors and trigger a defense against adopted microbes called effector-triggered immunity (ETI) [9, 11] (Scheme 1).

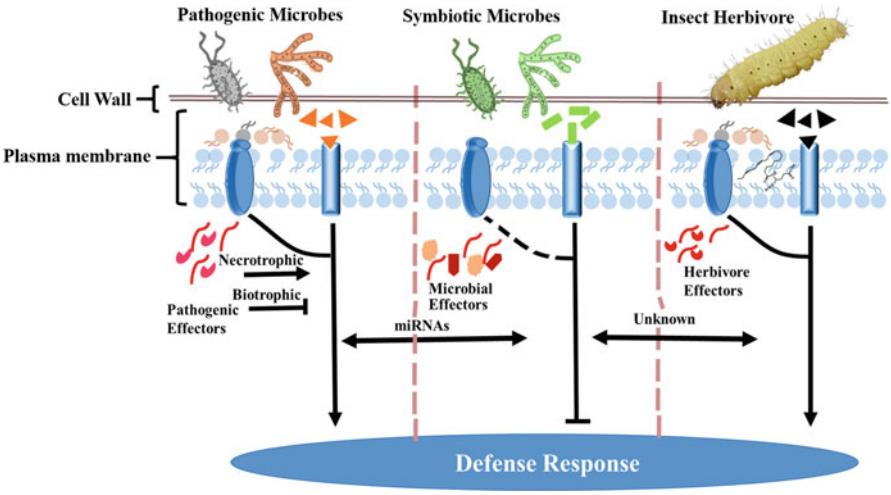
2.1 Early Response to Bacteria

The PRR flagellin-sensitive 2 (FLS2) is one of the most well-studied plant PRRs. FLS2 binds to bacterial flagellin (flg22) and promotes the formation of LRR receptor-like kinase (RLK) and brassinosteroid-insensitive 1 (BRI1), which acts as a co-receptor for flg22 and is essential for the full activation of MTI signaling [12]. It has been shown that the phosphorylation of the plant LRR kinase FLS2 is rapid, occurring within 15 s of stimulation with flg22 [13]. Other PRRs involved in the early perception of bacteria include the elongation factor-Tu (EF-Tu) receptor (EFR), which perceives bacterial EF-Tu and its peptide epitope elf18 [5]. The application of flg22 and elf18 induced a transient depolarization in *Arabidopsis* mesophyll cells and root hairs after a delay of approximately 1–3 min [14].

Plants perceive peptidoglycans (PGNs) which are a major constituent of bacterial cell walls. Plants employ several receptors to perceive PGNs. For instance, in *Arabidopsis*, PGN is perceived by two LysM-RLPs (*AtLYM1* and *AtLYM3*) [15] and in rice by two LysM-RLPs (*OsLYP4* and *OsLYP6*) [16]. The PGN-induced responses in *Arabidopsis* also require the LysM-RLK chitin elicitor receptor kinase 1 (CERK1), which failed to bind PGN itself [15].

2.2 Early Response to Fungi

Chitin is a major component of fungal cell walls and has been recognized as a general elicitor of plant defense responses for many years [17]. Different plants have evolved distinct mechanism of chitin perceptions. For instance, fungal chitin-related



Scheme 1 Shows how plants may differentiate between pathogenic, mutualistic microbes and herbivore injury. In pathogenic microbes, first plant cell wall-degrading enzymes disrupt cellular integrity. Cellular debris, ATP, and carbohydrates are sensed by damage-associated molecular pattern (DAMP) receptors (spherical membrane-bound structures) that initiate the early signaling to induce an immune response and instantly trigger signaling from microbe-associated molecular pattern (MAMP) receptors (rectangular membrane-bound structures) that perceive conserved pathogenic proteins. Plant defense signaling is further modulated by plant miRNAs and can also be manipulated by pathogenic effector proteins/molecules (pie shapes in pink) and small interfering RNA (siRNA, helical structures). In symbiotic microbes, similar cellular damage is not sustained, and therefore, the DAMP receptors are thought to be silent (dashed line) and consequently repress plant immune signaling. Concurrently, a set of mutualistic MAMP receptors also signal to repress cellular defenses. These two pathways are also modulated by plant miRNAs and by mutualistic effector proteins/molecules. In insect herbivore injury, the first herbivore injury causes mechanical damage that disrupts cellular integrity and followed by the action of elicitors. Similar to pathogenic microbes, signaling cascade cellular debris, ATP, and carbohydrates are sensed by DAMPs that initiate the early signaling to induce an immune response and instantly trigger signaling from herbivore-associated molecular pattern (HAMP) receptors (rectangular membrane-bound structures) that perceive herbivore-associated proteins and can also be manipulated by pathogenic effector proteins/molecules (pie shapes in red) and small interfering RNA (siRNA, helical structures). It is unknown whether the herbivore-associated pathways in cross talk with symbiotic or pathogenic microbes via plant miRNAs and by mutualistic effector proteins/molecules

PRRs are perceived by the LysM-RLK, chitin elicitor receptor kinase 1 (CERK1), and lysin motif-containing RLK5 (LYK5) in *Arabidopsis* [18]. Chitin induces *OsCERK1* activation in rice protoplasts early, within 3 min of treatment [19]. Moreover, increases in cytoplasmic Ca^{2+} concentration and *OsMAPK6* activation occur within 5 min of chitin treatment [20]. Chitin perception mechanism of *Arabidopsis* is different from rice, where *AtCERK1* binds directly to octamers of chitin, which in turn induces *AtCERK1* homo-dimerization and consequent immune signaling [21–23]. *Arabidopsis* might not use CEBiP-like LysM-RLPs to trigger immune responses upon chitin perception [22, 24].

As Y. Shen et al. [25] extensively reviewed about early signaling responses to fungal pathogens. Barley cultivars in North America were highly susceptible to the most devastating barley stem rust disease caused by *Puccinia graminis* f. sp. *tritici* (*Pgt*). In 1942, new resistant *Rpg1 gene* discovered has protected barley cultivars from severe stem rust losses for over 70 years [26]. The *Rpg1* gene encodes a constitutively expressed protein containing two tandem kinase domains: the protein kinase 1 (pK1) domain and protein kinase 2 (pK2) domain. The pK1 is a pseudo kinase, whereas the pK2 domain is catalytically active, and both domains are required for stem rust resistance. The pseudo kinase pK1 domain is associated with disease resistance, and the pK2 domain is involved in protein phosphorylation [27, 28]. The RPG1 protein is a functional kinase located in the plasma membrane, endomembranes, and cytosol. The resistance protein RPG1 disappeared rapidly (within 5 min) when barley seedling leaves were inoculated by avirulent and viable stem rust *P. graminis* pathotype MCCF. The disappearance of the RPG1 protein is due to phosphorylation and the phosphorylated status sustained for 20 h after inoculation. It is suggested that RPG1 protein phosphorylation is essential for disease resistance. The reciprocal responses of barley and stem rust belong to ETI of plant [25]. Interestingly, in the incompatible combination of wheat and leaf rust (Lovrin 10 and leaf rust race 260), the expression of the *TaCDPK2* gene was obviously increased at the levels of mRNA and protein, while the *TaCAMTA4* gene expression level started to decrease gradually after wheat leaves inoculated with leaf rust after 4 h [25].

2.3 Early Response to Virus

Unlike bacteria and fungi, viruses are not commonly regarded as encoding PRRs-PAMPs/MAMPs [29]. However, it was recently found that *Arabidopsis* mutants for LRR-RLK BAK1 are more susceptible to viral infection [30], suggesting that PRRs and unknown PAMP or DAMP might be involved in early virus perception. RNA silencing clearly represents a major plant immune strategy against viruses [31]. Thus, future research is required to understand systematically how these PRRs are involved in virus perception.

2.4 Early Response to Phytoplasmas

Phytoplasmas are plant pathogenic bacteria deviated from Gram-positive bacteria without cell wall, and they have significant genome reductions and are obligate intracellular pathogens of plants [32]. Phytoplasmas, unlike extracellular bacterial phytopathogens, do not require specialized secretory systems for pathogenesis; instead, they directly introduce their effector proteins inside host plant cytoplasm [33]. Phytoplasmas, transmitted by insect vectors to plants, have distinctly regulated effector genes for insect and plant colonization [34, 35]. Once translocated into the plant cytoplasm, effectors can traffic to different subcellular compartments,

including organelles and various membrane compartments [33]. A large number of effectors accumulate in the plant nucleus [36]. In systemically infected plants, phytoplasmas secret effector protein that trigger Ca^{2+} influx into the sieve elements detected as early as 24 h, conferring forisome dispersion, callose deposition, and probably cell wall thickening [37].

2.5 Early Response to Symbiotic Microbes

Beneficial microbes such as soil bacteria, epiphyte bacteria and arbuscular mycorrhiza provides beneficial effects on plant growth or/and stress resistance against plant pathogen and insect pests [38, 39]. Pathogenic as well as beneficial bacteria are initially recognized as harmful invaders in order to limit the microbe spread [2] (Scheme 1). FLS2 in plants can also detect flagellins of beneficial microbes to initiate plant-induced systemic resistances (ISR). For example, flagellin extracted from plant growth-promoting rhizobacteria (PGPR) *Pseudomonas putida* (KT2440) induces transcriptional and metabolic changes and systemic resistance in maize plants [40]. *Arabidopsis* fls2 mutant plant failed to close their stomata when treated with flagellin from PGPR *Bacillus subtilis* [41]. In the early symbiotic establishment between *Lotus japonicus* and *Sinorhizobium meliloti*, Flg22 induces defense-caused inhibition of rhizobial infection and delay nodule organogenesis [42]. Upon interaction of host plant and rhizobia, rhizobia secrete lipochitooligosaccharidic (LCO) nodulation (Nod) factors (NFs). NF recognition is crucial for the establishment of symbiosis between a host plant and rhizobia [43]. Indeed, PRR families are predicted to bind chitin-based molecules, including the bacterial NFs [44]. They all possess conserved LysM, chitin-binding LysM domains, but the specificity of each receptor for each chitin oligomer remains unclear.

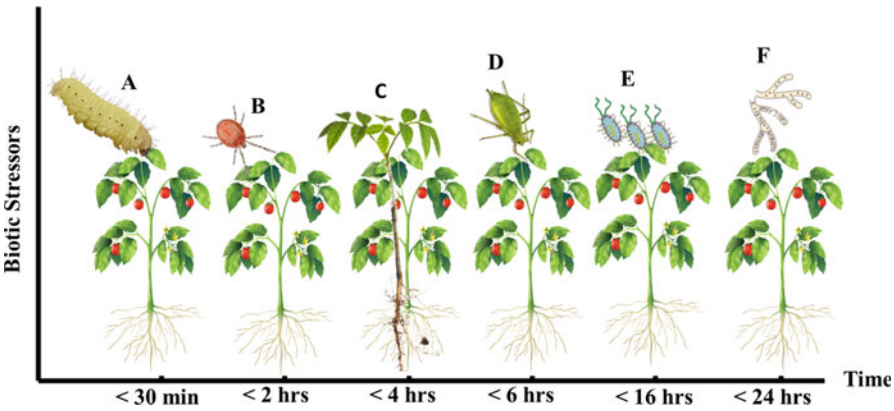
LysM-PRRs might be involved in the perception of fungal lipochitooligosaccharides (Myc-LCOs), which are symbiosis-mediating signals in the *arbuscular mycorrhiza* (AM) [45]. The formation of AM symbiosis is initiated when strigolactone hormones, secreted from host plant roots that stimulate hyphal branching and fungal metabolism, fungal short-chain chitin oligomers, as well as Myc-LCOs, elicit pre-symbiosis responses in the host plant [46]. Fungal LCOs have a striking structural similarity to rhizobial Nod-factor LCOs. Genome-wide expression studies demonstrated that defined sets of genes were induced by Nod- and Myc-LCOs, indicating LCO specific in early symbiosis perception [47].

Early in the molecular study of plant genes that regulate the transduction of signals associated with the presence of a microbe on or in plant tissues, some genes were found to be required for activating both fungal and bacterial symbiotic interactions [48]. This pathway called the common symbiotic signaling pathway (CSSP) has been extensively reviewed in [49]. This pathway includes RLKs and co-receptor proteins that perceive the presence of rhizobial bacteria or AM fungi and a series of relay signaling proteins that enter into the nucleus of the plant via nucleoporins, where they induce regular calcium spiking. Oscillations in Ca^{2+} activate a nuclear calcium−/calmodulin-dependent protein kinase (CCaMK) that

leads to the induction of gene expression needed for the establishment of mutualism. Pathogenic microbes might hijack CSSP during pathogen colonization of plant tissues. In a large screen of *Medicago truncatula* containing mutations in the CSSP, Rey et al. [50] found that a small number of mutants were impaired in both mutualistic and pathogenic symbioses. Therefore, when considering how plants respond to microbial presence, we must keep in mind that there are certain signaling pathways that have pleotropic effects on a variety of symbiotic interactions [48] (Scheme 1).

3 Early Response to Herbivores

MAMP/PAMPs early perception as has an analogous term for herbivore-associated molecular patterns (HAMPs). Several elicitors have been isolated from Lepidoptera and Coleoptera oral secretions (OS), from their salivary and ventral eversible gland secretions, and from the ovipositor fluids of these species [51–54]. HAMPs and PAMPs trigger Vm membrane depolarization, a transient intracellular Ca^{2+} influx, the activation of Ca^{2+}-dependent protein kinases (CDPKs), and MPK3 (wound-induced protein kinase, WIPK)/MPK6 (salicylic acid-induced protein kinase, NaSIPK) phosphorylation [8, 55–58]. Vm responses are much more rapid when *Arabidopsis* is damaged by insects [e.g., *Spodoptera littoralis* (30 min) and *Myzus persicae* (4–6 h)] than by bacteria [e.g., *Pseudomonas syringae* DC3000 (14–16 h)] [10] (Scheme 2). A growing body of evidence indicates the presence of mobile signaling molecules that travel from the wounded tissues toward systemic organs. Although the nature of these molecules remains unknown, their presence depends on the activity of an insect's oral secretions, which contain specific elicitors that are necessary and sufficient to initiate the response [1].



Scheme 2 Some examples of early responses to biotic stimuli. The response to some biotic stress may take hours. See text for further explanation. (**a**) Chewing insect herbivore response, (**b**) herbivore mites, (**c**) nearby plants, (**d**) sucking insect herbivores, (**e**) plant pathogenic bacteria, and (**f**) plant pathogenic fungi

Recently Camoni et al. [59] reported that the earliest event induced by *Spodoptera littoralis* feeding on leaves is the depolarization of the Vm. Although this herbivore-induced Vm depolarization depends on a calcium-dependent opening of potassium channels, the attacked leaf remains depolarized for an extended period, which cannot be explained by the sole action of potassium channels. The plasma membrane H^+-ATPase of *Phaseolus lunatus* leaves is strongly inhibited by *S. littoralis* OS. Inhibition of the H^+-ATPase was also found in plasma membranes purified from leaf sections located distally from the application zone of OS, thus suggesting a long-distance transport of a signaling molecule(s) [59].

OS originated elicitors includes: Fatty acid amino acid conjugate (FACs) (volicitin, linolenic and linoleic acids coupled with either glutamine or glutamate) [51, 54] is one of the model well studied elicitor. Though FAC-related pattern recognition receptors (FAC-PRRs) are unknown, strikingly most of the downstream early and late plant defense signaling is similar with MAMP/PAMP signaling. For instance, FLS2 detects bacterial invasion by recognition and direct binding of flagellin by bacterial flg22 epitope [12]. In *Nicotiana attenuata* virus-induced gene silencing of the FLS2 co-receptor, brassinosteroid-insensitive 1 (BRI1)-associated kinase 1 (BAK1) impairs OS-elicited JA production [60]. Silencing OS-elicited *N. attenuata* CDPK4 and CDPK5 strongly increases herbivore-induced jasmonic acid accumulations [58]. In *Arabidopsis*, *AtCPK28* acts as a negative regulator of PAMP-triggered immunity (PTI) signaling by phosphorylating *Botrytis*-induced kinase 1 (BIK1). BIK1 is rate limiting in PTI signaling that continuously turned over to maintain cellular homeostasis and suppressing flg22-mediated signaling [61]. In *Arabidopsis* sequential interaction of FLS2, BAK1, and BIK1, the NADPH oxidase is activated via phosphorylation resulting in increased production of superoxide (O^{2-}) and subsequent reactive oxygen species (ROS) such as H_2O_2 [62–64]. Furthermore, oviposition by *Pieris brassicae* induces immune responses in *Arabidopsis* that depend on the lectin-domain RLK (LecRLK) LecRLK-I.8 [52], and the LRR-RLK BAK1 participates in anti-aphid immunity in *Arabidopsis* [65]. Generally, these suggest that surface-localized LRR-RLKs and/or LRR-RLPs are involved HAMP.

4 Early Response to Neighboring Plants

Within hours, the most spectacular responses to environmental stimuli are those aimed to optimize light harvesting under a wide variety of suboptimal conditions. One of the most common threats to light harvesting in plants is the presence of neighbor plants, which can intercept sunlight [66]. Plant response to the proximity of competitors starts earlier, much before they become shaded. This depends on the ability of plants to perceive differences in the far-red (FR) light reflected by the leaves of the above canopy. This capability is known as the shade avoidance response and includes rapid photochemical responses and relatively fast morphological adaptation including reduced branching, reduced biomass, increased height, decreased leaf number, higher specific leaf area, lower chlorophyll a/b ratio,

decreased photoassimilation rates, and reduction in yield per plant [recently reviewed by 67]. SAR provides an example of integrated responses to light stimuli where photoreceptors (such as the phytochromes) rapidly interfere with transcriptional regulators and phytohormones to regulate plant growth and biomass production [68]. Similar mechanisms of shade avoidance are common in both monocots and dicots, where auxin and strigolactones inhibit axillary bud growth and play antagonistic roles with respect to growth-promoting cytokinins [69]. Therefore, perception of shade has a relatively rapid impact on the levels of hormones known to stimulate hypocotyl elongation. For instance, short-term (4 h) simulated shade treatments resulted in higher levels of the auxin IAA and brassinosteroids in a dynamic fashion, along with a mild but sustained increase in the levels of GA_4, the major bioactive gibberellic acid [70].

Plants can respond rapidly to the emission of volatile compounds from damaged or undamaged neighbor tissues and may alter their physiology in response to allelochemicals in their surroundings [71]. While soil allelochemicals necessitate a direct contact with the roots of neighboring plants (see above), volatiles emitted by neighbor plants have different lifetimes, can act at considerable distances, and require both root and shoot perception sites to deliver their information [72]. Volatile perception may involve different mechanisms of reception, including glycosylation, as recently reported [73]. There are several evidences of plant-plant signaling molecules, among which methyl salicylate and the green leaf volatile (GLV) *Z*-3-hexenyl acetate can induce long-distance responses. Methyl salicylate (MeSA) can be converted to salicylic acid upon plant uptake, and recently several plant proteins able to bind salicylate have been characterized [74] which could be related to MeSA signal perception. *Z*-3-hexenyl acetate was shown to induce long-distance responses in several plant species. *Chrysanthemum cinerariaefolium* wounded plants emit, among other volatiles, *Z*-3-hexenyl acetate and induce the biosynthesis of pyrethrins in volatile-exposed neighboring plants [75]. *Z*-3-hexenyl acetate emitted by herbivore-damaged tomato plants was also found to induce a rapid Vm depolarization and a significant influx on Ca^{2+} in leaves of receiver conspecific plants [76]. At the whole-plant level, plant volatiles can induce rapid decisions in plants in need to find their source of nutrition. For instance, parasitic plants can be guided toward host plant volatiles [66].

However, long-distance communication through volatiles is complicated by the presence of atmospheric oxidant (e.g., oxygen, ozone, and reactive N and S species) and temperature that may drastically change or degrade emitted molecules [77, 78].

Neighboring plants can also rapidly perceive root exudates that may provide a detailed layer of information regarding the competitive environment [79]. Among root exudates, a growing interest is toward strigolactones that plants exude into the rhizosphere. These molecules can stimulate interactions with arbuscular mycorrhizal (AM) fungi and improve plant fitness, but on the other hand, these molecules are exploited by parasitic plants and rapidly stimulate the germination of their seeds resulting in parasitization of the host plant [80, 81].

5 Conclusions

Plant responses to environmental stimuli are proportional to the intensity and the rate of the stimulus and can occur within seconds to hours or days. The concept of "early" is relative and depends on the dynamics of tissue and organ damage. Early cellular responses always occur within seconds to minutes, but the resulting responses of the signaling pathways depend on the intensity of the input perceived. Although calcium signaling is common to various responses to environmental stimuli, the effects of calcium on the signaling cascade and the eventual genetic response may differ in time depending on the type of stimulus perceived. In plant responses to biotic and abiotic environment, the term "early" is often used to indicate the first visible or detectable effect of a stress condition. Scheme 2 summarizes some of the biotic timing of response to biotic stimuli. The intensity and level of stress by the biotic stressor might differ, but the downstream signaling remains somehow similar.

In pathogenic microbes, first plant cell wall-degrading enzymes disrupt cellular integrity. Cellular debris, ATP, and carbohydrates are sensed by DAMP that initiate the early signaling to induce an immune response and instantly trigger signaling from MAMP receptors that perceive conserved pathogenic proteins. In symbiotic microbes, similar cellular damage is not sustained, and therefore, the DAMP receptors are thought to be silent and consequently repress plant immune signaling. Concurrently, a set of mutualistic MAMP receptors also signal to repress cellular defenses. In insect herbivore injury, the first herbivore injury causes mechanical damage that disrupts cellular integrity and followed by the action of elicitors. Similar to pathogenic microbes, signaling cascade cellular debris, ATP, and carbohydrates are sensed by DAMPs that initiate the early signaling to induce an immune response and instantly trigger signaling from HAMP receptors that perceive herbivore-associated proteins and can also be manipulated by pathogenic effector proteins/molecules and small interfering RNA. It is unknown whether the herbivore associated pathways in crosstalk with symbiotic or pathogenic microbes via plant miRNAs and by mutualistic effector proteins/molecules. Neighboring plants can also rapidly perceive root exudates and other seconder metabolites and detect these stimuli early and trigger cascade signals (Schemes 1 and 2).

The assessment of early responses might also be dependent on the experimental setup and the ease to quickly measure a response. It is still a matter of debate whether early responses measured under controlled conditions, where plants can be rapidly exposed to different environmental stimuli, will actually also occur naturally under field conditions. Nevertheless, the ability to identify early perception of environmental stimuli can significantly improve our capability to timely detect productivity and stress adaptation of nutritional and energy crop plants both at the local scale and the global scale. Herbivory and other biotic stresses are quickly sensed by plants, and timing of detection of plant responses might be crucial in rapidly changing pest and environmental conditions.

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Nitric Oxide as a Signal in Inducing Secondary Metabolites During Plant Stress

25

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Contents

Abstract

Secondary metabolites are the major defense elements of plants against biotic and abiotic stress conditions. They are diverse and valuable natural products induced by a variety of environmental and developmental cues. In recent years, NO has been successfully used as elicitor to stimulate secondary metabolite accumulation in plants. Emerging evidence has established the significant role of NO in plant growth and defense responses in plants. Several abiotic and biotic stress factors can induce NO-mediated regulation of the biosynthetic pathways of metabolites that can consequently alter their biological reaction toward the

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given stress. Moreover, exogenous treatments with NO donors also enhanced the accumulation of secondary metabolites, including phenolics, flavonoids, and caffeic acid derivatives in several species, suggesting the importance of NO accumulation for the secondary metabolic production. Complete elucidation of its role in the production of such secondary metabolites, which are pharmaceutically significant, is very essential for improving the large-scale commercial production and enhancing stress resilience in plants. Although several reports suggested the induction of secondary metabolites and NO against a range of stress factors, establishing link between NO and secondary metabolites under stress needed a deeper investigation. This chapter chiefly summarizes NO biosynthesis, signaling, and functions under abiotic stress in plants, highlighting what is currently known about secondary metabolite induction by NO in plants.

Keywords

Nitric oxide · Secondary metabolites · Abiotic stress · Biotic stress · Phenolics · Flavonoids

List of Abbreviations

ABA	Abscisic acid
AP2/ERF	APETALA2/ethylene response factor
APX	Ascorbate peroxidase
AsA-GSH	Ascorbic acid-glutathione
cADPR	Cyclic ADP-ribose
CAT	Catalase
Cd	Cadmium
cGMP	Cyclic guanosine monophosphate
c-PTIO	2-(4-Carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxid
Cu	Copper
DAF-2DA	4,5-Diaminofluorescein diacetate
eNOS	Endothelial NOS
GABA	γ-Aminobutyric acid
H_2O_2	Hydrogen peroxide
iNOS	Inducible NOS
L-NAME	L-N^G-Nitroarginine methyl ester; *N*(G)-Nitro-L-arginine methyl ester
MDA	Malondialdehyde
MeJA	Methyl jasmonate
MYB	Myb-related protein B
NaCl	Sodium chloride
NADPH	Nicotinamide adenine dinucleotide phosphate
nNOS	Neuronal NOS
NO	Nitric oxide
NO_2	Nitrogen dioxide
NOS	Nitric oxide synthase

NR	Nitrite reductase
O_3	Ozone
PAL	Phenylalanine ammonia-lyase
Pb	Lead
PCD	Programmed cell death
POD	Peroxidase
PSII	Photosystem II
PTMs	Posttranslational protein modifications
ROS	Reactive oxygen species
SNP	Sodium nitroprusside
SOD	Superoxide dismutase
ZN	Zinc

1 Introduction

Abiotic stress is defined as the harmful impact that nonliving factors including environment factors exert on the living systems such as plants and animals growing in the specific vicinity. Both plants and animals have evolved distinct mechanisms to survive abiotic stress imposed due to extreme climate. Plant survival is often challenged by a variety of different abiotic stress factors including drought, temperature extremes, heavy metals and salinity in soil, wounding, ozone, and UV-B stress. Water deficit and high temperatures are perhaps the two major abiotic stresses which are detrimental to crop growth and yield worldwide [1, 2]. Salts and heavy metal accumulation in soil is also prevailing in crop lands. Furthermore, recent years have seen the raising surface O_3 levels due to urbanization and industrial revolution which has also become toxic both for human health and vegetation [3]. On the other hand, the damage of the stratosphere ozone layer in turn causes an increase in UV-B exposure that leads to an increase of ion leakage, membrane protein oxidation, loss of photosynthetic efficiency, and ultimately global yield loss. Longer and severe stress episodes result in production of redox active molecules that in turn result in oxidation of proteins, lipids, and nucleic acids [4].

Given the sessile nature, plants have developed more complex mechanisms to sense and respond against the given stress condition. Plants respond to stress by activating tolerance mechanisms by perception and transmission of stress signals followed by a series of responses at multiple levels like morphological, physiological, biochemical, molecular, and anatomical adjustments [4]. These metabolic adjustments ultimately decide the stress tolerance or stress susceptibility of the plants. Although each stress induce a distinct defense response in plants, it is essential to understand the complete mechanism of plant defense to individual as well as stress in combination. Indeed in natural environments, plants may also be subjected to multiple stress responses at a time. Abiotic stresses disrupt the cellular redox homeostasis which leads to the oxidative stress or the generation of reactive oxygen species (ROS) [1]. Different plant groups may respond differently to the given stress dependent on the species, tolerance level, developmental stage,

and tissue affected by the stress. This may be due to the variance in metabolic adjustments to stress that are different in different tissues and genotypes of the same plant. The metabolic tuning of plants is usually triggered by number of defense molecules that enhance protection [5]. Although plant responses are unique to different extremes, they utilize the common components and signaling pathways to trigger defense. Recent research has revealed nitric oxide (NO) as one of the critical components in several plant acclimation responses to both biotic and abiotic stress conditions [2]. Literature demonstrated that various abiotic factors induce NO generation that lead to the activation of cellular processes for protection against oxidative stress and metabolic adjustments for survival.

1.1 Introduction to NO

NO is a lipophilic gaseous signaling molecule having versatile functions in both plants and animals. The first discovery in 1772 by Joseph Priestley described NO as an air-polluting "nitrous air" without any specific color and odor. After two centuries, Klepper observed NO emission by air purging of herbicide-treated *Glycine max* leaves followed by other observations on NO as a bacterial metabolic by-product [6]. A breakthrough study by Robert F. Furchgott, Louis J. Ignarro, and Ferid Murad turned up the signaling functions of NO in mammalian species and secured the Nobel Prize at Physiology in 1998. From that point, there were many parallel investigations on exploration of NO generation and NO signaling in both plants and animals. However, the investigations on NO in mammals were much rapid wherein the role of NO was well demonstrated in many physiological processes including muscle relaxation, neural communication, immune responses, and programmed cell death.

A bit later, NO has been accepted as multitasking molecule with innumerable functions even in plants. The lipophilic and diffusible nature of NO makes it perfectly suitable for several signaling processes in plants [7]. NO also plays an active role in modifying the activity of enzymes and some key signaling components via posttranslational protein modifications (PTMs). NO also plays a duel role as an antioxidant and as oxidant depending on the cellular concentration and plant species and many other factors. Endogenous NO levels have been reported to get triggered by abiotic stress conditions in diverse plant species. NO play a vital role in increasing plant adaptation to stressful conditions by modifying various physiological processes. There has been sufficient data suggesting NO as an endogenous signal that mediates plant responses to various abiotic stimuli. Nevertheless NO also acts as a critical messenger during stimulation of hypersensitivity response to pathogens.

1.1.1 Functions of NO

Albeit NO is recognized as a toxic gas for plant foliage by early discoveries, later it was treated as a powerful signaling molecule in plant defense during pathogen infestation. NO is actively involved in a plethora of plant development responses

including stomatal movement, seed germination [8], and floral transition besides having a significant role as anti-stress compound against a plethora of abiotic and biotic stresses such as drought, salinity, temperature extremes, UV-B, and heavy metal toxicity. Several reviews delineated the functions of NO in most of environmental abiotic stresses [2]. Besides, pharmacological studies using various NO donors and scavengers also demonstrated the pivotal role of NO in increasing plant tolerance to abiotic cues [2, 9]. Till date, it has been reported that exogenous application of NO donors could enhance stress tolerance in many species of plants including reed, sunflower, wheat, rice, bitter orange, tobacco, and *Arabidopsis*. NO is critical for stress tolerance by modulating osmolytes accumulation and metabolite reprogramming [10].

Besides, number of researchers reviewed the crucial role of NO in moderating various plant hormone-mediated development and stress responses [11]. The protective effect of NO in most reports has been attributed to its antioxidant role due to its ability to activate antioxidant enzymes [2]. There are several studies that support NO inducing stimulation of major antioxidant enzymes such as ascorbate peroxidase (APX), catalase (CAT), and superoxide dismutase (SOD) during stress conditions [12, 13]. Despite the emerging knowledge about NO-mediated plant stress responses including decreasing reactive oxygen species (ROS) levels and protecting membranes from oxidative damage, osmolyte accumulation, and regulation of various hormone-mediated signaling events, its functional status has been far from clarity. Nonetheless, the diffusible nature, short life, and complex chemistry in living systems of NO pose a great challenge to NO researchers [13].

1.1.2 Synthesis and Signaling of NO

In mammalian systems, NO is synthesized through well-characterized forms of nitric oxide synthase (NOS), iNOS, nNOS, and eNOS. Although the pace of investigations was bit slower in plants, initially two plant enzymes, nitric oxide synthase (NOS) and nitrate reductase (NR) [10], have been attributed for plant NO biosynthesis. Plants can also produce NO as a by-product of metabolic pathways including nitrogen fixation and respiration.

Later four major sources for NO generation have been deciphered in plants: nitrate reductase (NR) pathway, NOS pathway [14], and other enzymatic and nonenzymatic pathways [15]. Oxidative NO synthesis from *L-arginine* through NOS activity has been reported across the kingdoms including prokaryotes, unicellular eukaryotes, invertebrates, and mammals. However, the identification of NOS sequences from higher plants having high homology to already known NOS encoding genes [16] in other taxa is still awaiting. During the past decade, the first plant NOS-like gene (AtNOS1) in higher plants is identified in *Arabidopsis* having homology to a snail NOS. Additionally, the chlorotic symptoms of *Atnos1* seedlings disappeared by exogenous NO. Furthermore, the overexpression of AtNOS1 enhanced NO synthesis in *Escherichia coli* while proved to possess NOS activity by converting L-arginine to L-citrulline using commercial kits. However, its orthologs from maize and rice failed to show NOS activity indicating its function more as a regulator of NO rather than the actual gene coding for synthesis. Although few

other studies build pharmacological evidence for the existence of NOS like enzyme in various plant species, the purification of relevant protein is still underway [14]. Nonetheless, the identification of two genes in green algae *Ostreococcus tauri* and *Ostreococcus lucimarinus* share approximately 40% homology to animal *NOS* genes and also exhibit NOS-like activity [17]. NR-mediated NO synthesis is very common and known to involve in several physiological processes and plant defense against biotic and abiotic stress [16]. In several cases NO production in plant tissues occurs either through nonenzymatic light-mediated conversion of carotenoids or enzymatic catalysis through NADPH nitrate reductase. NR, in addition to its primary nitrate (NO_3) oxidoreductase activity, is capable of reducing NO_2 to NO with low efficacy [18]. Additionally, NO can also be produced through reductive pathways by assimilatory nitrate reductase, or through the mitochondrial electron transport system, or from xanthine dehydrogenase/oxidase [15]. The reductive NO synthesis from NO_2 can occur in cytoplasm, mitochondria, chloroplast, peroxisomes, and the apoplast of the plant cells [18]. Plant mitochondrial enzymes present in the matrix or the intermembrane space are also assumed to oxidize *L-arginine* to NO.

Undoubtedly, NO has the ability to modify the activity of enzymes and some key signaling components through posttranslational protein modifications including protein *S*-nitrosylation, carbonylation, and tyrosine nitration [2, 13]. While the NO-mediated protein modifications have been identified for distinct regulatory proteins such as antioxidant enzymes, there was less information on general mechanism by which NO is being sensed across multiple plant processes [2]. However, a study in *A. thaliana* suggested a unifying N-end rule pathway proteolysis mechanism involved in NO sensing in plants [2]. Sufficient data placed cGMP, cADPR, L-phenylalanine ammonia-lyase (*PAL)*, and *PR-1* as effectors of NO levels in plants [13].

In the past decade, researchers deployed different methods to elucidate NO-dependent processes including NOS/NR activity assays, NO-binding fluorescent dyes, and various pharmacologic approaches using NO donors including sodium nitroprusside (SNP) and *S*-nitroso-*N*-acetylpenicillamine and NO scavengers 2-(4-Carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide (cPTIO), and quantification of effectors by radioimmunoassay or liquid chromatography tandem-mass spectrometry [9, 15, 17, 19]. The recent use of targeted nanodonors and nanoshutters has enhanced the specificity while reducing the pleiotropic responses and artifacts encountered with pharmacological compounds [12, 16]. Numerous genetic studies have used *Arabidopsis* mutants with altered nitric oxide levels such as *noa1*, *nia1nia2*, and *nox1* to confirm the importance of NO accumulation in response to various stimuli [17]. Furthermore, the *nNOS* transgenic lines [20] also underlined the generation of NO as a vital response for increasing plant adaptation to stress [9].

1.2 Introduction to Secondary Metabolites

Plant secondary metabolites are the compounds that have vital role in plant interactions and defense. While our understanding on their role in fundamental plant growth and development is improving, diverse arrays of secondary

metabolites have been identified in higher plants. Most of them are synthesized from primary metabolites including carbohydrates, lipids, and amino acids [21, 22]. Secondary metabolites significantly contribute to develop specific aroma, tastes, and colors in plants that are in turn responsible for plant interactions with environment and defense against pathogens. In economic point of view, they are the major sources for natural products, pharmaceuticals, food additives, and flavors. The accumulation of secondary metabolites can be influenced by various genetic and environmental factors [23, 24]. Secondary metabolites including toxoids, polysaccharides, and flavones serve as key components for plant interaction with the biotic and abiotic cues in their vicinity. Indeed, these are the signals of plant communication during symbiosis, seed dispersal, and plant completion with other plants [25].

Secondary metabolites are not essential to life but essential for survival. Indeed the specific phytochemical profile of species can be used for systematic classification of species in chemotaxonomy. Majority of the plants have four chemically distinct metabolite groups such as terpenes, phenolics, nitrogen, and sulfur-containing secondary metabolites [25]. Terpenes constitute the largest group of secondary metabolites usually derived from acetyl-coA or glycolytic intermediates. Terpenes are structurally diverse group including monoterpenes, sesquiterpenes, diterpene, triterpenes, and polyterpenes that constitute toxins and feeding deterrents in plants. Carotenoids, insecticides like pyrethroid, and phytohormone abscisic acid are the most popular examples of terpenes in plants [26]. Phenols are aromatic compounds derived from the shikimic acid pathway, having a significant role in plant defense against various bacterial, fungal pests, and disease. Few examples of phenols which include lignin, flavonoids, isoflavonoids, and coumarin derivatives play effectively against a range of plant pathogens, protect cells from UV-B radiation and oxidative stress, and promote symbiotic associations. Phytoalexins, thionins, defensins, and glutathiones are the well-known sulfur-containing secondary metabolites [26–28]. They are useful in plant growth as source of reduced sulfur, in stress responses as volatiles defensive substances. The nitrogen-containing secondary metabolites including alkaloids and cyanogenic glucosides and nonproteins amino acids such as canavanine and azetidine-2 carboxylic acid are biosynthesized from common amino acids. These metabolites are mostly toxic and offer defense against pathogenic microbes and herbivoral animals and insects.

2 Production of Secondary Metabolites in Plants Under Abiotic Stress

Plants have potential to adopt some strategies to neutralize the effects of various abiotic stresses. External stress factors such as high and low temperature, salinity, alkalinity, UV, heavy metals, and drought can significantly affect the synthesis of secondary metabolites profiles (Table 1; [27]). The released secondary metabolites

Table 1 Representative studies on the effect of abiotic stresses on synthesis of secondary metabolites in plants

Stress	Plant species	Target metabolites	References
Salt	*Carthamus tinctorius* L., *Lycopersicon esculentum, Oryza sativa* L., *Solanum lycopersicum*	Proline, glycine betaine, total phenolic contents, total flavonoids, sorbitol, polyamines	[21, 23, 29]
Drought	*Labisia pumila*, *Oryza sativa*, *Salvia officinalis*, *Cichorium intybus*, *Papaver somniferum*, *Hypericum brasiliense*, *Brassica juncea*	Phenols, monoterpenes, essential oils, inulin, flavonoids, anthocyanin, polyphenols, rosmarinic, ursolic, oleanolic acids	[30–35]
Heavy metal	*Lepidium sativum*, *Abelmoschus esculentus*	Lepidine, thiol, proline, total phenolics, ascorbic acid content	[36, 37]
High temperature	*Dukus carota*, *Quercus rubra*, *Medicago sativa* L., *Camptotheca acuminata*, *Crucifers*	Terpenes-α-farnesene β-caryophyllene, terpenes-isoprene, quercetin, kaempferol, agmatine and putrescine, 10-hydroxycamptothecin	[38–42]
Low temperature	*Salix*, *Triticum aestivum*, *Medicago*, *Papaver somniferum*, *Catharanthus roseus*, *Pringlea antiscorbutica*, *Prisms sativum*, *Vaccinium myrtillus*	Terpenoids, putrescine, spermidine, alkaloids-vindoline, phenols-pelargonidin, agmatine and putrescine, flavonoids	[28, 43–45]
Light	*Vanilla planifolia*, *Zingiber officinale*, *Lactuca sativa*, *Ipomoea batatas* L.	Vanillin, gingerol, zingiberene, caffeoylquinic acids, Chlorogenic acid, hydroxybenzoic acids, flavonoids	[46–49]
UV-B	*Passiflora quadrangularis*, *Fagopyrum esculentum* and *Fagopyrum tataricum*, *Populus trichocarpa*	Flavonoids, phenolics	[50–52]

are involved in protective functions in response to both biotic and abiotic stress conditions. Abiotic stress-induced accumulation of phenyl amides, anthocyanin, and polyamines has been reported in the literature [22, 25]. The change in the accumulation and composition of secondary metabolites in response to stress factors has been considered as an adaptive strategy leading to tolerance. For instance, the enhanced synthesis of saponins in *Panax ginseng* [22], serotonin in cold-exposed *Datura* flowers, and enhanced lignification of cell walls in many plant species are the examples of stress-induced accumulation of secondary metabolites [43–45]. A number of stresses are capable of redirecting the metabolism toward the accumulation of biologically active secondary metabolites. Besides, a number of researchers have applied various elicitors for enhancement of secondary metabolite production in cultures of plant cell, tissue, and organ [21].

2.1 Influence of Temperature Stress on Secondary Metabolites

Both low and high temperatures effect the metabolic process in plants. Plants often face challenges with high and low temperatures. While high temperatures induce premature leaf senescence and reduce membrane integrity, the rate of photosynthesis and biomass production in plants and low temperature leads to osmotic injury, desiccation, oxidative stresses, etc. [27, 38, 39, 41, 43]. In order to maximize their temperature tolerance, plant species adjust the metabolism to either increase or decrease the secondary metabolites. Low temperature induces the synthesis of several types of cryoprotectant compounds including nitrogenous compounds like proline, glycine and betaine; sugar alcohols like sorbitol, ribitol, and inositol; soluble sugars like saccharose, raffinose, stachyose, and trehalose; and low molecular weight to maintain the osmotic balance [53]. Leaves of wheat and alfalfa accumulate putrescine and spermidine when exposed to a low-temperature stress. Temperature stress also modulates alkaloid and phenolic compound production in several plant species [40, 41, 45]. For instance, cold acclimation in apple tree was reported to be associated with a marked increase in the accumulation of chlorogenic acid. In several plants, the enhanced phenolic production in turn results in the cell wall lignification or suberinization [54]. Similarly, higher levels of phenolic acid, anthocyanin, flavones, and antioxidant capacities were observed in strawberry, sugarcane, and lettuce when grown under elevated temperatures [54]. In addition, the total phenol level and especially the geneistin levels were observed highest after cold temperature treatment in soybean roots [55]. However, the low temperature reduced the accumulation of alkaloids such as morphine and benzylisoquinoline in *Papaver somniferum* [44, 45]. The anthocyanin and flavonoid biosynthesis was also promoted by low temperature in *Zea mays* seedlings and leaves of *A. thaliana*, *Petunia hybrid*, and *Rosa hybrid* [55, 56]. It was further supported by the observed increase in the transcript accumulation of phenylpropanoid pathway genes including phenylalanine ammonia-lyase and chalcone synthase in *Arabidopsis*. Conversely, anthocyanin and carotenoid accumulation was reduced by high temperature in several species including *Vitis vinifera* and in *Brassicaceae*. This inhibition was partly attributed to the pigment degradation and reduced gene transcription under elevated temperature [56].

2.2 Influence of Salt on Secondary Metabolites

Salt stress is a global problem limiting agricultural production throughout the world [56]. Salt stress lead to cellular dehydration, ionic and osmotic stress in plants that subsequently results in accumulation or decrease of specific secondary metabolites. Salt stress is known to either induce or reduce the production of secondary metabolites to maximize the tolerance in plants [21, 23, 29]. Increased production of anthocyanins in *Vitis vinifera* cultures, polyphenols in *Aegiceras corniculatum*, tropane alkaloid in *Datura innoxia*, glycine betaine in *Triticum*

aestivum, vincristine in *Catharanthus roseus*, and polyamines in *Helianthus annuus* are some examples of salinity-induced regulation of secondary metabolites [24, 57, 58]. Similarly a positive correlation between proline accumulation and salinity tolerance has been reported in tomato [29]. Salt-induced ABA is also a player in decreasing photosynthesis due to stomatal closure and plant growth inhibition under salt stress. Furthermore, higher salt concentration in growth media resulted in accumulation of high levels of terpenoids, phytoalexins, and zealexins, while lower salt concentration substantially induced the content of kauralexins in maize roots [56].

2.3 Influence of Drought on Secondary Metabolites

Drought stress is the major abiotic stress that can impact food production across the world [59]. Active accumulation of compatible solutes and osmoprotectants is the most common drought-induced metabolic adjustment in majority of the plants [32, 60]. Besides the osmotic adjustment, reprogramming of plant metabolism also occurs in drought-stressed plants leading to multiple other changes in plant secondary chemistry [30, 33]. Drought is known to induce an increase in secondary metabolites such as phenols, saponin, anthocyanin, and flavonoids in several plant species [30, 34, 35]. For instance, moderate water deficit enhanced saikosaponins in *Bupleurum chinense* and salvianolic acid in *Salvia miltiorrhiza* roots [31]. The content of glycine betaine and the total alkaloids in *C. roseus* plants increased due to drought in comparison with the unstressed control plants [61]. Drought-induced changes in secondary metabolite composition, including elevated tocopherol and carotenoid contents, have been associated with improve photoinhibition tolerance in several plants. Hence, the plants or plant tissues with anthocyanin or flavonoids are protected from drought [25]. Drought stress is also known to change the ratio of chlorophyll “a” and “b” and carotenoids [33–35].

Drought stress also increased the production of rosmarinic, ursolic, and oleanolic acid in *Prunella vulgaris* and betulinic acid content in *Hypericum brasiliense* [34, 35]. Similarly, the accumulation of alkaloids including narkotine, morphine, and codeine in *P. somniferum* was significantly increased due to drought. Drought effects can be associated with drought severity and vary for different compound classes. For instance, the total inulin percentage in *Cichorium intybus* roots increased by mild drought stress, whereas severe drought stress decreased inulin yield [32]. Another interesting study suggested more complex and differential regulation of secondary metabolites synthesized via shikimate and isoprenoid synthesis pathways in eucalypts [62]. In these plants drought had no effect on isoprenoids, monoterpenes, and sesquiterpenes, while condensed tannins were enhanced, and concentrations of macrocarpals decreased due to drought [57, 62]. Thus, all these studies underscore the need of more focused studies on secondary chemistry under various ecotypes and drought severities [59].

2.4 Influence of Light on Secondary Metabolites

Light is an important physical factor that can affect the growth and metabolite production [46]. Light is a natural elicitor for many secondary metabolites such as gingerol and zingiberene production in *Zingiber officinale* cultures, foliar tannins, and a number of phenolic glycosides [47]. High light irradiation has seen to induce anthocyanin production in cell suspension cultures of *Perilla frutescens*, apples, and light-colored sweet cherry [46, 62]. The effect of light was also evidenced from the digitoxin accumulation in *Digitalis purpurea* L., enhanced ginsenoside contents in American ginseng plants, and increased artemisinin production in hairy root cultures of *Artemisia annua* [62]. Light not only has stimulatory effect on the formation of secondary metabolites, including flavonoid and anthocyanins, but also influences the secretion mechanism of secondary products [47, 49, 62]. It has been reported that photoperiod also affect the secondary metabolite content in some plants such as *Hypericum perforatum*, in which maximal production of metabolites occurs at flowering stage. Similarly, blue light has stimulatory effect on the vanillin content in *Vanilla planifolia* [46].

Among various physical variables, ultraviolet (UV) irradiation was considered to be the major inducer of secondary metabolites in several plant species including peanut, rice, maize, and basil [51, 52]. UV-B has been seen to increase flavonoids in barley, several *Passiflora* species [50]. UV-B significantly increased the quercetin concentration in *F. esculentum* [51] and kaempferol in *Populus trichocarpa* leaves [52]. In rice, UV-tolerant cultivar accumulated more C-glycosylflavones compared to susceptible cultivar. Increased UV-B exposure stimulates the total phenolic content as phenolics offer UV protection in plants. Similar to UV-B, UV-C irradiation also is shown to be the stimulus for phenylpropanoid pathway-derived compounds and flavonoid synthesis [52].

2.5 Influence of Heavy Metal on Secondary Metabolites

Heavy metal contamination in soil, air, and water may alter the chemical and metabolite composition of plants leading to poor production and quality [15]. Metals such as aluminum, cadmium, lanthanum, nickel, europium, and silver are known to influence secondary metabolite production due to either inactivation or stimulation of enzymes involved in their production [36, 37]. For instance, metal ions in the growth medium regulate the anthocyanin biosynthesis by inhibiting activity of PAL [15]. Cu^{2+} and Cd^{2+} have been shown to induce secondary metabolites such as shikonin, digitalin, and betalains [63]. However, combined Cd and Cu treatment reduced the production of total phenolics, flavonoids, saponin, and overall medicinal properties due to the inhibition of PAL activity in *G. procumbens* [15, 63]. Increases in heavy metal-induced secondary metabolite biosynthesis also result from increased synthesis of precursors [64]. At times heavy metal-induced stress activates the transcription of the genes encoding the secondary metabolites synthesis which

subsequently contribute to the defensive reactions of the plant [15]. In silver ion-exposed *Brugmansia candida* root culture, there is an increase in scopolamine due to the downregulation of the enzyme hyoscyamine-6-β-hydroxylase responsible for scopolamine release [65]. This can also be partly due to the metal-induced regulation of signaling molecules such as ethylene which in turn can regulate the production of tropane alkaloids such as scopolamine. Similarly heavy metals are also known to stimulate the activity of ethylene biosynthesis genes, 1-aminocyclopropane-1-carboxylic acid synthase, and oxidase either directly or through the jasmonate-mediated pathway [66]. In another study [67] also suggested a positive correlation between the increase in signal molecules with an increase of secondary metabolites under Cu^{2+} exposure. Similarly, the increase in the synthesis and metabolism of phenolic compounds under Pb stress was reported in *Phaseolus vulgaris*. The Pb-induced increase in phenolic content was thought to protect plants from oxidative damage and membrane lipid per oxidation. Plants growing in aluminum-rich soils also accumulate a lot of flavonoids in order to prevent oxidative stress [25]. It is clear from these studies that alteration in secondary metabolism may be a strategy of the plant to survive the phytotoxicity of heavy metals.

3 Abiotic Stress and Nitric Oxide

NO has gained significant attention in recent years due to its potentiality in enhancing tolerance of plants to various environmental stresses [2, 12, 13]. As a redox molecules, NO can function both as a positive and negative regulator of stress responses depending on the local concentration. Being a free radicle, NO plays a powerful role in activating ROS-scavenging enzyme activities and protecting from oxidative damage under abiotic stress. Studies in the recent past have established the role of NO in resistance to salt, drought, extreme temperature, UV-B, and heavy metal stress (Table 2; [12, 13, 74]). Although the complete mechanism by which NO reduces abiotic stress is yet to be deciphered, a definite role of NO is suggested in several physiological processes. In fact, an enhancement of endogenous NO accumulation has been observed in several plant species exposed to wide variety of stress responses providing evidence that endogenous NO could be actually involved in plant stress responses [2]. Although accumulation of NO during various stress conditions appears to be a general response in diverse plant species and tissues, its specificity has been established by using various inhibitors/scavengers such as c-PTIO or L-NAME which reversed these NO-mediated effects in many such studies [2, 69]. Furthermore, exogenous supplementation of NO donors including SNP, SNAP, and diethylenediamine have reported to offer protective actions against abiotic stress, while NO scavengers/inhibitors reversed these actions [12, 84]. Most of the abiotic stresses lead to oxidative burst that disrupt the cellular redox homeostasis. NO may act as a chain breaker and provide protection against oxidative damage under given stress. Being lipolytic small molecules, NO can easily cross cell barriers and enhance cell communications under stress. NO can directly or indirectly interact with a wide range of targets due to the number of posttranslation modifications such as *S*-nitrosylation and nitration [79]. Several transcription factors involved in the regulation of abiotic stress responses in plants

Table 2 Various studies describing the involvement of nitric oxide (NO) in plant abiotic stress tolerance

Stress	Plant species	NO response	References
Drought	*Arabidopsis thaliana*, *Medicago truncatula*, *Tagetes erecta*, *Oryza sativa*	Synthesis of ROS and NO, involved in ABA signaling, stomatal movement, late embryogenesis abundant protein expression, enhanced antioxidant defense and osmolytes, increased adventitious root length, reduced lipid peroxidation	[2, 20, 68–72]
Salt	*Cucumis satyas*, *Oryza sativa*, *Brassica nigra*, *Glycine max*, *Gossypium hirsutum*	Survival of more green leaf tissue, and increased quantum yield for photosystem II, increased germination rate and root growth, reduced lipid peroxidation, enhanced antioxidants, altered gene transcription, enhanced photosynthesis	[73–77]
Low temperature	*Helianthus annuus*, *Capsicum annuum*, *Oryza sativa*, *Citrus* sp., *Cucumis sativus* L.	Increase in endogenous NO production in wild types, decline the ROS level, synthesis of osmolytes, reprogramming of lipid signaling, negatively regulates sphingolipid phosphorylation, increases spermidine and spermine levels	[78–82]
High temperature	*Oryza sativa*, *Citrus* sp., *Cucumis sativus* L., *Festuca arundinacea*	NO acts as signal molecule for the stress response, protects the plant from heat stress-induced oxidative stress, plays an important role in H_2O_2metabolism ROS-scavenging enzymes, alleviated the expression of HSPs, and acts as signal molecule for the stress response	[12, 74, 78, 82, 83]
Metal	*Triticum aestivum*, *Glycine max*	Noticed that SNP pretreatment significantly reduced O_2-induced-specific fluorescence, increased the root elongation, reduced the NOS activity	[84–87]
Ozone stress	*Arabidopsis thaliana*, *Popules* sp.	Exogenous application or endogenous synthesis of NO reduces the damaging effects of ozone by activating active oxygen scavenging enzymes	[88, 89]
UV	*Betula pendula*, *Arabidopsis thaliana*, *Zea mays*	Increased accumulation of putrescine, spermine, and spermidine, reduced lipid peroxidation, activation of antioxidant enzymes, increased osmotic tolerance	[90–92]

including MYB family transcription factors and protein kinases are regulated through S-nitrosylation. It can also trigger several redox-based signaling while altering expression of several genes involved in plant defense. NO upregulated the activity and transcription of APX and GR, the two key enzymes in the ascorbic acid-glutathione (AsA-GSH) cycle in *Nicotiana tabacum* and *Cucumis sativus* leaves, and conferred resistance to abiotic stress [93].

It has been reported that even mild water deficit also leads to the accumulation of NO in cucumber roots [2]. Moreover, accumulation of NO as a result of application of exogenous donors in many reported studies also correlated well with the amelioration of drought stress, while the use of NO scavengers/inhibitors reversed this effect [68]. Exogenous NO improved drought tolerance by reducing stomatal opening, membrane damage, and lipid peroxidation in water-stressed plants [2]. Application of SNP enhanced plant tolerance to drought by inducing stomatal closure, reducing transpiration rate, thereby lowering water loss in leaves and protein synthesis, enhancing photosynthesis rate, and increasing the activities of ROS-scavenging enzymes [69].A good number of studies confirmed the generation of NO in guard cells in response to drought and ABA by using a NO-sensitive fluorescent dye DAF-2DA [70]. The increase in NO production under drought stress has been correlated significantly to the decrease in stomatal conductance in *Vitis vinifera*. Additionally, NO decreased drought-induced reduction in photochemical quenching during adventitious rooting in explants of *Tagetes erecta* [71]. Similarly, NO-treated *Dendrobium huoshanense* plants maintained high levels of antioxidant enzyme activities and less lipid peroxidation under drought stress [2]. Not only that, NO also help in maintaining high vacuolar concentrations of osmotically active solutes and amino acids under drought. NO promoted drought-induced free proline accumulation in *Oryza sativa*, *Ginkgo biloba*, and *Triticum aestivum* [72]. Similarly, accumulation of glycine betaine was also promoted by NO-mediated stimulation of *betaine aldehyde dehydrogenase* activity in the leaves of drought-stressed *Zea mays* [72]. Moreover, transgenic plants overexpressing the rat *neural nitric oxide synthase* gene in *A. thaliana* and *O. sativa* exhibit enhanced drought tolerance than their respective untransformed controls [2, 20].

More than 45 million hectares of cultivated land globally has been contaminated with high salinity limiting the plant water and mineral uptake. Previous research suggested that exogenous application of NO donors could enhance salinity tolerance in a number of plant species including *Phragmites communis*, *Lupinus luteus*, tobacco, sunflower, cucumber, wheat, and rice [73, 74, 76, 77]. In most cases, an enhancement of endogenous NO levels is followed by Na^+ exclusion and improved K^+/Na^+ ratios. On the other hand, *Arabidopsis noa1* mutant with lower NO level was more sensitive to NaCl further supporting the need of NO in salinity tolerance. Further SNP-induced antioxidant enzymes provided resistance to salt stress by alleviating the oxidative damage in many plant species including rice seedlings, cucumber, maize, etc. [73, 74, 76]. Besides, NO participates in enhancement of photosynthesis by inducing the photosynthetic pigments and adenosine triphosphate synthesis, by quenching excess energy, and by increasing in quantum yield of PSII by using exogenous NO in *Solanum melongena* seedlings under salt stress [75, 77].

Participation of NO in plant response to temperature extremes is also well documented in literature [12]. An increase in NO synthesis associated with cold acclimation was observed in *Helianthus annuus* and *Capsicum annuum* [12, 74, 79, 80]. Transgenic cucumber plants overexpressing *CsNOA1* constitutively had greater accumulation of soluble sugars and starch and a lower chilling damage index, while suppression of *CsNOA1* expression resulted in opposite effects [78]. Furthermore, exogenous application of an NO donor can induce cold acclimation through synthesis of osmolytes such as glycine betaine and proline and reprogramming of lipid signaling and composition [74, 81]. Similarly, high-temperature treatment increased NO levels in leaves of *Nicotiana tabacum* and *Medicago sativa* [9]. While exogenous NO has been able to reduce heat-induced cellular damage, depleting endogenous NO levels by cPTIO reversed these beneficial effects [12, 84]. SNP treatment recovered relative water content, chlorophyll content, and electrolyte leakage in heat-stressed *Zingiber officinale*, *Festuca arundinacea, Triticum aestivum*, and *Zea mays* [12, 82, 83]. NO plays a significant role in mitigating heat-induced oxidative stress in plants by maintenance of cellular redox hemostasis and through moderation of carotenoid content [68].

NO mitigate heavy metal stresses in plants mainly by upregulation of antioxidant defense, by regulating cellular free metal concentration, or by excluding the heavy metal in the root zone, thereby preventing the accumulation at toxic concentrations [15, 84–86]. SNP supplementation decreased Cd accumulation in roots and stems while increasing the photosynthetic and antioxidant activity in *Arachis hypogaea* [15]. In rice, exogenous NO treatment has increased Cd tolerance by increasing pectin and hemicellulose content in the root cell walls and decreasing Cd sequestration in leaf soluble fractions [85]. Moreover, the involvement of NO has also seen in protection of chlorophyll against Cd stress in *H. annuus* and Cu stress in *Lolium perenne* [86]. Similarly, NO treatment raised photosynthetic rate, antioxidant activity, and reduced MDA content in *Vigna unguiculata* and antioxidant gene transcription in *Triticum aestivum* under Al stress [87]. NO also plays a critical role in promoting antioxidant enzymes activities and inducing the activity of H^+-ATPase under metals stress in tomato plants. The role of NO in alleviating other heavy metals has been reviewed by few authors in recent years [15, 86].

Ozone exposure induced NO generation and flavonol accumulation in *Ginkgo biloba* cells [3]. Further, a study in poplar has indicated increased activity of phenylalanine ammonia-lyase (PAL) due to de-nitrosylation and also *S*-nitrosylation of nearly 172 proteins due to ozone fumigation [89]. Similarly studies indicate that upregulation of flavonoids and chalcone synthase gene responsible for flavonoid production by UV-B requires NO in *A. thaliana* and *Betula pendula* plants [90]. Pretreated with SNP prevented the oxidative stress progression in UV-B-exposed *Phaseolus vulgaris* seedlings by decreasing H_2O_2 content, increasing the thiol group content, and upregulation of active oxygen scavenging genes [91]. These studies suggest that UV-B-enhanced NO levels protect the microtubule organization as well as microtubule-related processes by in-plant cells against disrupting effects of UV-B [92]. All these evidence presents NO as a key regulator in maintaining cellular osmotic and redox status in plants under stress.

4 Role of NO in Inducing Secondary Metabolites

Biosynthesis of plant secondary metabolites is regulated by multiple endogenous signaling pathways. NO has been widely utilized as elicitor to stimulate secondary metabolite accumulation in several plants ([94, 95]; Table 3). Priming with SNP has enhanced the phenolic and flavonoid content in fenugreek seeds [116]. Similarly, SNP priming of ripe litchi fruits enhanced the shelf life due to the enhancement of the total phenolic content during postharvest storage [117]. NO is known to regulate the production of many pharmaceutically important secondary metabolites in plants [95]. *Rhodiola sachalinensis* A. Bor. is a perennial herb popularly known for its traditional medicinal properties in China. Nitric oxide induced the bioactive metabolites including salidroside in this endangered plant while increased the total content of phenolic and flavonoid compounds in lemon balm seedlings under in vitro conditions [101]. In another study, the effect of NO donor was studied on the content of secondary metabolites in *Calendula officinalis* L. SNP treatment had significant role in production of total phenolic and flavonoid content, antioxidant activity, and essential oil of capitule, while it had no effect on other pigment content. The application of NO donors for induction of the secondary metabolite production in plant cultures is also becoming increasingly popular [95]. To give few examples, SNP treatment induced catharanthine production in *Catharanthus roseus* cells [94, 96]. Similarly, the hypericin production by *Hypericum perforatum* was significantly enhanced by at least fourfold after eliciting with NO [96, 105]. The accumulation of secondary metabolite such as tannins, saponins, phenols, and total flavonoids is significantly enhanced by high doses of SNP in *Ginkgo biloba* callus cultures [106]. The accumulation of phenolic compounds and glycosides is subsequently followed by an oxidative burst and subsequent activation of specific enzymes activities such as PAL, SOD, and APX in *Gingko biloba* [118]. SNP treatment elicited the accumulation of secondary metabolites in *Echinacea purpurea* adventitious roots. Exogenous treatments with SNP also enhanced the accumulation of phenolics, flavonoids, and caffeic acid derivatives in this species suggesting the importance of NO accumulation for the secondary metabolic production [100]. Moreover, the involvement of NO was also suggested in the accumulation of artemisinin in hairy root cultures of *Artemisia annua* L. and taxol production from *Taxus chinensis* cell cultures [119, 120]. Many previous studies have shown that NO is being involved in elicited production of secondary metabolites such as ginseng saponin, hypericin, puerarin, catharanthine artemisinin, and taxanes in plant cell and tissue cultures [94, 96, 101–103, 105, 120, 121]. These metabolites are highly valuable components of pharmaceuticals and nutraceuticals. The production and isolation of secondary metabolites from *Ficus religiosa* L. in tissue culture are often challenged by callus browning [122]. Adding SNP to the MS medium along with 2,4-dichlorophenoxyacetic acid and 6-benzyl amino purine significantly reduced the accumulation of hydrogen peroxide and phenolic compounds in the callus tissues. Similarly, exogenous NO promoted callus induction and reduced browning of Chinese yam. Hence, complete

Table 3 Studies describing the role of NO and abiotic cues in eliciting the synthesis of secondary metabolites in plants

Plant species	Stress	NO elicitation	Target metabolites	References
Catharanthus roseus	–	SNP	Catharanthine	[96]
Scutellaria baicalensis	–	SNP	Baicalin	[97]
Tagetes erecta	–	SNP	Phenol and antioxidants	[98]
Sophora flavescens	–	SNP	Matrine	[99]
Onosma paniculatum Bur. et Franch.	–	SNP	Shikonin products	[100]
Rhodiola sachalinensis A. Bor. L.	–	SNP	Salidroside	[101]
Artemisia annua L.	–	SNP	Artemisinin	[102]
Atractylodes lancea	–	SNP	Volatile oil (β-eudesmol, atractylone, and atractylodin)	[103, 104]
Hypericum perforatum	High temperature	SNP	Improve hypericin production	[105]
Ginkgo biloba	UV-B	SNP	Phenols, acids, flavonoids	[106]
Zea mays	UV-B	cPTIO	Flavonoids	[92]
Pisum sativum L.	UV-B	NO	Cell wall polysaccharides	[107]
Taxus chinensis	UV-B	SNP, cPTIO	Flavonoids, condensed tannins, total phenolics, and taxol	[108]
Achillea species, *Ginkgo biloba*, *Vitis vinifera*	Drought	–	Total phenolic, flavonoid, soluble proteins, lignin	[109–111]
Spinacia oleracea, *Solanum lycopersicum*	Salt	NO	Total phenolics, flavonoids, osmolytes, carotenoids	[112, 113]
Vicia faba	Arsenic	NO	Photosynthetic pigments, phenols, phytohormones, minerals	[114]
Glycine max	Low temperature	–	Phenols, genistein, daidzein	[55]
Camptotheca acuminata	High temperature	–	Alkaloids (10-hydroxycamptothecin)	[41]
Helianthus annuus	High temperature	NO	S-nitrosothiols	[79]
Chlamydomonas reinhardtii	Cu^{+2}	SNP	Proline	[115]
Trigonella foenum-graecum	Oxidative stress	SNP	Phenolics, flavonoids	[116]

elucidation of its role in the production of such pharmaceutically significant secondary metabolites is crucial for improving the large-scale commercial production.

4.1 NO-Mediated Elicitation of Secondary Metabolites Under Abiotic Stress

Several studies (mentioned in above sections) have demonstrated the alteration in the secondary metabolite profile under abiotic stress in plants. NO can also increase production of secondary metabolites and activate plant protection systems even under stress conditions [94, 114]. Several abiotic and biotic elicitors can induce NO-mediated regulation of the biosynthetic pathways of metabolites that can consequently alter growth and development in plants [95]. However, very little is known on NO signaling in the biosynthesis of plant secondary metabolites under stress. Given the production of NO in plants in response to abiotic and biotic stresses [106, 107, 111, 123], it can be presumed that NO may have the most possible and prominent role in inducing secondary metabolites in response to stress. Hence, the elicitor or stress-induced NO production is essential for triggering the biosynthesis of critical secondary metabolites in plants [95]. Cu^{2+} stress could induce NO production and subsequent proline accumulation in *Chlamydomonas reinhardtii* and in roots of *P. ginseng* [115]. In another study, ultrasound treatment for 2 min resulted in a rapid and dose-dependent NO production in *T. yunnanensis* cell cultures which in turn stimulated the production of taxol and baccatin III [120].

An interesting study suggested that NO treatment created a strong demand for cysteine synthesis as a way to reduce oxidative stress. Cysteine synthesis is one of the rate-limiting steps for the formation of glutathione which is very crucial component in cellular redox responses. In agreement with that, active synthesis of amino acids specifically α-ketoglutarate-derived amino acids of the glutamate family was evident in response to NO treatment [94]. It is known from earlier reports that the metabolism of γ-aminobutyrate (GABA) is crucial plants exposed to low oxygen or high light condition. Exposure of plants to NO showed a moderate increase in the levels of GABA and 2-aminobutyrate and the significant increase for γ-hydroxybutyrate.

A recent metabolomic data suggested a significant increase in metabolites involved in purine and pyrimidine metabolism by 6 h after NO treatment. There was a significant increase in the levels of allantoin, guanine, urate, cytidine, cytosine-2′,3′-cyclic monophosphate, pseudouridine, uridine, and uracil by NO. On the other hand, NO treatment induced chlorophyll degradation as evident by an increment in the levels of pheophorbide, a breakdown intermediate product of chlorophyll in plants [19, 124].

NO is one of those key signaling molecules in elicitor-induced secondary metabolite biosynthesis in plant cells. Although pharmacological experiments with NO donor and scavenger showed that the occurrence of NO contributes to strengthening the transcription of genes encoding key enzymes involved in the biosynthesis of

those target secondary metabolites such as shikonin [100], little effort has been put onto revealing the signal transduction steps underlying NO activation of plant secondary metabolism. PAL is the critical enzyme that mediates the conversion of phenylalanine into trans-cinnamate, from which many plant phenolic compounds originate [125]. In several studies, more plant phenolic compounds are produced with increased PAL activity [120]. It has been clearly demonstrated that NO stimulates transcription of the PAL gene in plants [125]. Increased PAL production means greater efficiency in converting phenylalanine into phenolic compounds; and, therefore, in most cases, concentrations of plant phenolic compounds increase following the use of NO. In *T. chinensis* cell cultures, NO enhanced PAL activity while inhibiting the transcription of strictosidine synthase and tryptophan decarboxylase by inducing zinc finger-binding proteins [120]. Besides, exogenous NO donor SNP is known to induce the expression levels of 4-hydroxybenzoate metageranyltransferase and 3-hydroxy-3-methylglutaryl CoA reductase involved in shikonin biosynthesis in *O. paniculatum* cells [100].

Some abiotic stress-eliciting responses, including ROS production, lipid peroxidation, the activation of PAL, and osmolyte production, were also mediated by NO. For example, exogenous NO treatment enhanced the production of antioxidation-associated compounds, total phenolic content, proline, and flavonoids in salt-stressed spinach and tomato [111–113]. Furthermore, exogenous NO application has also increased the fresh and dry biomasses of edible parts compared to salt alone treated plants. Given the results in spinach, the authors have proposed the application of nitric oxide gas as an effective strategy for boosting biomass production and nutrition quality in spinach under salt stress. NO donor has also been proven to exert a protective effect against polyethylene glycol-induced drought stress in wheat seedlings by enhancing growth, relative water content, and reducing oxidative damage [2]. Similarly, heat shock in *H. perforatum* suspension cells induced NO production subsequently resulting in hypericin production [105].

The most common protective mechanism against UV irradiation is the biosynthesis of UV-absorbing secondary metabolites [106]. Several reports indicated the protective effect of NO against oxidative stress under UV-B irradiation [90, 91]. The role of NO in the regulation of flavonoid biosynthesis in *G. biloba* leaves under the UV-B was elucidated by [106]. Additionally, the sequential occurrence of NO production via increased NOS activity and increased chalcone synthase has been suggested [94]. A similar observation was noted in pea seedlings, where UV-B induced NR activity and NO production inhibited stem elongation due to the inhibition of xyloglucan-degrading activity [107]. In *Taxus chinensis*, spraying SNP and cPTIO had significant effect on the contents of photosynthetic pigments and taxol production [108]. Interestingly high levels of flavonoids, condensed tannins, total phenolics, and taxol were noted under UV-B+cPTIO treatment suggesting the requirement of balanced levels of NO in the secondary metabolism.

Use of fungal elicitors is one of the most effective strategies for inducing economically important secondary metabolites in plants. A study by [103] has shown that NO mediates violate oil accumulation induced by the endophytic fungus *Gilmaniella* sp. through salicylic acid and H_2O_2-dependent pathways in plantlets

of *Atractylodes lancea*. Furthermore pretreatment of plantlets with exogenous NO donor promoted volatile oil accumulation, while treatment with NO scavenger inhibited the burst of salicylic acid and volatile oil accumulation induced by the fungus. Likewise elicitation with another endophytic fungal *Cunninghamella* sp. also induced the NO-mediated accumulation of atractylone, hinesol, β-eudesmol, and atractylodin in suspension cells of *A. lancea* [104]. NO induced by cerebroside elicitor from *Fusarium* was involved in the regulation of artemisinin production by increasing the gene expression of 1-deoxy-D-xylulose 5-phosphate synthase and hydroxybenzoate meta-geranyltransferase in *A. annua* hairy roots as well [102]. It was reported that oligogalacturonic acid-induced NO accumulation could improve the transcription of squalene synthase and squalene epoxidase, two early enzymes for the synthesis of triterpenoid saponins in cell cultures of *Panax ginseng* [121]. Similarly, NO burst followed by the biosynthesis of torpinoid β-thujaplicin in elicited *Cupressus lusitanica* cells which has strong antifungal, antiviral, and anti-cancer activities [126]. NO was found to reduce the transcription of genes in the monoterpenoid indole alkaloids pathway and the octadecanoid-responsive *Catharanthus* AP2/ERF domain transcription through the inhibition of type-I protein prenyltransferase gene, leading to a downregulation of the catharanthine biosynthesis [96].

NO may also interact with other signaling molecules integral of plant defense system including jasmonic acid, ethylene, salicylic acid, and ROS while taking part in elicitor-induced production of secondary metabolites [94, 126]. Although these molecules operate through distinct defense signaling pathways, they are all known to interact with NO in mediating plant secondary metabolite production [126]. The combination of elicitation with various biotic, abiotic stresses, and other signal molecules implies NO as the keypoint in the signaling network leading to the biosynthesis of some secondary metabolites [95]. Jasmonic acid induce NOS activity and subsequent NO production leading to enhanced matrine accumulation in *Sophora flavescens* suspension cells [99]. Similarly, NO-mediated accumulation of fungal elicitor-induced puerarin production in *P. thomsonii* suspension cells occurs through both SA-dependent or SA-independent signaling pathways [94, 95]. Although a direct link between methyl jasmonate and NO is yet to establish, exogenous MeJA triggered a burst of NO during the accumulation of taxol from *T. chinensis* cell cultures. Furthermore the suppression of NO by its inhibitors also suppressed the MeJA-induced taxol production suggesting a central role of NO in taxol accumulation [120]. Similarly, NO acts downstream to MeJA during the accumulation of four tanshinone compounds in hairy root cultures of *Salvia miltiorrhiza* [127]. However, SNP supplementation along with methyl jasmonate leads to the marked decrease of the catharanthine production by repressing the transcription of its biosynthetic genes, while methyl jasmonate supplementation alone stimulated the transcription of catharanthine pathway genes suggesting an antagonistic relation between NO and MeJA [127].

NO acts synergistically with reactive oxygen species to stimulate ethylene biosynthesis and stomatal closure in defense response to UV-B irradiation in maize leave [92]. In another study, brassinolide pretreatment induced the production of NO

prior to the upregulation of cold-related gene expression and antioxidant enzymes activities in *Medicago truncatula* plants during cold stress tolerance. Further, brassinolide inhibitor reduced NO production and the expression of brassinolide-induced mitochondrial alternative oxidase, photosystem II efficiency, and homeostasis secondary metabolites accumulation [128]. This suggests that production of secondary metabolites or phytosignalling molecules may be the mechanism through which NO exerts its protecting effect from abiotic stress in plants.

5 Conclusions

Alteration in secondary metabolism is an effective strategy of the plants to survive and grow in adverse conditions [56]. Many studies indicated the influence of abiotic stress on the amounts of phenolic compounds, flavonoids, glucosinolates, antioxidants, osmolytes, carotenoids terpene derivatives, and phytohormones in plants. NO has been reported to be induced rapidly by abiotic and biotic elicitors in a variety of plant species. Although several studies evidence the role of secondary metabolites and NO in plant's response to various abiotic stress factors, the knowledge about NO-mediated secondary metabolome alterations in abiotic stressed plants is still in its infancy. Few recent studies have shown that exogenous addition of NO can enhance the effect of abiotic elicitors on plant secondary metabolite production. Moreover, different NO donors could be chemically synthesized to be used as a priming agents or elicitor for industrial production of important secondary metabolites in plant culture systems. The NO elicitation can be an effective strategy to significantly improve specificity and efficiency of the production of desired metabolites. Hence, complete understanding of the signal transduction pathways underlying NO-induced production of secondary metabolites not only advance our understanding but also is important for optimizing the commercial production of metabolites which are difficult to be obtained by chemical synthesis.

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Preharvest Methyl Jasmonate and Postharvest UVC Treatments: Increasing Stilbenes in Wine

26

Susana Cruz, Raúl F. Guerrero, Belén Puertas, María Isabel Fernández-Marín, and Emma Cantos-Villar

Contents

Abstract

In varieties of *Vitis vinifera*, a number of different stilbenes are present in several parts of the grapevine as constitutive compounds of the lignified organs (roots, canes, seeds, and stems) and as induced substances (in leaves and berries) acting as phytoalexins in the mechanisms of grape resistance against pathogens.

This chapter describes the strategies and recent advances regarding ways to increase the stilbene concentration in grapes through the use of a combination of elicitors. Special attention is paid to the treatment combining MEJA (methyl jasmonate)+UVC (Ultraviolet C light), which results in grapes enriched in stilbenes. The effectiveness of treatments is subject to many vinicultural

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factors, as is the transfer of stilbene compounds into the wine. Maximum skin contact with the must and minimum amounts of fining agent is recommended. However, the production of stilbene-enriched wines is a complex process which is difficult to standardize.

Keywords
Bioactive · Phytoalexins · *Trans*-resveratrol · ε-Viniferin · Induction · Dm · Stress · Elicitors · Biosynthesis · Functional

Abbreviations

BTH	Benzothiadiazole
C4H	Cinnamate 4-hydroxylase
CHIT	Chitosan
Dm	Time required for reaching maximum concentration of resveratrol
f.w.	Fresh weight
MEJA	Methyl jasmonate
OZ	Ozone
PAL	Phenylalanine ammonia lyase
STS	Stilbene synthase
TAL	Tyrosine ammonia lyase
US	Ultrasonication
UVC	Ultraviolet C light

1 Introduction

Stilbene are natural non-flavonoid phenolic compounds that are synthesized by a wide range of plant families: *Pinaceae, Moraceae, Liliaceae, Myrtaceae, Fagaceae, Gnetaceae, Cyperaceae, Dipterocarpaceae, Leguminosae*, and *Vitaceae*. Although polyphenols display enormous chemical diversity, stilbenes seem to constitute a rather restricted group of molecules, the skeleton of which is based on resveratrol (3,4′,5-trihydroxystilbene), with a structure consisting of two aromatic rings substituted by hydroxyl groups linked by an ethyl bridge, especially in *Vitaceae* and *Fabaceae*, and also based on pinosylvin (3,5-dihydroxystilbene) in *Pinaceae* [1].

In *Vitis* a number of different hydroxystilbenes are present in several parts of the grapevine as constitutive compounds of the lignified organs (roots, canes, seeds, and stems), and as induced substances (in leaves and berries) acting as phytoalexins in the mechanisms of grape resistance against pathogens.

As phytoalexins, stilbenoids are induced by infections and mechanical stress, such as that caused by UV damage or insects. Their production affords protection against many *Vitis* pathogens. Stilbenoids are, therefore, of great interest due to their activity defending vines from many devastating diseases and pests. In fact, stilbenoids have recently been reported to be effective against *Plasmopara viticola* [2, 3], and one of their potential uses is therefore as a naturally

Table 1 Stilbenoid in grape and wine

Compound	Grape (mg Kg^{-1} fw)	Wine (mg L^{-1})	Reference
trans-astringin	0.13–1.73	n.d.–38.1	[7–9]
cis-astriginin	0.02–0.29	n.d.–1.6	[8–10]
hopeaphenol	–	n.d.–2.7	[11]
pallidol	0.03–2.11	n.d.–9.2	[8, 9, 11]
trans-piceatannol	0.19–0.78	traces–5.2	[10, 12]
cis-piceid	0.39–6.77	n.d.–38.5	[9, 10, 13, 14]
trans-piceid	0.19–7.3	n.d.–50.8	[15, 16]
cis-resveratrol	n.d.–0.10	n.d.–23.2	[13, 17, 18]
trans-resveratrol	0.47–8.97	n.d.–36.1	[14, 17, 19]
trans-ε-viniferin	0.32–3.15	n.d.–4.3	[8, 9, 11, 18]
cis-ε-viniferin	0.10–5.28	n.d.–1.12	[8, 9, 20]
trans-δ-viniferin	0.02–0.66	n.d.–22.4	[8, 9, 18]

n.d. no detected

occurring pesticide for nonresistant species, such as Vitis vinifera L., the most widely cultivated grape species in winemaking.

Furthermore, stilbenes have been reported to possess health promoting compounds with cardioprotective, neuroprotective, and anticancer properties [4, 5]. *Trans*-resveratrol seems to be one of the most promising compounds due to its bioactivity. Other stilbenes, such as piceatannol and viniferins, are usually found in grapes and wine at a lower concentration than resveratrol. Although their bioactivity has received less attention as a consequence, some of their health-promoting properties are currently being investigated [6]. To sum up, stilbenes are of great interest thanks to their health-promoting properties.

However, dietary sources of stilbenes are rather scarce, resveratrol, for example, being found in small quantities in peanuts, berries, grapes, and wines. Grapes and wine are considered to be the most important sources of stilbenes in the human diet. The concentration of *trans*-resveratrol oscillates from traces to 8.97 mg L^{-1} in grapes, and from traces to 36.1 mg L^{-1} in wine (Table 1), while *cis*-resveratrol is usually detected in lower concentrations. *Cis*- and *trans*-piceid are also an important source of dietary stilbenes (up to 50.8 mg L^{-1}). Piceatannol and astringin are also usually detected in grapes and wine but in lower amounts. Vitrac et al. 2002 [7] found a high amount of *trans*-astringin in a red wine from AOC Bergerac (France). The dimers most frequently found in grapes and wines are *cis* and *trans*-ε-viniferin and *trans*-δ-viniferin.

A prospective study involving 40.685 subjects estimated the intake of stilbenes among the Spanish population. The authors established that main source of stilbenes in diet was wine (98.4%), followed by grapes and grape juices (1.6%). The main stilbenes ingested were *trans*-piceid (53.6%), followed by *trans*-resveratrol (20.9%), *cis*-piceid (19.3%), and *cis*-resveratrol (6.2%) [21].

The constitutive stilbene concentration in grapes and wine depends on many factors (Fig. 1). Viticultural factors include variety, rootstock, geographical location, meteorological conditions, fungal interaction pressure, and cultural practices [22].

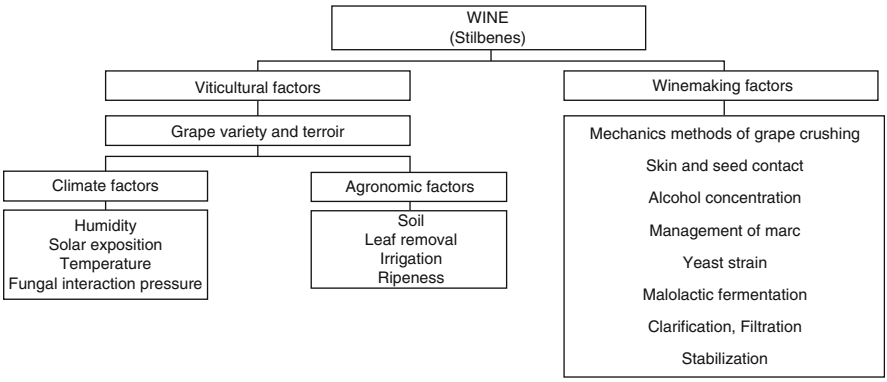


Fig. 1 Factors affecting stilbene concentration in wine

Studies conducted on 120 grape germplasm cultivars of Vitis for 2 years showed that berries of interspecific rootstock cultivars had very high levels of extractable resveratrol (<210 μg g^{-1} fw−1). The various genotypes of *V. riparia* tested usually contained high levels of resveratrol, whereas most genotypes of *V. vinifera* and their hybrids with *V. labrusca* usually contained relatively low levels (<2 μg g^{-1} fw−1) [23]. Moreover, red varieties contain higher stilbene concentrations than white ones [24]. Additionally, ripe grapes lose their ability to respond to elicitors.

Seasonal meteorological conditions, especially temperature, rainfall, and relative humidity during the last month before harvest, all affect stilbene synthesis because they are all related to fungal-disease pressure [22, 25]. Very few studies have been found with regards to soil. They are unequivocally linked with climate conditions.

Cultural practices may also affect the resveratrol concentration in grapes. No general recommendations can be given since each assay has been performed under specific conditions. For example, leaf removal at veraison increased the concentration of piceid in grapes from the Barbera cultivar but resulted in decreased resveratrol in the Croatina and Malvasia cultivars under cool meteorological conditions; leaf removal had no effect on the stilbene content of grapes in warmer and drier weather conditions according to a 4-year trial carried out in Piacenza viticultural area [26]. Indeed, other factors are considered that may interfere in the results, which should be discussed taking all these factors as a whole.

Moreover, winemaking techniques are also key to obtaining stilbene-enriched wines, as is discussed below.

The stilbene concentration in plants can be increased because they are phytoalexins and can therefore be induced by different stresses. The chemical structures of the most inducible stilbenes are shown in Fig. 2.

Plant stilbenes are synthesized via the phenylpropanoid pathway, where stilbene synthase (STS; EC 2.3.1.95) catalyzes the formation of simple monomeric stilbenes (e.g., resveratrol, pinosylvin, or piceatannol) from coenzyme A-esters of cinnamic

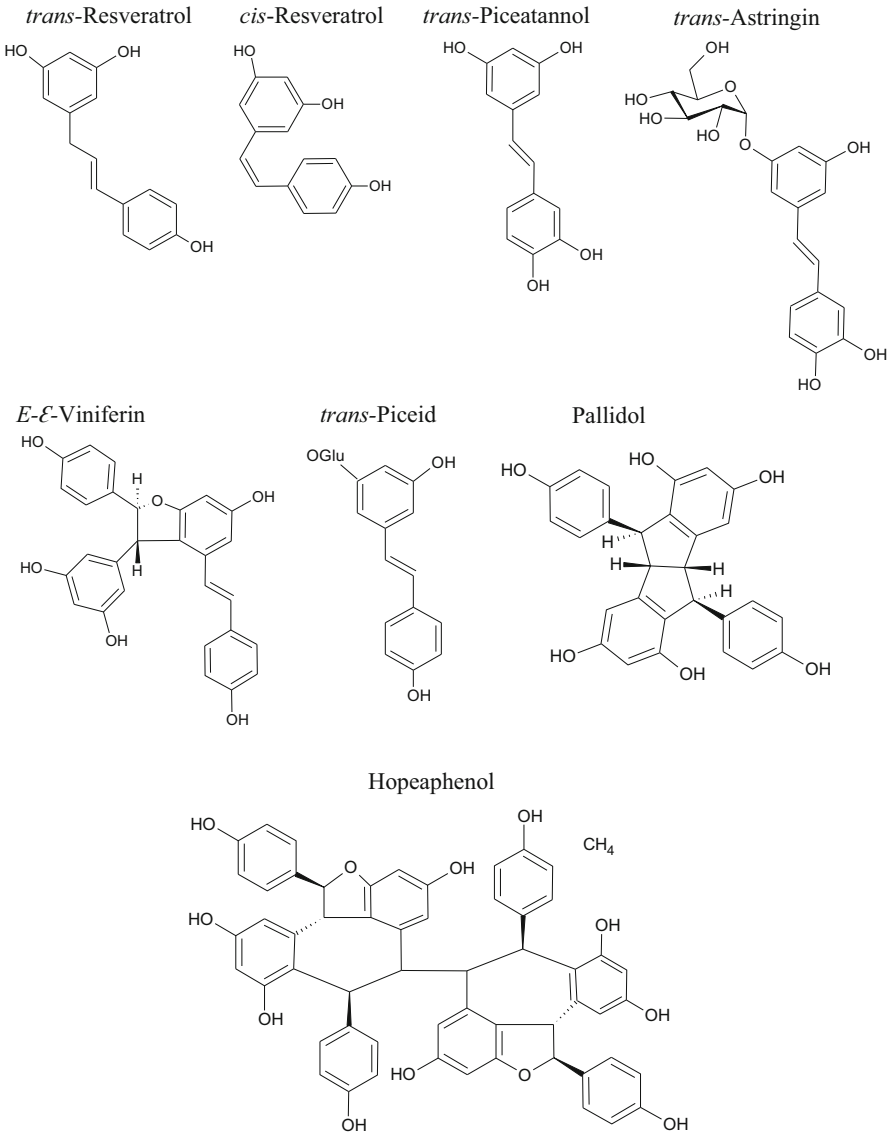


Fig. 2 Chemical structures of stilbenes found in wine

acid derivatives and three malonyl-CoA units in a single reaction (Fig. 3). The simple stilbene *trans*-resveratrol can be glycosylated, methylated, or polymerized by the action of specific enzymes and/or other mechanisms such as oxidation.

This chapter describes the strategies and recent advances regarding ways to increase the stilbene concentration in grapes through the use of a combination of elicitors. Special attention is paid to the treatment combining MEJA+ UVC.

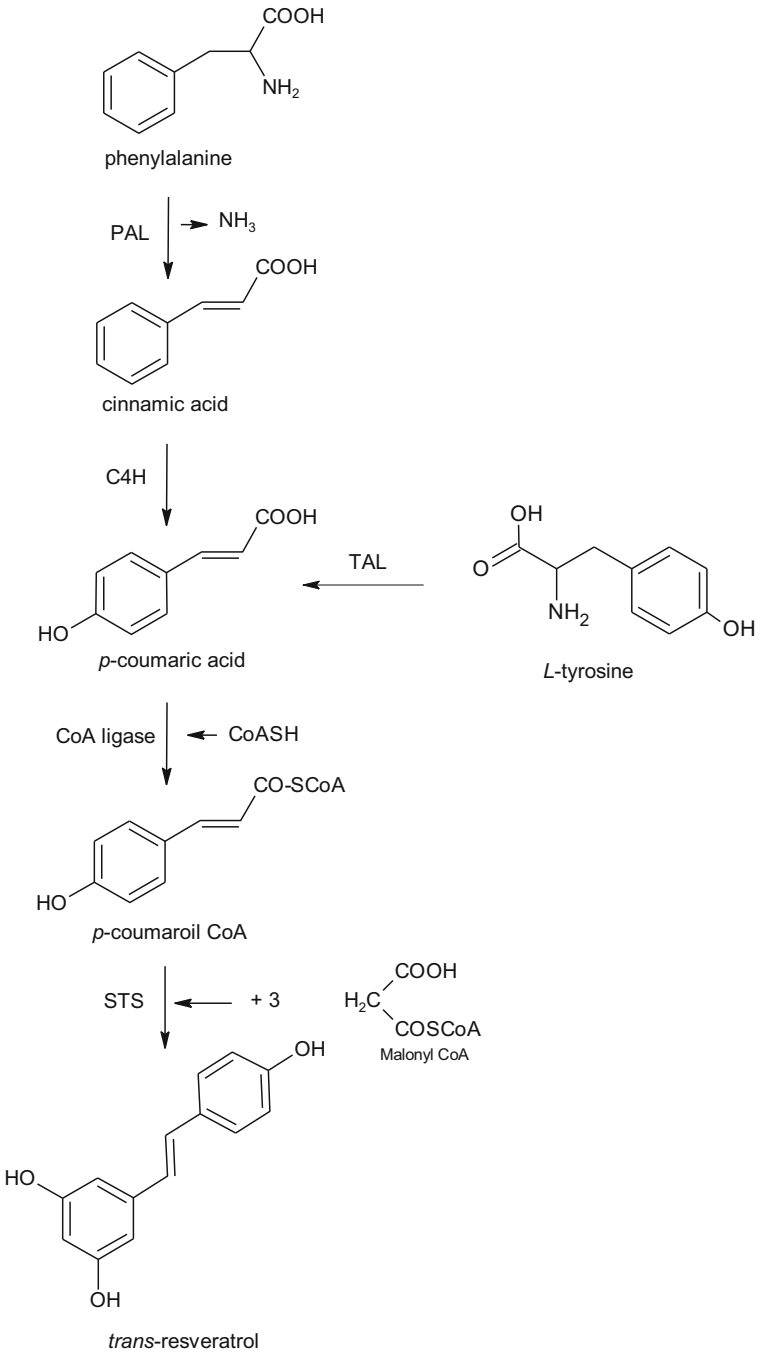


Fig. 3 Biosynthetic pathway for stilbene formation in plants

Moreover, ways to transfer stilbenes into wine to achieve the production of added-value wines (stilbene-enriched wines) are also reviewed and discussed.

2 Elicitors to Increase Stilbene Concentration in Grapes

Stilbenes are known to act as phytoalexins, plant defensive substances of low molecular weight synthesized de novo in response to stress. The process by which the grapevine is stimulated to produce secondary metabolites is called "elicitation," indicating an external stressful stimulus applied to the plant. Therefore, the stilbene concentration in grapes can be significantly increased by both biotic and abiotic stresses. The fungi that attack grapevines, such as *Botrytis cinerea* (gray mold), *Plasmopara viticola* (downy mildew), or *Erysiphe necator* (powdery mildew) are considered biotic stresses. Meanwhile, abiotic stresses can be classified as chemical and physical elicitors. Below is a review of how abiotic stress affects the stilbene content in grapes.

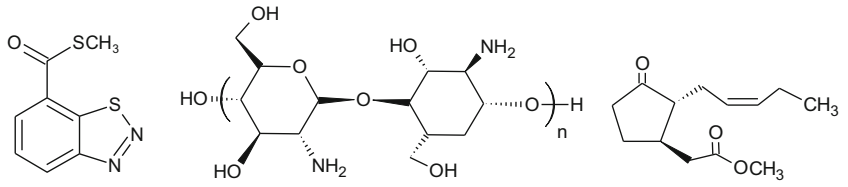
2.1 Chemical Elicitors

Many chemicals have been tested as elicitors in grapevines, the following standing out: benzothiadiazole (BTH), chitosan (CHIT), and methyl jasmonate (MEJA) (Fig. 4) [27–29].

BTH is a functional analogue of the hormone-like compound salicylic acid, which, in untreated plants, is required for the induction of defense genes [30, 31].

BTH has been shown to increase gray mold resistance in grapes by increasing the levels of phenolic compounds [32]. Preharvest treatment of Syrah grapevines with BTH (0.3 mM) significantly increased the resveratrol content in grapes by up to three times with regard to the control ones [33].

CHIT (β-1,4-D-glucosamine) is a polysaccharide obtained from the deacetylation of chitin and is a natural structural compound within the cell wall of several fungi and crustaceous shells. CHIT is described as having antimicrobial properties as well as being able to elicit plant defenses [34]. CHIT has been reported to induce stilbenes



(a) Benzothiadiazole (BTH) (b) Chitosan (CHIT) (c) Methyl jasmonate (MEJA)

Fig. 4 Chemical structures of (**a**) BTH, (**b**) CHIT, and (**c**) MEJA

in cell cultures [35] and grapevine leaves [36]. However, when used as a preharvest treatment, controversial results have been described [33, 37].

MEJA is the most active derivative of jasmonic acid. Both are endogenous plant regulators that act as signaling molecules upon biotic stress and are involved in plant defense mechanisms triggering the synthesis of secondary compounds [38].

Some studies have shown that the application of MEJA to grape bunches may exert a profound effect on the phenolic content of both the grapes and wine, particularly anthocyanins and stilbenes [39, 40].

In the case of stilbenes, MEJA treatment improved both the resveratrol and ε-viniferin content of Barbera berries in an accumulative manner until ripeness [41]. In contrast, more recent results have described how resveratrol was induced a few days after treatment but subsequently decreased throughout ripening until harvest [33]. In fact, the final level of stilbenes after MEJA treatment depends on other factors, such as variety, climate, and even viticultural conditions [42], which makes the treatment difficult to apply from a technological point of view.

Additionally, apart from the stimulation of stilbene compounds, other studies suggest that MEJA is also able to enhance wine quality, increasing the content of both anthocyanins, and therefore chromatic parameters and aroma compounds [43, 44].

2.2 Physical Elicitors

Many physical treatments have also been studied as elicitors in grapevines, the following being particularly significant: ultrasonication (US), ozone (OZ), anoxic treatments (AT), and UVC light [29].

US treatment has been proposed as a tool to produce a resveratrol-enriched grape juice [45]. In all the grape varieties tested, a significantly higher elicited amount of resveratrol was found in grape juice manufactured from fruit treated with US for 5 min followed by 6 h of incubation. The accumulation of resveratrol is transcriptionally controlled by the enzyme stilbene synthase and ultrasonication treatment was found to elicit the activity of stilbene synthase, demonstrating the underlying mechanism behind increases in resveratrol. However, in comparison with grape skin or wine, the amount of resveratrol in grape juice was much lower. Indeed, stilbenes are more soluble in alcoholic solutions such as wine than in aqueous media [46].

OZ treatment is also known to stimulate the synthesis of resveratrol in grapes under storage conditions. However, although OZ treatment (3.88 g h^{-1} for 5 h) increased the resveratrol concentration in grapes as much as UVC light did, the treatment decreased the quality of the grapes enormously [47].

AT of grapes placed in a vacuum chamber with nitrogen gas enhanced the resveratrol content [48]. After testing different times, 6 and 15 h were recommended to both improve resveratrol concentration and grape quality.

UVC light with a wavelength range between 200 and 280 nm is a germicidal, nonionizing radiation that has been used extensively to sterilize fresh fruit and

vegetables [49]. Moreover, UVC light is also very popular in the field of enhancing the production of resveratrol in grape berries and its derivatives, including grape juice and wine. In fact, UVC treatment is the most efficient elicitor at increasing the stilbene content in grapes, in particular *trans*-resveratrol.

The use of UVC light as an elicitor in grapevines was first described by Langcake and Pryce (1977) [50]. The authors observed an increase in *trans*-resveratrol concentration in leaves after irradiation (in vitro). Thereafter, many studies were developed in vineyards to determine the mechanisms and possible applications of this tool [51, 52]. However, the majority of the above studies were performed on leaves due to their availability during the vegetative cycle of grapevines.

In 2000, UVC light was applied for the first time in the postharvest treatment of table grapes [53]. Later, the treatment was optimized to maximize the resveratrol concentration in grapes [54]. The optimized treatment (1040w. 40 cm and 1 min) has been widely applied to different *Vitis* subspecies, varieties, areas of production, conditions, years, etc.

Regarding postharvest UVC treatment, two parameters should be taken into account. First, the maximum resveratrol concentration achieved, and second, the time required to reach this maximum concentration after the postharvest UVC treatment (called Dm). Obviously, the activation of plant defense mechanisms requires few days. Dm is a key factor to sure the quality of the grapes. If Dm is too long, grapes will lose quality, making them unsuitable for producing wine. Therefore, varieties achieving a high resveratrol concentration in a short time period guarantee quality stilbene-enriched grapes [24].

The induction capacity of grapes from three varieties of *Vitis vinifera sylvestris* (VS9, VS15, and VS16), seven of *Vitis vinifera sativa* (Merlot, Syrah, Graciano, Tempranillo, Palomino fi Graciano, Tempran, and Tintilla de Rota), and two hybrid direct-producer vines (Regent and Orion) after postharvest UVC treatment has been described for two harvests. Four compounds were identified in UVC treated grapes: piceatannol, *trans*-resveratrol, ε-viniferin, and δ-viniferin [24]. Varieties belonging to the sylvestris group and the Merlot variety presented high stilbene production. Syrah, *Vitis vinifera sylvestris* V15, Pinot noir, and Graciano stood out for their capacity to induce piceatannol, while Vitis sylvestris V9 and Syrah stood out for presenting the highest ε-viniferin concentrations after UVC treatment. Therefore, it could be established that the effectiveness of the UVC treatment depends on both variety and year, but not on the subspecies. As mentioned above, effectiveness is determined not just for the maximum concentration but also for Dm. Moreover, the authors concluded that from a technological point of view it is vitally important to consider the variability between years, since the number of days to reach the maximum concentration might also vary.

Terroir (climate and soil) is also considered a key factor for the effectiveness of postharvest UVC treatments [33]. Indeed, terroir factor was stronger than variety factor regarding stilbene induction capacity upon UVC treatment. In that study, the induction capacity of stilbenes was studied on four red grape varieties cultivated in four Andalusian terroirs. In agreement with previous data, the Syrah variety obtained the highest stilbene concentration, especially in Cabra (Córdoba) terroir

(up to 33 mg kg^{-1} f.w.) with limestone soil, a high average temperature and low average relatively humidity.

Therefore, the use of postharvest UVC light to increase grape stilbene content is quite difficult to standardize due to the numerous factors involved. However, certain conclusions can be reached: (i) each variety seems to be influenced to a different degree by the climate and harvest; (ii) varieties with high induction capacity and consequently a short Dm are highly recommended; (iii) Syrah is the particular variety proposed for projects aimed at producing wine enriched in stilbenes.

More recently, UV-C light application as a preharvest treatment tool to induce stilbenoid production was tested in open field experiments for table grapes [55]. UV-C light application preharvest day, output power, exposure time, and storage conditions were optimized.

UV-C light preharvest treatment was applied at different days before grape ripeness to establish the optimum application day to reach the maximum *trans*-resveratrol concentration. Grapes were illuminated with an UV-C light dose of 1866 J m^{-2} (1040 W, 1 min) on 7, 5, 3, and 1 days prior to the optimal harvest day. Maximum *trans*-resveratrol concentration was achieved after 24 h regardless of application day. An increase between 22- and 46-fold as compared with the control concentration was achieved. *trans*-Resveratrol concentration reached a maximal concentration in grape (19 mg kg^{-1} f.w. skin), 24 h after illumination (10.000 J m^{-2}), and subsequently declined. Preharvest UV-C light treatment might reduce the required time in 2 days to reach maximum *trans*-resveratrol concentration in comparison with postharvest UV-C light treatment. In 2002, a Dm value for Red Globe equal to five when postharvest UV-C light was established [56].

trans-Resveratrol was affected not only by the dose but also by how the dose was applied in terms of output power and exposure time. When postharvest storage was studied, *trans*-resveratrol increased for 3 days, after which time a reduction was observed. The mechanism for hormesis proposed by Luckey [57] suggested that low doses of UV-C could inflict repairable damage to DNA and this slight trauma would activate repair mechanisms for radiation-induced DNA damage. This suggests that sublethal radiation may stimulate vital processes inside the cells and create a positive change in the homoeostasis of the plant physiology. A dose over approximately 10,000 J m^{-2} approximately seemed to reduce *trans*-resveratrol concentration compare with lower dose [55]. Similar trends were found for ε-viniferin, whose concentration at days 3 and 4 reached similar levels to those of t*rans*-resveratrol. UV-C light increased its concentration between 5- and 31-fold depending on the treatment and sampling day. Maximum concentrations of 28,42 and 26,75 mg kg^{-1} f.w. skin were found on postharvest day 4 in grapes treated with 520 W for 10 min and 1040 W for 5 min, respectively.

Moreover, daily periodic preharvest UV-C light treatment showed a cumulative effect on grape stilbenoids, reaching a *trans*-resveratrol concentration around (120 mg kg^{-1} f.w. skin) [58]. The results for grape *trans*-resveratrol after daily periodic preharvest UV-C light treatment compared with the results after a single treatment demonstrated that a higher concentration was reached with each periodic treatment under comparable conditions. Periodic preharvest UV-C light treatment maintained a

high grape stilbenoid concentration over time, similar to that achieved by postharvest UV-C light treatment, but its maximal effects can be observed sooner, 24 h after each daily treatment. The variability of this UVC preharvest treatment regarding grape variety and season has not been studied yet, but it is expected to be highly variable.

2.3 Combination of Elicitors: Preharvest Treatment with MEJA Plus Postharvest UVC Treatment

All the above stresses or their combinations can be used to target increases in health-promoting stilbenes. A synergistic effect on phytoalexin production has been described between MEJA and ethephon [59], CHIT and UVC [33, 60], MEJA and UVC [33, 61], BTH and UVC [33], and MEJA and cyclodextrins [62, 63], among others [28].

Treatments with MEJA and UVC have been studied in *Vitis vinifera* L. cv. suspension cultures of Cabernet Sauvignon cells [64]. Both treatments improved both the production of stilbene within the cells and the accumulation of *trans*-resveratrol in the culture medium. UV-C irradiation for 20 min or MEJA at 100 μM was efficient in promoting stilbene accumulation. The combined treatment of UV-C and MEJA highly induced the production of total intracellular stilbene at the maximum of 2005.05 ± 63.03 μg g^{-1} dw and showed a synergistic effect on the accumulation of extracellular trans-resveratrol at 3.96 ± 0.2 mg l^{-1}.

From the above combinations, the preharvest MEJA + postharvest UVC treatments can be proposed as a promising treatment to increase the stilbene content in grapes [33]. Syrah grapevines were sprayed with MEJA (10 mM in ethanol) three times (20, 16, and 13 days before harvesting). A control was also treated with ethanol at the same time. Once ripeness was reached, grapes from both the treated grapevine and its respective control were harvested and UVC treated (Fig. 5). An increase in *trans*-resveratrol, piceatannol, and ε-and δ-viniferin content was observed in all the treated grapes, especially in the MEJA+UVC ones. Although the grape stilbene concentration reached with the MEJA-UVC combination did not exceed that reached by UVC alone, the storage period required after treatment to reach the maximum resveratrol concentration (Dm) was reduced by 3 days. This is an important finding because it demonstrates that the combination of MEJA with UVC accelerated stilbene biosynthesis, which is linked to the preservation of grape quality. Therefore, the MEJA-UVC treatment is suggested as an interesting application for stilbene-enriched grape production [33].

3 Stilbene-Enriched Wines

Numerous epidemiological studies have shown that long-term moderate consumption of wine is linked to a lower level of cardiovascular illnesses. A study conducted by Renaud and de Lorgeril [65] revealed that the incidence of coronary heart disease in France is about 40% lower than in the rest of Europe; this was termed the "French paradox," which appeared to be related to regular consumption of red wine.

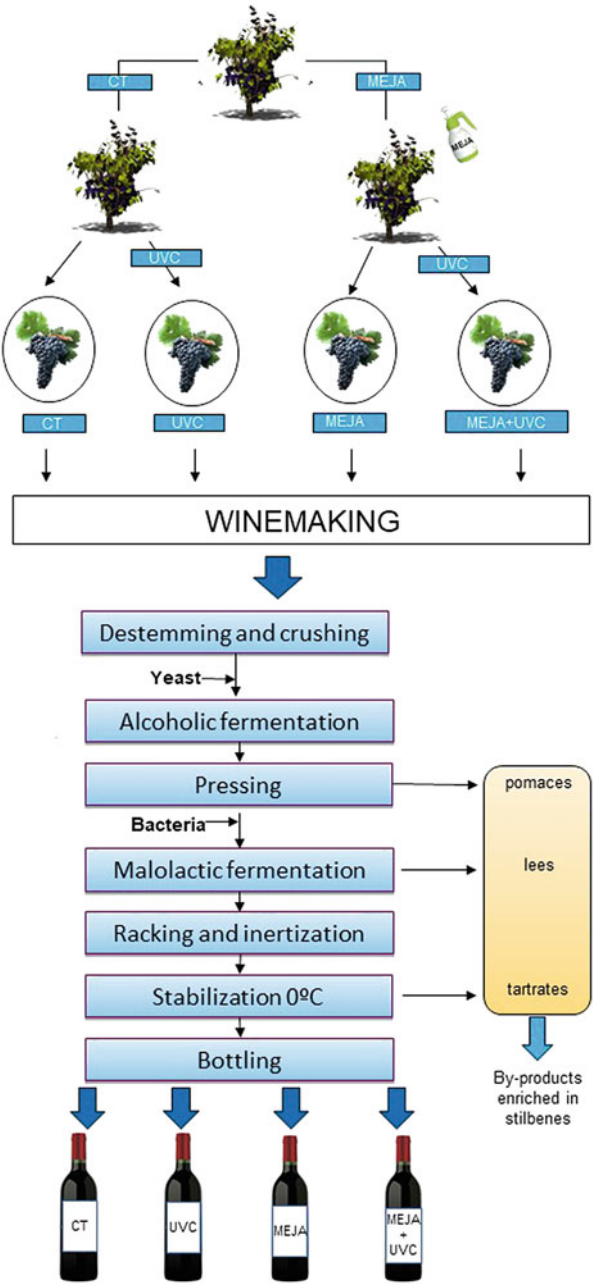


Fig. 5 Diagram of grape treatment and winemaking process

Numerous beneficial qualities with positive effects on health have been attributed to wine, particularly red, including antioxidant, anticarcinogenic, and antispasmodic properties; enhancement or activation of bile secretion; and antibacterial and

antihistaminic agents [66]. The finding that red wine presents more health-promoting activity than beer or spirits has led to research focusing its attention on phenolic compounds; within this group, stilbenes (in particular, *trans*-resveratrol) seem to show high bioactivity. Therefore, a great deal of effort has been devoted to increasing the stilbene content of wines.

Strategies for increasing stilbenes levels in grapes have been already described in the current chapter. Regarding factors which affect the stilbene concentration in wine, oenological practices such as skin contact maceration, yeast strain, fining agent, filtration, and ageing seem to be important (Fig. 1). In general, all the processes that maximize the extraction of phenols from skin are recommended [67]. Moreover, the use of yeasts which have the gene of overexpressed STS have been suggested [68]. However, this method is not allowed in Europe. On the other hand, the use of fining agents such as bentonite, casein, albumin, or PVPP reduced the resveratrol content in wines enormously [67, 69]. In fact, although PVPP is one of the most-widely used fining agents, it lacks selectivity and therefore it has a limited applicability. A new polymer (P-NIOA) has shown a similar removal to PVPP, but with a lower affinity to resveratrol [70]. Likewise, a filtration step may reduce resveratrol content by up to 58% [71], while ageing hardly affected resveratrol content in red wines aged in oak barrels [72]. To sum up, all processes involved in the final stage of wine production, but ageing, reduce importantly the content of stilbenes in wine (Fig. 5).

A study performed on stilbene-enriched grapes concluded that the concentration of *trans*-resveratrol decreased progressively during winemaking, especially during AF, probably due to the interaction with yeast and/or other organic compounds in the fermentation media [73].

In another more recent study, stilbene-enriched grapes obtained through a combination of MEJA+UVC treatments were used to make red wine following traditional methods to obtain a stilbene-enriched wine [39]. The results showed that wines whose grapes were treated first with methyl jasmonate (before harvest) and secondly with UVC light (after harvest) presented a twofold higher stilbene concentration than the control. However, the concentration in bottled wine was not very high (up to 2.32 mg l^{-1} of total stilbenes). The stilbenes were lost during the winemaking process, not only during alcoholic fermentation, as reported by previously [73], but also during the following steps. At pressing, at racking, and at cold stabilization wastes are generated (pomaces, lees and tartrates respectively) (Fig. 5). Above wastes are stilbene-enriched by products (up to 25 gr Kg^{-1} fw waste). They were even proposed as a valuable source for manufacturing nutraceutical products [39]. It is also remarkable that in the study by Guerrero et al. [73], the treated wines, with higher stilbene content, showed better chromatic properties and obtained the highest scores at tasting.

4 Conclusions

Stilbene-enriched wines are claimed to be a rich source of bioactives. They provide consumers with added value since their intake of stilbenes is significantly increased while the consumption of ethanol remains the same.

Many strategies have been tested to increase the stilbene content of grapes. Among them, the combination of preharvest MEJA treatment with postharvest UVC treatment on grapes is suggested to be the most powerful tool. Grapes treated in this way present a significantly higher concentration of *trans*-resveratrol, piceatannol, and ε-viniferin. The most difficult task is transferring those compounds into the wine. Stilbenes, as phenolic compounds do, interact with solids in the media (yeast, tartrates, and lees), precipitating and reducing their concentration in wine. In fact, winemaking by-products have been suggested as a valuable source of stilbenes for the manufacture of nutraceutical products.

To sum up, the production of stilbene-enriched wines is a complex process that is difficult to standardize. Many factors should be taken into account. Terroir and variety are key factors influencing both the constitutive stilbene concentration and induction capacity. The Syrah variety can be highlighted as a good candidate for undergoing induction experiments.

Regarding winemaking, some recommendations can be given. First, it is important to maximize skin and must contact during alcoholic fermentation. Secondly, post-fermentative maceration should be avoided as far as possible. Finally, the number of operations after fermentation (raked, filtrations, etc.) should be kept to a minimum.

Taking all the above into consideration, it is possible to produce stilbene-enriched wines, although it is difficult to make accurate predictions regarding their stilbene concentration due to the large number of factors involved.

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"Coffee Bean-Related" Agroecological Factors Affecting the Coffee

27

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Abstract

Coffee is the most consumed processed beverage aside from water, and green coffee beans are the most traded agriculture commodity after petroleum in the world. The agricultural production of green coffee beans and consumption of coffee have been increasing by 17% and 2% at an annual rate during the previous decades, respectively. The credit of increasing coffee production and consumption goes to its alluring organoleptic characteristics. The organoleptic or final cup quality characteristic of coffee is a multifactorial and complex trait, and both agricultural and postharvest processing factors influence this multifaceted trait significantly. Agroproduction technology of coffee influences 40% cup quality attributes of coffee beverage, whereas remaining 60% quality attributes are determined by postharvest processing technology. In this chapter, the relationship of organoleptic or final cup quality attributes with agricultural and environmental factors was reviewed. The analysis focused on how these factors affect the physical quality attributes of coffee beans in addition to the biochemical cup quality attributes. An overview of agricultural and environmental factors of coffee identified a critical impact of these factors in determining the physical and biochemical cup quality attributes. Geographical topography (especially altitude, slope of attitude, its steepness) was found to be the major element which also dictated the scope of influence of subsequent agricultural and environmental factors. Coffee verities or genetics, rainfall, frost, temperature, soil fertilization status, sun and shade ecosystems, and harvesting strategies played a decisive role in shaping not only the final physical and biochemical cup quality attributes but also in postharvest processing approaches. Each coffee variety (both *C. arabica* and *C. robusta*) is specified to a specific region with a set of its own inherent quality characteristics which played an important role in the production of certified specialty, organic, or other same kind of coffees. Moreover, there are still some bottlenecks that need to be addressed in order to fully understand the critical relationship of agricultural and environmental factors with final physical and biochemical cup quality attributes.

Keywords

Cup quality · Organoleptic characteristics · Sensory attributes · Preharvesting variables · Agricultural factors · Coffee body · Coffee biochemistry and coffee flavor

1 Introduction

Coffee is the world's leading hot beverage and also has a significant old history like alcoholic beverages. Botanical evidences suggest the Ethiopia as origin of coffee from where somewhat it was brought to Arab peninsula and started cultivation in sixth century [1]. Since sixth century many socioeconomic, cultural, and political factors like intercontinental trade, business, wars, and western colonization were the leading causes of transferring coffee consumption culture from one continent to another, and now recent data state that coffee production and consumption has multiplied many times in world with the annual production of 143.3 million bags (each 60 Kg) worldwide in 2015–2016 which is 0.7% more than the 2014–2015, whereas consumption rate surpasses the production rate with annual consumption of 154.3 million coffee bags having 2% per annum average growth rate [2]. Coffee is the main export of many developing countries including Brazil, Uganda, Burundi, Rwanda, and Ethiopia. Nearly 25 million people are engaged in the cultivation and production of coffee on their lands, and almost 500 million people are earning their livelihood directly or indirectly from the coffee business and trade [3]. In the crop year 2015–2016, the annual global export of coffee Arabica remains 70.3 million bags as compared to 40.82 million bags of coffee Robusta with annual increment of 1.7% [2]. Till July 2016, the retail average price of coffee (all forms) in major importing countries remains from 131 to 171.84 cent/lb. (US$, ICO indicator prices) [2]. Irrespective of the existence of nearly 80–100 species of coffee [4, 5], only two are considered obvious in trade and cup quality, namely, *Coffea arabica* (*C. arabica* L. (*Arabica coffee*) which accounts 70% worldwide consumption and *C. canephora Pierre ex A. Froehner* (*Coffee robusta*) which accounts remaining 30%; both of these coffee beans account for 99% coffee production all over the world [2, 6]. Latin America, Eastern Africa, Arabia, or Asia are the main producers of Arabica coffee beans, and western and central Africa, throughout Southeast Asia, and to some extent Brazil are the producers of Robusta coffee beans. Vast variations exist between two coffee varieties which do not only include different growing and cultivation conditions but also inclusively include physical aspects, composition, and their featured brews made after roasting [7]. Abovementioned production, consumption, and export and import data of coffee clearly depict the re-evolution of the coffee market and industry in the world after 1990s. Numerous factors like tumbling of international coffee agreement (1989), market freedom, ever changing lifestyle, food consumption and market trends, hope for sustainability, profitability, and more on that the health benefits of coffee consumption lead to the jumping of whole coffee sector again.

All the market available roasted and grounded coffees are the blend of coffee "Arabica" and "Robusta." Before roasting and grinding, green beans of "Arabica" and "Robusta" can be differentiated on the bases of size (grade), shape, color, weight, and other physical features. From compositional point of view, coffee "Robusta" has more acid contents and relatively more bitter in taste, neutral in pH, and low flavored which rendered it with less market share of around 30% as

compared to 70% share of coffee "Arabica" in world [8]. After the roasting, grinding, and fermentation, highly advanced analytical techniques, such as chromatography, spectroscopy (UV, NIR, MIR, visible, and Raman), Isotopic analysis, volatile analysis, proton transfer reaction mass spectrometry (PTR-MS), enzyme-linked immunosorbent assay, polymerase chain reaction, and thermal analysis, are the principal techniques that have been successfully applied for identification, differentiation, and authentication of coffee beans [9, 10]. Globally (specialty) coffee can be classified based on many factors like geographical locations of origin, cultural variations, taste preferences, production technology (soluble, insoluble, and freeze dried), bean stripping technology (dry, wet, or washed and semi-washed), extent of grounding the green beans, degree and the way of roasting and fermentation, way of water percolation and its duration, temperature of percolating water, application or absence of external pressure, and finally the combination of coffee beverage with milk, flavors, sugar syrup, alcohol, etc. [11–13]. But the name specialty coffee should not be mistaken and confused with all above types or with premier quality of coffees. The term specialty coffee actually represents all the well-differentiated coffee products beyond the abovementioned two varieties which coffee industries introduced after sabotage of international coffee agreement [14]. Now, specialty coffee has moved from niche to more than an industry. Many definitions about the specialty coffee already exist at professional and consumer level. According to Specialty Coffee Association of America (SCAA), Specialty Coffee Association of Europe (SCAE), and the Colombian Coffee Growers Federation (CCGF) extrinsic and intrinsic factors are involved in defining the specialty coffees. Extrinsic system refers to microclimatic production system which produces a sustainable coffee with sustainable intrinsic factors like taste, aroma, and flavor, etc. [15]. From the point of view of the consumer, coffee is considered specialty when it is perceived and valued by consumers for a set of unique characteristics that differentiate it from other conventional coffees [14, 15]. Specialty coffee also includes coffee beans production certified as organic coffee, fair trade, rainforest alliance, or eco-friendly which account for fertilizer, pesticide-free production system, etc., by consolidation of bio conservation, socioeconomic sustainable development, good agricultural practices, and farm management. Specialty coffee also has a core relationship with the Protected Designation of Origin (PDO) certification, a certification for protection and provenience of quality, which correlates the product with its specific regional culture and operating methods, atmospheric conditions, and raw material [15].

The word quality is difficult to define. But according to International Organization for Standardization (ISO), the quality is defined as "the ability of a set of inherent characteristics of a product, system or process to fulfill requirement of customers and other interested parties" [16]. These inherent characteristics can be referred to "attributes." These attributes are variable in "production-consumption cycle" of coffee. At farmer level, it is the combination of input cost and net profit; at business level, combination of attributes is wide, complex and involves physical features, defects percentage, regularity in provisioning, and price. At roasting level origin, moisture, price, organoleptic features, and repeatability of these features are important. Finally for consumer, the attributes for coffee include price, taste, flavor,

life style, trend, health effects, and environmental and sociological aspects. So, conclusively, coffee quality is assessed via its physical features (grade, color, density, defect ratio) in coffee producing countries, while cup quality is the only tool to judge quality in consuming countries. Since 2004, ISO also has made it mandatory to provide some information for standardizing the green coffee (ISO 9116) [16]. This information may include the harvest year, geographical and botanic origins, the moisture content, the bean size, total defects, and the proportion of insect-damaged beans.

Additionally, agro-production technology of coffee (from sowing to harvesting) is responsible for the 40% final cup quality, whereas remaining 60% cup quality is covered by primary and secondary processing steps [17]. Since 1990s vast research reports have been published in literature covering all these stages. But literature is still lacking a comprehensive review article stating all factors affecting all quality attributes from selection of land to end consumer. Keeping above all in mind, the objective of this review paper is to highlight all the elements which have direct or indirect influence on the quality attributes and parameters of coffee. This article will help us in ameliorating our overall existing understandings, practices, and processes which will conclusively lead to a positive quality uplifting effect over the improvement of espresso coffee.

2 Agroecological Factors

Coffee is a tropical bushy tree that is cultivated for its berries or cherries which have been processed trivially by dry or wet methodologies to get the final product called green beans: the basis of all coffee products. Cultivation of coffee plants is the first critical step in getting these immature and/or mature cherries (Coffee plant fruit) with the utmost desired characteristics, and selection of suitable piece of land is a prerequisite for this first critical step. The influence of all subsequent agro-ecological factors definitely is dictated by this piece of land. In other words, the extent or intensity of agro-ecological factors is also determined by the kind of cultivation land. These agricultural factors include altitude, irrigation, fertilization, climatic conditions, environmental factors, sanitary condition, insect and pest management, and finally harvesting strategies. These all agro-ecological factors found to have significant effects upon the vegetative and reproductive growth, flowering, fruit maturation, ripening, fruit and bean size, and finally the composition of coffee beans which ultimately have effects on the quality of coffee beverage [18]. Successively, a realistic and reliable monitoring system is also needed in this regard for the insurance of righteousness of each step and the timely corrective measures.

2.1 Altitude, Slope, and Slope Exposure

High plateau of continents and tropical forests (more than 1000–2200 mm) with mid-altitude regions (in Americas and Caribbean islands) are the natural habitats of

coffee Arabica, whereas lowland to mid-altitude regions (less than 900 mm) are the harbors of coffee Robusta. It is a common saying in coffee business that "Altitude determines the Attitude," attitudes of traders, buyers, investors, and at last consumers. The primary criteria for the selection of piece of land for cultivation of coffee are related to its altitude, latitude, slope steepness, and slope exposure towards sunlight. This primary criterion actually also defines the secondary criterion (i.e., environmental factors, fertilization, irrigation, sun and shade ecosystems, local technologies, and quality of harvesting strategies) which is also major concern for selection of a suitable piece of land for coffee growing and subsequent harvesting. Many authors categorically established the relationship between these primary factors (i.e., altitude, latitudes, steepness of slope, slope exposure) with quality of coffee cup and referred the effect of these primary factors as "*Terrain effect*" [19]. The cup quality or organoleptic characteristics of coffee are function of well-balanced combination of its volatile and nonvolatile components de novo synthesized from different pathways as a result of enzymatic reactions [20]. And these primary geographical factors (i.e., latitude, altitude, steepness of lope, slope exposure) affect the composition of these components or enzymatic actions on these components which result in the worldwide variation in coffee flavor, taste, aroma, and other sensory and organoleptic attributes [20–22]. Table 1 represents a relationship of the nonvolatile components of coffee beans with altitude which are also the prime source of almost 1000 kind of volatile compounds generated during the roasting and brewing of coffee bean grinds. Some of these components act positively (e.g., glucose, chlorogenic acid, trigonelline, caffeine, organic acids, fat) on the overall quality parameters, but some of the components are the source of declining the quality of coffee such phenolic compounds [23] and 5-caffeoylquinic acid (5-CQA) by causing unwanted degree of bitterness or astringency [24, 25]. Glucose, trigonelline, and chlorogenic acid are also believed to be precursors of volatile compounds which impart the aroma of coffee, and chlorogenic acid is also reported to employ a protecting action against microorganisms during maturation and harvesting while the moisture in air is high [26, 27]. However, some contradictory results also cited in literature stating trigonelline and sucrose having negative association with cup quality [28]. The lack of consensus among the works of Leonel and Philippe [28] and other authors (discussed below) is probably due to fact that studies of Leonel and Philippe [28] are carried out at lower altitudes (690–1293 m), while studies of all other mentioned authors were carried out at higher altitudes with different slope exposures, steepness of slope, and latitude. Some authors also claimed the sucrose in beans in a direct relationship with acidity of coffee beverage [8, 29]. Avelino et al. [30] reported a significant relationship between the altitude and quality of coffee beverage. He also found a considerable difference in the net contents of fat, sucrose, caffeine, trigonelline, and chlorogenic acid contents while comparing the cherries (Arabica) from two different altitudes of same mountaineer slope. Caffeine, trigonelline, and chlorogenic acid contents were reportedly found higher in the coffee beans from higher altitude, whereas sucrose contents were found surplus in coffee beans from the lower altitudes [30, 31]. In another report, working on the different altitudes in Honduras and Costa Rica, a positive relationship of

Table 1 Difference in the composition of coffee beans grown in the different altitudes of famous coffee grown areas of world

Country/origin	Altitude (m)	Species/variety	Fat	Sucrose	Chlorogenic acid	Caffeine	Trigonelline	Yield per tree (Kg)	References
			% of dry weight						
Costa Rica	1065–1250	Arabica	14.39	8.2	7.75	1.24	0.72	1–6.94	[30]
	1550–1780	Arabica	14.43	8.06	8.24	1.37	0.92	1.9–9.13	[30]
Heredia, Costa Rica	1200	Caturra and Catuai varieties	14.44	7.2	7.49	1.27	1.04	–	[8]
Honduras	600–1200	Caturra, Catuai, Pacas and Villa Sarchi varieties	14.1	7.7	7.4	1.3	0.95	–	[31]
Cuilapa, Guatemala	1100–1400	Catuai variety	15.47	6.95	7.71	1.4	0.95	–	[45]
Colombia	1373–2088 (Cauca)	Caturra	–	–	6.05	1.27	0.92	–	[34]
		Colombian	–	–	6.09	1.3	0.92		
	1385–2168 (Nariño)	Caturra	–	–	6.01	1.15	0.78	–	
		Colombian	–	–	6.02	1.19	0.75		
Central America	900	Caturra	14.07	7.39	7.61	1.11	0.84	–	[38]
	1200		15.12	7.73	8.14	1.17	0.78		
	1400		15.27	7.54	8.25	1.31	0.75		
	1600		15.31	7.15	8.46	1.25	0.99		

altitude-acidity, altitude-body (body is a sensory perception of the beverage heaviness on the tongue) altitude-fat whereas negative relationship of (excessive) yield-acidity was observed [30, 32]. But in both of these research works, the quantity of other components like peptides, free amino acids, and proteins has not been measured and compared as Montavon et al. [33] described that these components are also important in the final flavor, aroma, and taste of coffee. From Colombia, a research on the Caturra and Colombian varieties also showed that beside precipitation, dew point and drought period, and altitude and degree of slope also significantly affect the flavor development and acidity of coffee [34]. Barbosa et al. [35] in Brazil also carried out a work to study the relationship among altitude, latitude, and beverage quality and stated that altitude and latitude are inversely linked with each other with respect to cup quality. Certainly a high altitude is the foremost demand for a high quality of coffee but with a higher score of latitude can be compensated a lower score of altitude to produce better score coffee. A group of Brazilian coffee researchers, Ferreira et al. [36], recently, aimed at solving the dilemma of relationship between characteristic geographical location of coffee and its quality, tested the samples collected from fourteen (14) top quality coffee producing area (Matas de Manas), and concluded that all the geographical elements affect the beverage quality from intermediate level to strong level. Beside other features, altitude enormously affects the quality of grains (Physical quality) and beverage quality [37]. Hybrid and traditional coffee varieties were also checked to discover and compare the effect of various altitudes (900–1650 m) on them by keeping other variables (crop management, agronomic practices, soil, water, sunlight, shade, fertilization, etc.) almost constant in Central America and found the same trend that the level of chlorogenic acid, fat, and caffeine enhance with increasing elevation but decrease at higher elevation. The level of sucrose found to be unrelated to altitude in this work. Fat and sucrose contents differ significantly in both hybrid and traditional varieties at given elevation. Up to 1399 m, hybrid varieties tend to have more fat contents than traditional ones, but at all given elevation levels, sucrose contents found to be more in traditional varieties. No obvious effect of variety type was seen on caffeine or trigonelline concentration. The organoleptic appraisal exposed no variances between the F1 hybrids and the traditional varieties [38]. Different coffee varieties also detected to give variable results when grown on different positions and sides of altitudes. Silva et al. [39] noted that coffee Arabica varieties Catuai (red and yellow) showed both higher and lower scores of quality at lowest altitude (less than 700) which is against the previous findings in literatures (as mentioned above). This may be explained by the fact that slope exposure, steepness of slope, and soil characteristic may involve in this contradiction. The author and his coworkers declared Yellow Catuai as specialty coffee due to its uniformity in quality attributes and highest quality scores which is also not in agreement with respect to previous findings of Silva et al. [40], in which Red Catuai enjoyed the same results instead of Yellow Catuai. But Red Catuai in his work (Silva et al. [39] performed the same at all altitudes while Yellow Catuai had quality mean at lower altitude that was greater than Red Catuai. The Yellow Catuai had highest quality grades on southeast side, whereas Red Catuai got highest quality scores on northwest side. This may also due

to difference in the regional and soil characteristics. Production or yield of coffee was also observed to enhance with increasing per unit area of elevation along quality of coffee [41]. This increment in yield is due to better vegetative growth at elevating areas resulting in higher leaf to fruit ratio which accounts for more metabolism, biosynthesis, biomass accumulation (fat, caffeine, chlorogenic acid, trigonelline, etc.) in fruits or seeds eventually ameliorating the cup quality of coffee. Taveira [42] established the fingerprinting of coffee Arabica with respect to altitude and level of amino acids. The author cited that coffee Arabica which was cultivated above (1200 m) have higher level of gluconic acid, galacturonic acid, L-isoleucine, L-proline, putrescine, myo-inositol, and L-serine, whereas coffee grown below (1000 m) identified to be have surplus 5-CQA, glycerol 1-phosphate, L-valine, which may contribute to the bitterness, and those grown between 1000–12,000 m can be differentiated by L-aspartic acid, phenylalanine, fructose, oxalic acid, and galactinol [43, 44]. So these metabolites might be used as bio-marker for the identification and authentication of coffees or specialty coffees. Furthermore, there is still lack of substantial amount of literature about the relationship of altitude and metabolites like phenolic compounds, lactones, and various forms of sugars and chlorogenic acid. All well-differentiated coffees can be called specialty coffees. Daviron and Ponte [46] proposed the three bases for well differentiation of coffee, i.e., in-person service, material and symbolic attributes. Only material and symbolic attributes significantly affect the cup quality, whereas in-person services can only be evaluated between producer and consumer at time of consumption. Material attributes are related to intrinsic quality factors, whereas symbolic attributes are generated through the sign of geographical origin or sustainability certifications. The geographical features (altitude, latitude, slope steepness and slope exposure, climate, IPM, varieties, etc.) come into the category of symbolic attributes. Therefore, in order to produce the specialty coffees, it is very important to understand the complex relationship among the characteristics of geographical locations, coffee varieties, and climatic or environmental elements. In Honduras, a multifactorial analytical study, aiding in the regionalization of cultivated area in coffee producing elevated areas (726–1102 m), deduced that coffee (Arabica) grown above (1200 m) have higher cup quality and quite helpful for setting up the basis of specialty coffee (Arabica) production [32]. Luz et al. [47] and DaMatta and Ramalho [48] worked on the impact of climate on coffee quality for consecutive 4 years and discovered from quality grades that quality of coffee remained unaffected unless an untypical climatic change occurs and weather in each year did not tend to define the quality potential of coffee. Healthy coffee plant is the necessity of producing healthy, nutritious, safe quality-full coffee beans. Various factors in a specific ecosystem influence the (both positively and negatively) the degree of insect pest attack and disease occurrence. Altitude is also considered a decisive factor for the fate of insect pest diseases occurrence on coffee plant [49]. As with the variation of altitude, other relevant factors like rainfall, humidity of air, sunlight, humidity, etc., also vary, so it is the altitude which determines the kind of insect and pest attack on coffee plant. But unfortunately literature is lacking any data solely dealing the altitude and insect pest attack relationship with respect to quality of coffee beverages. Cerda et al. [50]

studied the role of altitude in triple interaction with (type) shade X management intensity on the regulation of pest disease manifestation and discovered a variable effect of variable elements (altitude, shade, and management intensity) on pest and disease regulation. Altitude was found to be having a positive relationship with certain kind of pest and diseases (e.g., coffee leaf rust) while leaving others unaffected. These may be due to variation in response to environment by various pests and disease. So now there is also a need to account in the other environmental factors (humidity, rainfall, temperature variation, soil fertility, etc.) while studying the sole relationship of altitude and pest and disease incident with respect to final cup quality. Additionally, literature is also devoid of the research on the relationship between the altitude and minerals (macro, micro, and minor), and the only available research work [51–56] about this has minor or irrelevant scope of work with respect to altitude. The other factors that are influential in the selection of land for coffee cultivation and how they affect the cup quality straightaway or circuitously have been summarized in detail in their individual sections.

Beside altitude and latitude, mountain sloppiness and steepness also carry weightage in determining the quality of cup as degree of sunlight being received by plants is determined by the sloppiness and steepness of terrain. As coffee trees are commonly planted on sloppy and steep tropical mountains so the exposure of slopes or slope exposure towards environmental and climatic factors also tends to affect the acidity, typicity, aroma, bitterness, and preference (degree of acceptance by panel of experts) of coffee. Generally coffee beans coming from east facing sloppy plots found to be rich in aroma, body, acidity, typicity, and preference than the west facing plots [57]. The reason behind this is that in tropical areas east side plot mostly received less sunlight as compared to west side plots due to cloudy phenomenon of tropical climate which affect their ripening processing by delaying it as slope exposure affects the quality of coffee beans before the start of ripening process of coffee cherries.

2.2 Coffee Varieties

It is a common slogan of roaster that "Variety is the spice of coffee." The choice of a good yielding and quality coffee variety (for both *C. arabica* and *C. robusta*) is the fundament of acquiring coffee with worthy and marketable inherent quality characteristics. At present, the generally adopted criteria for selection of a coffee variety briefly include yield, yield stability, plant vigor (stem diameter after 1 year), visual breeding score (combine score from yield, vigor, and plant shape for consecutive 3 years), growth habits, technical or quality features (beans size, %age of floating berries, %age of seed defects, caffeine contents, etc.) flavor characteristics via cupping and resistance to insect pests. Albeit, the flavor of same variety may vary from region to region as environmental factors, agronomical practices, farm management practices, and growing conditions may differ. Arabica (*Coffea arabica*) and Robusta (*Coffea canephora*) are two main varieties of coffee grown and traded globally. Arabica coffee is of highest quality, milder, and rich in flavors and aromas.

On the other hand, Robusta is a resistant variety, less susceptible to diseases, with a higher content of caffeine and lower quality than Arabica. It is used in different forms as soluble coffee, or a base for blends with Arabica coffee, and in the new trend of espresso preparations to produce the cream on top of the beverage [58]. Three genotype group has been discovered in *C. arabica,* namely, wild type (Sudan-Ethiopian origin), introgressed lines (from hybrid of timor), and nonintrogressed lines (typica and bourbon), whereas *C. robusta* has been find with two genetic classes known as Guinean group and Congolese group [59]. Table 2 represents the different group of varieties for the *C. arabica* grown around the world in the coffee producing countries. For *C. robusta*, most of its cultivars are still from unelected open-pollinated seeds [60], while selection of its varieties from clones and hybrids successfully achieved in past and still underway in many countries of Central and Latin America, Africa, Indonesia, etc., with or without compromising the yield, resistant against other insect, pest, and diseases [61]. Each coffee variety (both Arabica and Robusta) is specified to a specific region with a set of its own inherent quality characteristics which played an important role in the production of certified specialty, organic, or other same kind of coffees. As each variety of coffee is destined to a specific region or area, so the environmental, climatic, soil nutritional characteristics of that area offer a decisive role on composing the discreet profile of all compounds which play an important part in making that variety peculiar from others in terms of flavor, aroma, body, and taste as Steen et al. [62] described in his work that coffee varieties nearly have more than 800 compounds, but actually flavor, aromatic, or nonaromatic compounds are manifold of this figure, which may arise due to complex nature interaction among these $>$800 compounds during the roasting and brewing process. Nonetheless, literature still needs the data (or data available only focus the *C. arabica* hybrids or hybrids of timor) about profiling of flavoring, aromatic, or nonaromatic compounds among different group of varieties or with in the same variety to find out the causes of their enjoying discrete status. The only reason provided by the scientific reports on the variation of biochemical composition among different coffee varieties is their variation in genetic traits [38]. The genetic diversity of coffee varieties is the main constriction (especially for *C. robusta*) in the choice of coffee variety with its inborn attributes [63]. But this genetic diversity is also a boon for developing new varieties via hybridization with improved yield and insect pest and disease resistance as populations with reduced genetic diversity are more vulnerable to environment changes and thus lowering their chances of persistence [64]. In contrast, most of the *C. arabica* cultivars are derived from "typica" or "bourbon" genetic sources which resulted in low genetic diversity among cultivated arabicas [65]. So *C. arabica* plants are naturally vulnerable to various coffee diseases such as coffee leaf rust (CLR) (*H. vastatrix*) coffee berry disease (CBD) (*Colletotrichum kahawae*) and root-knot nematode (*Meloidogyne* spp.) [66]. But several breeding and genetic improvement programs are proved to be very helpful by diversifying the gene pool of *C. arabica* via interspecific hybridization with *C. robusta*, *C. liberica,* and *C. congensis,* and now many CLR, CBD, and nematode resistant varieties of *C. arabica* are under cultivation, but still the gene pool of coffee is lacking the drought and cool tolerance, herbicide and borer resistance traits [65].

Table 2 Botany, characterization, and global distribution pattern of selected mostly used coffee varieties/cultivars of Arabica

Species	Group of varieties	Botany	Specific characteristic(s)	Countries	References
Arabica	TYPICA	Pattern Variety	Average yield, elongated beans, large leaves, soft stem, good quality of coffee, susceptibility to insect, pest, and diseases, and horizontal branching pattern, e.g., Blue Mountain, Jamaique, Bergendal, BLP, Sumatra	Central America, Caribbean region, Colombia, Papua New Guinea, Indonesia, Pacific area, Cameron	[82]
	BOURBON	Natural Mutation (Typica)	Higher vigor, higher yield, broader leaves, round fruit, round beans, hard stem, erect branching, suited to deep lying sites, needs shade, ripens earlier than Típica, and susceptibility to all major insect pests and diseases, e.g., Red and Yellow Bourbon, Mibirizi, Jackson, Arusha	Brazil, Colombia, Central America, West Africa	[83]
	S-795	Kent X S288	Rust resistance	Indonesia, India	[84]
	JAVA		Vigorous growth with good vigor, resistances against coffee borer disease, elongated seeds and fruits, bronze young leaves	Ethiopia, Cameron	[85]
	MARAGOGYPE	Typica	Large fruit, large seeds, large internodes	Central America, Mexico	[24]
	CATURRA	Mutants of red Bourbon	Short stature, short internodes, round seeds and fruits, large leaves, high yield, soft stem, over fruiting, susceptible to insects pests and diseases	Colombia, Costa Rica	[86]
	MUNDO NOVO	Sumatra X Red Bourbon	Hard stem, higher yield, large leaves, cherries and seeds, open growth, suitable for organic cultivation in lower regions, suitable for	Brazil	[83]

			mediocre altitude too, e.g., LCMP 376-4, 501-5, 502-1, etc.		
	CATUAI	Yellow caturra X Mundo Novo	Short stature plant, high vigor, hard stem, normal yield, normal growth, and susceptible to all insect pest and coffee diseases, e.g., Red Catuai, Yellow Catuai, ouro verde	Brazil, Central America	[83]
	CATIMORE & SARCHIMORE	HdT X Caturra	Good resistance to various insect pest and nematodes of coffee plant, low yield, low cup quality, e.g., Colombia, T5157, I CAFÉ 95, CASTIC, AZTECA, Obata, Tupi	Portugal, Tanzania, Central America, Honduras, El-Salvador, India, Indonesia, Brazil, Costa Rica, Mexico, and Colombia	[87]
	ICATU	*C. arabica* X *C. Canephora*	Tall heighted, hard stem, higher yield, large leaves, seeds, cherries, resistance to common rust and nematodes of coffee	Brazil	[77]
	RUIRU 11	Catimor X selected progenies	Short stature, high yielding, resistant to coffee leaf rust and coffee borer disease	Kenya	[88]
	PACAS	Spontaneous mutation of Bourbon	Short size, short internodes, high productivity, high adaptability to local environment, better resistance against wind, sunlight, and droughts, lushy leaves	El-Salvador, Brazil, Costa Rica	[24, 34, 60]
	PACAMARA VARIETAL	Red Maragogipe X Pacas	Intermediate size, short internodes, high productivity, lateral branches, oval-shaped beans, high adaptability to local environment, better resistance against wind, sunlight, and droughts, lushy leaves, bigger beans size, susceptibility to insect, pest, and diseases, and better cup quality	El-Salvador	[24, 34, 60]

(*continued*)

Table 2 (continued)

Species	Group of varieties	Botany	Specific characteristic(s)	Countries	References
	VILLA SARCHI	Natural Mutation (Typica)	Short size tree, normal size cherries, red colored fruit, suitable for short to middle altitudes	El-Salvador	[24, 34, 60, 89]
	COLOMBIA	Hybrid	Poor root system, high yield, do not require shade, large beans size, suitable for monoculture, rust resistant, not self-proliferating, good cup quality	Colombia, Costa Rica	[24, 34, 60]
	YAPAR 95	Hybrid	Do not need shade, suitable for monoculture, not suitable for organic cultivation, require high fertilization, rust resistance	Brazil	[24, 34, 60]
	CARNICA	Hybrid	High yield, suitable for mid- to high altitude, resistance against low temperature and rust, susceptible to Cercospora, low cup quality	Latin America	[24, 34, 60]
	GEISHA	Arabica	Tall tree, large size fruit, elongated curvy thin shape beans. Required moderate heat for roasting, resistant against rust, good cup quality with floral aroma, and exotic sweet aftertaste with smooth silky mouth feel	Panama	[90]
	Blue Mountain	Mutation of Typica	Known to have some resistance to coffee berry disease	Jamaica, Hawaii, Kenya, Haiti, New papua guinea, Cameron	[24, 34, 60]
	Charrieriana	Not yet named	Newly engineered cameronian variety famous for caffeine free nature	Cameroon	[24, 34, 60]
	Maragaturra	Hybrid of Caturra and Maragogype	Intermediate characteristics of caturra coffee and maragogype coffee	Latin America, Indonesia, India	[24, 34, 60]

Despite limited reported gene pool of *C. arabica*, biochemical variation of key bioactive compounds is reported between traditional and modern Arabica cultivars. Modern cultivars have greater contents of total lipids, kahweol, malic and 5-caffeoylquinic acids, and trigonelline. Based on these facts, the kahweol and the kahweol/cafestol ratio could be used as a biomarker to differentiate between traditional and modern cultivars [67].

In Latin America, interspecific grafting is a common practice to prevent or reduce the root injuries which started after the outcomes of following authors Raghuramulu and Thimmaraju [68] who revealed that *C. arabica* is compatible to other coffee accessions which was a breakthrough in diversifying the genetics of *C. arabica* via using rootstock of other coffee varieties. The first reported work on the impact of grafting on beverage quality was published by Melo et al. [69] in which he found no caffeine difference between grafted and nongrafted plants. A comprehensive work in this scenario was done by Bertrand et al. [70] whose outcomes described complete compatibility of *C. arabica* (Caturra and Catimor) with *C. robusta* (T3446, T3718, T3561, T3757, T3561) and low or no compatibility with *C. liberica and C. dewevrei*. Rootstock and scion both found impacting, in one way or another, on viability, growth, yield, and cup quality. The rootstock provided a good growth, mediocre taped root system, high viability, strong stem, tallness but reduced yield. The drop in yield may be seen in the more bumper vegetative growth and tall strong stems, whereas low aluminum level in grafted plants may also a result of shallow root system. On the other hand, scion affects the number of beans and bean size, albeit, biochemical composition, and organoleptic qualities were found to be completely unaffected [70]. Interspecific grafting has also been exploited as genetic tool to produce decaffeinated or low caffeine coffee varieties. Caffeine is alkaloid stimulant with its controversial effects on the central nervous system, e.g., insomnia. Owing to debatable effects of caffeine on human health, the masses demand for the decaffeinated coffee hiked many times from last decade and currently decaffeinated coffee is the 10% of world coffee consumption. All the market available decaffeinated coffee has been produced by the artificial removal from the caffeinated coffee which is an expensive process and it also deteriorates the coffee quality by disturbing the biochemical composition of resulting decaffeinated coffee [71]. The limited genetic pool of coffee varieties, especially for coffee Arabica, is the biggest bottleneck in producing decaffeinated or low caffeine coffee varieties. However, the gene banks of coffee are still expanding due to collection of huge wild genetic resources of coffee plant across the Africa, Latin America, Madagascar, Indonesia, and Yemen by the international missions and consortiums of coffee research [72]. This huge diversity in genus *Coffea* reportedly have more than 21,000 accession worldwide now, and some of these have been investigated for improving the adaptability, morphology, yield, insect pest and disease resistance, and decaffeinated coffee production too [24, 38, 71, 73]. After the discovery of caffeine free (e.g., *Coffea pseudozanguebariae*) or low caffeine coffee varieties (*C. arabica*; AD0591, AD2491), interspecific grafting among different varieties was also performed, but rootstock was found un-influential for the low or decaffeinated scions. According to statistical data of Engelmann et al. [72] about the biodiversity of genus *Coffea* among the total

discovered and conserved *C. arabica* accessions, nearly 34% *C. arabica* genotypes are of low caffeine varieties. The caffeine contents in the *C. arabica* coffee varieties vary from 0.62% to 1.82% [74]. Nevertheless, interspecific grafting in *C. arabica* did not show expected results, but this vast genetic diversity in *C. arabica* with respect to caffeine contents provides a strong basis for intraspecific grafting and hybridization within in the *C. arabica* varieties. Still there is no study available about the intraspecific grafting intending to generate the low caffeine *C. arabica* hybrid varieties. Generation of caffeine free or low coffee varieties is also a way of improving the existing cup quality, organoleptic traits, and physical characteristics of *C. arabica* coffee since caffeine and chlorogenic acid involve in enhancing the bitterness, acidity of liquor, and smaller size green beans [71, 74]. Caffeine is found in a composite form with chlorogenic acid [75], but the quantity of chlorogenic acid in different varieties was found to be in a close proximity while caffeine's amount varies significantly exhibiting that only a fraction of chlorogenic acid is in complex with caffeine [76]. Chlorogenic acid made three quinic acid ester groups with caffeic acid, namely, caffeoylquinic acids (CQA) and dicaffeoylquinic acids (diCQA)] or with ferulic acid feruloylquinic acids (FQA) [24]. Each group has three isomers with 5-CQA being the amplest isomer. Caffeine production is a 94% genetic trait [77], but still the variation of caffeine among coffee varieties cannot be explained by the absence or presence of the governing gene [76]. The quantitative trait loci (QTL) coupled with principal component analysis (PCA) approached identified two independent locus *CQAL* and *RCQL* located on two linkage groups responsible for the variation of caffeine among coffee varieties. The level of caffeine enhances with level of CQAt (total CQA), but relationship between caffeine/CQAt is not absolute in all coffee varieties, suggesting that RCQL would regulate the ratios of caffeine and CQAt [75]. The QTLs involved in the yield and technological traits related to quality were also first time identified in the subgroups of pseudo-backcross individuals of *C. robusta* (Guinean 410 × Congolese A03): seven QTLs for yield, six QTLs for rate of pea berries, eleven QTLs for bean size, eight QTLs for 3-CQA and 4-CQA, and one QTL zone for each; caffeine and trigonelline, 5-CQA and bitterness were detected. For acidity and bitterness, male and female additive effect was also found. Up to 15% variability was detected in the QTLs for the organoleptic traits except 54.8% for acidity [78]. The detection of QTLs for organoleptic properties is a milestone for it can be used as an indirect way to improve these traits in the different varieties and used in the development of genetic maps to study the relative order of genetic markers and their relative distances for finally underway genome sequencing projects of coffee. A complete genetic linkage map for *C. robusta* and a partial linkage map of (*C. pseudozanguebariae* X *C. liberica*) are also reported and available [75]. Besides the development of cultivars with imparted desired biochemical composition, the cultivars with conferred preharvesting quality attributes are also crucial for safety and maintaining the quality parameters of coffee berries till before the start of harvesting. The different cultivars are developed for different cultivation systems. For conventional cultivations systems, large and tall tree cultivars (e.g., Mundo Novo) and for denser to super denser systems medium to small size cultivars with compact, cylindrical, or erect canopy (e.g., Catuai), for better

penetration of sunlight, have been developed in last two decades [79]. Tall cultivars are 30% more efficient against white frost as compared to dwarf varieties that are more resistant to windy frost. Coffee berries required a uniform pattern and time of maturation and harvesting for better end quality of product. Coffee cultivars are divided into four classes with respect to maturation time of berries. Each class suits to a specific region with a precise set of environmental factors. Early cultivars are considered best for the cold and frost prone areas, whereas late cultivars are suitable for mild hot to hot regions. Yellow fruit cultivars are better not to grow with manual or traditional harvesting systems due to confusion between ripen and unripen coffee cherries [80]. Coffee cultivars with deep root system and great number of root hairs are best suited to droughty, low nutrition, and acidic soil, and roots stock of such cultivars are best suited to developed drought resistant hybrids. Labor, equipment, and transport cost along rainfall damage due to rainy season can also be avoided by choosing a right coffee genotype suitable to that area [81].

Choosing a coffee variety is matter of balancing between two basic economic principles quantity versus quality. Mundo novo, Catuai, and Catimor are recognized for their high yield while varieties like typica, bourbon, and caturra are taken as high cup quality varieties. In every region of coffee production, each grower has his own set of concerns about coffee varieties and not even a single variety can address their all set of concerns. The selection of a coffee variety by growers mostly relies on the factors like its features, regional policy, and environmental characteristics. The coffee grower from reachable and well-managed areas are more concerned about the yield and marketability of a coffee variety as compared to poor farmers from remote areas who are more fretful about adaptability and yield stability [90]. In Central American and African coffee producing countries, growers usually prefer the traditional coffee varieties (Caturra, Bourbon, Pacamara, Catuai) over the inter-specific hybrids or introgressed (*C. arabica X C. robusta*) named "Hybrid of Timor" irrespective of the fact that Hybrid of Timor are resistant to rust and nematodes diseases but with low cup quality [8, 38]. In Central America, it is out of question to ignore the traditional varieties as these varieties are hallmark sign for good yield and exemplary quality among the common growers. So it is very important to describe here biochemical composition of beans from traditional varieties (TVs), hybrids from the cross of TVs, and *C. arabica* (F1-T/A) and hybrids from the cross of hybrid of timor lines with Arabica lines (F1-H/A). A research report published by Bertrand and his colleagues in 2006 revealed that elevation has more influential role on TVs than on others, whereas fat and sucrose are the two main constituents which found differ significantly among different varieties at same digits of elevation. At 1399 m altitude, the fat contents of these varieties were in the order of F1-T/A > F1-H/A > TVs where above 1400 m the order was somewhat like as F1-T/A = TVs > F1-H/A. Trigonelline and caffeine remained unchanged in all varieties at a given altitude, whereas chlorogenic acid contents differed only at intermediate elevations. Sucrose concentration was found in variation from low to intermediate altitudes in all varieties, but traditional varieties tend to have more sucrose concentration at these elevations. TVs have a general trend of accumulation of more fat and chlorogenic acid with the increase in altitude [32, 91] so does F1-T/A

but at lesser degree as they can accumulate more chlorogenic acid at lower elevations. So F1 hybrids are insignificantly affected by elevation. Additionally, TVs have high vigor and vegetative growth at high altitudes resulted in an increase in leaf to fruit ratio which in turn resulted in enormous supply of carbohydrates to berries with less nutrition competition. So this better supply of carbohydrates coupled with delayed berry ripening at higher elevations due to low temperature stems out as better cup quality conclusively. This issue was addressed by Bertrand et al. [91] by increasing the heterosis and homeostasis in F1 hybrids which give high vigor and leave to fruit ratio to F1 hybrids too which help in eliminating the variation of fat contents and consequently in the cup quality of these hybrid varieties. A near-infrared reflectance (NIR) spectrum of TVs is more modified by the elevation than new F1 hybrids which is confirmation of this work on F1 hybrids and can be used for identification and authentication of coffee varieties. Further, organoleptic properties do not differ at high altitude among all coffee varieties and with some exception F1 hybrids are equivalent to TVs. But at lowers altitudes F1-T/A and F1-H/A are considered superior than TVs [38]. As new varieties of coffee are being introduced, so genetic consistency should be maintained among the new and old varieties because genetic consistency is considered very important for the quality assurance of agricultural products [92]. Since 1950s most of the breeding and genetic improvement programs around the coffee producing countries mainly focused on generating high yielding and insect-, pest-, and disease-resistant varieties with rare focus on quality attributes. Breeders and geneticists started to focus the quality attributes of coffee varieties from 1980s with production of introgression lines. Literature is also full of conflicts over the quality equivalence of introgressed and nonintrogressed lines, while the reported efforts of exploiting heterosis and homeostasis are hopes for creating quality balance among the F1 hybrids [38, 59, 88].

2.3 Seeds and Seedlings Characterization for Cultivation

Vegetative propagation methods are mostly used for self-incompatible *C. robusta,* while true breeding *C. arabica* is propagated by means of seeds with a least fraction by microvegetative propagation [93]. As most of the coffee seeds' presowing handling, treatment, and storage studies focus only on seeds from *C. arabica* so this section will only address the seeds from Arabica coffee plants unless specified. Healthy seedlings are the guarantee of healthy plant and coffee cherries with desirable characteristics. The production of healthy seedlings solely relies on the vigor, viability, and germination of seeds. Several presowing management and storage studies have been conducted to ensure the preservation of vigor, viability, and germination of seeds. The quality of presowing seeds is generally measured in terms of germination percentage, percentage of seedling emergence (%E), and seedlings attained first true leaves (%FTL) [94]. Storage time, storage temperature, moisture contents, time of harvesting of seeds (intended for sowing), fruit maturity, and size are the important factors which considered crucial for selection or harvesting of seeds intended for sowing. Selection of right kind of seeds with

known moisture contents which are stored for recommended period of time must be authentic as controversies exist in the literature about storage of coffee seeds for midterm and long-term storage of seeds over the viability [95]. Coffee seeds have dawdling and irregular germination pattern and are prone to desiccation which made their potential storage a topic of scientific research. The productions of seedlings from seeds almost take 6 months (from Jun/Jul to Dec/Jan) and, even on this, uneven seedling emergence with low temperature weather of winter made it quite hard to get the high quality seedlings for planting in field. Despite shortening of germination test time span [96], it still needs whole 15 days to assess the seed quality, germination pattern, and seedling growth. The staging of whole germination process from imbibition to cotyledons leaves into seven stages made it easy to shorten this which also benefits us to get rapid quality evaluation results and official quality certificate, accurate characterization of coffee seed/seedling development, and standardize the seed quality assessments. Recently it has also been demonstrated commercially to confirm the germination and viability of seeds according to the criteria set by Rosa et al. [95] which showed that assessment of seeds lots at S1 stage of germination (14 days) has same outcomes as the germination test at 30 days [97]. Previously harvested or short term stored seeds (less than 3 year) with 12% moisture contents are viewed most suitable for the cultivation since these seeds exhibited higher germination percentage [96]. The parchment free seeds with water soaking treatment one day before the sowing showed quick emergence along high vigorous seedling growth which also lessen the plant nursery period by 4 weeks [98]. Soaking of the stored seeds may result in the brownish outgrowth due to fragmentation and decompartmentalization of the long dead seeds while nearly past dead seeds showed characteristic "blue green" color due to viridinic acid formed from the oxidation of chlorogenic acid, initiated by alkalization [99], catalyzed by enzymes, probably polyphenoloxidases and laccases [100]. So the appearance of this "blue-green" color may be used as marker for deciding the storage time of seeds and their application in the farm. Most of the coffee seed drying and storage studies used the parchment free seeds in lab, while on commercial scales coffee seeds are being sowed with the parchment as removal of parchment is not possible with adversely affecting the seed quality. About 9 to 10 months stored seeds with high vigor are also considered ideal because the emergence and germination process can start under more favorable climatic conditions and healthy seedlings can be obtained before the start of cold weather. The generally two sowing methods are in situ sowing and pregerminated coffee seed sowing, but direct sowing method is found to be more suitable for normal root growth and length with a normal pace of early growth and first true leaves (%FTL) [95]. Coffee seeds which are harvested at the time when cherries are fully mature are considered best as time of harvesting also affects the germination capacity and pattern [95]. On the opposite side, Guimarães et al. [97] claimed that seeds from coffee cherries which were harvested when the cherries were yellow-green stage exhibit maximum germination with suitable pattern. Maturation of seeds is important as only mature seeds are able to germinate in soil and water content of seeds at that time has critical value as mature seeds prone to desiccation are unable to germinate [101].

Coffee seeds (*C. arabica*) are no longer considered recalcitrant as they can survive below the threshold level (0.20 g H_2O/g dw) of recalcitrant seeds. Coffee seeds are now believed to have storage behavior defined as intermediate [102]. The minimum water content to which seeds of *C. arabica, C. canephora,* and *C. liberica* can be dried without damage is about 0.09 g H_2O.g-1 dw and 0.10–0.12 g H_2O.g-1 dw, respectively [102]. The water content of seeds and ambient temperature are interdependent and have been considered critical for the growth and survival of seeds in soil [103]. The seed water content decreases with lowering the temperature until chilling proving unfavorable for the viability of growing seeds. Generally, seed moisture content followed by storage temperature was the most important factor that influenced coffee seedling quality, whereas the fruit maturation stage least influenced quality. Some cultivars of *C. arabica* were found to be still possessing high germination rate (>85%) while storing at 10–15 °C when germination temperature range found suitable for higher germination is 20–30 °C [95, 102]. But Rosa and her coworkers [95] found that maturation stage has significant effects over standard germination rather than moisture content or physiological germination or their interaction between these two factors.

Immediately after harvesting, seed maturation stage does not affect the germination rate of dry seeds with moisture contents (10–12%). But in longer seed storage period, stage of harvesting found to be significantly affecting the physiological aspects of seed quality. Physiological quality of dry seeds was also found to be influenced more by storage temperature when dried up to 12% moisture contents. Actually there is a triple interaction of factors (e.g., storage time, storage temperature, and stage of harvesting) which found to play a crucial role in determining the quality of coffee seedlings. The premature harvested seeds with high moisture contents have a higher emergence speed indices than the cherry stage harvested stage with intermediate moisture (16–20%) contents that also produced poor quality seedlings. The seeds of either harvesting stage with mediocre moisture contents also have more impact on poor physiological quality and insignificantly affecting the coffee seedling production. The quality of seeds found to be synonymous if they are stored at 20 °C provided that they must be stored with 12% or 47% moisture contents irrespective of stage of harvesting. So the highest and lowest moisture contents (12%, 47%) of harvested coffee seeds have higher physiological quality as compared to the seeds with intermediate level of moisture [95]. The possible reason behind this oxidative or respiratory damage and deterioration is seeds with poorly activated injury repair mechanisms [109]. After the work on storage and drying by Rost et al. [95], this topic was further explored by addition of using the drying methods (e.g., slow and fast) [103] and somehow supports the results of Rosa and his team [95]. Physiological quality of seeds found to be compromised in both drying methods. Good percentage of viability and vigor was observed in seeds with higher (40%) and lower (12%) moisture dried by slow drying as compared to seeds with intermediate moisture level dried by fast drying that also lost the vigor. The microscopic rupturing of cell membranes may be the reason for this which is enormous at fast drying with intermediate moisture [104]. The fast drying process should be avoided in this regard as seeds may tend to have intermediate level of moisture content for longer period of

time, which may cause the respiratory damage and tissue injury lowering the viability, vigor, and physiological quality of seeds [105, 106]. The conflict in literature about advocating the usage of slow drying [107] or fast drying [95, 108] methods may arise due to using coffee seeds with different quality, ecology, variety, or genotypes, etc., in different studies as it is also documented that different species of *Coffea* have different level of tolerance to withstand desiccation process. The till known sequence of level of tolerance to desiccation is in the order of *Coffea racemosa* > *C. canephora* > *C. arabica* > *C. liberica* [109]. So we may assume that desiccation sensitive species may not possess the full range of desiccation tolerance mechanism by lacking sugar accumulation and glass formation upon drying and storing which suggested that desiccation tolerance mechanism may not involve in the water removal in coffee seeds as it occurred in desiccation tolerant seeds [106]. Therefore, the coffee seeds may not tolerate the desiccation up to <10–11% as reported by various drying and storage studies [110]. Even at intermediate moisture contents (<20%), water supply is restricted to major metabolism process, but still at the 11%, the loosing of viability is a sign of occurring the metabolic process. The literature does not have reasonable reason of this except the after ripening processes which are considered responsible for the "switching on" the certain genes during drying and storage of seeds [111]. The other reason for the desiccation tolerance is the induction of heat tolerant proteins, which are more significant in shade dried seeds and their abundance may be correspondence to the level of desiccation tolerance [112]. Santos and coworkers [103] studied some of the enzymes (i.e., esterases, catalases, peroxidases, β- mannanase, isocitrate lyse dehydrins, γ -aminobutyric acid GABA) of these "after ripening processes." Where the higher amount of these enzymes have been found in slow drying and high moisture content seeds. The transcriptomics study revealed the accumulation of catalases and peroxidases for germination and cope with the changing physiological conditions [112]. But the transcripts of these enzymes have not been seen beyond the low moisture level (12%), moisture level which may be to lessen the use of ending metabolic energy [113]. The activity of deterioration indexing enzyme "esterases" found in dried seeds before and after the storage. The activity of peroxidase enzyme found more in quick dried seeds, whereas no activity of superoxide dismutase was found in fresh and nondried seeds [112]. Isocitrate lyse and GABA plus dehydrins are symbolic enzymes indicating germination and water stress, respectively. The level of isocitrate lyse found to be higher at high moisture (>40%), whereas expression of GABA and dehydrins were high at onset of water stress (30% moisture) [114]. The desiccation causes the water and oxidative stress which create the reactive oxygen species (ROS, e.g., hydrogen peroxide) which lead to over-expression of catalases to balance the oxidative stress. However, the level of expression of Catalase and peroxidase is in complex configuration in endosperm and embryo, which may be due to maintaining the specific level of ROS [103, 112]. The other reason might be the expression of gene is controlled at various levels from DNA to phenotype and it is very hard to separately extract the RNA from endosperm and embryo for calculating the expression. A through separation of endosperm and embryo is also inevitable to study whether the stress-induced metabolic reactions

start earlier in embryo or endosperm. The accumulation of GABA at higher moisture contents (>50%) reveals its association with germination process instead of stress-induced enzyme while the accumulation of GAMA signals towards the increase level of expression of stress enzymes "dehydrins" [112, 113]. There is a need to design such a drying strategy that ensures the firmness of membrane and prohibits the resulting leaching of nutrients as nutrients are competitors with antioxidant system in seed and keep the metabolism process well organized [103]. Consequently, the drying method affects the quality of seeds in combination with final moisture of the seeds as slow drying with low moisture seeds (12%) gives good germination results.

Since coffee seeds are mechanically damaged during the coffee seed processing which not only lowers its quality in terms of germination and viability but also with respect to cup quality, so it is essential to partially cover this topic here by describing the damage, their causes and remedies, and the available test for identification of damaged seeds as detail description of this issue is beyond the scope of this review. Pulping is process of removing exocarp and endocarp to produce the seeds with parchment friction and attrition. Removal of pulp and parchment by peeling machines and palette scarifier, respectively, lowers the viability and germination and viability of seeds than unharmed seeds due to rupture of sensitive seed cell by external applied pressure, high drying temperature, and coffee seed borer. Due to high cost, labor requirement, and operational complexities, previously used tetrazolium test to determine mechanical damage has been replaced by the nondestructive LERCAFE test by immersing the seeds in cost-effective sodium hypochlorite. The least effective concentrations of sodium hypochlorite are 2.5% for seeds without parchment and 5% for seeds with parchments for 3 h at 35 °C, whereas slight higher concentration (3.5%) can also be used to lessen the time from 3 to 2 h. This test also has the capacity to distinguish the various kinds of mechanical damages. The occurrence of green stain in endosperm is symbol of high temperature drying, whereas a depression surrounded by a green ring is a sign of damage by coffee seed borer. However, sometimes the results of LERCAFE showed inconsistencies over seeds viability and germination results that is due to long period immersion (>5 h) of seeds in solution or pathogen infestation in endosperm of seed lots, whereas active chlorine usually reacts with the tissues of embryo to denote tissue damage by its coloration due to which LERCAFE showed superior results [115]. Since sodium hypochlorite is photo and thermo sensitive and contains a certain amount of active chlorine (almost 10% for commercially available), so its standardization by quantifying its active chlorine and immersion duration is very important for credible commercial applications. Sodium hypochlorite solution with 2% active chlorine contents and immersion period less than 5 h and more than 1 h found to be appropriate for viability and germination determination. The higher or lower treatment time and active chlorine contents than recommended may lead to extra or under-coloration of embryo tissues giving us a wrong clue about the viability and germination. However, activity of the active chlorine influences the certain antioxidant system of seeds too. The activity of esterase, esterase isoenzyme, and catalase found to be decreased or diminished, while the activity of superoxide dismutase and

alcohol dehydrogenase enzyme enhanced under various concentrations of active chlorine [115]. As discussed above, seeds with parchments are used for sowing on commercial purpose which hinders the normal germination process by providing the tight barrier. But this parchment also proved to enhance the viability of stored seeds as compared to hulled seeds irrespective the method of drying. Fifty percent hulled seeds lost their viability in the first 3 months of storage and in 1 year all the hulled seed lost their viability, whereas more than half seeds with parchment were still alive [116–118]. But the dark side of this is that parchment and other seed germination substrates may be the harbor of disease causing microbes and setting up these seedlings in field, opening up the plant for additional microbial load from surroundings. The pruning activities, manual or mechanical harvesting of fruits, wet or dry processing methods, etc., can vector these microbes not only from infected plants to healthy plants but also will make the healthy seeds infected [119]. The detailed and comprehensive reviews are available on the coffee microbiome, coffee microbial or fungal diversity, diseases, and vectors [120, 121].

As the living genetic resource, the germplasm is used in the production of improved coffee cultivars in terms of yield, yield stability, cup quality, and resistance against insect pest and diseases so we would also like to discuss the cryopreservation of coffee seeds. The work on the cryopreservation of germplasm of coffee is carried out since 1990s to avoid the issues like genetic erosion due to poor adaptabilities to new environments, labor cost, and large required space, to preserve the genetic resources of coffee. Cryopreservation is also a suitable technique to preserve the whole nonorthodox seed, and certain amount of work has also been carried out study the intermediate whole seeds of coffee (e.g., *C. arabica*) which are partially desiccation tolerant. Seeds are normally predried to a certain level of moisture before the cryopreservation. This may include shade or artificial drying. Abreu et al. [112] found that shade-dried seeds with 20% moisture contents can maintain the quality for 12 months. The precooling rate, drying rate, rewarming rate, initial seed lot quality, seed moisture contents, and relative humidity for drying are critical research factors addressed for the good viability and higher germination of desiccation tolerant seeds [122–124]. The gradual precooling of seeds down to −50 °C before immersion into liquid nitrogen showed higher rate of seed viability and germination than rapid cooling [122] while drying the seed with the 81% relative humidity which was found to be optimum for cryopreservation [122] contrary to previous findings of 78% of the same author [123]. But the reason behind the advantageous gradual precooling than rapid cooling is still a research topic to be resolved specifically in coffee seeds. As coffee seeds also contained polyunsaturated fatty acids (PUFAs) in fractional quantity, might be the cracking of lipid glasses, formed due to transition phase of materials; in the membrane lead to the lower the viability in rapid cooling. The simple and inexpensive procedure for the precooling was outlined by placing seeds in the folding of ice in simple dry ice bath where the precooling rate is 10 °C/min which is also thought to be optimum. The other prejudicial factor is rewarming or thawing rate which is no longer considered detrimental as coffee seeds are dried to a moisture extent 17% or 20% that is equal to unfrozen water content in seeds before the immersion in liquid nitrogen [123]. The aberrant findings of Vasquez et al. [124]

of least seed viability percentages even drying the seeds till recommended extent (20%) may arise due to environmental factors, from seed development to seed drying, affecting the tolerance of seeds against desiccation and liquid nitrogen [122, 123].

Geostatic methods (e.g., spatial variability) have also been used to typify the physiological study of coffee seeds. Various studies [125–129] also successfully define the region for the quality seedling production. Despite the advances in quantitative soil mapping techniques, most soil maps continue to be produced using conventional techniques. The usefulness for decision making of such maps is restricted [34]. Furthermore, as discussed in Sect. 2.1, most of genetic and breeding improvement programs only focus on the yield, yield stability, cup quality, and insect pest resistant coffee varieties; little attention was paid to the genetic transformation of existing coffee cultivars for production of desiccation tolerant coffee seeds genotypes. The least amount of works available focus only on the non-Arabica coffee cultivars, e.g., *C. pseudozanguebariae* X *Coffea liberica var. dewevrei* [122, 123]. The backcross progenies of these least variable parents showed a transgression in the course of most delicate parent without a discrete level of desiccation tolerance. Further, seed desiccation tolerance is a declared quantitative polygenic or multifactorial trait, but initial seed viability or moisture [130], initial desiccation rate seed size, and tree fertility not affect the understudy trait [123]. The mapping of QTLs responsible for desiccation tolerance is still underway after their successful application on other important agricultural crops. In genus *Coffea*, most of the discovered genetic accessions belong to the *C. robusta* and desiccation tolerance of this species supposed to be equivalent to *C. arabica*. The simplified methods and protocols from simple cryopreservation to inheritance in breeding and from breeding to molecular level (QTLs mapping) are also now available. So it is now prerequisite and vital to thoroughly explore globally discovered genotypes of *C. robusta* along *C. arabica* to finally desiccation tolerant seeds hybrid. If this milestone is achieved, it will have a great impact not only on research and development but also on the cheap preservation of seeds at commercial level for the quality seedling production.

2.4 Soil and Fertilization

Among various reasons of lower cup quality of coffee, under or no fertilization of coffee fields is also a leading factor for this cause. A study by Sivetz and Foote [131] showed that nutrient deficiencies may decrease cup flavor. On the other hand, Pochet [132] demonstrated a very clear and positive link between the organoleptic qualities and low soil fertility. To deal with low soil fertility issue, the conventional ways of fertilization are also same for coffee fields, i.e., organic and inorganic or mineral fertilization. This section is also highly linked with topographic conditions, management practices, organic matter and herbicide applications, shade, rainfall or irrigation, planting and intercropping schemes, and agroforestry systems which collectively determine the fate of soil by affecting its organic matter contents

(OM), soil available moisture, porosity, aeration, texture, acidity, cations, cations exchange capacity (CEC), and soil microbial diversity. Unfortunately the studies published so far on the topic of soil and fertilization of coffee fields mostly focus on the yield, growth, soil fertility, soil erosion, soil acidity, soil toxicity, etc., like issues and literature still lacks the data on the direct relationship of soil nutrients or fertility with cupping quality of coffee. So this section will take into account all these factors which proposed to affect the yield directly and cupping quality indirectly as Castro-Tanzi and his coworkers proposed that yield has significant positive but weak correlation with coffee quality irrespective of a very few contrary findings which may be due to possibilities of these studies in high density plant populations or agroforestry systems (e.g., *Inga edulis*) where no effect of fertilization is seen [133].

The proper and sustainable supply of nutrients should be at the priority as nutrition of coffee plant determines the coffee bean size (grade) and its biochemical composition. Even after the addition of OM or litter, nutrient pool of soil is not considered sufficient for the sustainable supply of nutrients due to which inorganic fertilizers have to apply to fulfill the needs of coffee plant. The consumption of inorganic fertilizers by 1ha fully matured and highly productive coffee plants is estimated up to 135 kg N, 34 kg P_2O_5, and 145 kg K_2O [42]. The exact amount of nutrients required by plant may vary from region to region and largely depends upon the soil topography, rainfall, site-specific characteristics, agro-ecosystem, type and density of coffee and other plants, seasonal variations, and cultural practices [134]. According to the extent of requirement, nutrients are divided into two large group called macronutrients and micronutrients. The macro- and micronutrients include N, P, K, Ca, Mg, S and Fe, Mn, Zn, Mo, Cu, B, Al, respectively. The soil often lacks the required quantity of major macronutrients which have to be replenished by inorganic fertilizers. Fortunately the pool of micronutrients is sufficient according to the plant demands and any fluctuation in the level of macronutrient level may also lead to disturb the micronutrients level which can result in the toxicity of some nutrients (i.e., Al toxicity). N endowed green color to chlorophyll for photosynthesis and food preparation and directly influences the vegetative growth, flowering and bearing capacity of coffee fruits, amino acid formation, and composition. The possible fluctuation in the sustainable supply of N may upset the amino acid or protein composition of coffee beans and hence lowering the cup quality of beverage. N also keeps the fruit/leave ratio and acts as preventive measure for the die back diseases in coffee plant. The soil N contents are in positive relationship with soil OM contents [135]. The provision of OM or litter rich in N contents also favors the microbial activities and microbial diversity as microbes also need N for reproduction so, consequently, lower the decree of minerals leaching. The data showed that increment in soil N is in positive relationship with yield and soil N uptake efficiency hike by rise in soil Ca(O) suggesting soil Ca concentration as a "marker" for yield [136]. It may be due to neutralization effect of Ca, Mg, Na carbonates which pacify the rising acidity due to addition of mineral fertilizers Chadwick and Chorover [138] by increasing the CEC [137]. The reason of pH drop in coffee fields and then forests and grass lands may be due to excessive inorganic fertilization application leading to severe acidification of soils [133] while some authors still found no drop in

acidification of soils even fertilization rate kept constant and reason behind this may be the mineral saturation of the agro ecosystem before the initiation of study [138]. Contrarily, the application of theses macronutrient fertilizers (N, P, K, etc.) increased the availability of micronutrients (Cu, Zn, Mn, Fe) due to lower pH effect of fertilizers. The availability of micronutrients is also positively linked with SOC and pH. Depending on the soil fertility status, the nitrogen fertilization rate varies from 600 to 800 Kg/ha which some authors claimed quite high and a lower N fertilization rate (i.e., 200 kg/ha) is quite sufficient to get maximum yield [139, 140]. Excessive application of nitrogen, while it intensifies the production, has also been described to decrease bean density and quality. In South America, Dessalegn [141] stated that coffee grown with substantial application of nitrogen fertilizer had inferior, nimbler, and thinner body than that from unfertilized fields. Pinto et al. [139] documented that higher (above 300 Kg/ha) and three applications of N per annum lead to 57–105% leaching of fertilizer in the form of NO_3-N due to its higher mobilization rate through which it exited the root zone of coffee plant. The efficient N recovery has been achieved by decreasing the N fertilizer and application rate. The highest N recovery (up to 61%) is showed by 200–300 Kg/ha N rate with the seven split applications. Paulos et al. [142] also reported the reduction in yield with further incrementing the N after 300Kg/ha. Regarding soil nutrients, P is the second most important nutrient after N and most of agro-ecosystem soils are considered lacking the proper amount of this nutrient irrespective of addition of litter from shading or other tress well known for their positive effects on P cycling in soil [143]. These results showed us crucial role of other factors like soil conservation and management practices, site-specific characteristics, features and kind added OM, microbial diversity and activity [144, 145]. Flowering and fruiting ability of the coffee plants is largely expedited by the P. The role of P is also obvious in healthy and strong root system of coffee plant and adequate supply of P is essential for plant vigor, strong wood, sound fruit formation, and early maturity of coffee cherries and bumper yield [134]. A 50% increase in yield is observed by increasing the level of P from 0 to 33 Kg/ha. No further increase in yield with increasing P input has been observed. As P is immobile nutrient, so 45 cm from trunk and 15 cm depth are the optimal horizontal or vertical distances of application [146]. It is also registered that combined interaction of NP showed better results than alone. No association has been stated between phosphorus and the physical and organoleptic quality of the bean. K also has crucial role in berry development, cherry maturation, and activate various physiological enzymes important for the good quality coffee production [134]. A high concentration of calcium and potassium in beans has been linked with a bitter and “hard” taste.

C. arabica is a highland (as discuss above) plant where the soil erosion is more obvious with its overwhelming effects on plants making them more vulnerable to insect pest diseases as C. *arabica* plants are already more prone to insect pest attack as compared to *C. robusta*. This pronounce vulnerability of coffee plants may lower the cup quality by various means, i.e., affecting the coffee vegetative or fruit cherry development, maturation, and ripening stages. On the other hand *C. robusta* is cultivated in the lowland areas of Africa and North America. The average farm

size is 5 ha in these regions and coffee farming is quite devoid of fertilization due to poor subsistence of farmers [147]. These farmers only use organic fertilizers, e.g., animal or poultry manure for fertilization and manual weeding for plant protection. This is the most acceptable reason of yield reduction in east and central African countries where up to 70% record yield drop was documented in last three decades. The soils of these fields found quite low in N, Ca, and Mg near the critical value and P above the critical level as these organic fertilizations are unable to supply the required amount of these core nutrients. Soil, soil fertility, and fertilization are the challenging factors to carry over with its direct or indirect influences not only on coffee productivity but also on beverage quality as world's best coffee farming system are said to be at high altitudes with sloppy fields. These sloppy fields at high altitude are also the center of high natural runoff and minerals leaching losses collectively called soil erosion which broaden the importance of farm management practices here [148–150]. The organic matter (OM) in the top crust (30 cm) of soil is the first hit of this soil erosion where most of the feeder roots are located, which means direct and immediate influence on the nutritional status of coffee tree. The soil crust must consist of 80% OM and the amount of soil OM contents depends on various ecological factors like intercropping, type of intercropped crop, absence or presence of shading plants, pruning, pruning extent, plant density, weeding, rainfall, wind, runoff, etc. Soil OM contents have a positive association with soil N, P contents, and pH, whereas OM contents of agroforestry or intercropped systems are always higher than the mono-crop system. In the mono-crop systems, OM contents of Robusta fields found to be higher than that of the coffee Arabica fields [135] and the reason behind these findings is the difference in the height of cultivation of both crops as coffee Arabica always planted at higher elevation where there is greater runoff and leaching affects as compared to lower altitudes. On the other hand, in the intercropped or agroforestry systems, coffee Arabica tends to store more OM contents than Coffee Robusta fields [135]. The fall in the level of OM contents leads to compactness of surface soil affecting the root growth, lessens the aeration, soil pH, soil porosity, soil water retention capacity, availability, and recycling of nutrients, enhances the leaching of nutrients, and makes the plant more vulnerable to insect pest and diseases. The lowering of pH is the most devastating outcome of lower soil OM contents, which in turn affects the nutrients uptake by plant, nutrients assimilation, OM mineralization, and microfaunal (microbes and friendly insects or worms) activities. Mulching is highly recommended by several authors in order to conserve the OM level of soil and increase the soil minerals contents too [151–153]. N, K, Ca, P, and Mg are the basic required crop nutrients which also showed a rise in level due to act of mulching. In addition to these cat ions, OM is also important for incrementing the cationic exchange capacity (CEC) of soils, which in turn improves physical characteristics, soil microorganism diversity, and soil water relationship [147]. The choice of organic material for mulching depends upon the several factors like availability, cost, season, ease of handling and transportation, land, soil type, etc. Owing to biological nitrogen fixation features, leguminous crops are considered best for mulching irrespective of the fact of their competition with main crop and lowering the soil pH due to N mineralization

[154]. Some authors also reported the additional advantages of mulching with leguminous crops like greater flowering and obvious vegetative growth of coffee plant and weed control function [155]. The degree of effectiveness of mulching is site specific and depends upon soil permeability, soil organic carbon (SOC), percentage of soil surface cover, interaction among these variables, and other agro-ecological conditions. The mulching improves the SOC and water aggregate stability index (WAS) and reduces the soil bulk density (BD) and Interrill and rill erodibility of coffee fields at high altitudes [154]. The increase in BD following mulching as reported by some author [152] may be due to incorporating the mulch material with soil instead of layering which affects the porosity and hence the BD of soil. Tumwebaze and Byakagaba [135] also reported that BD has a positive linkage with pH and N and P contents of soil. The authors also noted BD is highly crop and crop production system dependent. BD is higher in mono-crop systems than agroforestry or intercropped systems, and Robusta coffee soils also have greater BD values than Arabica coffee soils which may be due to their cultivation at lower altitudes.

The outcomes of mulching in coffee fields largely found to be depended upon the type of its application, superficial or buried mulching. Superficial mulching gives delayed results (e.g., soil physical properties improvement, increasing SOC), while buried mulching resulted in fast improvement in soil but less soil protecting capability in the starting [156]. Buried mulching also reduced the runoff depth by 40.2% as compared to superficial mulching which exhibited (50–87%) rise in infiltration rate and runoff values are smaller in both types of mulching [157]. Crusted soils promote runoff rate and superficial mulching does not leave positive effect on crust soils over runoff rate. In addition, soil conditions significantly affect the runoff and infiltration in comparison to poor relation of soil class with them. Both kind of mulching give rise to 53–87% reduction in soil losses, whereas highest soil losses were achieved with smallest particle size mulch material. The highest rate of erodibility was seen in sandy and very fine sandy soils due to their ease of detachment because of weak association with organo-clay complex. Thus, sandy and silty soil (i.e., silt, silty loam, loam, silt clay loam) soils are highly vulnerable to runoff and acts of mulching offer resistance to erosion by strengthen the bond between finer particles and organo-clay complex [154].

Besides mulching, high density planting was also proposed as counter measurement for soil erosion and its detrimental consequences. Soil erosion is mainly considered responsible for soil acidification in coffee fields along nutrient leaching. Soil erosion was found to be more obvious in less denser planting systems, whereas high density planting systems contributed in enhancing the soil OM contents, exchangeable cations (Ca, Mg, K), CEC, extractable P, water storage soil crust and lowering the soil acidity, Al and Fe toxicity, and microbial population [158]. The control of soil erosion is also differing with respect to the cropping or plantation system. In agroforestry system, litter size is found to be more important erodibility factor [14] as compared to intercropped system where gradient slope has marked influence on both shaded and sun coffee plantations [148]. In agroforestry system of coffee, the threshold levels of litter cover and slope gradients are 30% and 60–65%,

respectively. So to limit the erosion, coffee should be planted on altitudes with slope gradient less than 30% and with 60–65% litter cover by any plant residue [14]. Agroforestry system not only lowers the temperature of microclimate by 4.1 °C below the canopy of trees which helps in delay ripening of coffee cherries, a prerequisite for fine quality coffee production, but also lowers the soil temperature up to 3.1 °C and conserves the soil moisture contents up to 6.4% more than the full sun cropping systems [159]. The rate of nutrients mineralization and leaching is also detected at lower level in the shaded or agroforestry systems which ensure the sustainable supply of nutrients to plant round the years [160, 161]. Soil moisture and soil temperature are main drivers directly affecting the soil texture, microbial activities, rate of mineralization, minerals uptake by plant, BD, soil porosity, and soil CO_2 efflux. Conservation of soil moisture in agroforestry system assures the continuous supply of water which is crucial for the good bean size, another factor for good cup quality of coffee, and therefore reduces the threats of economic loss [159]. The disturbance of the carbon pool partitioning for metabolism due to soil drought condition may lead to the overexpression of invertase to buffer the sugar concentration under limited photosynthesis which eventually disturbs the soluble sugars and amino acids level throughout the plant and coffee cherries, thus affecting the cupping quality of beverage [162]. After the reported results over the reduction of yield seen in agroforestry or shaded planting system with leguminous species, Soto-Pinto et al. [163] revealed that 30–45% shade or canopy cover is the optimal level in agroforestry or shaded planting system without affecting the yield and quality attributes of coffee fruit. The richness and density of shading trees are constructively correlated number of strata and negatively related with sunlight below canopy cover and radius of central bush of coffee tree. The independence of coffee yield, soil fertility, pH, and nutrient contents from shade type is symbol of involvement of other factors, i.e., environmental conditions, management and agronomical practices in yield, and quality attributes of coffee [155].

Owing to lack of harmonization between mineralization and plant uptake, OM contents of soil cannot cope with the nutrients demand of coffee plants for sustainable coffee yield with good quality characteristics [147]. To withstand with their nutrients demands, tropical coffee plants are also in symbiotic relationship with vesicular arbuscular mycorrhizal fungi (VAMF), which in exchange of carbohydrates provides the coffee plants not only with macronutrients (i.e., P, N) and micronutrients (i.e., B, Zn, Cu) [164, 165] but also abridged the water use and nutrients loss [166], increases the plant resistance against soil pathogens [167] and drought, and improves the soil texture by diversifying the soil microflora aiding in rapid mineralization of OM [168]. To improve the yield and cupping quality of coffee, it is very important to get insight into the diversity and composition of VAMF communities and environmental drivers which affect, in either way, their diversity and composition. The literature is full of controversies about the environmental drivers which determine the VAMF abundance, diversity, and composition of their communities [169]. The influential environmental drivers which determine the fate of VAMF abundance, diversity, and composition may include OM, soil P and N level, soil pH, elevation, spatial location, soil texture, soil moisture, management

practices, host species, and shade. More specifically, soil P level found to be an indicator of VAMF diversity, whereas soil pH, soil inorganic N and P, soil moisture, and shade exhibited a significant relationship over the VAMF community composition [169–171]. So the controversies existed in literature may arise due to different site-specific conditions, soil texture, spatial location, and nature of work, etc.

Sustainable organic coffee production required a sustainable organic matter specifically rich in macronutrients especially N and K. Livestock dung, composted and crop residue, farmyard, and organic matter from natural system are the common sources of OM. Despite fulfilling the OM contents of soil and its associated benefits, the natural supply of NPK from all these kind of OM is $<10\%$ which is insufficient to refurnish the exhausting soil and satisfying the nutrients demands of coffee plant [172]. On other side, long term dependence only on mineral or inorganic fertilizers may not be sustainable because of rendering the soil highly acidic, degrading, devoid of soil microbial diversity, and having unstable soil aggregates. So, recently, a new integrated approach of using both inorganic fertilizers and organic manure showed promising results under the liberal supply of irrigation water. This integrated fertility management system showed a comparable growth pattern to inorganic fertilization by supplying the nutrients to coffee plant in lag phase during which organic manure alone failed to immediately fulfill the coffee plant's needs [172]. But scientific literature still needs the data on consequences of this integrated fertility management system on yield and quality. Industrial production of coffee also resulted in the millions of tons production of coffee waste (i.e., husk, pulp and peel) which is highly vulnerable to our environment due to its high contents in tannins and caffeine [173]. Besides this, coffee waste found to be rich in organic carbon ($>50\%$) and the other nutrients that are usually lacking in coffee soils (i.e., total N 1.27%, K 2.46%, and with C/N ratio 40.02) [174]. So this coffee waste is under investigation for sustainable coffee production after its successful application in mushroom cultivation. Santos et al. [175] failed to get significant results of using untreated coffee waste in coffee fields due to high C/N ratio, higher fruit and leave drop, and higher occurrences of fungal infestation. Compositing, and oxygen driven fermentation procedure by the microbial communities, was also investigated as a favorable tactic for lowering the C/N ratio and hiking the level of N and K [176]. The application of composite material also reportedly improves the site-specific features, soil physical characteristics, and soil texture [175]. Compositing approach is considered a good approach for recycling of natural nutrients but like integrated fermentation management system, effects of composite material application on beverage quality are still unknown.

2.5 Rainfall, Irrigation, Temperature, and Climate Change

This section basically deals with impact of rainfall, its correlating ambient day temperature, drought, and solar radiation. But as fluctuations in temperature and erratic rainfall pattern are generally perceived in the category of climate change [177], so it is reasonable to discuss the impact of climate change here too. Coffee is a

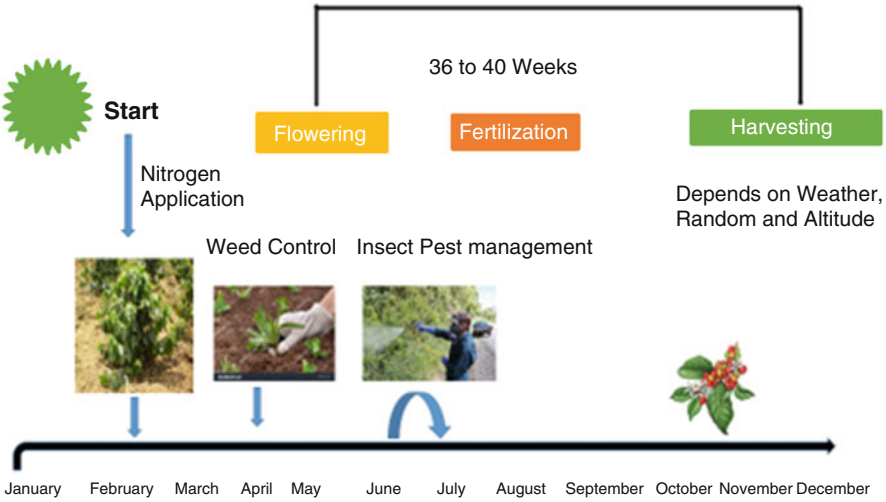
crop of high altitude, sloppy mountainous, and hilly inter-tropical areas with distinct dry and wet seasons. In these regions, wet season is characterized by precipitation rate of around 1500–2000 ppm per annum and consists of 7–9 months with atmospheric humidity near or equal to saturation, whereas in dry seasons the rate of precipitation befalls below 50 ppm and entailed only for 3–4 months concurring with the coolest period. Rainfall and sunshine distributions have a strong influence on flowering, bean expansion, and ripening. The susceptibility and rainfall requirement of high altitude grown *C. arabica* differs from low to mid-heighted hilly areas cultivated *C. robusta* due to difference in effective rainfall, evapotranspiration rate, topography, soil moisture holding capacity, soil texture, soil infiltration rate, and soil runoff. During rainy seasons, the level of rainwater exceeds the evapotranspiration and evaporation as compared to dry periods where water demand is high than water availability which can compromise the yield and fruits' physical and compositional quality by affecting its vegetative and fruit growth, development and maturation stages, in case water requirement is not met by irrigation. Although Robusta can withstand high rainfall greater than >2000 ppm, this high rainfall throughout year may limit the yield and quality of beans due to absence of required dry spell, less humid environment, temperature required for onset of flowering, beans development, bean size, and for cherry maturation stages [178]. The numbers of flowers determine the quantity of fruits on a tree which in turn influence the beans size (grade) and physical quality attribute of coffee beans. Onset of flowering is of much importance that an earlier or delayed flowering may result in earlier or delayed subsequent phases including harvesting during which they may be devoid of optimum required temperature, rainfall, humidity, nutrients, etc., and hence devastating the quality of coffee. Further, the earlier or delayed harvesting may worsen the successive drying processes, luckily if harvesting did not coincide with rainy period, due to lack of facilities in short period of time [179]. After this the other drivers which govern the flowering and beans quantity and quality too are (a) 30 days water stress per quarter of year required for flowers buds to get mature, (b) a certain level of humidity and temperature less than 24 °C suitable for anthesis, (c) "star flower" phenomenon which happens due to failure of pollination if humidity is less than 20% and temperature is above 24 °C, and (d) stormy and strong windy weather during flowering [178, 179]. In developing countries or irrigated coffee fields, flowering induction and extent of flowering can be controlled by deliberately irrigated water stressing after the heavily irrigation. Masarirambi et al. [178] recommended the 25 L water per plant with irrigation timing 25cb for optimum amount of flowering and ripe berries. Higher number of irrigation timings (i.e., 30cb, 35cb) may result in the higher number of ripe berries unsuitable for immediate successive operations. In Asia pacific, Amarasinghe et al. [180] also registered how to reduce irrigation water consumption by starting the irrigation in January after one to one half month of dry stress for flowering induction. Amarasinghe and his team concluded that reduced irrigation supply up to 150 mm (one round in 4 dry months) can receive an acceptable yield (i.e., 4000 kg/Ha) and any additional irrigation beyond 287 mm would increase the production cost along yield and quality drawbacks. After flowering induction and flowering quantity, precipitation, humidity, and temperature

influence the fertilization and coffee vulnerability to insect pest and diseases. Rainfall encourages maintaining the soil moisture level necessary to uptake the nutrients from soil after first fertilization. In case of delayed or no precipitation, nutrients cannot be part of plants to be mature and fruits badly affecting both the yield and quality of coffee fruit and beans [178]. But during coffee fruit maturation and ripening stages, a high precipitation (300 mm/month) and high temperature (>24 °C) may enhance the crop susceptibility to fungal diseases [181]. Barbosa et al. [182] also noted that among various other reasons of low scores of cupping quality of coffee high humidity index and rainfall during fruit ripening are major causes. Oberthür et al. [34] registered the ties between the various environmental factors and inherent coffee characteristics. He defined that annual precipitation, dew point, diurnal temperature ranges, and altitude favor the development of acidity, whereas dew point, daily mean temperature, and altitude support the flavor development in mid- to high altitude ranges with less degree of slope. On the other hand, in the same ranges of altitudes but with high degree of slope, he discovered that mean annual diurnal temperature range, dry months, annual precipitation range, and degree of slope are key factors for acidity while annual rainfall rate, dry months, and slope degree are critical for flavor determination. Trigonelline and caffeine contents of coffee beans are found less in the mid- to high altitude regions having less annual precipitation rate, high diurnal temperature ranges, and mean annual temperature [34]. Camargo et al. [183] also previously quoted that regions with relatively high mean annual temperature usually produced under-quality coffee beans as we know, now, that altitude and temperature are in negative relations to each other too. The coffee from mid-heighted altitudes with rainfall below the 1500 mm tends to be slightly acidic due to high contents of lipids, chlorogenic acid, and sucrose while at the same time, a contrary relationship was detected between lipid contents and rainfall [32]. As discussed above a high rate of rainfall, generally more than 1500 mm, is considered fatal due to deteriorating the physical bean quality and beverage quality and creates the favorable conditions for rust attack. Lipid believed to play a role in conservation of liquor quality during roasting because lipids form a protective layer on bean surface preventing the escape of volatile compounds [24]. Rezende et al. [184] also reported the retardation of coffee bean maturation while irrigation from May to July instead of good productivity trait.

Despite the positive role of drought in flowering synchronization and fruit setting, any drought or restricted irrigation conditions just before or during seed development can accompany a significant fruit drop and undersized beans [178, 183]. Prolonged drought stress results in stomatal control of transpiration rate than the general considered osmotic adjustments traits. The resilience to gas exchange with well relative water contents and growth, rather than elastic or osmotic adjustments, showed us the “avoiding drought” somewhat than tolerating it [185, 186]. Abiotic stresses, i.e., drought, temperature, frost, etc., may lead to osmatic and oxidative stresses, which may result in the generation of reactive oxygen species (ROS). These ROS can initiate the damage of tissues not only in vegetative parts but also in fruits, seeds, and reproductive parts via lipid peroxidation, bleaching of pigments and protein, and inactivation of various physiological important enzymes. The saturation

or unsaturation of fatty acids, to cope with abiotic stresses, may lead to changes in the fatty acids composition and eventually lowering the beverage quality [185].

Coffee is an evergreen perennial crop with water demand more than usual seasonal average rainfall with dry spells and routinely dry season of several weeks. The relationship of quality coffee beans production with required seasonal or and weather conditions is elaborated in Fig. 1. The soil moisture depletion during dry spells and season may adversely affect the growth of plant, fruit maturation, and ripening. So soil moisture deficit during this period of time must be dealt by irrigation to supplement the water. The coffee crops which received supplemented irrigation water exhibited 50–60% higher yield than the un-irrigated coffee fields [187]. The rate of irrigation varies from year to year and may depend upon planting systems, plant density, rainfall frequency, soil moisture holding capacity, dry spells, length of dry season, and method of irrigation. The quality of irrigation water may



Coffee Production is highly dependent on a regular sequence of weather events. Idea Climate for Arabica Coffee are

- Dry period of three months trees in order for them to flower properly.
- A **Good Soaking** to commence flowering but not continuous rain which will affect the fruit setting.
- **Avoiding Extreme Temperature,** which can cause range of physiological problems include flowering abortions
- **Regular Rainfall** throughout the berry development stage.
- **A Dry period** coming up to harvest.
- **A Dry Period** around harvest which will be ideal for picking and sun drying (this is for the ideal scenario but can't be true for all the producing countries.

Fig. 1 How climate affect coffee production?

also matter in deciding the final beverage quality but any direct study relating the quality of irrigation water and cup quality is still missing from the scientific database. However, application of treated waste water resulted in the insufficient supply of nutrients due to causing poor soil infiltration, reducing SOM and CEC, and increasing soil sodicity [188]. Surface irrigation, overhead irrigation, and ground level irrigation are the basic kind of irrigation systems with their own pros and cons related to operation, cost, energy, labor requirement, crop protection, evaporation, and soil texture effects [60]. However, drip irrigation (in trickle level irrigation) is considered superior than others due to fewer chances of rust occurrences, maintaining high nutrition and phytosanitary conditions, high productivity, 35–70% high plant growth, height, and dilated plant crown [187]. As this section of review will only discuss the irrigation role on the yield and physical quality of fruits and final beverage quality by supplementing the soil moisture deficit, so detail discussion on irrigation methodologies is not proper here. After different irrigational techniques, there are also different schemes of providing this supplemental irrigation water among which deficit supplemental or deficit irrigation scheme is a conventional approach to avoid drastic reduction in yield and physical and cupping quality of coffee with tremendous up to 20–50% water use efficiency for crop yield [190, 191]. Shimber and his coworkers [190] also discovered that raw and physical quality of green beans in terms of size, shape, color, odor, and conformity highly improved by a recent kind of deficit irrigation type called partial root drying (PRD) as compared to well water and normal deficit irrigation. The sensory and other organoleptic characteristics of brew from PRD were also found significantly better than from well water and normal deficit irrigation's coffee beans. This improvement may be linked with altered hormonal changes and higher total soluble solid contents; however, author did not specify the other climatology factors (e.g., precipitation rate, fertilization, mean sunlight value and temperature, shade, altitude, slope, topography, soil texture). Partially, this deficiency, recently, has been made up by the work of Liu et al. [192] by discovering the relationship of different rate deficit irrigation (DI) and nitrogen fertilization on yield and beverage quality. Liu and his colleagues revealed that 80% to 40% DI (80% to 40% of full irrigation, FI) reduced the tree height, branch length, and trunk diameter by 5.6–21.2%, 5.7–16.5%, and 5.1–8.3%, respectively. The short stature and short length of branches means less number of flowering and fruits which definitely will produced altered sized berry seeds worsening both the physical and brew quality. The rate of nitrogen from low to high (N_H, 140 g N plant^{-1}), middle N (N_M, 100 g N plant^{-1}), and low N (N_L, 60 g N plant^{-1}) increased the tree and branch length 5.1–8.5% and 9.1–9.8%, respectively. The DI (low to high) cut the annual dry beans yield up to 60% and N fertilization rate (low, medium high) ameliorates the dry beans yield by up to 46.2%. The counter effect of N (till 0.2 g kg^{-1},) on DI, in improving quality traits and yield, may occur due to changes in carbon assimilation and no stomatal conductance [193]. Continuous or well water irrigation also hinders the coffee maturation and ripening process and it is well documented that these irrigation schemes produced immature coffee berries [194] presenting a serious quality threat towards whole upcoming process activities [195]. The pattern of irrigation, in combination with other environmental

factors, played a critical and significant role within and among the different vicinities on physical quality and biochemical composition of brew. The extent and pattern of irrigation and rainfall profoundly affects the nitrogenous compounds (e.g., amino acids, proteins, protease, total nitrogen, and PPO) and contents and mass of coffee beans while highest quantities of these nitrogenous compounds are detected in less irrigated and restricted rainfall areas [189, 192]. There is direct relationship between the extent of restriction of water supply and protein, lipids, crude fiber, chlorogenic acid, and caffeine contents of coffee beans. The low to high (80 to 40 of FI) restricted supply of water can increase the contents of protein, crude fate, and caffeine by 9.4–14.7%, 26–14.1%, and 15.5–18.3%, respectively [192]. The very high application of N in restricted supply of water may further aggravate the worsening of final cup quality as the work of Liu et al. [192] showed that N application (low to high) in restricted supply may further increase the protein and chlorogenic acid contents by 7.1–26.2% and 6.4–37%, respectively. Further, proteolytic activity involves the lysis of proteins into smaller polypeptides, peptides, and amino acids in coffee seeds [196] which in turn contributes in Maillard reaction along carbohydrates [197]. So any variation in the rate of proteolytic activity can result in the variation of amino acids, polypeptides, and peptide contents which in turn would upset the Maillard reaction outcomes like its specific color or aroma and flavor, eventually deteriorating the cup quality of coffee. The literature further indicates that despite good yield, full irrigation (FI), with even high N fertilization, had lower contents of protein and chlorogenic acids which harm the quality of coffee. But the combination of moderate DI (80% of FI) with high N application cannot only give comparable high yield but also improved nutritional quality of coffee [189, 192]. Some authors also claimed low yield under full irrigation system [198] which may be due to waterlogging which created the hypoxia or anoxia like conditions in soil in which aerobic metabolism of plant suffer and consequently producing fermentative pathways products to fulfill the energy needs.

Volatile compounds have pronounced impact on the sensory and organoleptic characteristics of coffee. However, out of hundreds volatile compounds, only a few are key to determine the final quality of coffee [199]. Nearly 300 volatile compounds were detected in coffee green beans out which 200 still exist in coffee beans after roasting [200]. These volatile compounds are the sources of body (i.e., aroma, acidity, fruitiness earthiness, etc.) and sensory attributes of coffee. Bertrand et al. [201] first time in literature determined the direct and detailed relationship of these volatile compounds with climate (i.e., temperature, rainfall, solar radiation). Out of 44 volatile compounds, they enlisted 21 institutes to be in strong relationship with changing temperature, rainfall, and solar radiation which belong to the alcohol, aldehyde, ketone, lactone, furan, phenol, pyrazine, pyrrole, hydrocarbon, aliphatic acid, and sulfide classes of chemicals. Alcoholic, aldehyde, ketonic, and sulfide class of compound noticed to play generous roles in changing the body and sensory quality of coffee with respect to changing temperature. Alcoholic and aldehydes (except ethanol) compounds found to have direct relation with temperature, while sulfides and ketones possess negative relation. The majority of temperature positively linked compounds (i.e., butan-2.3-diol, butan-1.3-diol, 2-butoxyethanol, 2-

ethylhexan-1- ol, 3-methylbutanoic acid, benzaldehyde, hexan-2-ol, acetic acid, 2-phenylethanol butan- 2-one, benzyl alcohol, gamma butyrolactone and butan-2,3-dione) are also positively associated with the earthy flavor while five cold climate positively linked compounds (namely, ethanol, dimethylsulfide, butan-2-one, 2-methylfuran and acetone) are in direct relationship with fruity flavor. Some of other major volatile compounds remained unaffected but play a critical role in final cup quality, including 2-furanmethanol, ethanol, toluene, 3-methylfuran and hexanal, 2-methylpropan-1-ol. The literature cited that high temperature (>23 °C) during fruit development brings about the changes in the expressions and activities of genes and enzymes of these volatile compounds leading to disturb the "standard" concentrations of these volatile compounds and eventually hampering the body and sensory attributes of coffee [201]. Working with the *cafés-terroir* in Honduras, Avelino et al. [31] noted that the effect of temperature is conditioned by the latitude and altitude and that those attributes jointly favor coffee quality, producing the local characteristics of taste and aroma. Bertrand and his coworkers also deduced that temperature plays a more vital role in deciding the cup quality of coffee than rainfall as rainfall linked to four (2-ethylhexan-1-ol, 3-methyl-2-butenoate, methane, and gamma valerolactone). But, unlike their claims, rainfall always played its overall indirect critical role in maintenance and keeping the temperature and humidity under certain required limit all year round. Average daily temperature also found to alter the "standard" concentrations of isomeric compounds of major coffee compositional components (caffeine, chlorogenic acid, lipids, sucrose, polysaccharides) but keeping the overall net concentration of major component unaltered [202]. The isomers of same compounds respond differently to the temperature, i.e., 5-CQA is negatively correlated with temperature, but the reverse trend was observed for 3-caffeoyl quinate (3-CQA) and 4-CQA content. Similarly in lipids, stearic acid (about 7%) stored more in warmer conditions than linoleic and palmitic acids (35–45% each) [202]. After the discoveries of Bertrand et al. [201] and Joet et al. [202] on coffee volatile compounds and temperature role in controlling the biosynthetic pathways of certain important fatty acids, Abreu et al. [203] in the same year work on unfolding the relation of amino acid profile and proteinase activity with temperature as the previous revelation from Montavon et al. [33, 197] and Ludwig et al. [196] has showed the varying activity of proteinase and protease enzymes of coffee seeds from hotter to cold coffee fields (immaturation stage to maturation) responsible for varying quality due to variation in the ratios of free amino acids being taken part in Maillard reaction liable for coffee aroma, color, and flavor. Abreu and his colleagues noted a higher concentration of free amino acids at immature stage (both from hotter and colder regions) of coffee seeds then at mature stage, but seeds from hotter regions found to have higher amount of amino acid. Additionally, hotter side coffee seeds found to have higher amount of asparagine (Asn), aspartic acid (Asp), and lysine (Lys) at immature stage and at maturation stage more variety of amino acids (i.e., methionine (Met), glutamic acid (Glu), Asn, Lys, leucine (Leu), alanine (Ala), tyrosine (Tyr) and gamma-aminobutyric acid (GABA)) added to seeds from hotter regions. Abiotic stresses (like drought, high temperature) also lead to increase the production of nonprotein amino acid GABA due to higher dehydrin

gene expression [204, 205]. Due to deamination of these amino acids during roasting, ammonia from these amino acids will combine with sugar degradation products that gives pyrazines that are responsible for flavor and aroma of coffee. Thus, the variation in the amount of pyrazines due to variation in the amount of amino acid can interfere the quality attributes of coffee too [203].

Climate change can be defined as any significant change in climate, such as temperature or precipitation that lasts for an extended period of time, typically decades, whether due to natural variability or human activity [206]. So we can say that climate change is anything to do with the changing rate of precipitation, dry spells and season or rise or fall of temperature and humidity, etc. By 2050, globally, rise in temperature (by 2 °C) will result in some increased seasonality of precipitation in different cropping regions which consequently affected the crop quality and yield and may increase the chances of insect pest infestations [207]. Climate change can be resulted in the loss of specialty coffee certifications such as "Denomination of Origin" (DO) certifications as currently coffee suitable areas will not be capable of producing high caliber quality coffee. A detailed direct and indirect influence of climate change is summarized in Table 3. Hike in temperature will consequently rise the soil temperature which in turn results in higher rate of evaporation and OM breakdown leading to poor soil structure and high probability of soil erosion. Erratic rainfall pattern will affect the induction of flowering, number of flowering, fruit set,

Table 3 Direct and Indirect effect of extreme or unusual meteorological events on the coffee Arabica

Hazards	Direct effect on the tree	Indirect effect
Temperature Hazards	$\geq$ **23 °C**: ripening of the fruits increases which leads to progressive quality loss $\geq$ **25 °C:** Reduction in photosynthesis $\geq$ **30 °C:** Depression in growth of tree Extreme changes in temperatures can cause abnormalities in leaf, stem, and flower or even abortion	An increase in the infestation of pests or disease
Heavy rainfall, hailstorm, and winds	Damage to tree, increased fruit falling especially close to harvesting time Soil erosion, landslides, subsidence, wash-away of agrochemical applications	Land sliding, erosion in soil, subsidence and removal of agrochemical applications Damage to infrastructure like road lead to increases in costs
Infrequent and unseasonal rain	Great flowering rate	Possible enhancement of some diseases Drying difficulties during postharvest
Persistent rain	Can reduce flowering effect, setting of fruit, lowering photosynthesis because of reduced access to sunlight	High humidity may favor some fungal diseases and can cause mortality of some pests such as Coffee Berry Borer (CBB)
Drought (prolonged)	Weakening the trees, causes wilting, and increases death of younger plants	Under extreme stress, plants are more susceptible to drought

United Nations Development (UNDP [206])

fruit development, and maturation. Rainfall with storm hailing further can damage the fruit amount and size and can further worsen the drying, processing, reducing quality, and marketability of coffee. Temperature variations can lead to change the insect pest dynamics. Coffee Robusta is more adaptable to climate change, as compared to coffee Arabica, and can bear up to 22–26 °C. However, temperature below 6 °C is considered fatal for Robusta too [208]. Selected cultivars of Arabica also showed reasonable and satisfactory results in terms of yield and quality, but these cultivars are highly susceptible to frost and temperature below 18 °C. Recently, the impact of changing climate on coffee suitability and quality is projected by Läderach et al. [209] by using the two niche models, namely, MaxEnt (Maximum entropy) and CaNaSTA (Crop Nice Selection for Tropical Agriculture) in Nicaragua. MaxEnt can predict the crop suitability while CaNaSTA is specializing for crop performance (i.e., quality) predictions using the limited input data. These models predict up to 90% reduction in the coffee suitability at elevations lower than 800 masl while mediocre elevations will face 20–25% coffee unsuitability [209]. The chosen quality attributes are acidity and flavor, as specialty coffee of this region is famous for its acidity and flavor and also exhibits association with the climate change. By 2050, CaNaSTA predict lowering the suitability of coffee production with high acidity and flavor and lower to mid-elevated area will be the first target of this change. New areas heighted >1500 masl are predicted suitable for future coffee production by CaNaSTA [209]. This climate change can also lead to cropping distribution pattern in Latin America [210], East Africa, [211], and Asia Pacific [212]. Rise in temperature and altered rate of rainfall would be the basic cause of shift of coffee production from lower altitudes to higher altitudes where temperature and rate of precipitation will be bit more suitable for berry maturation and fruit ripening with lower incidence of rust attack [213]. This shift will not only put natural forestation at higher elevation in pressure leading to further climatic changes, but there is also a 30–40% shift in coffee production from one developing country to another affecting socioeconomic condition of that country as all coffee producing countries do not have required higher elevation coffee fields [213]. Owing to climate change, North America and Latin American countries including Brazil can lose the competitiveness of high quality or specialty coffee production while provision of opportunity of high quality coffee production may be granted to Asia Pacific countries. Some authors also claimed climate change a step in providing favorable climate for rearing the coffee berry borer, coffee leaf borer, and other insect pests of coffee offering a serious threat towards the yield and high quality coffee production [214]. Climate change has provided the newly adopted insect pests a new habitat to generate and expected to affect the distribution, demography, and life cycle of many insect pests. Jaramillo et al. [215] predicted that even 1 °C increase can favor the higher development of coffee berry borer (*Hypothenemus hampei*, *H. hampei*) both in altitudinal and latitudinal ranges. The severe case of altitudinal migration, recently, has been seen in Indonesia, Uganda, and Tanzania where *H. hampei* have been observed migrating at the rate of 300 m/decade [216], and if these rates of migration continue, it may take 30 years (by 2050) for *H. hampei* to reach East Africa, the hub of quality Arabica production [217]. The numbers of generations also

increase (1–4.5 to 5–10) as response of raising temperature so partially suitable areas for *H. hampei* may turn into favorable habitat. Encroachment of coffee crop, as a result of climate change and insect pests, to higher elevated areas will not only cause deforestation but also a future threat to sustain the current coffee quality Bunn et al. [178], food security, biodiversity, and carbon (C) [218]. The ecological niche modeling studies for the mapping of new and loosing areas predicted that 83% of future coffee producing area will not be suitable for Arabica coffee to produce coffee with present existing typical cup quality as compared to only 17% incompatible for Robusta. Moreover, Arabica could lose up to 56% existing cultivation area with only a gain of 9% totaling 19.4 million hectares. On the contrary, by 2050, Robusta is expected to get double new cultivable area than the lost [177, 218]. Despite future availability of enough cultivable area for coffee to meet demands, 14–65% of future arable area is currently under forestation and other food crops alarming the food security status. The expansion of coffee tillable land will also cause the expansion of the distribution of coffee insect pests (especially *H. hampei*) from current 50–57% to 77.7–93.02% [218], another indication of quality threat to coffee beans. Further, there are also other conjunctive hurdles in re-colonizing the coffee at higher elevation, such as lack of infrastructure required for transformative agronomic, harvesting, and postharvesting operations, new kind of coffee predators, i.e., coffee leaf rust even now more prevalent in higher elevations too, higher rate of soil erodibility, hike in input cost, unavailability of higher altitudes in some major Arabica coffee producing countries, e.g., Brazil, Ethiopia, Colombia [209]. Several authors stated the adaption and mitigation strategies to cope with the climate change and its impacts on coffee productivity, quality, and socioeconomic conditions. The detail of these adaption and mitigation strategies is listed in the Table 4 (also add crop insurance, build coffee collaboration network, restructuring of coffee chain, financial transfer tools (payments for ecosystem or watershed services) and policy making).

2.6 Sun Versus Shade or Agro-Ecosystems

Coffee Arabica and coffee Robusta accounted for world's 99% coffee production. Both coffee varieties have natural inclination and adaptability to produce superior quality coffee under the shade only as two varieties historically evolved from understory forests of Africa [185]. In Latin America, in 1950s, most of farmers began to produce sun grown coffee, in order to meet the global consumption demand, by substituting the shade with other costly inputs like fertilizers, herbicides, pesticides, large plant spacing, etc. [219]. The outcomes of this trend are taken to be severe as it not only left its toxic effects on soil and water, irrespective of high yield, but also occasionally produced low quality coffee. The choice of sun or shade grown coffee systems (*agro-ecosystems*), with their own pros and cons too, largely depends upon the understanding the prevailing environmental factors (resources, conservation, biodiversity, stability, organic production, byproducts, etc.), framers priorities (yield or quality, etc.), regional policies (eco-friendly coffee production, organic

Table 4 Options and acclimatization to different strategies

Level of acclimatization	acclimatization	Examples
Plant	Strategies to acclimatize plants to climate changes	Introduction of more varieties, i.e., pest- or drought-resistant Pruning Grafting
From field to Farm	Actions required to enhance the endurance of the farm, mainly done by changes in the way farmers manage their production practices	Improvement in pest management system Improvement in soil and water management Modifying fertilization practices Establishing cover crop Mulching Planting trees Establishment of windbreaks Introduction of solar driers Modification of planting dates and driers
Farming system and household practices	Measures to prepare the household against the potential negative effects of drastic climate change	Income diversification (off and on farm) Ease access to financial services Train farmers to apply adaptation strategies Ease framers' access to seasonal forecasts and other meteorological information Motivating men and women to work together to address challenges in better way
Topography and terrain	Actions that enhance the resilience of the coffee farm's landscape	Engage in plantations or re-plantation at massive scale, i.e., afforestation and re-afforestation
Reconstruction of coffee chain	The whole coffee chain should be reconstructed in order to create flexibility in accepting the hybrid-resistant coffee varieties with varying quality features	Drought-, insect-, pests-resistant varieties and caffeine-free varieties
Financial transfer tools	The financial assistance or financial transfer facility must be provided to those farmers involved in environment friendly activities	Payments for ecosystem or watershed services
Collaboration networks	Coffee collaboration networks should be built in order to cope with climate change in mutual way by disseminating the technical knowledge inclusively other measures	Coffee collaboration networks at district level, provincial levels, national levels, and finally at international levels
Crop insurance	Crop insurance should be introduced both by governmental and private sectors	Coffee crops should be insured against any calamity, insect pest attack, and resulting yield quality deterioration

(*continued*)

Table 4 (continued)

Level of acclimatization	acclimatization	Examples
Improving environment/ framework conditions	Measure which creates and enables the operating environment of farmers or increases the framework conditions in which farmers do their business	Improving farmer's organization to facilitate and enhance reach to weather related information and other relevant support services Enhancing access to early warning system, improve local ownership (climate maps, local export committees, adaptations as part of local development strategies, etc.)

coffee, certifications, specialty coffee, etc.), input costs, selling prices, etc. The benefits of shade grown coffee systems are found to be significantly linked with amelioration of other environmental factors like biodiversity, soil and water conservation, optimal temperature, and humidity maintenance, etc. [220]. But we are unlikely to add unnecessary details here about these improvements and the pros and cons of sun versus shade gown coffee systems as it will mask the true objective of this section and additionally literature and coffee productions manuals are full of details on this topic. The species which are chosen for the purpose of shade by farmers largely depend upon their side benefits and ease of management, but up to 60% shade trees belong from leguminous family [221]. Table 5 represents the general criteria for the selection of a tree species for shade purpose. After elevation, shade is considered core element in the bigger size (AA or AB) quality production of green coffee beans due to provision microclimatic conditions of optimum cooler climatic temperature and humidity and eventually longer or delayed ripening procedure [22, 45, 92]. The credit of high quality coffee production by delayed ripening may goes to accumulation of desired components in well-desired ratios with other contents [22, 32], while higher sucrose, trigonelline, and chlorogenic acid contents in sun grown coffee are the symbol of incompletion of these processes in beans due to rapid ripening of flesh of fruit in sunlight [29, 222]. So the conversion of coffee cherries to green beans was significantly greater for shaded coffee than for full sun coffee [210]. Larger leaf area to fruit ratio under shade is also an indicator of better bean filling capacity leading to better cup quality. As in coffee producing and exporting countries, physical quality of berries and green beans really matters as the price and quality of their product depends upon the grade (size) of berries or green beans. Moreover, Van Der Vassen and his colleagues noted that bean size and density is often correlated to aroma, flavor, and superior beverage quality. Even small variations in bean size can affect a number of sensorial attributes, e.g., through different responses to roasting [18]. Yield and quality are the only two tools which determine the extent of profit of framers. Various agricultural management practices such as fertilization, fruit thinning, pollarding, weeding affect both these tools [223]. Agricultural and management practices are considered playing supportive role to

Table 5 Trivial information of tree characteristics conferring suitability as shade species for coffee in Amor de Dios, Chiapas, Mexico

Characteristics	Acceptable	Unacceptable
Tree-crown/branch extension	Open/medium-sized branches	Dense crown/very short or large branches
Moisture maintenance	Yes	Not
Growth	Fast	Slow
Coffee yield (close to the tree)	High	Low
Climate inside the plantation	Fresh	Hot
Litter contribution decomposition rate	High amount/medium rate	Low amount/low or high rate
Height of tree	Manageable	Unmanageable
Size of leaf and distribution	Small and scattered	Very large and dense
Disease and infestation	Low	High
Species and weed control	Important	Not important
Resistance to strong winds	Resistant	Not resistant
Hardness of branch	Nonfragile	Fragile
Strength of root	Strong	Weak
Additional benefits	Given	Not given
Foliage density	Moderate	Excessive
Deciduousness	Perennial	Subdeciduous or deciduous

shade in betterment of the fragrance aroma and flavor of coffee [223]. Leonel and Philippe [28] documented the obvious role of shade on organoleptic quality (body, acidity, flavor, and preference) only with high fertilization levels. Vaast et al. [222] noted that fruit thinning has insignificant relationship with overall yield as both kinds of trees, full loaded and thinned (1/4% to ½%), showed alternate bearing pattern of berries, but thinning to quarter resulted in the most balanced production of berries. Same authors noted that fruit thinning is also responsible for fast ripening of the remaining berries, both in shade and sun, which is detrimental to the cup quality of coffee. Fruit thinning only improves the bean size in the highly productive year, whereas in recessive yield year, beans size did not differ significantly among the fruit thinned and unthinned trees. In comparison to fruit thinned tress, high fruit bearing tress showed a 30% drop in starch (but total soluble sugars remained unaltered), erratic leaf mineral content variations, and high probability of leaf rust occurrences [224]. Shade reduced the difference of productivity between two successive years, whereas full sun grown coffee showed significant variations from one year to next year [22]. Shade also increases the leaf to fruit ratio, but this ratio also pursuits the alternate bearing pattern of productivity each year. Shade also increases the bean size and weight and amends the biochemical composition of beans [225]. Fat and caffeine contents were higher in beans of shade-grown plants, whereas chlorogenic acid, sucrose, and trigonelline contents were higher in beans of sun-grown coffee beans owing to which sun-grown coffee showed more bitterness and astringency as compared to acidic high flavor shade-grown coffee [22, 222]. However, same kinds of results have not been registered for all kind of cultivars of coffee under shade. A

positive relationship between sucrose and shade was detected for Catuai cultivar [45], and negative relationship was detected for Catimor cultivar [222]. So light regime affects the sugar metabolism differently in different cultivars of coffee. The cultivars possessing the introgressed genome from *C. robusta* via the Timor hybrid showed the reduction in sucrose in shade environment as compared to cultivars introgressed from *C. arabica* [222, 226, 227]. The high sucrose content in former mentioned introgressed cultivars is due to enhanced sucrose synthase enzyme activity in mature endosperm because of higher expression of its second gene *CaSUS2* under shade [226]. However, we cannot ignore the variation in response of different cultivars towards different topographic and climatic conditions other than shade [204]. The existing controversies in literature over the other important compositional contents, i.e., fat, trigonelline, chlorogenic acid, etc., of coffee may also be referred towards the same set of reasons as described above. Although, regardless of cultivar type, light regime significantly affects the reducing sugars and sucrose of green beans, total sugar contents of coffee beans remains unaltered [226]. The sugar or carbohydrate state varies with the development stage of coffee seeds where high to low ratios of reducing sugars/sugars was detected from immature to mature stages. The larger beans size of shade grown coffee is also credited to the maintenance of high reducing sugar/sugar ratio in perisperm. The increased size in perisperm and endosperm may be due to high cell divisions or increased cell size or both of them and may need further histological studies to confirm this. Further, besides studying the main compositional components, variations of other components, e.g., amino acids, polypeptides, tannins, phenolics, minerals, also needed to get a comprehensive view. All beneficial effects of shade are also site-specific and under influence of changing environmental gradient. From lower to mediocre elevated areas, where the temperature is high, shade influences and improves both the physical and sensorial quality of coffee, but at higher altitudes, only sensorial quality is significantly under influence of shade and physical quality (i.e., bean size) is enormously under the influence of temperature and altitude [18, 30, 31]. Bosselmann et al. [18] did not detect a significant difference of bean size between shade- and sun-grown coffee beans at higher altitudes except an insignificant tendency of shade towards a size increment. On the contrary, at high altitudes, shade has a destructive relationship with body, fragrance, acidity, sweetness, and preferences [18, 45], and sunlight regime improves the body, aroma, sweetness, and other sensorial attributes of coffee [18, 30, 31]. The significant negative effect of shade at higher altitude may be attributed towards lowering of temperature below the lower side of threshold level of optimum temperature range (i.e., 18 °C) [228, 229], as temperature is already in optimum range at higher altitudes and sun regime tends to produce superior quality coffee beans while shading at this elevation can lead to drop in temperature [18]. Moreover, in addition to decreased temperatures, shade also influences the number of beans in each plant. Floral initiation is light dependent and fewer flowers are developed in shade, allowing more assimilates for each individual bean in the plant [57]. Overall, the shade or agroforestry system is basically chosen for the marginal coffee production areas to protect the coffee crop from adverse climatic condition. The level of shading, in these areas, should neither

be excessive nor too low for protection and production of coffee [230]. Finally, the major influence of agroforestry system on coffee physiology is lined with limit temperature fluctuations (by as much 4–5 °C), maintaining required relative humidity, and decreases the wind speed and aerodynamic roughness of the cropped area.

Shade and yield also have controversial relationship in literature as some authors [22, 222, 231] claimed a negative while others claimed an insignificant relationship [210, 232, 233]. This controversy is likely due to the difference in shade type and extent, coffee varieties, vegetative stage, ecological conditions, water and nutrients availability, etc. [210]. The other possibilities of low yield under shade are designated to (a) less response of coffee trees to available input or due to competition, (b) lower level of carbon assimilation, (c) greater inducement to somatic growth than flower buds, and (d) fewer nodes per branch and flower buds at existing nodes [22, 228, 229]. However, Haggar and his working team showed that comparable yield to full sun-grown coffee can be achieved via intensive organic system, i.e., by application of fertilizer and other inputs to shade-grown coffee. Shading effect was found to be unobvious during the early vegetative growth stages and after drastic pruning till high yielding stages [231]. Despite intensive work on shade or agro-ecosystems in coffee, nobody still registers the mathematical relationship of yield versus sun-grown coffee or yield versus shade or shade versus increased bean size. For all additional aspects of shade other than physical cup quality of coffee, the readers are referred to study the already available reviews on shade and coffee such as Beer [234], Beer et al. [233], and DaMatta et al. [235].

2.7 Insect Pests and Diseases

As a perennial crop, coffee plants (both Arabica and Robusta) are considered an attraction for a wide range of insects, pests, and diseases (IPDs). These IPDs negatively affect the plant, plant health, plant nutrition, plant growth and development, yield, and physical and chemical quality of product. But in this section we would like to solely relate the IPDs occurrences and resulting deteriorating physical and chemical quality of product. This section will not cover the origin, life cycle, epidemiology, etc., of IPDs; however, a little detail, where necessary, about agricultural or management practices may be mentioned surfacely for the cause of preserving physical and chemical quality of cherries. Most of the occurrence and intensity of IPDs largely depends upon the kind of coffee production system [236]. Highly diverse coffee-based agroforestry system is the habitat for many plant friendly birds and ants important in bio-control of coffee berry borer (*Hypothenemus hampei*) [237–239]. Additionally, shade prevents the various IPDs like coffee berry disease (*Colletotrichum kahawae*), coffee blight (*Phoma costarricencis*), coffee brown eye spot disease (*Cercospora coffeicola*), coffee rust, and die-back from becoming epidemic by providing and modification of microclimatic conditions and host physiology changes [228, 236, 240, 241]. On the opposite side, diverse agro-ecosystems may play a conductive role in the eruption of American leaf spot disease (*Mycena citricolor*), white thread blight (*Corticium koleroga*), pink disease

(*Erythricium salmonicolor*), and infection diseases [242–244]. Most of coffee IPDs epidemics are site specific and erratic climatic conditions favor the eruption of IPDs to some extent. Briefly structural factors such as susceptibility, aging, and other one-off factors like erratic meteorological variances and socioeconomic conditions related to farm managements are considered the important drivers for the coffee IPDs epidemics [242]. Most of coffee IPDs deteriorate the quality and yield of coffee plant by attacking or affecting the flowers, fruits, foliage, transport system, or organ system of plant. Each part of plant has its own significance in healthy and quality berries and beans production. Flower IPDs (e.g., antestia bug) damage the flower and flower buds turning them into brown or black entity which finally fall off [245]. From small green berry stage to mummified berries, various berry IPDs (Antestia bug and coffee berry borer) strike causing a 9–60% drop in yield and darkening of beans [246]. Berry pulp is an excellent source of sugars for most of berry pests and fungi which can consume that sugar rich pulp and produce alcohols and acid that not only can percolate into the coffee beans but also affect macro- and micronutrients transportation between pulp and beans and hence alter biochemical composition of beans [247]. Leaves or foliage are the food or energy hub of whole plant from where not only each component of coffee beans but also "food" for whole plant is being transported. Any IPDs targeting foliage will, definitely, affect the developing and maturing berries and beans in terms of their size, appearance, weight, color, and compositional contents. It is also generally assumed that 1% damage of foliage can result in 1% reduction in yield. Organ or transport system of plant also has primary importance as roots absorb the required nutrients from soil and stem and branches are the prime in transporting the nutrients and food. Any outbreak of root or stem IPDs will distort the supply of nutrients and food towards leaves and berries, respectively, and consequently compromising the yield and quality of fruit. Here now we are describing the IPDs impacting on these three plant systems. Ants, mites, grasshoppers, locusts, crickets, termites, aphids, scale insects, mealy bugs (root, sucking, leave, capsid bugs), scale insects (star or fringed), thrips, beetles, weevils, borers (leaf, twig, stem, berry borers), fruit flies, chafers, leaf miners, moths, leaf eating caterpillars, Skeletonizer, loopers, etc., are the major described IPDs of coffee in literature [248]. Grasshoppers (*Zonocerus variegatus* and *Zonocerus variegatus*), locusts (*Phymateus viridipes*), aphids (*Toxoptera aurantii*), and crickets (*Gryllus bimaculatus*) are not considered as "pests" of coffee now, but their large populations can damage the branches of plant bearing flowers or fruits [60, 249, 250]. The honeydew from aphids is used as a food source for other pests, which can cause fungal epidemics. The scale or wingless insects (i.e., coccus spp. and *Asterolecanium coffeae*), ants (*Atopomyrmex nzocquerysi*), and mealy bugs (i.e., *Planococcus kenyae*) suck the sap from the plant tissues and thus disturb the supply of sugars and nutrients to vegetative and reproductive parts of plants [251] due to which plant growth may be affected and coffee beans may be undersized. The honeydew of scale mealy bug insects is also used by sooty fungus (Leaf Rust *Hemileia* and *Diacanthodes* sp.) which lowers the whole plant photosynthesis rate by making a dusty layer at surface of leaves. This reduction in photosynthesis rate may lead to lower supply of carbohydrates and nutrients to coffee cherries and consequently

abnormal sized coffee cherry production. The scale insects, bugs (Pentatomid and Mirid), and thrips are more dangerous at small green berry stage as these insects assault on green berries causing berry destruction and drop-off [248]. Ants are nectar thieves and flower predators, lessen the pollen viability via antibiotic secretions, and are also less efficient pollinators as compared to winged insects, but ants may amplify the pollination by attacking the pollinators which can increase their movement and hence pollen transfer rate among flowers may rise [252]. Sucking bugs (*Antestiopsis* spp., *Lamprocapsidea cofeuei*, and *Habrochila* spp.) are considered more serious in lowering the physical quality of bean in Africa. These bugs, as name indicated, also suck from growing tips and flower buds leaving blackened flower buds or drop the immature berries and rotten the beans. Sucking bugs not only destructively target the physical quality of beans but also inject the toxic saliva and fungal spores while sucking sap and making the berries shrink [245]. This fungus usually transmitted from infected plant to healthy plant and also caused the taste defect in coffee after processing [253]. Termites (i.e., *Nasutitermes Termitidae* and *Macroterrne Termitidae*) are beneficial in softening the soil and fastening the water infiltration, but as termites feed on dead bark tissues so termites can lead to death of weak coffee tress [248]. Coffee thrips (i.e., *Diarthrothrips coffeae*) are no longer considered as serious pests; however, irrespective of minor pests, their rare epidemic eruption is also reported in literature [248]. Beetles or weevils (i.e., Systates spp.), Coffee Leaf Skeletonizer (*Leucoplema dohertyi*), and caterpillars (i.e., *Parasa vivida*) eat on green leaves (some leaving only veins and compartmented the leaves), while chafers (i.e., *Pseudotrochalus* spp.) strike on and eat roots and other vegetative parts of tree. Eating of leaves and roots can seriously affect the fruit filling, development, size, and biochemical composition of beans [254]. Larvae of coffee leaf miners (i.e., *Leucoptera caffeina*) produced blotch like brown lines on upper and lower side of leaves causing the premature death of leaves while carrying the full fruit load and ultimately death of stem tip called overbearing die back [251, 255]. This, consequently, also destructively affects the physical and cup quality of coffee. When the berries are fully ripened, matured, and stable, no insect can attack them except coffee fruit flies (*Trirhithrum coffeae Bezzi*) which lay their eggs in the berry pulp and consumption of the berry pulp and nutritional material affect the beans' nutritional composition deteriorating the cup quality during brewing [256]. After sucking bugs, borers are considered more fatal pests of coffee. These insects are named after their target sight of boring or appearance, i.e., twig borers (*Xylosandrus compactus*) bore in twigs, stem borers (Ancylonotus *tribulus*) bore in stem, berry borers (*Hypothenemus hampei*) bore in berries, white borer (*Monochamus leuconotus*), black head borer (*Apate monachus*), etc. [257, 258]. Borers generally tunnel the targeting site, make wide and long galleries, and laid the eggs on bark or fruiting branches. The larvae can reach the heartwood and root that leads to death of whole or affected part. The berries affected by berry borer are usually light in weight and undersized and not only fetch the fewer prices but also contaminate the whole harvesting lots [251, 258–260]. The epidemic form of Coffee Berry Moth (i.e., *Prophantis smaragdina*) was observed in intensive coffee production systems where its larvae feed on flowers and hole the berries. The larvae move from one

berry to another in a cluster leaving behind brown, hollow berries and beans. As compared to Coffee Berry Moth, the larvae of Coffee Berry Butterfly (*Deudorix lorisona*) make a single hole in berries and eat the beans [248].

Nematodes are the Arabica coffee loving parasites since most of the Robusta varieties are resistant to some extent. Even in case of nematodes attack, the symptoms are not enormous and yield and quality of Robusta coffee are not compromised [59, 60]. Nematodes (Root Lesion Nematodes: *Pratylenchus* spp. and Root-knot Nematodes *Meloidogyne* spp.) generally dwell in soil or roots of plant, suck the sap, cause lesions or open wounds providing sites for infection by fungus and bacteria, weaken the plant, prevent the nutrient absorption even in fertilization application, and fallen the yield and quality of beans [261, 262]. There are also reports of obliteration of feeder roots and necrosis [248]. The reduced supply of nutrients leads to chlorosis and necrosis of leaf tips and stems and up to 40% reduction in yield [248]. Bertrand et al. [70] demonstrated that interspecific grafting of *C. arabica* with rootstock from *C. robusta* is a significant approach to protect the tree roots from root knot nematodes with lowering the physical and cup quality of coffee. Further, the invasion of fungi (i.e., *Fusarium* spp., *Meloidogyne* spp., *Rhizoctonia* spp., and *Phytophthora* spp., etc.) results in withering and finally death of plant [263, 264]. In fungal diseases, the most common fungal diseases in coffee plantations and fruits are coffee berry diseases (CBD), coffee wilt disease (CWD), coffee leaf rust (CLR), grey leaf rust (GLR), root rot disease (RRD), Phtiriosis, coffee canker, American leaf spot (ALS), and Brown eye spot (BES) [248, 251]. All the fungal diseases result in the foliage loss, blockage of sap supply, ratting of fruits, etc., but the mode of action of each fungi and periodic symptoms may slightly vary as follows. CBD (*Colletotrichum kahawae*) is characterized by color spots on berries. The lighter spots or scabs are not taken as serious, while darker spots or wounds are considered as serious disease. The fungi feed on the pulp leaving behind empty, dry, wrinkled pouches which usually fall off. CBD can attack on all mature and green berries without any visual impact on leaves or branches [265, 266]. RRD is caused by any of these fungi, i.e., *Rosellinia bunodes, Rosellinia pepo, Phellinus lamoensis, Leptopows lignosus,* etc., and involves rotting of roots and leaves, withering of foliage, decaying of branches, and loss of yield and quality [267]. In CWD (causal organisms: *Fusarium xylarioides, Gibberella xylarioides,* or *Carbuncularia xylarioides*), fungal mycelia occupy the sap vessels thus blackening them causing yellowing and drying of leaves which finally fall and tree die. Berries of affected trees turn red and appear to ripen prematurely. Dark brown-black necrosis of younger branches may be pronounced and laterally restricted, while a similar necrosis may also be apparent along leaf veins [268]. Coffee canker also causes the withering of plant, but in this the causal fungi (*Ceratocystis fimbriata*) decay the woody tissues of plant [269]. CLR (*Hemileia vastatrix*) is the cause of physiological activity loss in affected plants leading to withering and loss of survival. Brown lesions first appear on the downside of leaves through which fungi attack and penetrate, producing orange color under-spore at later stage, causing the lesions to become necrotic [242]. CLR attacks are more austere on high yielding plant, and in most of the cases, the attacks are undetected due to disease and plant interaction

[236, 270]. As compared to CLR, GLR (*Hemileia coffeicola*) is less epidemic and involves the disruption of photosynthesis and phosphorus storage also causing withering of plant [269]. ALS (*Mycena citricolor and Omphalia pavida*) is considered as serious problem in high quality coffee producing areas of altitude 1100–1500 m with high humidity in Central America. This disease is known to occur on all parts of tree including berries except roots. Numerous round light brown lesions with 5–10 mm diameter hole in leaf and no surrounding chlorosis appeared that also lead to withering of plant and falling of fruit [243]. ALS and BES can also reduce the productivity up to 35% due to infection and falling of fruits [248]. Coffee Ringspot Virus is the only viral disease causing necrotic ring spot and paling along the leaves. This virus completely destructs the physical quality of berries by making a depression [271].

There are only two ways to control/mange the coffee IPDs epidemics: (a) preventive managements and (2) supportive managements. Preventive managements include every activity leading to the prohibition of occurrence of coffee IPDs like avoiding the coffee cultivations at those areas where climatic changes are more erratic or where the climatic conditions support the coffee IPDs epidemics. Preventive managements also include the breeding and introduction of durable IPDs-resistant varieties in coffee IPDs epidemic areas [242]. The supportive management includes action after the occurrence of coffee IPDs but before reaching their epidemic stage. These supportive managements include developing the ecological control of coffee IPDs. The short time ecological controls may apply the chemical fungicides, but these short term measures must be complemented with long term measures like enhancing the multiple ecological control mechanisms by developing new best practices, new best shade systems, bio-fungicides, cheap biological control methods, etc. The supportive measures also involve improving the socioeconomic condition of farmer for better agriculture practices, academia-farmer linkage, technology transfer and improved, updated and regular extension services.

2.8 Harvesting

Coffee harvesting is laborious and challenging activity in green coffee production and processing. Generally, the widely used two methods for coffee harvesting are (i) selective picking and (ii) strip picking [272]. Selective picking is an arduous hand picking process in which only matured cherries are harvested individually for further processing, whereas strip picking is the machine or harvester aided activity in which all sort of/entire crop (fully matured, matured or under ripe) cherries are harvested/picked. Selective picking is highly desirable but time intensive and costly method for fine quality Arabica bean production of high quality green coffee. Contrarily, mechanical picking method is more cost effective and suitable method to those areas (e.g., Brazil) where cherries get mature uniformly [272]. For selective picking method, the extent of picking times depends upon species, farm, tree, and agro-ecological conditions. In this method, normally three to seven pickings are required for coffee Arabica. Harvesting at maturation stage is the most crucial step

influencing the final cup quality. Early picking of matured Arabica red cherries gives the best physical and biochemical cup quality of coffee [38]. Low contents of caffeine were also found in overripe and under-ripe cherries [273]. The selective picking cherries, at fully mature stage, are used for wet processing, while cherries with varying maturations stages are recommended for further drying process [274]. Harvesting methods directly or indirectly influence the dry bean size, weight, color, primary and secondary defects (full black, fungus attacked, insect damaged, and foreign matter, etc.), odor, and roast and cup quality. Selective picking at maturation and further appropriate postharvest treatments gives the best coffee beans size [38]. Strip picking and other poor harvesting practices may result in reduction of bean quality and uneven bean distribution. Uniform bean size is not only a criterion for fetching high price in international market [275], but also uniform beans give uniform roast free of burnt, over-roasted or under roasted roast. Coffee from selective harvesting have superior quality (Grade 2) considered equivalent to specialty coffee, while strip harvesting usually gives lower (grade 5) quality coffee. Selective picking also gives uniform beans weight and color as mature cherries have 20% more weight than under-mature cherries [89]. Inclusion of under-ripen or overripen cherries not only gives varying weight beans but also nonuniform/uneven colored roast [276]. Primary and secondary defects cause off-flavor in coffee and least average number of cherries with primary and secondary defects was found in washed coffee from selective harvesting. This may be due to the fact that strip harvesting may harvest the broken, faded, foxy, insect pest damaged, and fungal-infected cherries. Similarly properly harvested and postharvest managed cherries tend to produce better aroma and odor [273, 276]. Selective harvested coffee beans are also resistant to roast volume change and roasted bean weight loss, whereas highest roast volume change and roasted bean weight loss was observed for strip harvesting coffee. The reason behind this is the nonuniform cherry size due to which there are more chances of moisture and internal composition losses, volatile compound losses, and gas expansions [5, 277]. High acidity coffee is considered as premium and fetch premium. High acidic coffee has a sharp, pleasing, snappy flavor, which gave more intense aroma and better quality to the resultant beverage [24]. Selective harvesting and further postharvest processing via wet processing produces such kind of high quality acidic coffee. Breaking of mucilage layer, covering the parchment, in fermentation process, while wet processing, may be the other reason of high quality acidic coffee. It is also reported that selective picking with wet processing gives good cup quality features such as acidity, body, and flavor. High cup quality in terms of flavor for selective picking may be due to the fact that mature cherries possess highest amount of sucrose which contributes to the higher brix of coffee with other optimum taste attributes [89]. The choice of harvesting method depends upon the way of processing after harvesting (dry, wet, or semi washed), availability of skilled labor and expertise, climatic and seasonal conditions, marketability, profitability, variety of coffee, local policies, and farmer socio-economic condition. Normally selective picking is the choice, where wet or semiwet processing is adopted, to maximize the percentage of mature cherries. The choice of harvesting method is also not made

rationally and may depend upon long instituted practices and traditions. Countries of Arabica naturals use strip harvesting, whereas countries of washed coffee used selective hand picking. During last three decades, large wheel- or tractor-driven harvesters have been used for strip harvesting with a potential damage to plant. The self-propelled harvesters also have inbuilt winnowing system to simultaneously pick the cherries from ground and removing the dust, dirt, leave, sticks, sand, and other foreign material. However, growers must be vigilant to cope with drawbacks of mechanical and strip harvesting, e.g., capability of separation of immature or damaged cherries from fresh, mature, and fully ripen cherries.

3 Future Prospective

Coffee quality is a multidimensional and complex trait and, collectively, all agriculture factors significantly influence in shaping both the physical and final cup quality attributes of coffee. However, despite intensive research during past decades in this vast area, there are still some gaps or bottlenecks that need to be fill or address. Substantial research work still needs to be carried out in establishment of detailed relationship of altitude, latitude, and steepness of slope not only with all final good cup quality attributes and volatile and nonvolatile biochemical components of coffee but also with other agriculture factors which are allegedly being dictated by these geographical topographies. Metabolite profiling or fingerprinting of coffee varieties with respect to varying geographical topographies would be highly appreciable in selecting the right kind of variety for specific regions. Diversifying the limited genetic pool of coffee Arabica will also help to develop the coffee varieties with desirable organoleptic traits. Climate change is major threat to specifically existing coffee Arabica cultivars. Any transgenic coffee program to make coffee Arabica cope with climate changes without compromising the physical and cup quality attributes could be an initiative. However, any such coffee gene transgenic technology should be devoid of antibiotic marker genes. Elucidation of metabolic pathways of both key volatile and nonvolatile coffee components in relation with altitude, latitude, and coffee fruit maturation stages can also be future research topic. However, this perspective research should also focus on identifying the role of enzymes and genes in different maturation stages and geographical localities. Moreover, the great deal of "omics study" approach is inevitable in concluding (i) the storage studies of coffee seeds under various storage strategies, (ii) getting insight into symbiotic relationship of coffee plant with VAMF, and (iii) coffee fruit development and berry maturation investigation under various environmental factors (e.g., under or high fertilization, mulching, integrated fertilization system, drought or raining or irrigation, sun and agro-ecosystems, frost, IPDs). A mathematical model study program also needs to statistically measure and unlock the relation of sun and shade ecosystems with physical and biochemical attributes of coffee. The short time ecological controls of coffee IPDs can be replaced by research and development of multiple ecological control mechanisms, developing new best practices, new best shade systems, bio-pesticides, cheap biological control methods, etc.

4 Conclusion

In conclusion, numerous agricultural, environmental, and harvesting variables influence the physical and organoleptic attributes of coffee. Geographical topographic factors are important for obtaining the specialty coffees, but these factors are not the only factors in determining the final physical and cup quality attributes of coffee. Different (transgenic)coffee verities give different specialty coffees on same locality with their own set of inherent quality characteristics and biochemical compositions which definitely signify the importance of coffee varieties. Sustainable and balanced supply of macronutrient is important not only to make sure the availability of micronutrients but also for desired bumper yield, early development, and maturation of coffee cherries, fruit and flowering setting, vegetative growth, fruit/leave ratio, coffee berry size(grade) and its biochemical composition, immunity against coffee IPDs, soil conservation, and abundance and diversity of VAMF. The pattern and extent of irrigation, rainfall and temperature, or solar radiation influence the nutrient absorption and hence the mass and volatile and nonvolatile compounds composition of coffee seeds by effecting the various physiological and enzymatic reactions responsible for the formation of these volatiles and nonvolatiles. The development of integrated soil fertility approach with recommended figures of mulching, shade (agro-ecosystem) irrigation, and harvesting could be an promising initiative in creating the microclimatic conditions suitable for getting coffee(beans) with desired physical and sensorial scores.

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Part V

Secondary Metabolites in Insect–Plant Interactions

Diversity of Floral Glands and Their Secretions in Pollinator Attraction

28

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Abstract

Floral glands that produce substances related to the attraction and reward of pollinators are crucial for the reproductive success of angiosperms. These structures may include nectaries, osmophores, elaiophores, and resin glands and are quite diverse in flowering plants. This chapter presents the diversity of morphologies and substances produced by the floral glands and how they improve the pollinator's attraction. We also describe how some angiosperms and floral visitors may have coevolved leading to specific pollination systems in some groups of plants. The integration of morphological, chemical, and ecological studies allows for a better understanding of the relationships that evolved between flowers and pollinators along their evolutionary histories. These comprehensive approaches provide opportunities to dissect the evolution of secondary metabolites produced by specialized secretory structures in flowers, including the origin and subsequent modification of these glands and their produced compounds.

Keywords

Elaiophores · Flowering plants · Nectaries · Osmophores · Pollination · Resin glands · Secretory structures

List of Abbreviations

CRC	Crabs claw
ER	Endoplasmic reticulum
TS	Transversal section

1 Introduction

The majority of flowering plants rely on animals to transfer pollen grains from one flower to another enabling successful reproduction [1, 2]. Pollinators visit the flowers searching for resources used as nutrients, for breeding, or for the construction of nests. Therefore, flowering plants have developed different strategies for attracting pollinators, such as a variety of colors, shapes, and/or the production of resources by specialized structures [3, 4].

The main specialized structures present in flowers are glands (secretory structures), which produce attractants or resources for the visitors. The relationship

between the flowers and the pollinators is mostly mutualistic, where the flowers offer the resource and the pollinators guarantee the transfer of pollen grains to the stigmas. Thus, the substances produced by these secretory structures may play a central role in pollination. Flowers that do not offer any kind of resource are not dependent on animals for their reproduction and, in most cases, are wind or water pollinated. Such abiotic pollination occurs in only 30% of flowering plants [5]. Therefore, knowledge about floral secretory structures is fundamental for understanding how the relationships between pollinators and flowers have evolved.

The main resource offered by flowers to their pollinators is pollen, but additional resources, such as nectar, oils, perfumes, and resins, are often present, which may also act as attractants [4, 6, 7]. The secretory structures responsible for the production of these substances include nectaries, osmophores, elaiophores, and resin glands [8–13]. The purpose of this chapter is to describe these floral glands that serve in support of pollination and the diversity of compounds that they produce and to discuss some of the involved functional and evolutionary aspects.

2 Floral Nectaries

2.1 Nectary Structure and Nectar Production

Floral nectaries are glands responsible for the production of nectar, which is the main food resource offered to potential pollinators [10, 14]. They may vary in location and morphology but are similar regarding their anatomical structure [10, 11, 15]. Floral nectaries generally comprise an epidermis, a nectary parenchyma, and a subnectary parenchyma (Fig. 1a–c).

The epidermis may be composed of elongated secretory cells in palisades or of small nonsecretory cells. When cells are nonsecretory, stomata are often present, which is a very common path for the release of nectar [10, 11, 14–16] (Fig. 1d). Stomata involved in nectar release have been called nectarostomata and are characterized by having lost the capacity to regulate their aperture [17–20]. Their position on the nectary may be homogeneous, occupying the entire gland, or the stomata may also be restricted to some special locations (e.g., on the top surface of the nectariferous disk in *Spondias*, Anacardiaceae) [21]. In cases where the epidermis is secretory (or nectariferous) or does not display stomata, the nectar is released through the cell wall and cuticle [22–24]. However, the release of nectar through stomata and cuticle may take place simultaneously [10, 11, 22].

Different families of plants, such as Anacardiaceae, Bignoniaceae, Caprifoliaceae, Fabaceae, Malvaceae, and Orchidaceae, have characteristic receptacular nectaries, but in some species they are completely absent or replaced by secretory trichomes (e.g., *Anacardium* of Anacardiaceae, *Adenocalymma* of Bignoniaceae) (Fig. 2a–c) [10, 21, 25–29]. A unique characteristic of nectariferous trichomes is the presence of transfer cells, characterized by a thicker anticlinal wall with irregular ingrowths, which are important to the facilitation of transmembrane flow of solutes [28, 30].

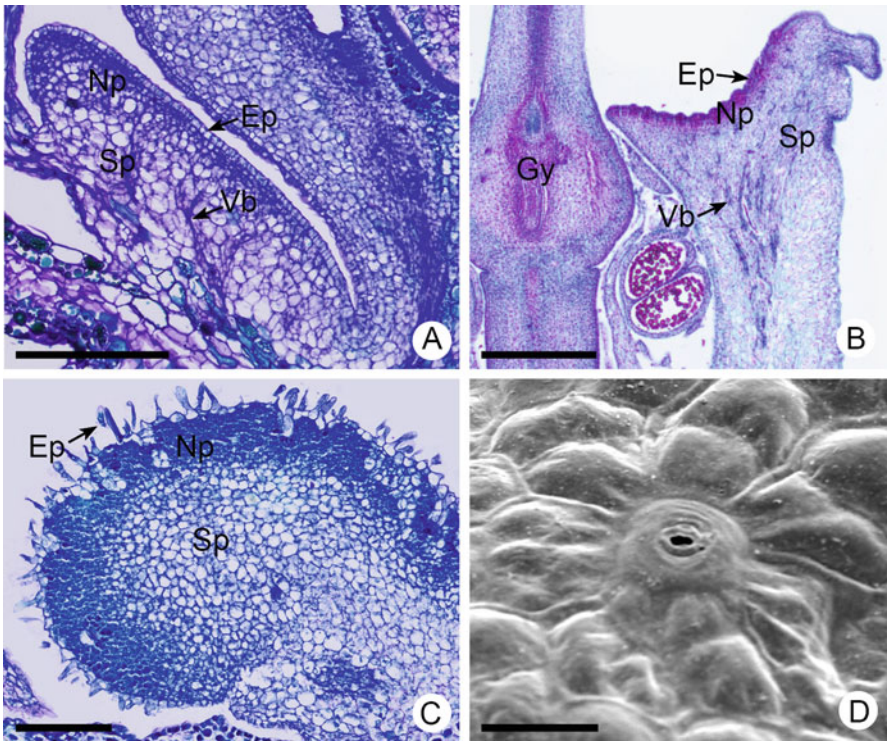

Fig. 1 Structure of floral nectaries. (**a**) *Schinus molle* (Anacardiaceae): receptacular nectary with uniseriate epidermis, nectary and subnectary parenchyma, and vascular bundles in the subnectary parenchyma. (**b**) *Euphorbia sipolisii* (Euphorbiaceae): nectary consisting of epidermis and nectary and subnectary parenchymas. (**c**) *Spondias tuberosa* (Anacardiaceae): the nectary consists of a papillose epidermis; vascular bundles in the parenchyma are absent. (**d**) Stomata on the nectary surface of *Schinus molle* (Anacardiaceae). *Ep* epidermis, *Gy* gynoecium, *Np* nectary parenchyma, *Sp* subnectary parenchyma, *Vb* vascular bundles. Bars: (**a**, **c**) 100 μm, (**b**) 500 μm, (**d**) 10 μm. (Photos: (**a**, **c**, **d**) Elisabeth D. Tölke, (**b**) Karina B. Gagliardi)

The nectary parenchyma has typical features of secretory tissues, such as small cells with a dense cytoplasm and large nuclei, presence of small vacuoles, and thin cell walls (Fig. 1a–c) [10, 11, 15]. Starch grains are quite common in this tissue and are related to the production of sugars that eventually compose the nectar and/or provide the energy necessary for the secretory process [16, 20, 31–34]. Starch grains are often numerous before anthesis and rare after anthesis, indicating their consumption during the secretory process [21, 35–41]. Druses have often been found in floral nectaries, more likely related to the transport of sugars through the inhibition of ATPase but also to the formation of thin cell walls in the nectary parenchyma due calcium sequestration and herbivory deterrence [22, 42–46].

The subnectary parenchyma is nonsecretory and has larger cells compared to those of the nectary parenchyma (Fig. 1a–c), bigger vacuoles, intercellular spaces,

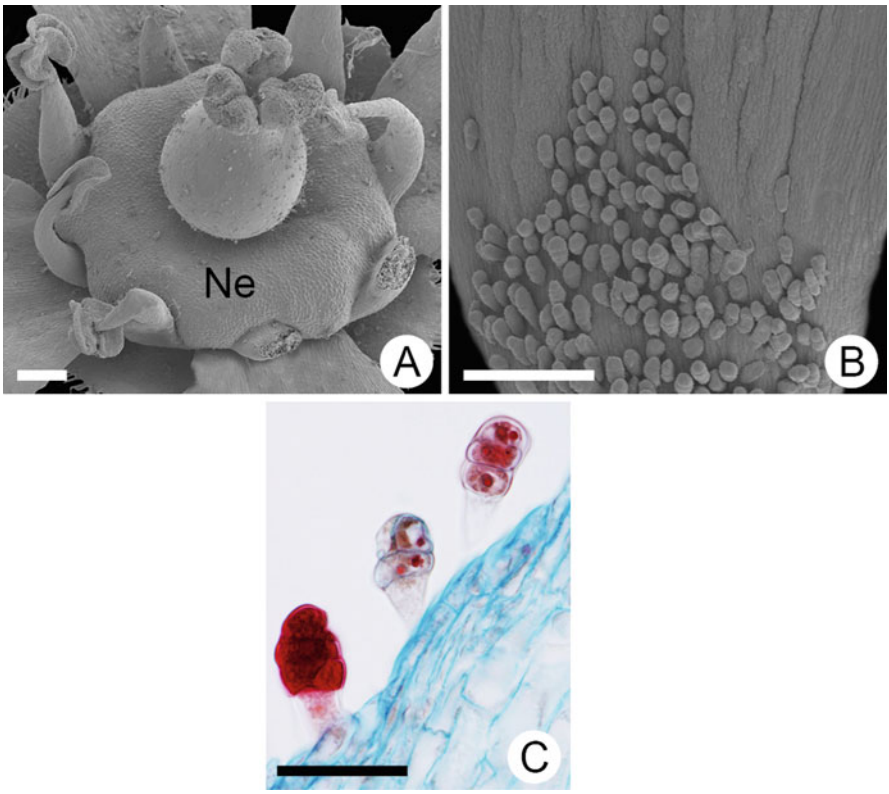

Fig. 2 Structure of floral nectaries. (**a**) Receptacular nectary in *Schinus latifolia* (Anacardiaceae). (**b**, **c**) Nectariferous trichomes at the base of petals of *Anacardium humile* (Anacardiaceae). *Ne* nectary. Bars: (**a**) 200 μm, (**b**) 100 μm, (**c**) 50 μm. (Photos: Elisabeth D. Tölke)

and less dense cytoplasm [10, 11, 14–16]. Vascular bundles are usually present in the subnectary parenchyma (Fig. 1a and b) and may contain phloem or xylem or both [14–16]. The vascular supply in some cases is correlated with the sugar concentration in the nectar. Nectaries that secrete very concentrated nectar are vascularized by phloem only, while low sugar concentrations occur in nectaries vascularized equally by xylem and phloem or only by xylem [25, 37, 47–49]. The vascular branches may reach the nectary parenchyma and sometimes even the epidermis, but generally just the phloem elements are found among these tissues [16, 44, 50].

The ultrastructure of nectariferous tissues is quite similar in species from different lineages of plants and have numerous vacuoles that increase their size along the secretory process, dense cytoplasm rich in ribosomes, highly developed rough endoplasmic reticulum, active dictyosomes, numerous mitochondria, and plastids sometimes containing large starch granules (Fig. 3a–d) [10, 11, 15]. The vacuoles are small and numerous in early phases of the secretory process in the course of which

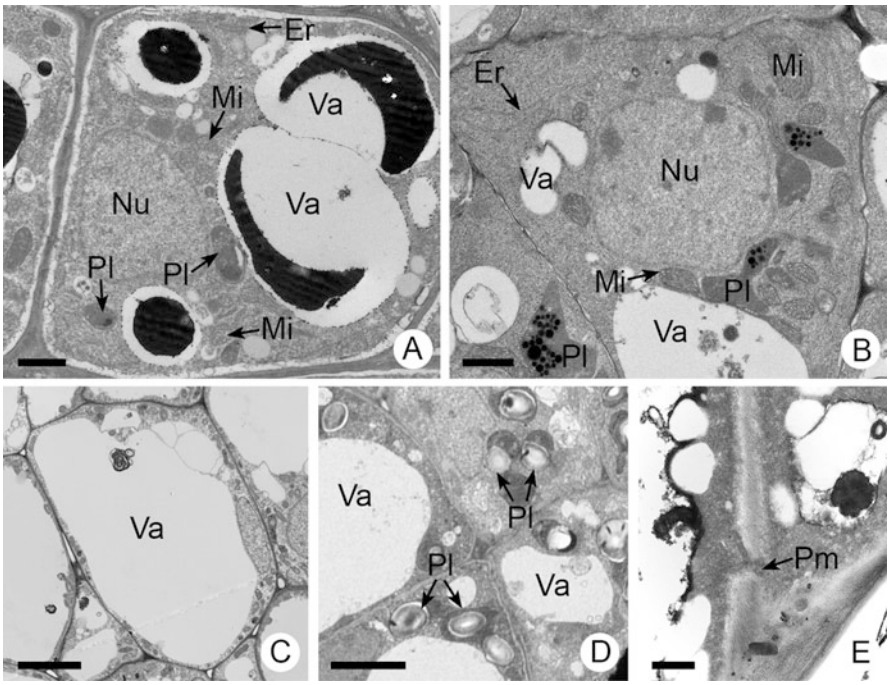

Fig. 3 Ultrastructure of nectaries. (**a**) Trichomatous nectariferous cell of *Anacardium humile* (Anacardiaceae) showing vacuoles of different sizes and dense cytoplasm containing rough endoplasmic reticulum, mitochondria, and plastids. (**b**) Nectary parenchyma cell of *Spondias dulcis* (Anacardiaceae) with similar organization as in *A. humile*. (**c**) Nectary parenchyma cell of *Schinus molle* (Anacardiaceae) containing a single huge vacuole that occupies almost the entire cell lumen, forcing the organelles into the periphery. (**d**) Plastids containing large starch grains in the nectary parenchyma of *Tapirira guianensis* (Anacardiaceae). (**e**) Plasmodesma in the nectary of *S. molle*. *ER* endoplasmic reticulum, *Mi* mitochondria, *Nu* nucleus, *Pl* plastid, *Pm* plasmodesma, *Va* vacuole. Bars: (**a**, **b**, **d**) 1 μm, (**c**) 5 μm, (**e**) 500 nm. (Photos: Elisabeth D. Tölke)

they merge with each other. Thus, there are only a few huge vacuoles in the protoplast at the final stages of secretion, sometimes reduced to a single one that nearly fills up the entire cell lumen (Fig. 3c) [10, 11, 15]. Some slight variations observed in the ultrastructure of these cells (e.g., presence or absence of amyloplasts, number of mitochondria, number and appearance of vesicles) are mainly related to the mechanism of transport of the sugars that eventually compose (*i*) the nectar, (*ii*) the secretory process, and (*iii*) the source of nectar carbohydrates [10, 11, 16, 23, 24, 51].

Sugars may be transported through the apoplast or symplast, and the two processes may possibly take place simultaneously [10, 11, 16, 22, 44, 47, 50, 52, 53]. Recently, a new model of transport of nectar was proposed, in which the final nectar moves by a pressure-driven mass flow in the nectary apoplast while sugars that become part of the nectar diffuse from the sieve tubes through the symplast to the secretory cells, where nectar is formed and sugars cross the plasma membrane

by active transport [51]. According to this model, there is no combination of the apoplast and symplast mechanisms. Current evidence suggests, however, that pre-nectar is mainly transported through symplast via plasmodesmata into vesicles where its composition changes to then be released by exocytosis (Fig. 4) [11, 54, 55]. Indeed, plasmodesmata (Fig. 3e) have been shown to occur in great quantity, this being evidence for symplast transport of sugars [10, 11, 16, 22]. Nectar secretion by nectariferous trichomes excludes an apoplastic transport of nectar due to the presence of barriers in the external cell walls in the stalk and intermediate cells of the trichomes [55].

Nectariferous cells remain intact after the release of nectar, the latter taking place via two different mechanisms – granulocrine or eccrine secretion [10, 11, 15, 16, 23, 24, 56]. In granulocrine secretion, molecules are grouped and transported in ER- or dictyosome-derived vesicles that fuse with the plasmalemma and release the molecules to the outside of the protoplast, while eccrine secretion involves transport of individual molecules across the plasmalemma (Fig. 4) [10, 11, 16, 23, 24, 56]. Parenchyma cells rich in endoplasmic reticulum cisternae, dictyosomes, and vesicles are evidence of granulocrine secretion, while cells poor in endoplasmic reticulum and dictyosomes have more likely an eccrine mechanism (Fig. 4) [10, 11, 16, 23, 24, 56].

Finally, the source of nectar carbohydrates may be immediate photosynthesis by the nectary itself, mainly by the subnectary parenchyma, or by any other part of the plant, or may result from starch storage in plastids present in the parenchyma cells (Fig. 4) [16, 39, 51]. While both mechanisms may occur, the absence of starch grains confirms that the sugars of the nectar result from photosynthesis, supplied in most instances by the phloem that reaches the nectary parenchyma [16, 51].

2.2 The Chemical Constituents of Nectar

Nectar chiefly consists of sugars, especially of the disaccharide sucrose and the monosaccharides fructose and glucose (Fig. 5) [57]. Also, there are minor amounts of other monosaccharides (mannose, arabinose, xylose) and disaccharides (maltose, melibiose) as well as oligosaccharides and sugar alcohols (Fig. 5) [57–59].

Possible correlations between the type and composition of nectar sugars and the kind of associated pollinators have long been discussed. Flowers with high-sucrose nectars are more likely to be visited by bees, butterflies, moths, and hummingbirds, while flowers with high-hexose nectars are usually pollinated by small, unspecialized insects, passerine birds, or Neotropical bats [57, 59]. There are certain phylogenetically restricted trends in the sugar composition of the nectar within each of the families Asteraceae, Gesneriaceae, and Scrophulariaceae, yet their flowers are visited by a diversity of generalist pollinators [60–64]. In Anacardiaceae, also with a more generalist pollination system, there seems to be no clear pattern of nectar sugar composition, here apparently affected by various environmental factors [21, 41]. On the other hand, plants with similar, more defined concentrations of nectar tend to have the same kind of more specialized

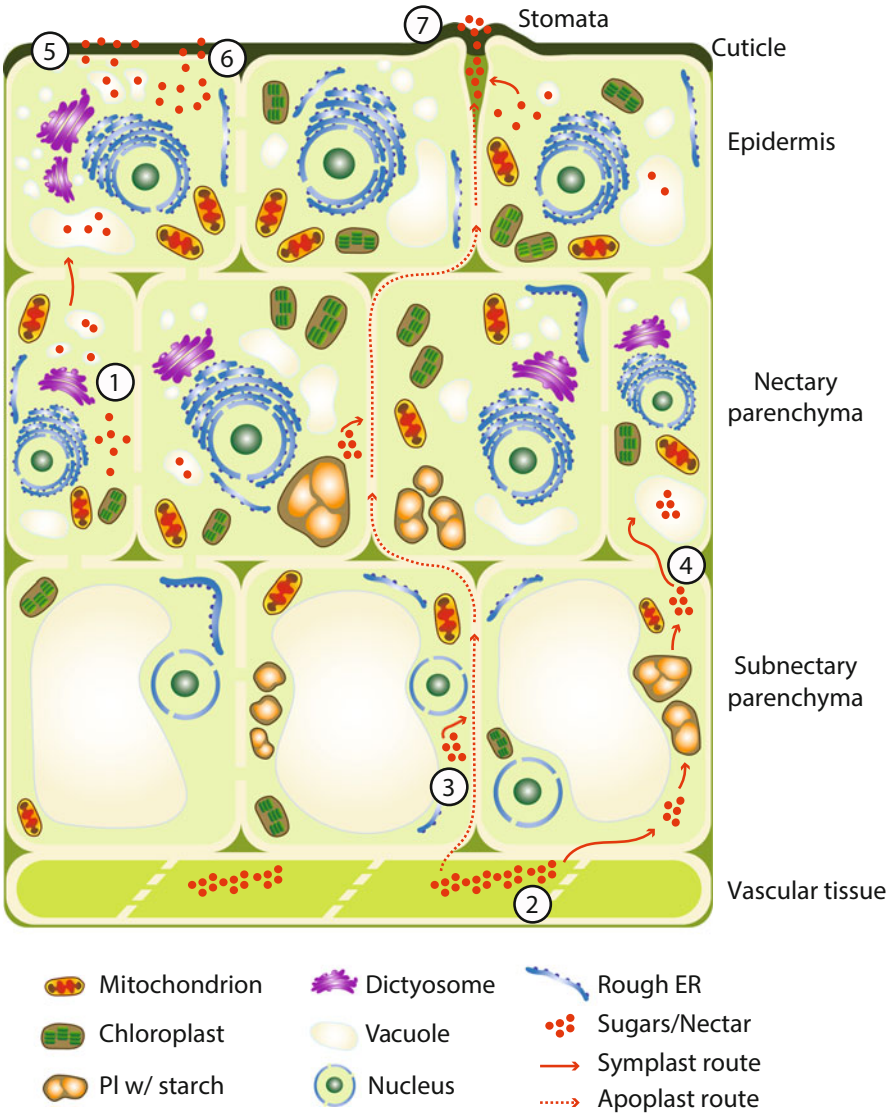

Fig. 4 Nectar production, storage, and secretion. Nectar production may occur through different mechanisms: (1) production in nectary parenchyma by the organelles therein, such as rough endoplasmic reticulum and chloroplasts. Afterward the nectar may be modified by the enzymes present in the cytoplasm and stored in vacuoles; (2) production from the sucrose of the phloem. (3) The sucrose may be broken down into glucose and fructose, further modified, and transported outside the nectary or (4) may be stored first in amyloplasts and/or vacuoles. The secretion of nectar may be released (5) through granulocrine or (6) eccrine mechanism or (7) by nectarostomata. The representation of the three mechanisms in the same schematic drawing does not imply that all these mechanisms occur simultaneously

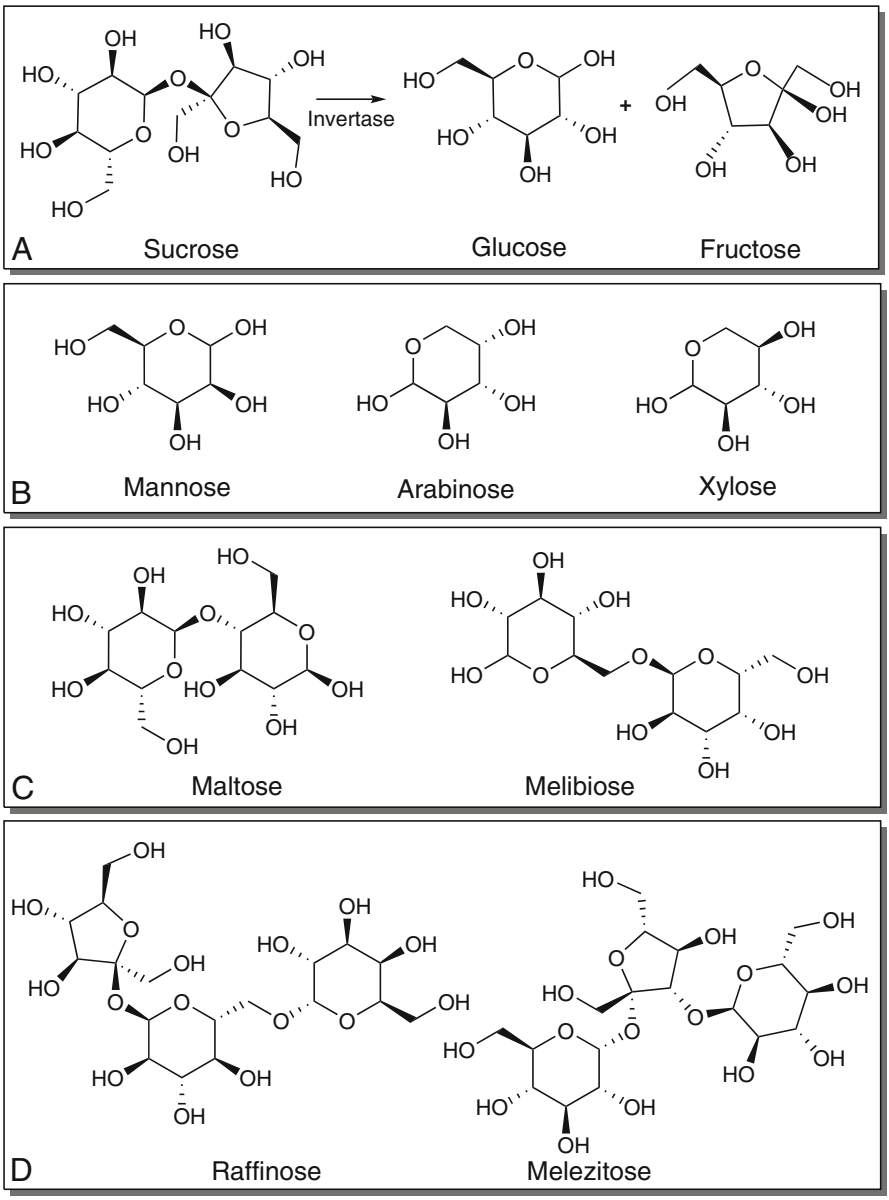

Fig. 5 Sugars of floral nectar. (**a**) Main sugars of floral nectar. The monosaccharides glucose and fructose result from the breakdown of sucrose by invertase. (**b**) Other monosaccharides occurring in floral nectar. (**c**) Disaccharides. (**d**) Oligosaccharides are rare (occur in some families, such as Myrtaceae and Orchidaceae)

pollinators. Highly concentrated nectars are related to insect-pollinated flowers, whereas flowers pollinated by birds and bats generally produce more dilute nectars [57–59, 65]. Thus, in most cases, morphological floral constraints play a greater role in limiting the access of a flower to interested visitors (potential pollinators, nectar robbers, etc.), rather than the sugar composition of its nectar.

Amino acids are the second-most common components of nectar [57, 66, 67]. The amino acid concentration and composition may vary significantly within a population and even within a single plant [68–70]. However, the overall amino acid composition is generally more highly conserved than the individual amino acid concentrations [71]. Amino acids contribute to the taste of nectar and are important in regard to the types of pollinators that visit or avoid the flowers [71–75]. Some proteins (nectarins) also occur in nectar; these have only been studied in a few genera (e.g., *Nicotiana*, *Allium*) [76–78].

Small amounts of other substances also occur in nectar, such as lipids, organic acids, phenolic compounds, alkaloids, and terpenoids [57]. In some species, these substances, especially lipids, may even comprise a large proportion of the solutes of nectar, which is then referred to as mixed secretions [10, 21, 29, 41, 57, 59, 79, 80]. Lipids often provide a particular flavor and odor that can be essential for certain pollinators; in addition, they are twice as energy-rich as sugars and an important food resource [81–83]. The major lipids in nectar are fatty acids of different chain length, but recently volatile oils, such as monoterpenes, have also been identified, produced either by the nectary itself or by microorganisms that inhabit the gland (Fig. 6) [84–87]. While lipids are linked to the attraction of pollinators, such compounds as alkaloids, phenolics, coumarins, and saponins may be toxic and/or repellent to some groups of floral visitors [57, 67, 88–90]. The main alkaloids thus far identified are nicotine, anabasine, caffeine, and amygdalin, which have been detected in nectar of different families of angiosperms (e.g., Rosaceae, Rutaceae, Solanaceae) (Fig. 6) [91]. Regarding phenolics, most of them are flavonoids, such as quercetin, kaempferol, myricetin, and isorhamnetin, but aurones may be also found in some species with colored nectar (Fig. 6) [92, 93]. Therefore, the interactions between the substances present in nectar and the pollinators are complex and not only related to alimentary reward but also to modulating insect behavior [94, 95].

2.3 Evolution of Floral Nectaries and Nectar

Floral nectaries are enormously diverse and have evolved independently several times within angiosperms; in all cases, nectar is related to interactions with pollinators [14, 95]. The shape, structure, and location of nectaries vary greatly among plants [10, 14, 56, 96–100], each lineage with unique peculiarities. Variations within the same family or genus are common, depending on phylogenetic and/or ecological constraints [14, 82, 101].

Beetles seem to have been the chief insects to pollinate early angiosperms, and it has been claimed that pollen was the only attractant and reward [102–105]. However, recent evidence has shown that the first reward to pollinating insects in

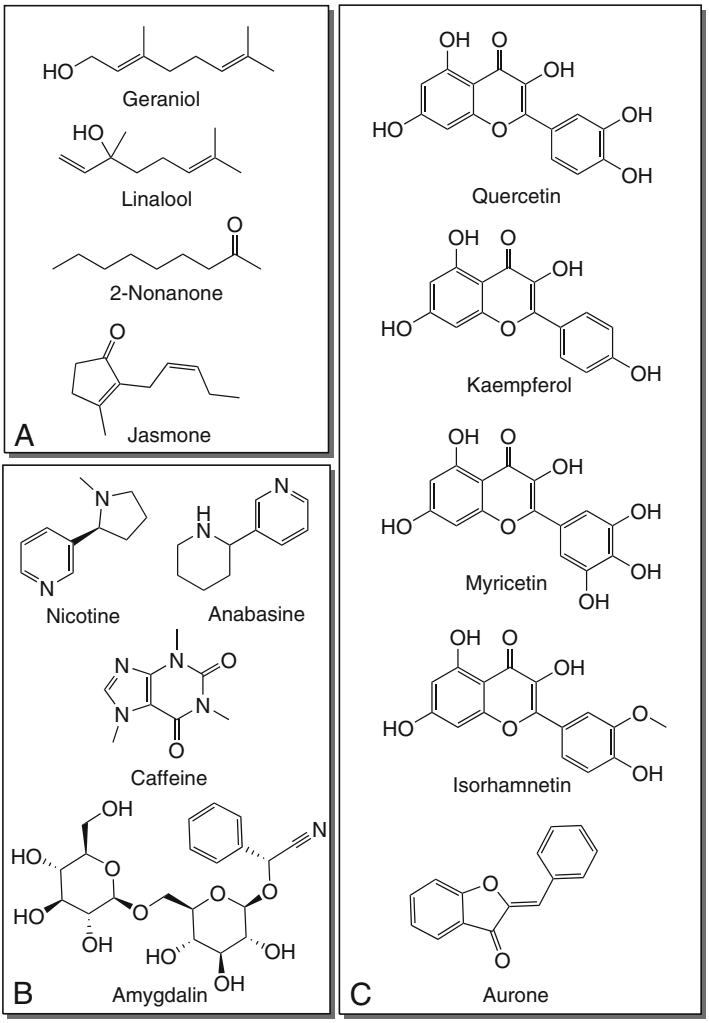

Fig. 6 Terpenoids (**a**), phenolics (**b**), and alkaloids (**c**) identified in floral nectars

early angiosperms was floral secretion and not pollen [9, 105–107].Gymnosperms (e.g., Cupressaceae and Pinaceae) produce ovular secretions that are rich in sugars, comparable to the nectar of angiosperms, suggesting that ancestral populations of gymnosperms were already insect-pollinated [107]. Nowadays, the large fossil record of insects and plants has reshaped our understanding of pollinator evolution, and it is widely accepted that the first flowering plants had a generalist pollination mode and that pollination by beetles, moths, and flies all evolved in angiosperms simultaneously [108–112].

In the evolutionary history of angiosperms, floral nectaries first appeared in Nymphaeales (Fig. 7) [105, 113–116]. The floral nectaries of early angiosperms

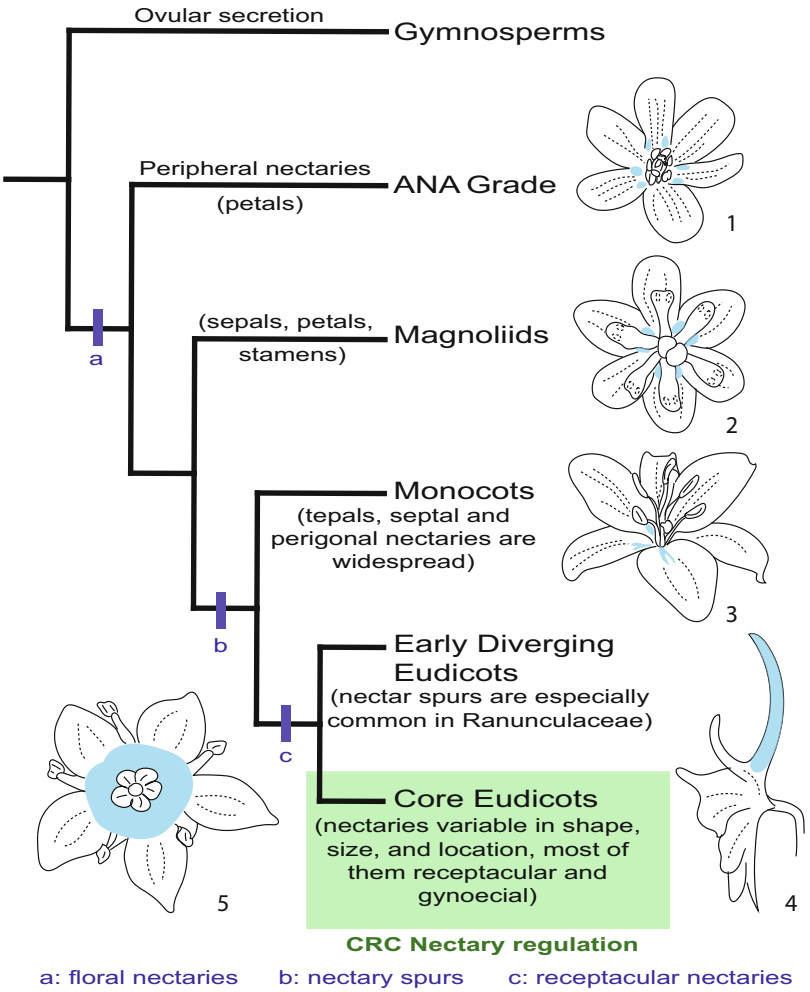

Fig. 7 Evolution of floral nectaries. The hypothetical tree shows the main synapomorphies of each large clade of angiosperms. Line drawings of (1) Cabombaceae (petal nectary), (2) Lauraceae (staminal nectary), (3) Liliaceae (perigonal nectary), (4) Ranunculaceae (nectary spur), and (5) Rutaceae (receptacular nectary) exhibit the floral nectary in *blue*. The development of floral nectaries in core eudicots depends on CRC (*crabs claw*) expression

are structurally simple. The flowers of Nymphaeaceae bear a central nectary on the petals, while the flowers of Cabombaceae produce nectar through glandular trichomes [105, 115–117]. Although other early-branching lineages as Amborellales, Austrobaileyales, and Chloranthales are pollinated by insects, no floral nectaries have been reported so far in those orders [111, 113–115, 118–123]. Nectar is produced in most of the families of magnoliids (e.g., Winteraceae in Canellales; Aristolochiaceae in Piperales; Lauraceae and late Monimiaceae in Laurales;

Annonaceae, Magnoliaceae, and Schisandraceae in Magnoliales); however, nectary location and structure are quite diverse, including petal nectaries, large glands on the base of filaments, and stigmatic nectaries (Fig. 7) [14, 105].

Septal nectaries are widespread in monocots, although absent in the largest family – Orchidaceae (Figs. 7 and 8a) [14, 99, 113, 124–127]. They rarely occur outside monocots and are a result of incomplete fusion of a small region of the carpel margins [9, 14, 99]. Perigonal nectaries are second-most common type of nectaries in monocots and may be epidermal as well as trichomatous [99]. Some authors suggest that perigonal nectaries may have evolved from septal nectaries by heterochrony, but further studies are necessary to confirm this hypothesis [14, 128–130]. The absence of the septal nectaries or the emergence of perigonal and staminal nectaries are related to alternative pollination modes (e.g., buzz pollination in some Asparagales) and attraction (e.g., floral deceit in Dioscoreales) [14, 99, 128–130].

Receptacular nectaries first emerged in the early divergent eudicots. However, nectaries on petals, staminodes, and carpels also occur in this lineage (Fig. 7) [14, 105]. Within the order Ranunculales, nectar spurs are very noticeable; these are extensions of various parts of a flower that produce and store nectar (Fig. 8b–c) [14]. In Delphinieae (Ranunculaceae), for example, the nectar spur is formed by the postgenital fusion of two primordia of the internal perianth whorl and is linked to the pollinator's proboscis length [131]. Therefore, morphological variation of these structures is highly correlated with the type of pollinators and the reproductive success of individual plants [132].

Distinct receptacular nectaries are common in the core eudicots (Figs. 7 and 8d), but in flowers with inferior ovary or narrow corolla tubes, they seem to become lost in the course of evolution [105]. The presentation of nectar through these structures has many advantages, like the increase of the nectar volume and its constant production during anthesis, improving the attraction of pollinators [105]. Developmental studies have demonstrated that CRC (*crabs claw*) expression is required for nectary development in core eudicots [133] (Fig. 7). This gene is linked to the regulation of carpel development in early lineages of plants, but its expression in nectaries of derived lineages suggests a tendency of change from the typically peripheral perianth position of the nectary in basal taxa to central positions associated with reproductive organs in eudicots [133].

3 Osmophores

The presence of odor is related to the production of volatile oils by secretory structures located inside or on the surface of vegetative and/or reproductive organs [134]. In some plants such as *Ceropegia* (Apocynaceae), *Aristolochia* (Aristolochiaceae), and species of Orchidaceae and Araceae, the odor production is restricted to certain areas of the floral organs, where the cells generally differ structurally from their neighbors.

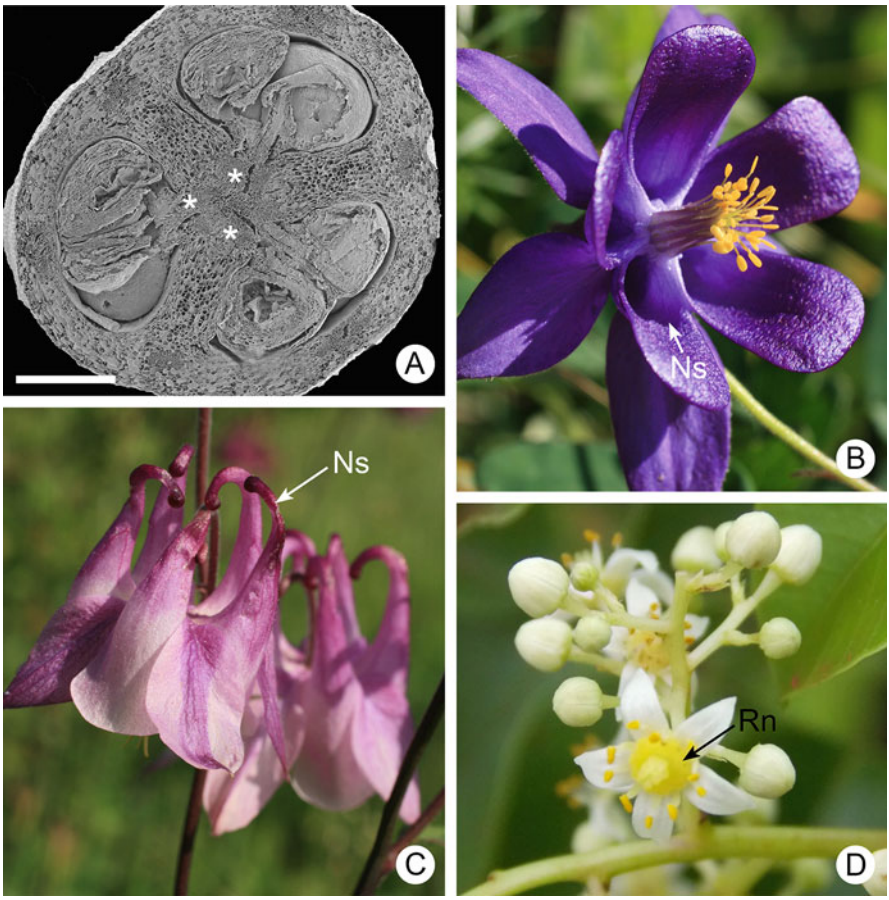

Fig. 8 Floral nectaries in monocots and eudicots. (**a**) Septal nectary (*) in *Habranthus tubispathus* (Amaryllidaceae). (**b**) Nectar spur in *Aquilegia einseleana* (Ranunculaceae). (**c**) Nectar spur in *Aquilegia vulgaris* (Ranunculaceae). (**d**) Receptacular nectary in *Spondias tuberosa* (Anacardiaceae). *Ns* nectar spur; *Rn* receptacular nectary. Bar: (**a**) 500 μm. (Photos: (**a**) Nathália Streher, (**b**–**c**) Reinhard Jahn, and (**d**) Elisabeth D. Tölke)

In general, petals are the main floral structures that emit odorous substances, but sepals, stamens, pistils, and nectaries can also emit specific volatiles [135].Some remarkable examples are the osmophores (scent glands) on *i*) the adaxial surface of petal lobes, like in the family Apocynaceae [136]; (*ii*) on the abaxial face of the labellum of some species of Orchidaceae (*Cyclopogon elatus*) [137] or the hypochile of the labellum of *Stanhopea graveolens* [138]; or (*iii*) appendages of dorsal sepals of *Bulbophyllum wendlandianum* [139]; or (*iv*) on the spadix appendix as well as the club-shaped organs located directly above the female flowers of certain Araceae, as *Sauromatum guttatum* [140].

3.1 Osmophore Structure, Odor Production, and Release

Osmophores vary in surface morphology, being either glabrous or possessing trichomes, and the epidermal secretory cells may have different shapes, either with papillae (Fig. 9a) or without, then simply cubic. Furthermore, the cuticle can vary from striated (Fig. 10a) to smooth (Fig. 9a), be composed of epidermal tissue (Fig. 9b) or epidermal plus subepidermal tissue [8], sometimes with one or more layers of subepidermal secretory parenchyma cells.

The cytoplasm of both the epidermal cells and the secretory parenchyma, when both constitute the gland, is rich in ribosomes (Fig. 10a–d) and organelles like elaioplasts (Fig. 10b and c), smooth endoplasmic reticulum (ER) (Fig. 10b and c), and mitochondria. Intense secretory activity of elaioplasts and ER may be observed in osmophore cells (Fig. 10b and c), as reported for Apocynaceae [136], and in studies with Araceae [141–143], Orchidaceae [139, 144–147], and Passifloraceae [148]. The secretion (scent) produced by osmophores is mainly composed of lipids, which are directly related to plastids and ER, since these organelles are responsible for the production of many types of lipids in the cell [149]. The energy reserve for the secretory process may be found in the form of starch grains (Fig. 10c) within plastids of the epidermis or in the parenchyma [150].

One of the main features of osmophore cells is the prominent vesicle population (Fig. 10a) in the early secretory stages. The synthesized lipids are packaged and transported through the cytoplasm of the secretory cells mainly via these vesicles. In a similar way, the secretion is usually transferred from one cell to another through vesicles that merge with the plasma membrane (exocytosis), being encompassed by the adjacent cell in the reverse mechanism (endocytosis) (Figs. 10d and 11). However, some osmophore cells have plasmodesmata through which the secretion can pass to the adjacent cell (Fig. 11). The last step in the path of the secretory process is the release of the secretion to the outside. Once again, granulocrine release is the most common mechanism, i.e., the epidermal cells have secretory

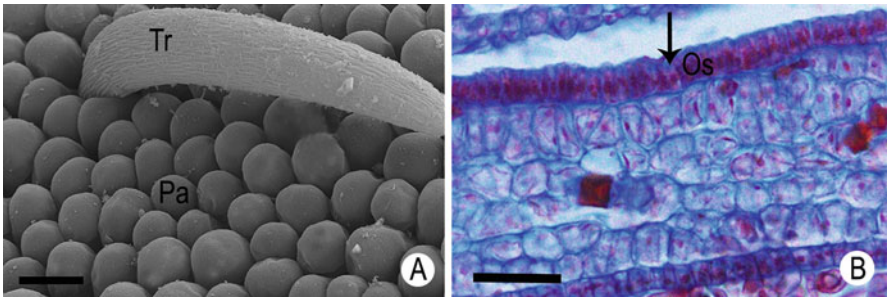

Fig. 9 Structure and shape of floral osmophores. (**a**) Papillate osmophore. Epidermis with smooth cuticle and some trichomes between the papillae (*Ditassa gracilis*, Apocynaceae). (**b**) Epidermal osmophore (*Joannesia princeps*, Euphorbiaceae). *Os* osmophore, *Tr* trichome, *Pa* papillae. Bars: (**a**) 20 μm, (**b**) 50 μm. (Photos: Natalie do Valle Capelli)

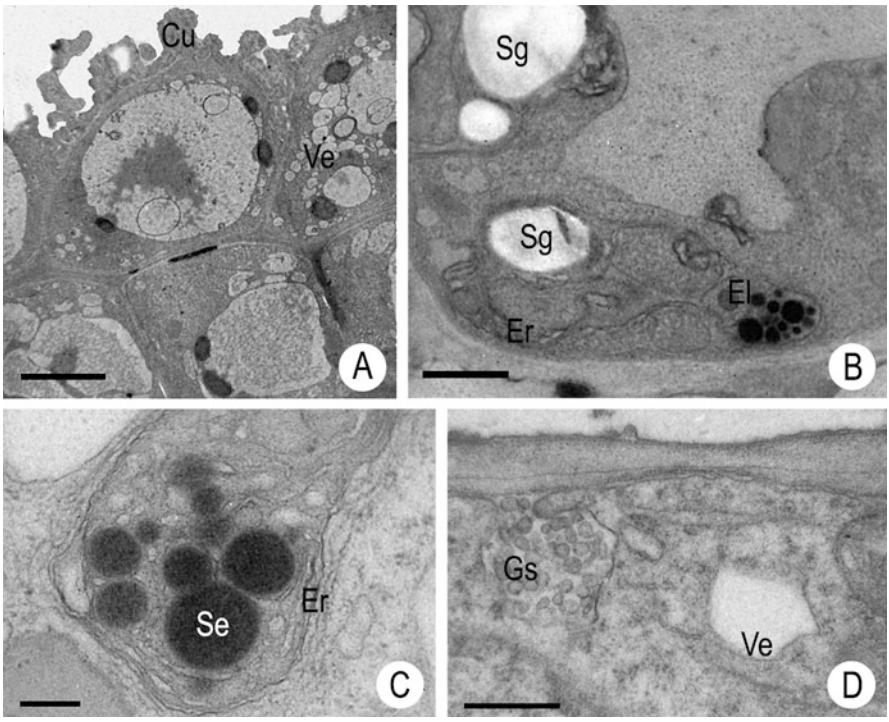

Fig. 10 Ultrastructure of osmophores. (**a**) Osmophore cells of *Aspidosperma australe* (Apocynaceae) showing vacuoles of different sizes; dense cytoplasm. (**b**) Osmophore epidermal cell of *Tabernaemontana catharinensis* (Apocynaceae) containing rough ER, plastids containing starch grains, and elaioplasts. (**c**) Association between elaioplast and rough ER to produce secretion (*T. catharinensis*). (**d**) Secretion being encompassed by the adjacent cell (endocytosis) (*Stapelia hirsuta*). *El* elaioplast, *ER* endoplasmic reticulum, *Gs* granulocrine secretion, *Se* secretion, *Sg* starch grain, *Ve* vesicle. Bars: (**a**) 2 μm; (**b**) 1 μm; (**c**) 0.2 μm; (**d**) 0.5 μm. (Photos: Natalie do Valle Capelli)

vesicles which fuse with the plasma membrane in the distal portion of the cell, transferring the secretion to the periplasmic space (Fig. 11) [139, 144, 145, 147, 151]. Then, the secretion crosses the cell wall and cuticle, reaching the gland surface and escaping to the atmosphere. Diffusion of the secretion across the cuticle can occur due to the lipophilic nature of cutin, aided by the secretory flow, the pressure exerted by the protoplast, and/or the possible presence of microchannels in the cuticle [23].

3.2 The Chemical Nature of the Odor

The floral odors produced by osmophores are usually composed of isoprenoids, terpenoids, benzenoids, phenylpropanoids, fatty acid derivatives, and various nitrogenous and sulfur-containing compounds. These are generally of low molecular

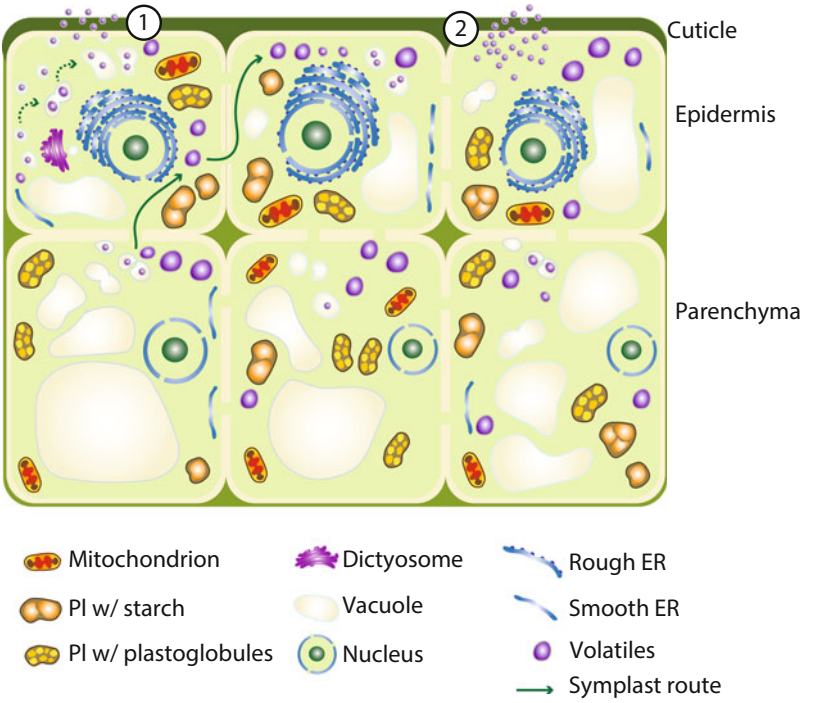

Fig. 11 Volatile oil production and secretion. Scheme of osmophore composed of epidermis and parenchyma, where the oils are produced in plastids and smooth ER. During secretory activity, the exchange of secretions between the secretory cells may occur via vesicles (exocytosis/endocytosis) and/or through the symplast route via plasmodesmata. Starch is commonly found within plastids, and oil droplets may be transported across the cytoplasm packed in vesicles or free in the cytosol. In the epidermis, the release of volatile oils may occur either through the (1) granulocrine or (2) eccrine mechanism. The representation of the two mechanisms in the same schematic drawing here does not necessarily mean that both mechanisms occur simultaneously

weight, low polarity, and low vapor pressure – properties that facilitate volatility. Some compounds are present in most floral aromas, while others are found only in certain species [152]. Despite the greater or lesser similarity between the chemical compositions of the volatile oils of each flower, there must be a synchronization between the moment of an insect's activity and the emission of the odor, besides other parameters involved in floral development [153].

The chemical composition of scents plays an important role in the communication between organisms. The typical odor of a flower may result from the majority of volatile terpenes and/or their combination with amines and ammonia [8, 144]. The function of the emitted volatiles is quite diverse. The most important function is communication. Odors have a significantly better transmission range than visual cues. The composition of floral scents is not conservative in all species of a genus. For instance, *Clarkia breweri* (Onagraceae) has fragrant flowers, while *Clarkia concinna* is scentless [154]. In addition, the quality and quantity of odor emission

can also vary between individual plants of the same species and even between individual flowers of an inflorescence. In *Cimicifuga simplex* (Ranunculaceae), two of the three subspecies do not produce odor and are pollinated by bees, while the third one emits methyl anthranilate and isoeugenol, which specifically attract butterflies for pollination [155, 156]. Similarly, the fragrant *C. breweri* is pollinated by moths, whereas other species of *Clarkia* are not fragrant and they are self-compatible, pollinated by oligolectic bees [154].

3.3 Odor Dynamics and Presentation

The release process of odors by flowers can be highly complex, exhibiting different patterns of dynamic emission and chemical composition of volatile compounds. The composition and amount of volatiles emitted may vary depending on the stage of floral development or the time of the day (some plants emit volatiles during the day, while others emit them at night) [157]. For example, *Stephanotis floribunda* has a peak of emission of linalool and methyl benzoate around midnight, while the emission of 1-nitro-2-phenylethane reaches the highest levels in the morning [158, 159]. *Nicotiana sylvestris* emits phenylpropanoids at night, while the emission of terpenoids does not oscillate [160]. In general, specific daytime emission of odor compounds accurately correlates with pollinator activity. Plants that mainly emit odor during the day are predominantly pollinated by bees, bumblebees, and butterflies, whereas flowers that mainly emit odor at night are pollinated by moths and bats [161]. This emission of daytime or nighttime odor appears to be regulated by light intensity and/or temperature. However, a circadian clock is involved in *Cestrum nocturnum*, *Nicotiana suaveolens*, *N. sylvestris*, *Rosa hybrida*, *Antirrhinum majus*, and *S. floribunda* allowing a precise time of emission independent of environmental signals [157]. The complex regulation of scent emission is related to the morphology, anatomy, and cellular characteristics of the osmophore [162]. In some flowers in which anthesis occurs at a given time of day, other processes must occur simultaneously with scent release in order to ensure pollination, such as the precise time of nectar and pollen presentation, thermogenesis, and/or movement of organs [153, 163, 164]. Floral scents are particularly important in flowers with nocturnal anthesis, where olfactory signals attract pollinators over long distances [153].

3.4 Evolution of Osmophores and Floral Odor

Floral scent emission is clearly affected by environmental factors, such as temperature, irradiance, and air humidity [165–167], as well as biotic processes, such as pollination and herbivory [168–175]. Hence, the high environmental plasticity of floral scent is likely to deflate estimates of floral scent heritability. On the other hand, it is often argued that the observation of a low heritability could be caused by genetic variation having been depleted by strong selection

in the past [176, 177]. In *Brassica rapa* the heritability of floral scent is correlated with the pleiotropic responses of various plant traits [178]. These latter authors observed the alteration of the entire floral scent bouquet after only three generations in response to artificial selection of a single compound.

A study of the divergent evolution in plants of *Brassica rapa* compelled by pollinators was conducted, in which the plants were developed in a phytotron under standardized conditions [179]. The replicates were preserved as isolated lines during 11 generations to be able to evaluate independent, repeatable evolutionary changes. In this study they used three pollinator treatments: bumblebees, hoverflies, and hand pollination. Pollination was performed 23 days after seeding out in a flight cage in the greenhouse under standardized conditions, with bumblebees and hoverflies. Pollinators were let to forage on fast cycling *B. rapa* plants of the control group of the respective generation. Before pollination, pollinators were hungry. Five pollinators were added individually and sequentially; each insect was allowed to visit a maximum of three different plants before removing them from the cage. Floral scent was measured before pollination 19–21 days after sowing out, and the quantification of volatiles was conducted by gas chromatography with mass-selective detection. Hoverfly-pollinated plants showed a significant decrease in the emission of four scent compounds: methyl salicylate, *p*-anisaldehyde and indole, and benzyl nitrile. In bumblebee-pollinated plants, the total amount of scent emission per flower almost doubled, as more than half of the analyzed volatiles showed increased emission.

In addition, plants with floral scent may have different pollination syndromes within the same group or may be related to a larger type of syndrome. Studies addressing these relationships are very informative, providing excellent opportunities to determine whether pollinator changes are correlated with parallel variations in the chemistry of floral bouquets [152].

3.5 Floral Scent Production in Deceptive Plants

Just as there are animals that collect resources in flowers without an effective pollination, there are also plants that attract pollinators without offering any sort of resource, a phenomenon known as deceptive pollination [180–183]. Floral deception occurs in more than 30 families of angiosperms (e.g., Aristolochiaceae, Apocynaceae, Araceae, Berberidaceae, Bignoniaceae, Iridaceae); however, Orchidaceae is the family with the highest number of species with this condition [182, 184, 185]. In deceptive pollination the flowers mimic floral signals of rewarding plants (food deception) or mating signals of receptive females (sexual deception) to attract pollinators [186]. These signals may include visual and olfactory cues. Therefore, these flowers do not synthesize nectar, oil, or resins, while scents may still be produced [183].

The production of floral scents by osmophores in deceptive plants may be essential to an efficient pollination, especially when other visual and/or morphological cues to attract floral visitors are lacking [187–191]. Several experiments

demonstrate that in deceptive species the reduction of the quantity of floral scents is responsible for a decrease in the number of pollinators, confirming their importance [188, 191, 192]. The perfume may act in both, food and sexual deception, providing an olfactory cue to these animals, which may be searching for food rewards, nests, and/or a sexual partner [193, 194].

In food-deceptive pollination, bees are the most common agents, mainly attracted by the visual and olfactory features of the flower, but there are also records of Coleoptera, Diptera, and Lepidoptera involved in this kind of pollination, since the scents produced by the osmophores in this case are mainly monoterpenes and sesquiterpenes, like those described in nondeceptive species [183, 186, 194, 195]. Regarding sexual-deceptive pollination, the flowers of some members of Orchidaceae mimic sexual partners of potential visitors, having flowers that are morphologically similar to the female and producing scents that are perceived as pheromones by the pollinators [183, 186, 196]. The scents produced by the flowers are very specific, each species of orchid emitting the particular scents that attracts a specific pollinator, ultimately involving a process referred to as pseudocopula [197–199].

Some deceptive species with flowers that bear strong colors and scents can attract insects, mainly beetles and flies, that oviposit within the flower while simultaneously leading to pollination [183, 200, 201]. In one of the most elaborate examples of this kind of pollination, several species of *Aristolochia* and *Asarum* (Aristolochiaceae) and *Ceropegia* (Apocynaceae) attract and temporarily entrap pollinators – their flowers usually have strong odors that simulate decaying organic matter [8, 153, 202–207].

Some species of Orchidaceae in the genera *Satyrium* and *Dracula* also attract flies by emitting strong fungal scents [201, 208, 209]. For instance, the flowers of *Dracula chestertonii* emit a strong mushroom-like scent attracting female flies; the scent is composed of typical mushroom constituents such as oct-1-en-3-ol, oct-1-en-3-one, octan-3-ol, and octan-3-one (Fig. 12) [210]. In *Satyrium pumilum* the most

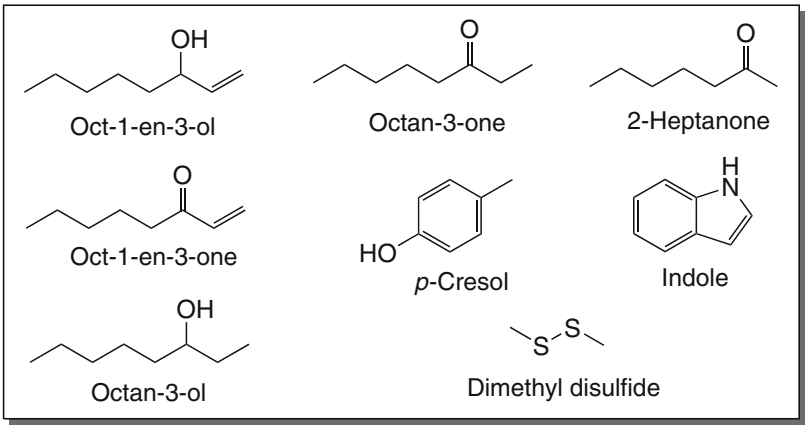

Fig. 12 Volatiles from flowers of various deceptive species

important scent compound is dimethyl disulfide (Fig. 12), a compound also identified in an unrelated deceptive species of Solanaceae – *Jaborosa rotacea* [201, 211]. Flower scent dominated by oligosulfides and fatty acid-derived acids is associated with carrion mimicry, whereas scent mainly composed of *p*-cresol, indole, and 2-heptanone is associated with dung mimicry (Fig. 12) [152, 201, 212–214].Carrion and dung mimicry on the basis of similar compounds has evolved independently in different plant lineages.

4 Elaiophores

Elaiophores are secretory structures involved in the production of nonvolatile lipid rewards to pollinators. These structures were discovered about 50 years ago by Stefan Vogel [215], described and reported for the first time for Malpighiaceae, Krameriaceae, Scrophulariaceae, Iridaceae, and Orchidaceae [216], and are usually located on sepals, petals, or stamens (Figs. 13 and 14).

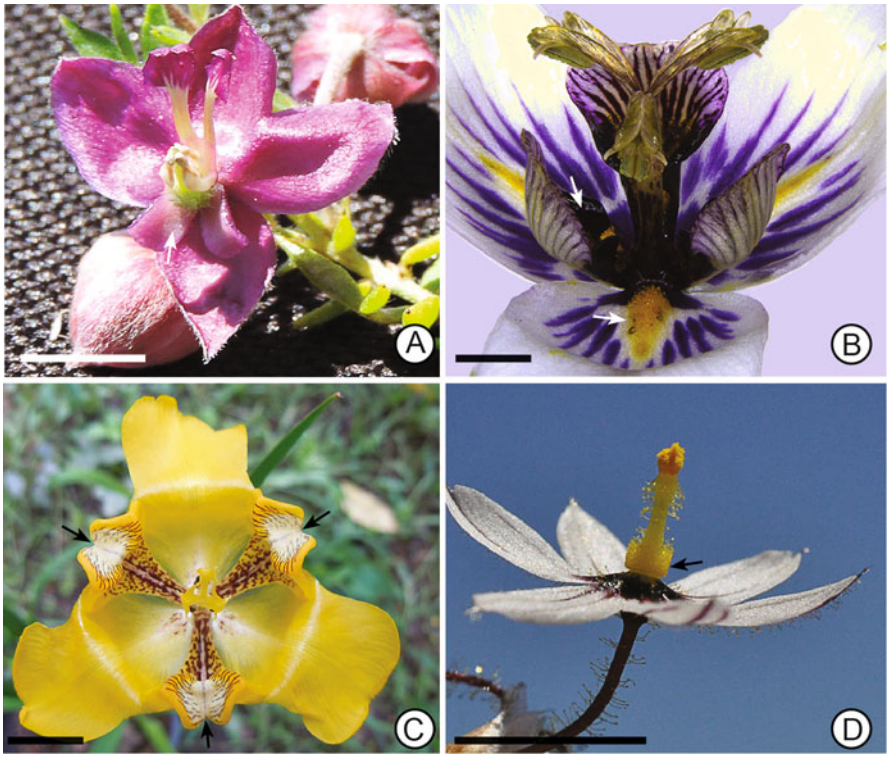

Fig. 13 Elaiophores in flowers. Glandular regions marked by *arrows*. (**a**) *Krameria grandiflora*. (**b**) *Herbertia zebrina*. (**c**) *Cypella aquatilis*. (**d**) *Sisyrinchium scariosum*. Bars: 10 mm. (Photos:(**a**) D.L. Borges, (**b**, **c**) T. Pastori, and (**d**) L. Eggers)

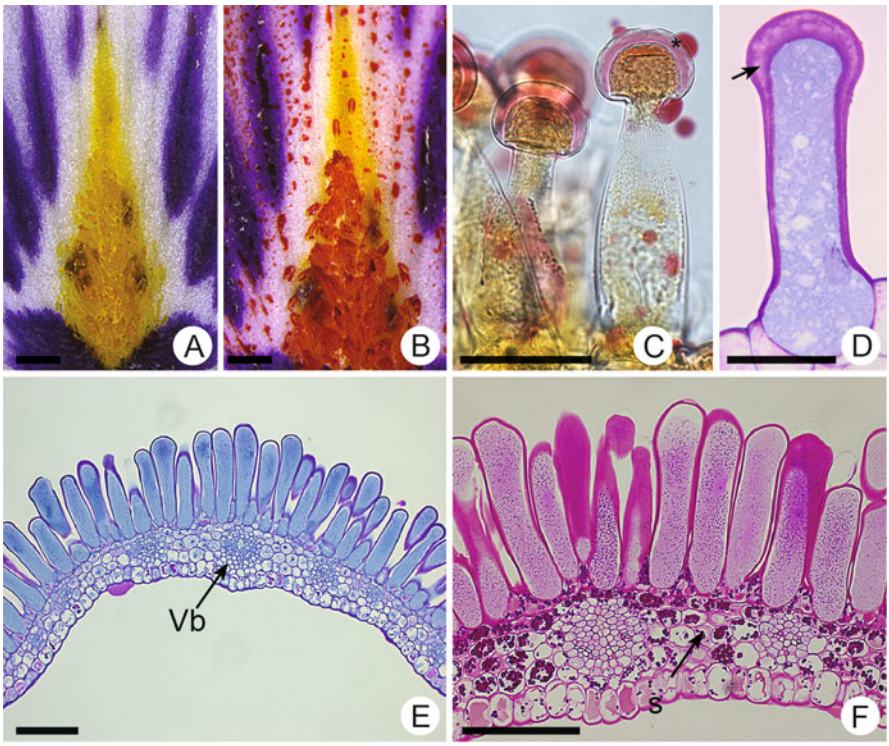

Fig. 14 Glandular trichomes on the outer tepals of *Herbertia zebrina* at anthesis. (**a**) Unstained material and (**b**) stained with Sudan Red 7B. (**c**) Trichomes of *H. zebrina* in transversal section (TS) stained with Sudan Red 7B at anthesis, showing the oil secretion released to the outside (*asterisk*). (**d**) Unicellular glandular trichomes in TS stained with Toluidine Blue at pre-anthesis, showing the subcuticular space (*arrow*). *Cypella magnicristata* in TS stained with Toluidine Blue (**e**) and PAS-Schiff (F) at pre-anthesis. Note the trichomes and vascular bundles in the elaiophore area and the strong starch accumulation in the parenchyma cells near the elaiophore area. *S* starch, *Vb* vascular bundles. Bars: (**a**, **b**) 1 mm, (**c**, **d**) 50 μm, (**e**, **f**) 200 μm. (Photos: Tamara Pastori)

4.1 Structure and Location of Elaiophores

Anatomically, elaiophores are subdivided into two broad categories: epidermal and trichomatous. Epidermal elaiophores consist of secretory epidermal cells, which are generally elongate and accumulate the oil under their cuticles, forming "blisters" [11, 56, 216–218]. These secretory structures are documented in two families of eudicots, Malpighiaceae and Krameriaceae, and two families of monocots, Orchidaceae and only one species of Iridaceae [219–222]. Epidermal elaiophores are positioned on tepals or petals (Fig. 13a) and/or sepals [223–228].

Trichomatous elaiophores consist of hundreds to thousands of glandular trichomes, uni- or pluricellular, which in most cases form a very dense surface (Fig. 14a) [11, 56, 218, 220]. Lipid production is continuous, and secretions are

generally unprotected, although in some cases lipids may accumulate in a subcuticular space [229, 230]. Trichomatous elaiophores occur in five orders and nine families of angiosperms and quite variable regarding their location [221, 222]. In monocots the elaiophores are unicellular and occur only in two families of Asparagales: Iridaceae and Orchidaceae. In these families, trichomatous elaiophores are distributed on tepals (Fig. 13b and c) or on the staminal column as in *Sisyrinchium* (Iridaceae) (Fig. 13d) and *Grobya* and *Ornithocephalus* (Orchidaceae) [216, 221, 222, 226, 228, 230–235]. In eudicots, trichomatous elaiophores are multicellular and located mainly on sepals or petals (e.g., Calceolariaceae and Cucurbitaceae), inside the tube of the gamopetalous corolla (e.g., Stilbaceae and Solanaceae), or on spurs (*Colpias* and *Diascia*) and on the staminal column (*Lysimachia*) [128, 216, 217, 219, 236–242].

4.2 Floral Oil: Production and Chemistry

Aside from being either epidermal and trichomatous, the ultrastructural characteristics of secretory cells of elaiophores are very similar [226, 227, 235, 243–245]. These cells with their machinery for lipid production are quite abundant and characterized by the presence of plasmodesmata connecting all glandular cells, dense cytoplasm, extensive endoplasmic reticulum, abundant mitochondria, numerous plastids with lipid inclusions, lipid droplets in the cytoplasm, elaioplasts, and conspicuous nuclei [226–228, 234, 237, 243].

The floral oils released by elaiophores are usually colorless or yellow and odorless [216, 218]. They are composed of complex mixtures of mostly nonvolatile lipids but also small amounts of aldehydes, amino acids, carbohydrates, phenolic compounds, hydrocarbons, and ketones [216–218, 246]. Studies on the chemical composition of floral lipids are scarce, but generally these nonvolatile lipids consist of fatty acids and/or glycerides, and their composition varies within plant families and among genera and species [216, 217, 224]. All chemical analyses available have shown that glycerides generally occur in the form of monoglycerides or diglycerides and rarely triglycerides. The most common free fatty acids found in floral oils are myristic, palmitoleic, palmitic, oleic, stearic, and eicosenoic acids (Fig. 15) [216, 242, 246–252].

The synthesis of lipids is complex and occurs through different routes involving fatty acids and acylglycerols (we here present a general scheme of synthesis; see Fig. 16). In general, the synthesis of lipids depends on the available carbohydrate sources (sucrose and/or glucose). The carbohydrate may be transported directly from the phloem to the secretory cells via symplast or apoplast and/or may become synthesized in the parenchyma of the elaiophore. Additionally, the carbohydrates may be temporarily stored in vacuoles and/or plastids (Fig. 16). Lipid synthesis occurs first through the glycolytic pathway, where the sucrose is first degraded into pyruvate molecules [253, 254]. Long-chain fatty acids with 16 or 18 carbons in length are synthesized in plastids and then transported to the

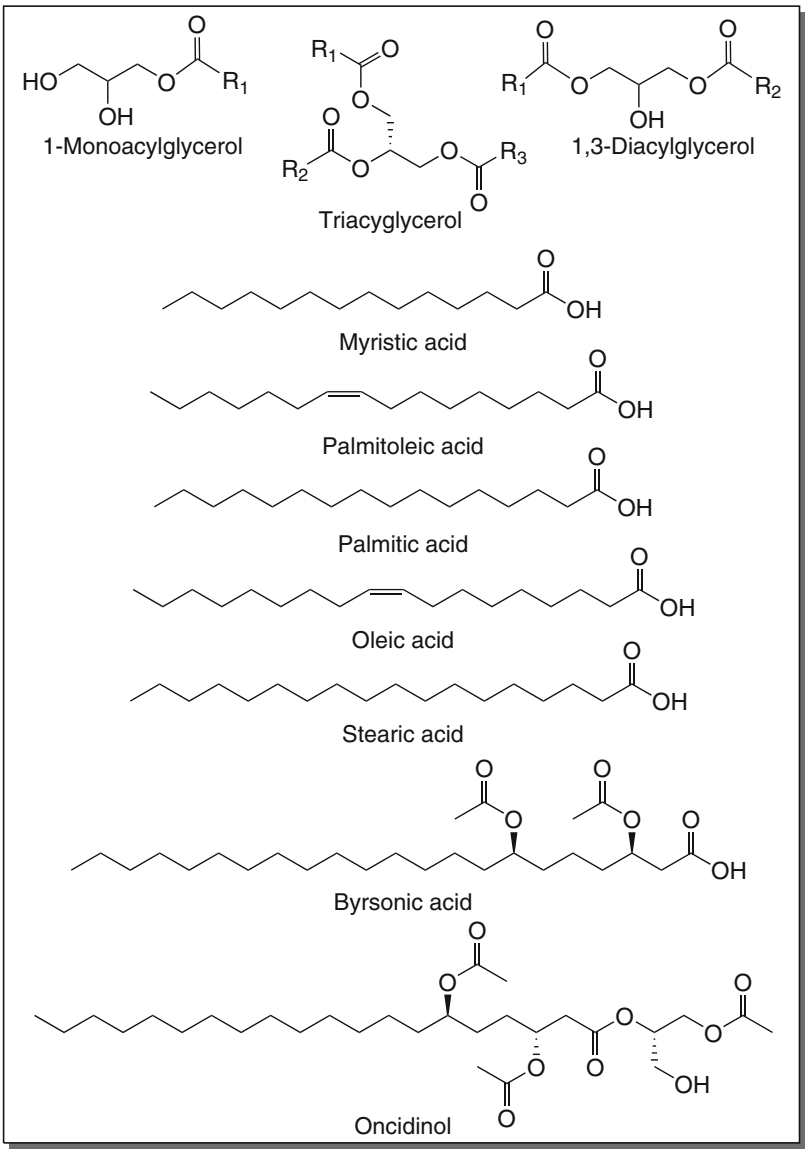

Fig. 15 Nonvolatile lipids of floral oil. The general structure of mono-, di-, and triglycerol; the free fatty acids most often found in floral oils, myristic, palmitoleic, palmitic, oleic, stearic, and eicosenoic acid, and structure of byrsonic acid and oncidinol

endoplasmic reticulum (ER) (Fig. 15) [253–255]. The elongation stage of fatty acids occurs mainly in the membranes of the ER and due to their hydrophobic nature requires facilitated transport, since they are immiscible in the cytoplasm [253, 255]. Upon their synthesis, the lipids may be stored within the

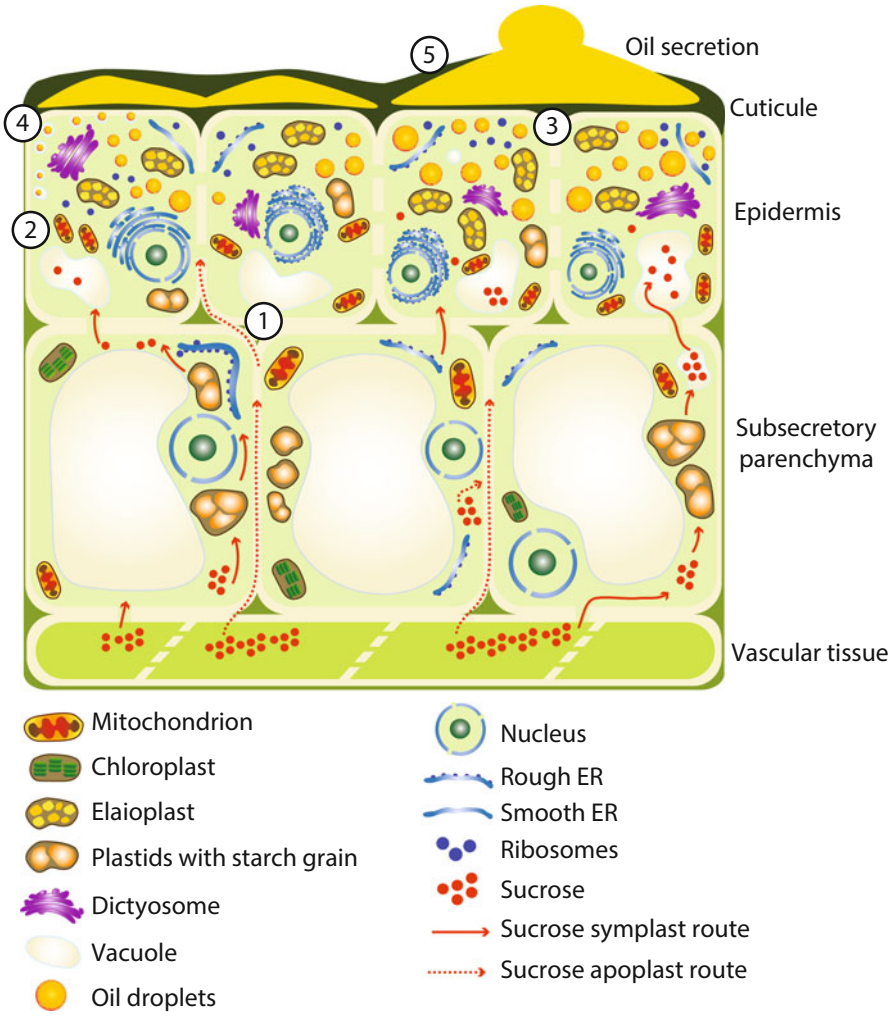

Fig. 16 Nonvolatile lipids – production, storage, and secretion. Nonvolatile lipids are produced in specific metabolic pathways and involve the plastids and the endoplasmic reticulum. The production of the lipids depends primarily on sucrose molecules, which can be synthesized (1) in the elaiophore region, or be transported to the subsecretory and secretory parenchyma and epidermis (symplast or apoplast pathway). This sucrose may be stored in vacuoles and/or amyloplasts or enter into lipid metabolism, where it is degraded into pyruvate molecules via the glycolytic pathway; (2 and 3) in elaiophores, the biosynthesis of fatty acids and glycerol occurs in the secretory epidermis; (4) the secretion of lipids may occur though the cell wall by the granulocrine mechanism and (5) can be accumulated in the cuticle, forming blisters, or be released from the cell wall

plastids (plastoglobuli) before being transferred to the cytosol. The secretion is located and visible in the cytoplasm as lipid bodies are free in the cytosol or packed in vesicles [254].

Elaiophore secretions are released to the surface of the gland by two main pathways: granulocrine and eccrine [256]. The observation that vesicles and multivesicular bodies are mainly located close to the plasma membrane and the presence of a large periplasmic space indicate that the secretion is likely released by the granulocrine mechanism [256]. Recent studies also have shown that lipid release occurs via secretory vesicles in most species [226, 227]. Although some studies have reported the possible occurrence of eccrine secretion [256, 257], it is difficult to prove the occurrence of this type of mechanism. Upon reaching the periplasmic space of the cell, lipids cannot be transported by facilitated diffusion through the cell wall, and the most likely hypothesis is that the protoplast exerts pressure on the hydrophobic material accumulated in the periplasmic space and thereby forcing it to cross the cell wall [23].

The accumulation of lipid secretions under the subcuticular space is commonly observed both in trichomatous and in epidermal elaiophores [216, 230, 258]. In some species, lipids are released only through cuticle rupture caused by contact with pollinators [216, 231, 237]. In others, the secretion may permeate the cuticle through microchannels, not forming subcuticular accumulation [231].

4.3 Lipids as a Specialized Reward to Pollinators

Floral oils are made available to highly specialized pollinators by only a few angiosperms. Only bees collect floral oils, and of these only about 500 species, i.e., less than 2% of global bee species, actively collect floral oils [216, 218, 259]. Oil-collecting bees belong to the tribes Ctenoplectrini, Centridini, Tapinostapidini, and Tetrapediini (Apidae), and Macropidini and Redivivini (Melittidae) [259]. In Tapinostapidini there are about 12 genera with 95 species collecting floral oils; the largest group of oil-collecting bees (about 230 species) is in the genus *Centris* (Centridini) [216, 221]. Oil-collecting bees have specific morphological adaptations and behavior, for example, in *Centris*, where the oil-collecting apparatus is located on the front pair of legs and the animals have soft and absorbent hairs [217, 260].

Floral lipids with their high nutritional value, as compared to the carbohydrates of nectar [216–218, 259, 261], are mainly used as larval food by bees in combination with pollen [216, 217] and possibly also in adult nutrition [218]; in addition, they are used for the construction of nests [261].

4.4 Evolution of Oil-Offering Flowers

Studies on oil-offering flowers have mainly focused on collector observations, on their morphology and on the distribution of elaiophores in flowers. Integrated studies have shown that there are many independent interrelational transitions regarding the presence of elaiophores in angiosperms and bee collector behavior [128, 216–221, 262]. Both the production of floral oils by angiosperms and the behavior of oil collection by bees are polyphyletic [221, 262].

Oil-offering flowers evolved independently at least 28 times in 11 families of angiosperms [221]. In most of these families, floral lipids occur in single lineages, while in Orchidaceae and Iridaceae, elaiophores developed independently several times during evolution [221]. In Orchidaceae, Renner and Schaefer [221] recorded 12 independent transitions to elaiophores, suggesting that there could be many more such transitions in that family. In Iridaceae, there is indication of four independent transitions to elaiophores and many reversions [221, 233, 263, 264]. Reversions to an absence of elaiophores have been identified in various families and may have occurred 36–40 times in angiosperms [221], exceptions being Stilbaceae and Malpighiaceae and the genus *Krameria* (Krameriaceae) and *Calceolaria* (Calceolariaceae) where the presence of elaiophores is probably common to all members [223, 239, 265].

Oil-collecting bees display specific behavior and morphological adaptations, and this evolved independently at least six times: in Centridini, Tapinostapidini, Tetrapediini, *Ctenoplectra*, *Macropis*, and *Rediviva* [221, 262, 266]. However, it is difficult to estimate the number of total transitions because some genera are nonmonophyletic and estimations based on the behavior of oil-collecting bees indicate that gains and losses are probably equal, depending on the adopted scenario [221, 267]. Clearly, besides the behavioral feature of collecting floral oils, there are morphological relationships between bees and angiosperms. For example, species of *Epicharis* and some species of *Centris* have specialized morphological adaptations for collecting floral oil from Malpighiaceae [216, 260].

The interactions between plants and pollinators are at the origin of many events of speciation and coevolution. These interactions are probably responsible for most of the diversity within angiosperms. On the other hand, very specific interactions, characterized by a high degree of reciprocal evolution, are rare in most ecosystems [254]. Thus, the availability of such highly specialized floral resources, as lipids, can drastically reduce the diversity of floral visitors and pollinators and consequently have a negative effect on the plants' ability to expand and diversify. Bees, however, rarely collect rewards exclusively from a single species and normally obtain sources from a broad variety of different species [218, 220]. In addition to the specialized morphological adaptations needed to collect this type of resource, specific behaviors are also necessary that generate adaptive costs. This could explain why this specialized relationship between bees and flowers with floral oils is fairly rare and could be the reason for the many reversals [221].

5 Resin Glands Related to Pollination

5.1 Resins Produced by Flowers and Inflorescences

Resins are complex mixtures of substances composed of liposoluble volatiles and nonvolatile terpenoids and/or other secondary metabolites, such as phenolic compounds, that are secreted by specialized structures inside or on the surface of the plant [268, 269]. They are commonly found in vegetative organs but have rarely

been reported from floral structures. While some inflorescences and flowers do produce resins, they are not necessarily involved in the pollination process. Resin glands serving in support of pollination are found only in a few genera from different lineages of plants, such as *Clusia* and *Chrysochlamys* of Clusiaceae, *Clusiella* of Calophyllaceae, and *Dalechampia* of Euphorbiaceae [270–272]. Here the resins are nonnutritive rewards for certain groups of solitary bees that use them to build their nests [217, 273, 274]. In addition to the structural function, these resins are waterproofing and have antimicrobial, antifungal, and antiviral properties that reduce the risk of pathogens in nests and thus may help to protect bee larvae [270, 273, 275].

5.2 Resin Glands

The structure of resin glands varies greatly between genera and even among species of the same genus. In *Dalechampia* (Euphorbiaceae), for example, parts of the bracts of the staminate inflorescence are modified in secretory structures, forming a resin-secreting gland [270]. These resin glands are laminar structures that have a palisade uniseriate epidermis, covered by a cuticle, and responsible for resin secretion in the inflorescence throughout the development of the pseudanthium [276, 277]. Unlike in *Dalechampia*, the floral resin in *Clusia* (Clusiaceae) is produced and stored in great quantity in secretory ducts, which exist in all vegetative and reproductive parts of the plants. Flowers with floral resin glands are marked by secretory resin ducts that are distributed abundantly on filaments and connectives. These resin ducts are either simple or branched, and some anatomical studies of *Clusia* have revealed that the terminal staminal ducts are externally covered by a single layer of epidermis, which breaks down during anthesis, releasing the resin on the surface of the filaments [278]. Rupture points on the connective surface associated with the subepidermal resin ducts were also observed in some species of *Clusia* [279, 280]. This is understood to be a very particular and complex case of a floral resin gland, since the resin is produced and stored in internal secretory structures and posteriorly released to the external surface during anthesis. In some *Clusia* species (such as *C. insignis* and *C. lanceolata*), the release of resin above staminodes, in female and some male flowers, prevents the mixing of pollen with resin, while the opposite occurs in those species that release the resin above the stamens (such as *C. burchellii* and *C. nigrolineata*) [281] (Fig. 17a–d). These slight differences and the role of the resin in the transfer of pollen from one flower to another are still under investigation.

Resin glands like those present in flowers of *Clusia* were also found in the spathe of *Philodendron* (Araceae) [13]. In this case, the secretory ducts form a complex anastomosed system, and the cells of the epithelium separate at the ends of the ducts, allowing the resin to leak out and accumulate inside intercellular spaces below the epidermis [13]. The release of the resin is by either rupture of the epidermis covering the duct or from stomata that coincide at the end of the duct. Although present in the inflorescence, the resin secretion is not related to the attraction of pollinators (here, scarab beetles); the resin is crucial for the pollination process, however, since it helps in the adhesion of the pollen grains to the body of the beetles [282, 283].

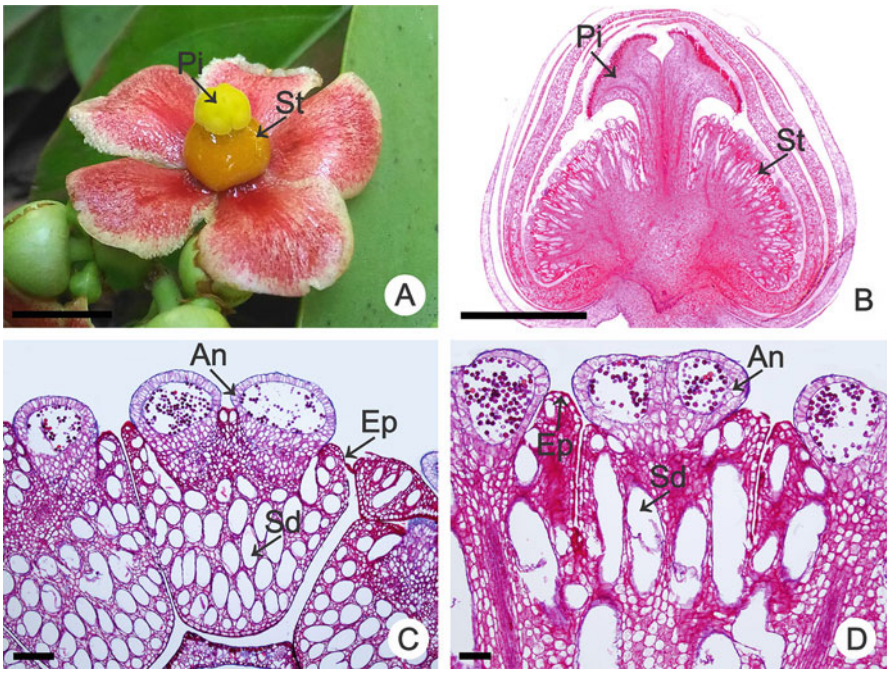

Fig. 17 Floral resin glands of *Clusia burchellii* (Clusiaceae). (**a**) Flower in vivo with stamens covered with resin. (**b**) Overview of longitudinal section of the flower. (**c**) Cross section of stamen showing secretory ducts in the connective. (**d**) Longitudinal section of stamen showing the secretory ducts ending in the subepidermal layer of the connective. *An* anther, *Ep* epidermis, *Pi* pistillode, *Sd* secretory duct, *St* stamen. Bars: (**a**, **b**) 5 mm, (**c**) 200 μm, (**d**) 100 μm. (Photos: Ana Cláudia Alencar)

Ultrastructural studies of floral resin glands are scarce; it has been shown, however, that the subcellular features of these glands are similar to those described for resin glands in general [10, 13, 279, 284]. The predominant organelles are plastids, mitochondria, and smooth endoplasmic reticulum, which are compatible with the synthesis of terpene resins [10]. The epithelial cells of some species of *Clusia* show all these characteristics and a prominent vacuole, sparse dictyosomes, and oil bodies inside plastids, small vacuoles, and vesicles free in the cytosol, depending on the composition of the resin and its secretory pathway [279] (Fig. 18). Plasmodesmata are not always present, and the production of vacuoles, in some cases, is observed only during the secretory period, like in *Philodendron* (Araceae) [13].

The mechanisms of resin release involve granulocrine and/or eccrine processes (Fig. 18) [10, 11, 13, 56, 279]. Oil droplets and resin components can easily cross the plasma membrane and cell walls, characterizing the eccrine process of secretion, while some vesicles may fuse to the plasma membrane allowing some secretory components to exit the cells through exocytosis, characterizing the secretory process as granulocrine [279].

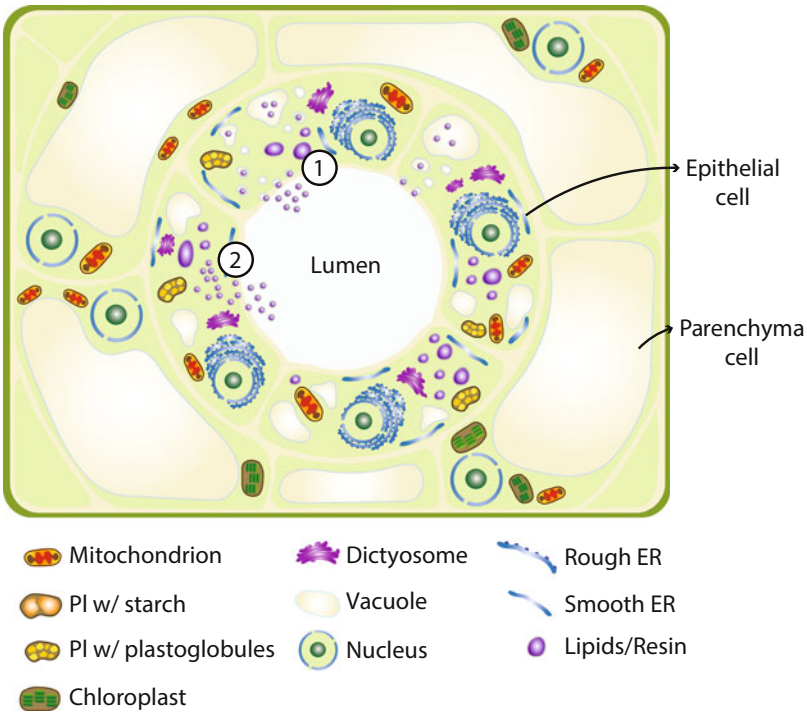

Fig. 18 Resin production, storage, and secretion. (**a**) Epithelial cells secreting resin into the lumen. The secretion of resin may be released through (1) granulocrine or (2) eccrine mechanism

5.3 Floral Resin: Characteristics and Histochemistry

Resins usually have amber coloration, but their composition may determine different colors or even its total absence [269]. They are usually are composed of a diverse mixture of terpenes, phenolic compounds, and several other classes of compounds like polysaccharides and fatty acids, and there is variation in their composition between groups of plants [269]. In addition, in *Dalechampia* (Euphorbiaceae), fatty acids are found aside from terpenoids and phenolic compounds [273, 276, 277]. Detailed chemical studies have only been performed in *Clusia* and *Chrysochlamys* of Clusiaceae, where the main constituents identified were benzophenone derivatives, as 7-epi-nemorosone [281, 285]. Other studies have identified terpenoids, lipids, mucilage, proteins, and phenolic compounds as resin components [269, 273, 279]. Despite the variation in the composition of resins, the main components in all the hitherto investigated groups of plants are terpenes.

5.4 The Evolution of Floral Resin Glands Related to Pollination

Resin glands that serve in support of pollination, until now, have only been documented for two orders of angiosperms, Alismatales in monocots and Malpighiales in core eudicots. Evolutionary studies focusing on the floral resin glands of Araceae (monocots) are lacking; however, Armbruster [273] suggested some hypotheses about how this evolution occurred in Malpighiales. The first hypothesis presumes that the resins in *Dalechampia* (Euphorbiaceae) and *Clusia* (Clusiaceae) may have originated as a system of defense against insect attacks. That author also hypothesizes that bees may have been attracted to resiniferous flowers (or inflorescences) by chance and afterward, through coevolution, the flowers increased the amount of resin secreted concomitantly with the raise of resin utilization by bees. Finally, he also suggests that the resin has originated suddenly, without a previous function.

Phylogenetic studies of *Dalechampia* have lead to the hypothesis that early divergent species of the genus were pollinated by male euglossine bees, which collected fragrances, or by bees that collected pollen [286]. The shift from a pollen or fragrance reward to resin may have occurred because those species apparently produced resin through both the sepals of the pistillate flower and the protective bracts of the male inflorescence; thus the resin secretion in *Dalechampia* most likely originated as a mechanism of defense and, secondarily, took on a reward function [286]. In contrast, the presence of isoprenylated benzophenones in the resin and the exudate present in the secretory ducts of all other vegetative organs of Clusia suggests that this resin has the same composition or the composition is very similar in the different organs and that both secretory ducts have defense functions in the plant. Later, bees started to inflict injuries to the plant to collect the exudate for use in the construction of their nests. Therefore, the reward feature of flowers to pollinators may have arisen from wounded flowers and, consequently, leading to the reproductive success of some species [273, 287, 288].

6 Conclusions

Angiosperm flowers reached an extraordinary degree of morphological diversity in the different plant lineages whose evolution may have occurred in synchrony with the evolution of pollinators or may have been subject to adaptive factors through time. In order to better understand the interrelationships of floral evolution, one will need to consider the various morphological modifications and developmental constraints in the particular phylogenetic contexts in which flowers originated. Regardless of the factors involved in this diversification, the mechanisms of attraction and reward of pollinators played a key role in ensuring the species' perpetuation and reproductive success in different environments. The floral glands are fundamental in these two processes. Although the types of floral glands are different depending on the group, their structure, ontogenesis, and secretory activity are directly related

to the floral development and the moment of pollen release and stigma receptivity. This synchrony is even more remarkable when we consider that some species have diversified their floral resources and may have two or more types of floral glands, increasing the possibilities of crossbreeding by different groups of pollinators. These factors associated with the existence of internal glands, such as the resin ducts of *Clusia*, which release their secretion on the surface of the androecium during anthesis, demonstrate the high degree of specialization some flowers reached during plant evolution and how complex the analysis of pollination biology can be.

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Sugar and Polyphenolic Diversity in Floral Nectar of Cherry

29

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Contents

Abstract

Cherries (*Prunus avium* L. and *Prunus cerasus* L.) are economically important fruit species in the temperate region. Both are entomophilous fruit species, thus need pollinators to give high yields. Since cherry's flower is easy-to-reach, bees and other pollinators can smoothly collect nectar as a reward for doing transfer of pollen to receptive stigma. Nectar in cherry is usually attractive for insects,

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especially to honey bee (*Apis mellifera*) who is the most common pollinator. Nectar is predominantly an aqueous solution of sugars, proteins, and free amino acids among which sugars are the most dominant. Trace amounts of lipids, organic acids, iridoid glycosides, minerals, vitamins, alkaloids, plant hormones, non-protein amino, terpenoids, glucosinolates, and cardenolides can be found in nectar too. Cherry flower may secrete nectar for 2–4 days and, depending on the cultivar, produces up to 10 mg nectar with sugar concentration from 28% to 55%. Detailed chemical analysis of cherry nectar described in this chapter is focused on sugar and phenolic profile in sour cherry. The most abounded sugars in cherry nectar was fructose, glucose, and sucrose, while arabinose, rhamnose, maltose, isomaltose, trehalose, gentiobiose, turanose, panose, melezitose, maltotriose, isomaltotriose, as well as the sugar alcohols glycerol, erythritol, arabitol, galactitol, and mannitol are present as minor constituents. Regarding polyphenolics, rutin was the most abundant phenolic compound followed by naringenin and chrysin. Cherry cultivars showed different chemical composition of nectar which implies that its content is cultivar dependent.

Keywords
Prunus avium L. · *Prunus cerasus* L. · Flower · LC/MS · HPAEC · Polyphenolic profile · Sugars

1 Introduction

Cherry is the common name of several species of the genus *Prunus* originated from the common ancestor in area between the Black Sea and Caspian Sea in Asia Minor [1]. Among cherries, the sweet cherry, sour cherry, flowering ornamental cherry species, and a few other *Prunus* species used as rootstocks for cherries are considered important [2]. Cherries are members of the *Rosaceae* family, *Prunoideae* subfamily, and genus *Prunus* and are further placed within two subgenera *Cerasus* Pers. and *Padus* (Moench) Koehne [3]. The *Cerasus* Pers. subgenus and *Cerasus* Koehne section contain the diploid sweet cherry ($2n = 2x = 16$), and the tetraploid ($2n = 4x = 32$) sour cherry and ground cherry.

Cherries are one of the oldest fruit crops known to mankind. It is believed that Theophrastus has mentioned cherries roughly 300 years BC [4]. Another earlier writing suggests that Lucullus brought cherries back to Italy when he returned from the Pontu region (in present day Turkey). Archaeologists have discovered fossilized cherry pits in Stone Age caves and dwellings of western Switzerland, Bourget (France), and Parma (Italy) [5] that places cherry into the Neolithic Period (about 4000–5000 years ago).

Both sweet and sour cherry production, as the most economically important among cherries, has increased significantly during the past two decades in the traditional leading cherry-producing countries. The annual global sweet cherry production (average 2014–2016) is about 1.7 million tons and shows a slightly increasing tendency. The leading sweet cherry-producing country is Turkey,

followed by the USA, Iran, Italy, Spain, Chile, and Ukraine. Sour cherry is often called the fruit species of Eastern Europe because the most important producing countries are located in this part of the world. Global production is about 1.3 million tons (average 2014–2016). In countries where there is a keen interest in sour cherry-based products, such as the eastern European countries, production is usually machine harvested and is increasing slightly. The world's leading sour cherry-producing country is Turkey, followed by the Russian Federation, Poland, Ukraine, Iran, the USA, Serbia, and Hungary [6].

Cherries are a deciduous fruit tree, having an attractive appearance during bloom time. The cherry fruit is a nutrient dense food with relatively low caloric content and significant amounts of important nutrients and bioactive food components including fiber, polyphenols, carotenoids, vitamin C, and potassium [7]. Sweet and sour (syn. tart) cherry ripen first among stone fruits, followed by apricot, peach, and plum. Because sweet cherry is first on the fresh market, it is in high demand in the late spring and early summer. The majority of sweet cherries are consumed fresh with the remaining 20–25% processed as brined, canned, frozen, dried, or juiced. In contrast, 97% of tart cherries are processed primarily for cooking and baking and the confectionary industries [7, 8].

Pollination is a crucial part of growing quality cherries because most of the cultivated varieties of sweet cherry are self-incompatible. To set fruits, they require pollen from suitable pollinating cultivars. Thus for the commercial production of sweet cherry, a good orchard design, with enough pollinizers have to be planted [9]. Besides, pollinating insects should be present for adequate transfer of compatible pollen to the stigma. Among sour cherry cultivars, there are more and more self-compatible ones; however, foreign pollination can improve quality even at these cultivars.

According to recent research, cherry flowers are very attractive to various insects [10]. They observed activity of 31 species of insects belonging to 5 orders and 13 families of class. Honey bees (*Apis mellifera*) have been assumed to be the main pollinators in cherry [11], due to their high demand for pollen and nectar and their hairy body, which collects and disperses the pollen [12–16]. However, honey bee is not active on temperature below 12 °C or in rainy weather conditions. In that case, pollination can be also successful since other insect species belonging to the *Bombus*, *Andrena*, and *Osmia* spp. could maintain their activities on lower temperatures and during rainy days [17]. Landscapes with wild bee habitats enhance pollination, fruit set, and yield of sweet cherry, presumably due to their higher pollination efficiency [18]. Therefore, it is very important to attract honey and wild bees to proper pollination of these crops, especially commercial crop production.

2 Role of Nectar

Plant species that depend on insect (or other animal) pollinators for their reproduction have put lots of effort in many floral traits such as floral display, flower architecture, color, scent, and nectar [19]. To attract pollinators, plants offer different types of rewards, where floral nectar represents the main plant reward for many

pollinators [20]. Floral nectar composition, its quality, and chemical and physical features varies widely between species and type of nectary and most probably are related to different consumers and ecological factors (abiotic and biotic).

Flowers often have specialized structures that make the nectar accessible only for animals possessing appropriate morphological structures, and there are numerous examples of coevolution between nectarivores and the flowers they pollinate. The main function of nectar compounds is related to the attraction of pollinating insects. It is well-known that honey bee chemoreceptor can detect volatile substances, contained in the nectar of crop plants at distance of about 2 km [21]. In this way, pollinators are unintentionally mediating the transfer of pollen to receptive stigma, becoming a key attribute for increasing cross-pollination [22, 23]. Although floral nectar production represents a high cost for the plant, it ensures a higher possibility of fruit/seed set, higher reproductive success and gene transfer into next generation. The production of nectar (when starch granules in the parenchyma are broken down) often peaks when anthers start to shade pollen and when the stigma is the most receptive. Generally flowers secreting more nectar show more successful pollination events [24].

It is proved that secreted nectar volume correlates with flower size, which is probably due to the pleiotropic effects, where larger flowers have larger nectaries and more space for nectar [25, 26]. The amount of nectar reward is positively correlated with the number of pollinator visits, the number of flowers visited within a plant, and the duration of the visit within a flower [27]. Generally, energy received from nectar per insect (or other pollinator) must be enough to attract pollinators, but still need to encourage movement of the pollinator from flower/plant to another one. This means that nectar volume is correlating with the body size of the pollinator [28].

The attractiveness of nectar to pollinators depends on taste [29], but odor and color play an important role too [30, 31]. Characteristics such as volume, concentration, color, and taste may be related to the concentration and composition of dissolved sugar (especially glucose, fructose, and sucrose). But also other components, including minerals, phenolic compounds, and amino acids, may make a cardinal contribution to its attractiveness to honey bees [22, 32–34]. Bees prefer bright flowers, while visual and chemical associations are pushing it to navigate within the field [35].

Nectar concentration is highly influenced by geographical distribution, thus environmental factors, especially light, water, nutrients, CO_2 concentration, temperature, humidity, soil moisture, and wind [36]. Besides, nectar composition can vary between the two sexual phases of a given hermaphrodite flower [37], phenology phase, among flowers on different plants [38] and individuals, populations, cultivars, or subspecies of the same species [39]. Physiological factors such as flower age, health of plants, and damage to floral parts also affect the quality and composition of nectar.

3 Nectar Productions in Cherries

Cherry flowers are allogamous, actinomorphic, and are arranged in racemose clusters of 2–5 flowers. The sweet and sour cherry flowers are from 2.5 to 4.0 cm in

diameter, white, hermaphroditic, and are attractive for pollinators [40]. The cherry flower structure is usually characterized by stamina standing far from the pistil, thus insects can touch the stigma only during nectar collection, passing along the pistil [41]. In cherries like in most of the temperate fruit trees, sieve tubes more or less directly supply secretory parenchyma cells called the "nectariferous tissue" with pre-nectar prior to nectar secretion. The nectary is receptacular, covering the whole surface of the receptacle [42].

For the protogyn, sour cherry varieties is very important to know the periodicity of nectar production and the synchronization of the endogen rhythm with stigma receptivity and anther dehiscence. As it was stated in [43], dichogam flowers produce nectar periodically by 12th hours, the homogam ones by 6th hours, and the time of maximum production is synchronized by the stigma receptivity and anther dehiscence. In the hybrids of sweet and sour cherries, 3-h gaps can be observed in nectar production [43]. Additionally, the change of pollination strategy for the protogyn sour varieties: (i) stigma exerted – wind pollination, (ii) state of pollination chamber – beetle pollination, (iii) opening of anthers – pollination by bees and other insects was observed [44].

A sour cherry flower may secrete nectar for 2–4 days and, depending on the cultivar, produces 0.2–9.0 mg nectar. Generally, autochthonous landraces like "Cigánymeggy" or "Oblačinska sour cherry" type yield less but more concentrated nectar, with sugar values of 0.1–1.8 mg/flower/day, while cultivated varieties produce more but rather dilute nectar [45]. Among sour cherry cultivars, "Meteor korai" and "Debreceni bötermö" are one of the best nectar producers giving 10.27 μl and 7.21 μl of nectar respectively; with 13.96% and 16.6% of sugar, respectively [46].

The nectar of early blooming fruit trees such are cherries is important for honeybees in the brood rearing season, but rarely can provide unifloral honey, as well [45]. Sweet cherry blossom is more attractive for bees than sour cherry blossoms primarily because the nectar of sweet cherries is much richer in sugar (55%) than that of sour cherries (28%). But, sour cherry cultivars produce a significant amount of nectar at night [47], thus attracting night insects.

If a successful fertilization should be achieved even at self-incompatible cherry and sour cherry cultivars, all details of their pollination biology should be known, including the sugar and polyphenolic composition of nectar, as ones of the primary attractants [46, 48].

4 Nectar Compositions

The number of papers related to the examination of the chemical composition of the floral nectar is not large, although it is much more available for floral nectars than for extrafloral nectars. Mainly studies have focused on the qualitative aspect. The main components of the nectar, sugars and amino acids, were the most examined, while other solutes were not subjected to the research to that extent. This is rationale since the nectar is predominantly an aqueous solution of sugars. Also, sampling is not easy considering the duration of secretion (few hours to several days), and the amount of

nectar produced (less than 1 μL to few ml proportional to the nectary parenchyma volume).

Sugars, proteins, and free amino acids are the three major components of floral nectar among which sugars are the most dominant [49]. Nectar is highly variable at any taxonomic scale indicating that plant phylogeny can be a stronger determinant of nectar composition [50]. But, pollinator type can also shape the composition of nectar because different pollinators show preferences for solutions of different viscosity and/or sugar composition [51]. In general, insect pollinated flowers, like in cherry, produce relatively concentrated nectar.

Secondary metabolites and volatile compounds in flower nectar are appearing in low level. Compounds belonging to the secondary metabolism are either synthesized in the nectaries themselves or can also be derived directly from the phloem. They can have a range of effects on pollinator preference and performance, from fully negative to positive. More often, these compounds are usually regarded as "toxic compounds" and are involved in antimicrobial defensive functions, protection from nectar robbers, and pollinator attraction [29, 52]. On the other hand, secondary metabolites can significantly stimulate bees to feed, while indirectly, pollinators can have benefits from them by reducing gut pathogen loads [53, 54].

The phenolic compounds in nectar have several roles in attraction and/or repelling honey bees (phenolic compounds can give an astringent taste, thus inhibiting herbivores) in nutrition of pollinators, in oxidation prevention of other nectar substances, and in providing an aggregate value to honey commercialization by the certification of the botanical origin [29, 55]. In some cases, nectar constituents may also help defend the flower against invaders, which allows flower to promote out crossing and achieve its ultimate goal, and that is to set a fruit/seed [52]. Besides polyphenolic compounds, some amount of abscisic acid (ABA) can be found. The role of this plant hormone is the protection of plants in conditions of environmental stress, especially in reducing the penetration of UV-B ultraviolet radiation [56]. Also, jasmonic acid, its precursors and its derivatives, have been identified as a hormone that affects the secretion of floral nectar and defense responses [57, 58].

Volatile compounds, important cues that help insects locate flowers, mediate plant response to pathogen infection, plant-parasitoid signaling in response to herbivory, and plant-pollinator communication during flowering. Most of the floral fragrance compounds are terpenoids (most common monoterpenes), simple aromatics, amines, and hydrocarbons [59].

Amino acids are contributing to the taste of nectar and are important source of nutrients for animals, especially for those that are exclusively dependent on nectar for their nutrition, such as butterflies [60]. As it was stated in [34], phenylalanine is the most abounded one in nectar (which generally has a strong phagostimulatory effect on honeybees), followed by tyrosine, threonine, histidine, and aspartic acid. Also, it seems that some amino acids, like asparagines, are avoided by all guilds and bee families, while glycine-threonine, H-serine, serine, β-alanine, valine, leucine are bypassed by most bee families. Besides, some level of non-protein amino acids can be detected in nectar. Those compounds can be toxic and found in seeds which serve as deterrents to insect feeding. However, β-alanine, ornithine, homoserine, and γ-

aminobutyric acid (GABA) are also accumulated in nectar but are non-toxic [49]. According to the mineral analysis of nectar ion composition, concentration of K^+ is the highest, following by Na^+. Some levels of Ca^{2+} and Mg^{2+} have been also detected. According to [61], potassium and sodium chloride deter honey bees. Proteins/enzymes, or so called "nectarin" in floral nectar includes invertase, transglucosidase, transfructosidase, phosphatase, tyrosinase, alliinase, nectarin, I-superoxide dismutase, and others, playing important role in hydrolysis of sucrose, polymerization of glucose and fructose molecules, possibly defense and many more. Trace amounts of lipids, organic acids, iridoid glycosides (catalpol), vitamins, alkaloids (anabasine, gelsemine, nicotine, and caffeine), terpenoids (thymol), glucosinolates, and cardenolides can be found in nectar too.

Recently, a gene that encodes an apoplastic invertase of Arabidopsis has been discovered. This gene represents the first gene whose function is required for floral nectar secretion [62].

Chemical screening is usually done by standard chromatographic techniques hyphenated to spectral methods. New technologies and advanced techniques conquer difficulties in analyzing small fluid volumes, enabling more detail identification and quantification of nectar components.

Most of the individual studies on nectaries, nectar, and nectar consumers were included in a comprehensive book review [63]. Cherry nectar properties and chemistry were not examined to a great extent, and just a few papers discussing the composition of sour cherry floral nectar were published so far. As for the sweet cherry, no available data could be found. Therefore, presented results on cherry nectar included in this chapter rely on just a few published papers [46, 64] where nectar sugar profiles of sour cherry cultivars were reported. Most of the data on phenolics were drawn from the study carried out on "Oblačinska" sour cherry clones [64].

4.1 Nectar Carbohydrate Profile

Nectar carbohydrate profile is prevailed by three sugars, the disaccharide sucrose and its monosaccharide units, fructose, and glucose. Nectar components are believed to originate from phloem sap that is enzymatically processed and transformed within nectaries [65]. Since the phloem sap contains mostly sucrose, chemical reactions must occur to produce glucose and fructose in the nectar. The relative amounts of each are determined by hydrolyses of sucrose catalyzed by transglucosidases and transfructosidases localized in the nectaries which occur before or during nectar secretion [66].

The total sugar concentration in floral nectar can range from 5% (w/v) to 80% (w/v) [67] and may differ among individuals, populations, cultivars, or subspecies of the same species [38, 39, 68, 69]. Also, amounts and relative concentrations of the major constituents, glucose, fructose, and sucrose may vary among species from almost all sucrose to all hexose. According to [61], sucrose, maltose, glucose, fructose, trehalose, and melezitose are sweet for bees; while lactose, melibiose, raffinose, xylose,

and arabinose are tasteless; mannose and galactose are toxic to bees; where, gentiobiose and cellobiose and repellent to bees.

The nectar composition can vary greatly depending on plant species and environmental conditions [38], as well as on floral sexual phases [70], and flower position within inflorescences [71]. According to [72], between-plant variability of nectar sugar composition can be due to a casual selection of flowers of different ages, because in some cases, sucrose breakdown in nectar can be related to flower age. But this cannot be applied on the results of the recent investigation reported on sour cherry [64] were the flowers were in the same phenophase code [65], BBCH scale [73]. On the other hand, some authors considered nectar sugar composition as it is conservative taxonomic character [26, 74].

So far, sugar composition of sour cherry nectar was only explored by the two research groups. One research group was investigating sour cherry cultivars in Újfehértó, in the eastern Hungary in the period 1997–2000 [41, 46, 47]. The following sour cherry cultivars were examined: "Újfehértói fürtös," "Pándy 48," "Érdi jubileum," "Meteor USA," "Montmorency," "Debreceni bőtermő," "Nefris," "Sárándi S/Gy," "Korai pipacs," "Mej Djuk," "Kőrösi korai," "Érdi nagygyümölcsű," "Kántorjánosi 3," "Oblacsinszka," "Érdi bőtermő," "Cigány 404." Three sugar components (glucose, fructose, and sucrose) were determined by thin layer chromatography and quantitative evaluation was carried out by densitometry (CAMAG TLC Scanner II). The cultivars "Újfehértói fürtös," "Pándy 48," "Érdi jubileum," and "Érdi bőtermő" yielded nectar with high sucrose content in each season, even under varying climatic conditions, and are valued from an apicultural point of view [46].

Subsequently, in order to determine the floral insect attraction, the floral secretory product of the two cultivars, an autofertile cultivar (":Újfehértói fürtös") and an autosterile cultivar ("Pándy 48") were studied [41]. The nectar of both studied cultivars contained all three major sugar components: sucrose, glucose, and fructose. The nectar sugar composition of "Újfehértói fürtös" varied to a great extent according to the seasons. The phenomenon was explained by the great fluctuation in air temperatures, which influenced the sugar production of the cultivar to a great degree. "Pándy 48" yielded nectar with quite stable concentration and composition in the studied four seasons.

The other research group studied nectar of the most planted sour cherry cultivar in Serbian orchards, "Oblačinska" sour cherry, an autochthonous cultivar [64]. Investigation included 16 nectar samples of "Oblačinska" sour cherry clones. Both the content of sugars and sugar alcohols were studied using high performance anion exchange chromatography with pulsed amperometric detection (HPAEC/PAD). Carbohydrates were separated on a CarboPac® PA10 pellicular anion-exchange column. Total of 14 sugars and 6 sugar alcohols were determined, showing great variability in carbohydrate profiles among studied genotypes. Nectars from several sour cherry clones stood out based on the notably different concentrations of the individual sugars and sugar alcohols. Such an unequaled nectar composition was related to the assumption that Oblačinska sour cherry is not a cultivar but mixture of many different genotypes.

As reported in [41], during 4-year study sucrose level in cultivars „Újfehértói fürtös "and „Pándy 48 "ranged from 18–59 mg/mL and from 27–59 mg/mL,

respectively. As expected, fructose, glucose, and sucrose were found to be the major constituents of all investigated "Oblačinska" sour cherry nectar samples [64]. Based on the total sugar content found in nectar, certain clones have been singled out as the most concentrated (up to 97.6 mg/mL), while the others had dilute nectars (23 mg/mL of sugars). Averagely fructose, glucose, and sucrose amounted 36.8%, 28.9%, and 30.9% of the total content of all carbohydrates, respectively, and this was in line with the other results [47]. The ranking based on fructose content takes into consideration human sensation of taste. Also, fructose in the concentration range from 15 mg/mL to 60 mg/mL in 14 sour cherry cultivars was reported [41], while in "Oblačinska" sour cherry nectars fructose content was up to 34 mg/mL [64].

On the basis of the sucrose/(glucose + fructose) quotient [20], the nectar of "Újfehértói fürtös" belonged to the sucrose-rich group each year, like the majority of sour cherry cultivars, whereas the secretory product of "Pándy 48" could be classified into the sucrose-dominant category in one of the seasons [46]. According to the proposed quotient, one "Oblačinska" sour cherry clone was hexose dominant [$S/(G + F) < 0.1$], four clones were hexose rich [$S/(G + F) = 0.1–0.49$], while other 11 were sucrose rich [$S/(G + F) = 0.5–0.99$] [64]. These results are in accordance with some previous results [46]. Proportions of sucrose over fructose and glucose have been linked with different classes of pollinators and found to be important in plant-mutualism interactions [75].

The sucrose-dominant nectar composition of 45 species belonging to tribe Sinningieae (*Gesneriaceae*) was also documented [76]. Several authors have suggested that sucrose-rich nectar is mostly found in flowers pollinated by insects with long mouth parts, whereas hexose-rich nectar has been found in flowers pollinated by short-tonged insects [20, 32, 77–79]. On contrary, an analysis in *Antirrhinum* and *Lycium* has revealed constant sugar composition despite a large variety of pollinators [78, 80].

According to [64], other carbohydrates, including the monosaccharides (arabinose and rhamnose), the disaccharides (maltose, isomaltose, trehalose, gentiobiose, turanose), the trisaccharides (panose, melezitose, maltotriose, isomaltotriose), as well as the sugar alcohols (glycerol, erythritol, arabitol, galactitol, and mannitol) were present as minor constituents in sour cherry nectars. The presence of mannitol, melezitose, panose, and maltotriose was not confirmed in some of the studied nectars. Minor sugars identified in nectars of some flowers, such as arabinose, galactose, mannose, gentiobiose, lactose, maltose, melibiose, trehalose, melezitose, raffinose, and stachyose can be toxic to potential pollinators [68, 81–84].

Among minor constituents, isomaltose, maltose, and sorbitol were the dominant in comparison to other components. For the sorbitol (a polyol with low molecular weight, highly soluble, and non-reducing compounds), results were fully expected, because this sugar alcohol is the main photosynthetic product and the primary translocated carbohydrate in *Rosaceae* [85]. Although has no influence to insects' preference, sorbitol is a frequent constituent of Mediterranean nectars [22]. Also, if it can be found in the fruit, it improves the sweet taste and texture of the mesocarp [86]. But also, its accumulation is considered as an adaptive response of plants to drought, salinity, or chilling stress [87].

Maltose is pretty rare or absent in nectars, and although it tastes sweet to honeybees, it is usually less attractive for them than sucrose [49, 88]. Earlier it was believed that maltose is synthesized in nectar itself [89], but its presence in nectar is due to the fact that maltose is a degradation product of starch (obtained from chloroplasts during starch degradation in night), while nectar secretion in flower starts with starch degradation [90]. Also, maltose is a major product of catabolism of starch in guard cells which can be found within the flower [91]. In regard to disaccharide isomaltose, it is more related to honeydew then to nectar, so that is the reason why honeydew honeys had significantly higher mean values of this sugar than the blossom honeys. In unifloral cherry honey, the concentration of the isomaltose was around 0.80% [92].

4.2 Phenolic Compounds in Cherry Nectars

Phenolic compounds are widespread natural constituents and their main function is to protect plants against various biotic and abiotic stresses. Their multiple roles in floral nectars and the relationship with pollinators were outlined in the literature [28]. Mainly, phenolics are associated with functions such as attracting pollinators or repelling nectar thieves, maintaining nectar in a microbe-free state, being important components of floral scents. Their role in cherry pollination could be the same, although yet not proved. Some phenolic compounds together with some other constituents may accumulate in floral nectar due to passive absorption by the nectar [30].

Although numerous flavonoids have been described in literature, their presence in floral nectar was not studied extensively. The same applies for the composition of cherry nectar topic. Often phenolic composition was reported only qualitatively [93], where floral nectar chemical compositions of 29 species native to Argentinian Patagonia and phenolic composition measured on qualitative scale were reported. A scarce number of papers show that nectar phenolic profile is characterized both by various phenolic aglycones and their derivatives. In rosemary nectar, kaemferol-3-sophoroside and quercetin-3-sophoroside were identified as the most abundant among 15 different flavonoids [94], while in Portuguese heather nectar (*Erica* sp.) flavonol aglycones quercetin, kaempferol, myricetin, and isorhamnetin were identified [95]. The occurrence of flavonols in higher plants was associated with lignification in cell walls and with UV absorption of flowers, as nectar guide [96]. Also, functional roles of flavonols as developmental regulators and/or signaling molecules in plants were discussed [97].

Although nectar composition of various sour cherry cultivars was examined [46], studies on floral nectar in terms of detailed phenolic characterization were not performed until the investigation of phenolic diversity in floral nectar of different "Oblačinska" sour cherry clones [64]. The phenolic complexity of sour cherry nectar was apparent and the qualitative phenolic profile was shown to be characterized mostly with flavonol glycosides. All identified glycosides were derivatives of kaempferol, quercetin, and isorhamnetin [64] (Table 1).

Table 1 Quantification of flavonol glycosides identified in floral nectars of "Oblačinska" sour cherry clones. The relative content of flavonol glycosides (in this table) was expressed as rutin equivalents (RE) per mL of nectar (μg RE/mL)

Name of identified compound	Relative content (μg RE/mL)
Kaempferol 3-*O*-(2″-*O*-hexosyl)hexoside-7-*O*-rhamnoside 1	0.002–0.197
Quercetin 3,7-di-*O*-hexoside	0.002–0.029
Quercetin 3-*O*-(2″-*O*-hexosyl)hexoside-7-*O*-rhamnoside 1	0.022–1.465
Isorhamnetin 3-*O*-(2″-*O*-hexosyl)hexoside 1	3.285–4.066
Quercetin 3-*O*-(2″-*O*-hexosyl)hexoside	0.001–0.245
Kaempferol 3-*O*-(2″-*O*-hexosyl)hexoside-7-*O*-rhamnoside 2	0.002–0.170
Isorhamnetin 3-*O*-(2″-*O*-hexosyl)hexoside 2	0.089–6.247
Quercetin 3-*O*-(2″-*O*-hexosyl)hexoside-7-*O*-rhamnoside 2	0.001–0.038
Quercetin 3-*O*-hexoside	0.002–0.225
Kaempferol 3-*O*-(6″-*O*-rhamnosyl)hexoside	0.024–6.084
Isorhamnetin 3-*O*-(6″-*O*-rhamnosyl)hexoside	0.067–6.104
Quercetin 3-*O*-pentoside 1	0.001–0.022
Kaempferol 3-*O*-hexoside	0.001–1.382
Isorhamnetin 3-*O*-hexoside	0.001–1.639
Kaempferol 3-*O*-(6″-*O*-acetyl)hexoside	0.001–0.177
Isorhamnetin 3-*O*-(6″-*O*-acetyl)hexoside	0.004–2.331
Quercetin 3-*O*-pentoside 2	0.001–0.503

Further, sour cherry nectar phenolic profile was characterized with the presence of rutin, pinocembrin, and galangin, detected in all nectar samples, while gallic acid, hesperetin, and naringin were found in some samples. In earlier work, rutin was shown to act as a feeding stimulant for some insects [98]. Also, recently was proved that rutin has the highest antimicrobial (especially antibacterial) activity in honey [99] so there is a possibility that its function in nectar is the same. Pinobanksin, naringenin, and chrysin were detected in variable amounts in sour cherry nectar. Table 2 shows the content of phenolic compounds (average values). Naringenin plays an important role in plant development and it was reported to show bactericidal and/or bacteriostatic activity [100], and antimicrobial effects against yeasts [101], but shows low activity as feeding stimulant in insect-plant interaction [102]. Moreover, naringenin influenced bee foraging behavior as deterrent [103], but no relationship could be underlined between its level and yield efficiency (yield per trunk cross sectional area), of "Oblačisnka sour cherry" clones that were studied by. As a matter of fact, group of clones that stored high content of nagingenin is showing all kind of yield effectiveness, which stands the same for the group of clones with very low level of this flavanone [64, 104].

The positive influence of abscisic acid in nectar to the immune response of worker honeybees and larvae after being parasitized with *Varroa destructor* was described previously [105]. In plants abscisic acid regulates fundamental physiological functions and accumulates in response to different environmental stresses [106, 107] and can be found in phloem and xylem sap and in nectar [95, 108]. In honey, this

Table 2 Quantification of phenolic acids and flavonoids in nectars of "Oblačinska" sour cherry clones (μg/mL)

Compound name	Content (μg/mL)
Gallic acid	0.005–0.010
Caffeic acid	0.003–0.015
Rutin	0.096–6.472
Naringin	0.026–0.092
(−)-*cis*,*trans*-abscisic acid	0.005–0.331
Naringenin	0.009–4.076
Pinobanksin	0.005–0.128
Hesperetin	0.002–0.006
Chrysin	0.030–1.597
Pinocembrin	0.010–0.764
Galangin	0.014–0.719

phytohormone comes mainly from nectar [109]. The content of this phytohormone in "Oblačinska" sour cherry clones varied from 0.005 to 0.331 μg/mL [64].

Regardless of the similar chemical structure, only certain flavonoids are capable to absorb light in the visible region of spectra, thus rendering color. Flavone glycosides and flavonol glycosides absorb near 350 nm, but their role in the floral pigmentation is not predominant, as they are weakly colored. Usually flavonoids accompany carotenoids which are dominant in yellow pigmentation. The early work on the flower *Rudbeckia hirta* indicated flavonols as pigments responsible for ultraviolet absorption in nectar guides for bees and other insects, and it was the first interpretation of ultraviolet absorption in a nectar guide in chemical terms [96]. Due to chemical modifications at the C-8 and C-6 position on A-ring, flavonols become yellow hydroxyflavonols [110]. Also, *O*-glycosylation at the 7,4′-positions or *O*-methylation at the 3′- or 3′,5′-positions may contribute to the yellow color [110]. Also, other authors reported flavonols importance for nectar guides, such in [111] who isolated the pigment from the petals of *Brassica rapa* and identified it on the basis of MS and NMR spectroscopic data as isorhamnetin 3,7-*O*-di-beta-D-glucopyranoside.

Although some species use colored nectar as a signal for pollinators [112, 113], we assume that this could not be the case with the nectar of the "Oblačinska" sour cherry. However, based on identified phenolic compounds, certain conclusions can be made. Namely, the presence of various flavonols in nectar of "Oblačinska" sour cherry could be the reason for its pale yellow color. Several derivatives with *O*-glycosylation at 7-position were identified (Table 1). Also, isorhamnetin which is 3′-methoxylated derivative of quercetin was typical flavonol in all nectars. Of all the quantified flavonols, the largest amount of izorhamnetin 3-*O*-(2″-*O*-hexosyl) hexoside 2 was found in nectars along with rutin and therefore this specific compound could be the one that contributes to the nectar color the most.

Finally, comparison of polyphenolic profiles of "Oblačinska" sour cherry fruits [114] and nectar of the same sour cherry clones revealed some disagreements. The fruit clones stored some of the phenolics not found in the corresponding nectar, such as gallic acid, naringin, and naringenin. The opposite was found for hesperetin,

where some quantity of this flavanone was found in nectar clones but not in the fruits. Finally, rutin was one of the most abundant compounds determined in the fruits of the same "Oblačinska" sour cherry clones and its content was highest in clone II/2, in both nectar and fruit [64, 114]. As it was stressed out, no matter that floral nectar is secreted through intrafloral nectaries as a phloem solution [115] and cherry fruit is formed form ovary within the flower, it seems that those two processes are quite different and fully independent. In fact, this result is expected, because deciduous fruit trees, to which sour cherry belongs, have accumulated necessary minerals and organic compounds by the end of the previous growing season and use these reserve nutrients to support initial growth and development in the following spring. Thus, during flowering time (when leaves are just started to expand and are without photosynthetic competence), reproductive development is under total reliance on reserves stored within the tree [116]. On the contrary, during sour cherry fruit development (which occurs ≈55 days from pollination to fully ripe fruit), leaves are fully developed and are having the main role as the main source of photo assimilates [117].

5 Conclusion

Except few studies, not much was done in the analysis of cherry nectar. Sweet cherry was not an object of any study so far, so the results of this chapter are based on sour cherry nectar. According to the chemical analysis of our model plant's ("Oblačinska sour cherry") floral nectar, it can be concluded that selected clones of this cultivar showed different sugar and polyphenolic profile, where constituents showed big variation. In sugars, fructose, glucose, and sucrose were the most abounded, while arabitol, rhamnose, arabinose, turanose, gentiobiose, panose, melezitose, and matotriose, together with galactitol and mannitol, were in minor quantities. Regarding polyphenols rutin, naringenin and chrysin were found in the highest levels. Only rutin, pinocembrin, and galangin, together with ( )-cis, trans-ABA were detected in all nectar samples. Probably the cause of unequaled nectar composition (both for polyphenolics and sugars) in sour cherry is its hybrid origin (segmental allotetraploid between *Prunus cerasus* and *Prunus fruticosa*) and unstable inheritance.

In the future, nectar chemical composition, could be a breeding aim for creating a cultivar that will attract pollinators the most, and thus ensuring high yields, or have components that can protect plant from economically important bacteria/viruses/fungi. Besides, this chapter would like to support and encourage scientists to analyze nectar for all other components and connect it with the pollenizer preference.

Also in the following years, nectar of sweet cherry, and other minor cherry species like European dwarf cherry (*Prunus fruticosa* Pall.), mahaleb cherry (*Prunus mahaleb* L.), and duke cherry (*Prunus* × *gondouinii* Rehd.), and/or other agricultural plants, should be analyzed in details and connect it with honey quality.

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Pollinator Trapping in Carnivorous Plants

30

Kazuki Tagawa

Contents

Abstract

Carnivorous plants use insects not only as prey for nutrient supplementation but also as pollinators for sexual reproduction. Consequently, when these plants have flowers and trap leaves simultaneously, there is a risk that they will trap mutualistic pollinators. Pollinator trapping can have various fitness consequences for carnivorous plants depending on which factors are limiting their fitness at a given time. Thus, plants that are pollen limited will be negatively impacted by pollinator trapping, whereas those that are nutrient limited will benefit from this. Carnivorous plants have evolved diverse characteristics to manage pollinator trapping based on these fitness-limiting factors. In this chapter, I discuss these characteristics with a particular focus on visual and chemical traits resulting from the

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production of secondary metabolites and biological factors to gain an understanding of the evolutionary ecology of pollinator trapping in carnivorous plants.

Keywords
Carnivorous plants · Pollinator · Pollinator–prey conflict · Anthocyanin · Predator–prey interactions

1 Introduction

Carnivorous plants are an intriguing group of angiosperms that reverse the usual trophic interaction between plants and animals, with the plant acting as the predator and the animal becoming the prey [1], and consequently have attracted the attention of scientists since Darwin's time [2]. Carnivorous plants mainly use their trap leaves to capture and digest small invertebrates and absorb nutrients such as N and P from them [3], which increase plant growth [4] and seed and fruit set [5–7]. This trait allows them to live in nutrient-poor habitats (e.g., wetlands, alpine areas, and tropical forests) [8] and has evolved at least ten times independently, with 800 species in 19 genera, 12 families, and 5 orders having been identified to date [9].

In addition to the predator–prey interactions between carnivorous plants and insects, many carnivorous plants have entomophilous flowers and rely on insects for pollination to facilitate sexual reproduction and outcrossing. In such a situation, carnivorous plants face conflicting pressures, as there is the possibility of capturing mutualistic pollinators in their trap leaves and consuming them. Thus, carnivorous plants face a trade-off between the pollination service and nutrient uptake. This issue was raised as the "prey/pollinator paradox" by Juniper et al. [10] and discussed as the "pollinator–prey conflict (PPC)" by Zamora [11], and comprehensive reviews of the PPC have since been written by Jürgens et al. [12] and Cross et al. [13].

When carnivorous plants rely on insects for their pollination and pollinator/pollen limitation exists, pollinator trapping will reduce the fitness of the plants, leading to a PPC occurring. By contrast, those plants that do not rely on insects for their pollination and are not pollinator limited will not experience a PPC. Furthermore, when nutrient limitation rather than pollinator limitation is the fitness bottleneck, trapping pollinators will increase the fitness of carnivorous plants [12]. Therefore, the consequences of trapping pollinators will vary depending on the breeding system and the factors that limit the fitness of carnivorous plants, which has led to the evolution of diverse characteristics of the trap leaves and flowers.

Two major characteristics of carnivorous plants will affect the efficiency (or risk) of trapping pollinators. The first is the degree of spatial or temporal separation between the flowers and trap leaves. If trapping pollinators reduces the fitness of carnivorous plants, it will be adaptive for the flowers and trap leaves to be separated spatially or temporally to reduce the risk that pollinators are captured by chance, for example, by producing a long flower scape to spatially separate the flowers from the rosette-shaped trap leaves (e.g., *Drosera* spp. and *Pinguicula* spp.) [8] or by producing the flowers and active trap leaves in different seasons (e.g., *Sarracenia* spp.)

[14]. However, if pollinator trapping increases the fitness of carnivorous plants, it may be evolutionary favorable to use a strategy that increases the efficiency of trapping pollinators, for example, by producing the flowers and active trap leaves simultaneously and having them in close proximity to each other.

The second characteristic that will affect the efficiency of trapping pollinators is the expression of visual and/or chemical signals in trap leaves through the production of secondary metabolites. If trapping pollinators reduce the fitness of carnivorous plants, it may be advantageous to separate the groups of insects that are visiting the flowers and trap leaves through the production of attractants or repellents. Three mechanisms can be used to achieve this: (1) attracting pollinators specifically to the flowers, (2) making the trap leaves unattractive to pollinators, or (3) making the trap leaves attractive to non-pollinating insects specifically [12]. On the other hand, if trapping pollinators increase the fitness of carnivorous plants, it may be adaptive to use a strategy that exploits the perceptual biases of pollinators visually and/or chemically to attract them to the trap leaves [15, 16], and pollinators may accordingly evolve to recognize the cues of the trap leaves to avoid being trapped.

In this chapter, I focus on pollinator trapping by carnivorous plants and the secondary metabolites that affect their trapping efficiency. In Sect. 2, I provide an overview of empirical studies that have examined pollinator trapping by various types of carnivorous plants, and in Sect. 3, I summarize the visual and chemical signals that promote or suppress pollinator trapping. In Sect. 4, I consider the relationships between carnivorous plants and co-occurring noncarnivorous plants and discuss how co-occurring plants affect the efficiency of pollinator trapping by carnivorous plants. Finally, in Sect. 5, I discuss the counteradaptations of pollinators to carnivorous plants.

2 Empirical Studies on Pollinator Trapping in Carnivorous Plants

There are five main types of trap leaves in carnivorous plants: adhesive/flypaper traps, pitcher/pitfall traps, snap traps, eel traps, and suction/bladder traps [9]. Among these, adhesive traps, pitcher traps, and snap traps catch terrestrial invertebrates, including flower-visiting insects, and so will be the focus of this section.

The mechanism of capturing prey varies greatly among these three types of trap leaves. With adhesive traps, a large area of the trap leaf is exposed to the outside to allow any insects that are passing by to be trapped [17]. By contrast, pitcher and snap traps guide insects inside the pitchers and laminar lobes, respectively, meaning that it is more difficult for them to capture passing insects, so these types of trap leaves may face stronger selection pressures than adhesive traps to develop traits that attract insects. Consequently, the types of trap leaves may affect the mechanism that is used to promote or suppress pollinator trapping. An overview of previous studies on pollinator trapping by each type of carnivorous plant is provided below.

2.1 Adhesive Traps

In the genus *Drosera*, the degree of pollinator trapping has been investigated for eight species: four species that separate the rosette-shaped trap leaves from the flowers through the production of long flower scapes and four species that have stems supporting both the flowers and trap leaves in close proximity (two of which have small, round trap leaves and two of which have vertically developed, linear trap leaves) (Table 1). Six of these species use large- to medium-sized Diptera (Syrphidae, Bombyliidae, Calliphoridae, Tachinidae, and Muscidae) as pollinators, while the remaining two use Coleoptera, and all eight species tend to take small Diptera (mainly Nematocera) and terrestrial-dwelling invertebrates as prey. Therefore, regardless of the degree of separation of the flowers and trap leaves, many of these species do not trap any pollinators or only trap pollinators at quite a low level.

The exception to this is *D. makinoi*, in which effective pollinators, including large Diptera and Hymenoptera, are captured throughout the flowering season [22]. The flowers and trap leaves are remarkably close to one another in this species, so it is considered that pollinators visiting the flowers are accidentally trapped due to the morphological characteristics of the plants. However, since *D. makinoi* is self-compatible and self-pollinated flowers can make similar amounts of seed as outcrossed flowers (Watanabe, unpublished data), pollinator trapping may not reduce the fitness of this species. Interestingly, *D. toyoakensis*, which has similar morphological characteristics to *D. makinoi*, has a relatively low level of pollinator trapping (see Sect. 6), and the inbreeding coefficients of these species reflect this situation (*D. makinoi*, 0.497; *D. toyoakensis*, 0.260; Watanabe, unpublished data).

In *Drosophyllum lusitanicum*, the main pollinator fauna is Coleoptera (*Enicopus* sp., 56.6% of flower visits) and Hymenoptera (*Panurgus* sp., 25.6% of flower visits), while the main prey fauna is small Diptera (83.5%), so the main pollinators were not trapped [23].

In the genus *Pinguicula*, the degree of pollinator trapping has been investigated in *P. vallisneriifolia* [11]. According to this study, the degree of pollinator trapping and its fitness consequences varies depending on the shadiness of the microsites where the plants grow. Large Diptera (*Bombylius* sp.) and Hymenoptera (*Lasioglossum* sp. and *Anthophora* sp.) visit flowers only in sunlit sites and are not captured by trap leaves. By contrast, small Thysanoptera (*Taeniothrips meridionalis*) and Coleoptera (*Eusphalerum scribae*) visit flowers and are frequently captured by trap leaves regardless of the shadiness of the microsites. Thus, the trapping of Thysanoptera and Coleoptera at shaded sites may lead to a reduced fitness because there are no other efficient pollinators at these sites, whereas the trapping of these insects at sunlit sites may have no effect on fitness because the more efficient dipteran and hymenopteran pollinators remained untrapped. The main pollinators of other species in the genus *Pinguicula* include Hymenoptera (for *P. ionantha*, *P. lutea*, and *P. planifolia* [24]; and *P. longifolia* [25]), Diptera (for *P. alpina* and *P. vulgaris* [26]), and Lepidoptera (for *P. moranensis* [27]), while the main prey are tiny invertebrates such as Collembola, Acarina, and Nematocera [28–31]. Consequently, *Pinguicula* spp. do not capture medium–large pollinators.

Table 1 Pollinator trapping in the genus *Drosera*

Species	Reproduction	Flower-trap separation	Pollinator species and abundance	Prey species and abundance	Pollinator trapping	Ref.
Drosera anglica	SC	*Long*	Diptera (95%): Syrphidae (66%), Bombyliidae (10%), Muscidae (10%), Calliphoridae (7%), Tachinidae (3%), Dolichopodidae (3%), Hymeroptera, and Thysanoptera	Diptera (Nematocera, 56.2–84.2%)	Minimal: only an individual of *Thrips* sp. (Thysanoptera)	[18]
Drosera arcturi	SC	*Long*	Diptera (94%): Syrphidae (72%), Tachinidae (14%), and Muscidae (8%)	Most common prey were Diptera (40%) and Coleoptera (23%). Other prey included Lepidoptera, Hemiptera, and Thysanoptera	Minimal: 4.3% of prey included in the three major pollinator families (Syrphidae, Tachinidae, Muscidae)	[19, 20]
Drosera auriculata	SI	*Short*	Diptera (100%): Syrphidae (76%), Tachinidae (14%), and Muscidae (10%)	Most common prey were Diptera (48%; Sciaridae, Chironomidae, Culicidae) and Hemiptera (16%). Other prey included Lepidoptera, Trichoptera, Coleoptera, Hymenoptera, and Thysanoptera	Minimal: 3.3% of prey included in the three major pollinator families (Syrphidae, Tachinidae, Muscidae)	[20]
Drosera cistiflora	Unknown	*Short*	Coleoptera: Scarabaeidae (in excess of 58%) and Chrysomelidae (18%)	Prey were tiny, averaging less than 2.0 mm in length. Most prey were Diptera (97%)	Non	[21]

(*continued*)

Table 1 (continued)

Species	Reproduction	Flower-trap separation	Pollinator species and abundance	Prey species and abundance	Pollinator trapping	Ref.
Drosera makinoi	SC, autonomous (Watanabe, unpublished data)	*Short*	Diptera: *Mesembrius flaviceps* and *Sphaerophoria menthastri*	Most common prey were Diptera, Hymenoptera, and Lepidoptera	Exist: 4 of 5 pollinator species were trapped	[22]
Drosera pauciflora	Unknown	*Long*	Coleoptera: Scarabaeidae (in excess of 58%) and Chrysomelidae (18%)	Prey were tiny, averaging less than 2.0 mm in length. Most prey were Diptera (72%). Other prey included crawling invertebrates such as beetles, arachnids, and ants	Minimal: Only an individual of Chrysomelidae beetle	[21]
Drosera spatulata	SC	*Long*	Diptera (100%): Syrphidae (69%), Tachinidae (25%) and Muscidae (6%)	Most common prey were Hemiptera (24%), Hymenoptera (wasps/ants: 19%), and Coleoptera (18%). Other prey included Diptera, Lepidoptera, and Thysanoptera	Minimal: 3.8% of prey included in the three major pollinator families (Syrphidae, Tachinidae, Muscidae)	[19, 20]
Drosera toyoakensis	SC, autonomous (Watanabe, unpublished data)	*Short*	Diptera: *Sphaerophoria menthastri*, *Eupeodes corollae* Lepidoptera: *Eurema hecabe*, *Zizeeria maha argia*	Most common prey were Diptera (mainly Nematocera), Hymenoptera, and Lepidoptera	Minimal: 1 of 4 pollinator species was trapped at 1 of 6 seasons	[22], Tagawa et al., unpublished data

The finding that the main pollinators of *Drosera* spp., *Drosophyllum lusitanicum*, and *Pinguicula* spp. (i.e., large Diptera and Hymenoptera) are not captured by adhesive trap leaves regardless of their degree of separation from the flowers has two possible explanations. First, the adhesive traps only have a limited retention capacity [32], meaning that large pollinators can escape after being trapped [18]. In a comparison of the insect fauna that was captured by the trap leaves of *Pinguicula nevadense* and model traps, Zamora [29] showed that *P. nevadense* captured prey only below a specific size threshold (ca. 4 mm), while the model traps captured prey that were 0–20 mm long, despite the composition of prey being similar for *P. nevadense* and the model traps, suggesting that size is the principal factor determining the actual prey of carnivorous plants. Similarly, Gibson showed that body size determines whether prey can escape from the trap leaves of *Drosera filiformis* and *Pinguicula lutea*, with prey of <5–10 mm being unable to escape. Second, it is possible that prey can recognize the visual and/or chemical signals of the adhesive traps and avoid landing on these, as discussed in detail in Sect. 3.

2.2 Pitfall Traps

Carnivorous plants in the genus *Nepenthes* produce dioecious flowers and cannot be self-pollinated. Therefore, when pollinator/pollen limitation exists, the trapping of pollinators will lead to a reduction in fitness. The degree of pollinator trapping has been investigated in *N. gracilis* growing naturally in Sumatra [33]. Flowers of this species are pollinated by visiting pyramid moths (*Herpetogramma* sp., *Pleuroptya* sp., *Pagyda* sp., *Ambia* sp., and *Pycnarmon* sp.) at night and calliphorid flies in the evening, while ants are captured as the main prey with no pollinators being taken. The pollinator fauna of several *Nepenthes* species has been shown to include Diptera (for *N. rajah* [34], *N. kinabaluensis* [34], *N. villosa* [34], *N. macfarlanei* [35], and *N. mirabilis* [36]), Diptera and Hymenoptera (for *N. curtisii* ssp. *zakriana* and *N. reinwardtiana* [34]), and Coleoptera (for *N. rafflesiana* [37], mentioned in [35]). *Nepenthes* spp. trap a broad range of insect taxa, but ants are usually abundant among the prey items taken [38, 39]. *Nepenthes rafflesiana* has an ontogenetic pitcher dimorphism, whereby the aerial (upper) and ground (lower) pitchers have different morphologies and trap different types of insects, with many ant individuals being trapped in both types of pitchers and a guild of generalist pollinator insects (e.g., Lepidoptera, Thysanoptera, and Coleoptera) being trapped only in the aerial pitchers [40, 41]. This trapping of generalist pollinator insects in the aerial pitchers has been shown to be due to the secretion of volatiles [42] (see Sect. 3 for further discussion). Therefore, there is a possibility that pollinator trapping could also occur in the aerial pitchers.

In the genus *Sarracenia*, the degree of pollinator trapping has been investigated in *S. gracilis* [7]. The pollinators of *S. gracilis* include the hymenopterans *Augochlorella aurata* (1.5 ± 0.48 visits/flower/h) and *Bombus affinis* (0.5 ± 0.25 visits/flower/h) and the dipteran *Fletcherimyia fletcheri* (0.3 ± 0.18 visits/flower/h), while the prey species are mainly amphipods and ants. Therefore, pollinators are not

trapped by this species [7]. It has also been shown that *Bombus* spp. are the main pollinators of *S. flava* [43] and *S. alata* [44], and ants are the main prey of *S. alata* [45, 46] and *S. purpurea* in some habitats [47, 48], with Diptera being taken by *S. purpurea* growing in a different habitat [49]. Therefore, pollinator trapping is unlikely to occur in *Sarracenia* spp. However, *Bombus* spp. are frequently trapped by an *S. purpurea* population that has been introduced from North America to Britain, despite these insects being major pollinators of this population, with one study showing that 49/170 pitchers trapped more than one *Bombus* individual [50]. This difference in pollinator trapping between native and introduced populations may reflect the coevolutionary history between *Sarracenia* and *Bombus* (see Sect. 5 for a detailed discussion).

2.3 Snap Traps

The degree of pollinator trapping in *Dionaea muscipula* was investigated by Youngsteadt et al. [51], who showed that the major pollinators of this carnivorous plant that have high visiting frequencies and pollen loads are the sweat bee *Augochlorella gratiosa*, other bees (e.g., *Lasioglossum creberrimum*), and beetles (*Typocerus sinuatus* and *Trichodes apivorus*), while the major prey species are terrestrial invertebrates, including spiders (40%), ants (26%), and beetles (11%). Thus, there is very little overlap between the prey and pollinator fauna, and none of the major pollinator species are trapped. This low level of pollinator trapping may be due to the spatial separation of the flowers and rosette-shaped traps [51].

3 Carnivorous Plant Signals and Pollinator Trapping

3.1 Visual Signals

Some species of *Drosera*, *Dionaea*, *Nepenthes*, and *Sarracenia* produce red trap leaves through the expression of the secondary metabolite anthocyanin [52, 53]. These red trap leaves are conspicuous to the human eye due to their strong contrast against the green background [2]. It has been hypothesized that the red coloration of the trap leaves functions as a signal to attract insects effectively because, in some carnivorous plants, the color of the trap leaves changes from green to red under nutrient-deficient conditions (see [54] for experimental studies on *Drosera spatulata* and *Dionaea muscipula*). In a pioneer study on this hypothesis, Schaefer and Ruxton [16] showed that the prey capture rate of *Nepenthes ventricosa* was higher in pitchers that were artificially colored red than in those that were artificially colored green. However, while this study clearly demonstrated the attractiveness of red traps, its ecological validity was questioned because the experiment was conducted in Germany, which is outside the natural range of *N. ventricosa*, and the composition of prey that were trapped in the experiment (mainly Diptera) was not consistent with the compositions in the native habitats of this species (mainly ants) [55].

Later studies using actual carnivorous pitcher plants and artificial pitcher models did not support the attraction function of red pitcher traps, with the redness of the traps having no effect on the quantity of ants trapped by *Sarracenia alata* [45] or *Nepenthes gracilis* [56]. Bennett and Ellison [55] also showed that the attraction of ants as prey in *Sarracenia purpurea* was not affected by the redness of the trap leaves but rather the presence of nectar [55]. These results are reasonable considering that ants have dichromatic color vision based on two spectrally distinct photoreceptors ([57, 58] but see [59, 60]), which will make it difficult for them to forage based on color. In addition, experiments using artificial adhesive traps showed that red traps caught fewer prey individuals (mainly Diptera) than green traps [19, 61, 62], suggesting that prey species can recognize the difference between red and green traps and avoid landing on red traps. Since many insects, including Diptera, do not possess red color receptors (only possessing blue, green, and ultraviolet receptors [57]), they may identify and avoid red traps based on other color-related features, such as the intensity of reflected light [16, 63].

These findings indicate that the production of red traps is likely to have associated costs, including a reduction in the number of trapped prey and the costs associated with anthocyanin biosynthesis [64]. So, what are the benefits of having red trap leaves? In general, anthocyanin provides plants with protection from light stress under intense light conditions [65], direct protection from herbivores and pathogens [66, 67], and honest signals indicating the degree of chemical protection [68, 69]. Since many carnivorous plants grow in sunlit habitats [8, 70] and an in situ experiment showed that the trap leaves of *Drosera rotundifolia* became redder when the canopy light transmission was increased through the removal of vegetation [71], it may be reasonable to consider that red trap leaves function to reduce light stress. Red trap leaves may also provide defense against herbivores since herbivorous insects of carnivorous plants exist [72, 73]; indeed, it has been shown that the red pitchers of *Nepenthes gracilis* experience less herbivory than the green pitchers [56].

In addition to these general functions, Jürgens et al. (2015) proposed the hypothesis that the red coloration of the trap leaves reduces the risk of trapping pollinators [19], demonstrating that half the number of prey individuals was captured in artificial red traps than in green traps, and 25% of the number of pollinator individuals was captured in artificial red traps compared with green traps. These results suggest that the red color may function as an honest signal to pollinators, and that pollinators avoid landing on red trap leaves.

This raises the question of why insects avoid landing on red trap leaves. First, insects may learn the danger of trap leaves in relation to the red color. Large insects that are trapped by chance will be able to escape from the trap leaves (see Sect. 2) and learn to avoid landing on other trap leaves with similar characteristics. Second, insects may have an inherent tendency to avoid landing and foraging on red leaves because the red color that is produced by anthocyanin signals the existence of direct and/or indirect chemical defense – consequently, insects also avoid landing on the red leaves of noncarnivorous plant species [69].

3.2 Chemical Signals

Some carnivorous plants emit characteristic scents from their trap leaves that can be detected by humans [10]. The flower-like scents of *Drosophyllum lusitanicum* [23] and *Sarracenia* spp. [74] and the honey-like sweet scents of *Drosera indica* and the related species *Drosera finlaysoniana* [75] and *Drosera toyoakensis* (Tagawa, personal observation) are particularly strong. The chemicals secreted from the trap leaves are mainly terpenoids, benzenoids, and aliphatic compounds, which are the same chemicals as are usually secreted from the flowers and fruits of angiosperms [76]. Therefore, it has been considered that the chemicals that are secreted from the trap leaves function to attract insects in the same way as those secreted from the flowers and fruits.

The relationship between the quantity of secreted chemicals and the efficiency of prey trapping has been investigated for some carnivorous plant species. *Dionaea muscipula* has been shown to secrete more than 60 kinds of chemical substances from the trap leaves, including terpenes, benzenoids, and aliphatics, most of which are also secreted from the flowers and fruits [77]. In one experiment, the odor of *Dionaea muscipula* effectively attracted starved but not non-starved *Drosophila melanogaster* under light conditions, suggesting that this carnivorous plant attracts insects through "food smell mimicry" [77]. *Sarracenia flava* and *S. leucophylla* secrete 47 kinds of chemical substances, most of which are also secreted from the flowers and fruits, and the amount of secreted chemicals per unit time was found to be positively correlated with the number of attracted blowflies [78]. In *Nepenthes rafflesiana* var. *typica*, which has ontogenetic pitcher dimorphism (see Sect. 2), the composition and quantity of chemicals produced differ between the ground and aerial pitchers, with the ground pitchers secreting some aliphatics and terpenoids but being poor in benzenoids and the aerial pitchers secreting larger amounts of odors and a larger spectrum of volatiles, including some terpenoids and benzenoids that are usually found in flower scents [42]. In accordance with the scent profiles of these pitchers, the aerial pitchers have been shown to trap larger numbers of more diverse prey, including generalist pollinators (e.g., Lepidoptera, Thysanoptera, and Coleoptera), than the lower pitchers, suggesting that the upper pitchers effectively attract flower-visiting insects by emitting chemical substances that are similar to the flowers [42]. To confirm the fitness consequences of trapping flower-visiting insects, additional studies are required on the pollinator fauna of focal carnivorous plants – if the focal plants attract and trap insects that usually visit the flowers but are not pollinators, this will have a positive effect on their fitness.

If pollinator trapping negatively affects the fitness of a carnivorous plant, it will be adaptive to secrete different chemical substances from the trap leaves and flowers to attract (or repel) different insect species [12]. El-Sayed et al. [20] showed that in *Drosera auriculata*, which produces flowers adjacent to the trap leaves, the trap leaves are made unattractive to pollinators through the emission of typical chemical substances. Furthermore, there is no overlap between the chemical substances that are emitted from the flowers and trap leaves, with the flowers mainly emitting 2′-aminoacetophenone (34% of the total chemicals produced) and 2-phenylethanol

(30% of the total) and the trap leaves mainly emitting plumbagin (74% of the total) [20]. To confirm the effects of attraction chemicals, the same authors applied flower- and/or trap-derived chemicals to clear sticky models and counted the numbers of trapped insects. They found that significantly fewer pollinators were trapped on the models with both flower and trap chemicals and only trap chemicals than on the models with only flower chemicals, while larger numbers of insects other than pollinators were trapped on the models with both flower and trap chemicals than on the models with either trap or flower chemicals alone [20]. These results suggest that the chemical substances produced by the trap leaves of *D. auriculata* repel only pollinators, which prevents pollinator trapping.

Unlike visual traits, the chemical traits of plants, including the types and amounts of chemicals secreted, can vary plastically over a short period of time. Indeed, in *Dionaea muscipula*, the amounts of terpenes, benzenoids, and aliphatic compounds that are secreted from the trap leaves repeatedly increase and decrease during the day, with lower levels of secretion at night than in the daytime [77]. Similarly, *Drosera toyoakensis* secretes larger amounts of honey-like odors in the morning (Tagawa et al., unpublished data). Therefore, if the fitness consequences of pollinator trapping vary over time, it is possible for the chemical traits to match this. On a short time scale, it would be adaptive for carnivorous plants to emit attractive chemicals from their trap leaves when the flowers are closed and to avoid emitting these or emit deterrent chemicals when the flowers are open. Future studies would be useful to clarify the relationship between the quantitative/qualitative changes in chemical substances that are emitted from trap leaves and changes in the capture rates of insects, including pollinators.

In addition, it may be adaptive for carnivorous plants to change the types and/or amounts of trap chemicals produced and to control the numbers of trapped insects, including pollinators, depending on their nutritional state. When nutrients are deficient and limiting fitness, it may be advantageous to attract and capture as many insects as possible, including pollinators, while the opposite may be true when pollination and outcrossing limit the fitness. In accordance with this hypothesis, the amount of emitted terpenes was found to decrease with an improvement in N status in *Dionaea muscipula* [77]. However, this decrease in emitted terpenes did not affect the attractiveness of the plants to the prey species *Drosophila melanogaster*, possibly because they can recognize benzenoids and aliphatic compounds, the profiles of which did not change with N addition [77]. Therefore, additional experiments are required using actual pollinator and prey species of *Dionaea muscipula* [51]. It is also known that the microorganisms that live in pitcher traps alter the chemical profiles that are emitted from the traps, leading to quantitative/qualitative changes in the trapped insects. When opening the trap entrance, *Sarracenia purpurea* emits chemical substances that are known to act as general attractants to flowers and fruits. However, as the amount of trapped prey increases, dimethyl disulfide (DMDS) appears to be secreted, which is generated during the decomposition of proteins by bacteria, resulting in changes in the prey spectra from generalist flower-visiting insects to insects that are usually attracted to decaying organic matter [78]. This change may be adaptive for carnivorous plants as it shifts the strategy from trapping

prey that include flower-visiting insects to trapping only non-flower-visiting insects after an improvement in N status.

4 Effects of Co-occurring Plants on Pollinator Trapping by Carnivorous Plants

Plant–plant interactions in relation to pollinators may vary from competition to facilitation [79, 80]. Some plants compete for pollinator services and/or interspecific pollen transfer [80], while other plants help each other through the joint attraction and/or maintenance of pollinators, which is bidirectional and brings fitness benefits to all of the interacting plants [81]. In addition, unidirectional facilitation can occur, which brings fitness benefits to only one of the plant species involved in the interaction. Unidirectional facilitation occurs by one of two mechanisms: the "magnet species effect," whereby plants that have a great reward and high attractiveness enhance the local abundance of pollinators, bringing benefits to other plants growing sympatrically [82], and "Batesian floral mimicry," whereby deceptive plants that do not reward pollinators gain pollinator visits by mimicking rewarding models [83].

The relationship between carnivorous plants and noncarnivorous plants growing sympatrically can also vary from competition to facilitation. El-Sayed et al. [20] hypothesized that flowers of *Drosera* spp. (*D. spatulata* and *D. arcturi*) visually mimic the flowers of the noncarnivorous plant *Donatia novae-zelandiae* and attract pollinators efficiently. *Drosera* spp. do not emit chemical substances from their flowers, whereas *Donatia novae-zelandiae* emits large amounts of chemicals. Therefore, the authors argued that *Drosera* spp. exploit insect search images to attract insects without secreting any chemical substances from the flowers, reducing the physiological costs of producing them [20]. This relationship could contribute to the pollination efficiency of carnivorous plants through either the magnet species effect or Batesian mimicry. The selection pressure to reduce the cost of chemical production may be high for carnivorous plants growing in nutrient-poor habitats, making it adaptive to exploit insect pollinators or prey that are attracted by other plants.

Like the flowers of noncarnivorous plants, the trap leaves of carnivorous plants attract insects using visual and/or chemical signals. Therefore, competitive to facilitative relationships may exist between the trap leaves of carnivorous plants and the flowers of noncarnivorous plant growing sympatrically. In terms of facilitative relationships, two situations can be considered: (i) the trap leaves of carnivorous plants attract insects, which increases the visitation rate to flowers of plant species, or (ii) the flowers of noncarnivorous plant species attract insects, which increases the trapping rate of carnivorous plants. The former relationship may be unlikely in the case of carnivorous plant species with a high trapping efficiency, resulting in facilitation being unidirectional, with the presence of flowers of co-occurring plants positively affecting the fitness of carnivorous plants. In addition, the trapping of insects by carnivorous plants may reduce the local abundance of insects, negatively affecting the fitness of co-occurring plants. Tagawa et al. [22] examined unidirectional facilitation between carnivorous plants and co-occurring noncarnivorous

plants as maintained by the magnet species effect and hypothesized that the existence of flowers of co-occurring plants increases the number of insect individuals trapped by carnivorous plants. In support of this hypothesis, the carnivorous plant species *Drosera makinoi* and *Drosera toyoakensis* have been shown to trap more prey individuals when the flowers of noncarnivorous plant (*Eriocaulon decemflorum* for *D. makinoi* and *Lysimachia fortunei* for *D. toyoakensis*) are near to them compared with when they are absent, and these increased prey include pollinators of co-occurring noncarnivorous plant species. These results suggest that the flowers of co-occurring plants increase the density of flower-visiting insects around carnivorous plants (i.e., the magnet species effect), resulting in carnivorous plants experiencing increased numbers of prey. In the future, studies are needed to quantify how the presence of carnivorous traps affects the number of pollinator visits and the fitness of co-occurring plants, as well as to investigate interspecific pollinator–prey overlaps and the consequences of these.

5 Evolution of Pollinators to Avoid Carnivorous Plants

In predator–prey interactions, the predators evolve to capture the prey more efficiently, and the prey evolve to avoid predation by the predators [84]. Therefore, it may be adaptive for potential prey insects to have traits that help them to avoid being predated on by carnivorous plants. Two types of traits could be used to avoid predation: the ability to sense trap leaves to avoid landing on them and the ability to escape after being trapped. The ability of prey to escape after being trapped will partly depend on their body size [85] (see Sect. 2), so it is considered that small insects are more likely to have developed the ability to sense the presence of trap leaves and avoid landing on them.

This raises the question of whether the selection pressure would be great enough to drive the evolution of a trait that allows insects to avoid being trapped by carnivorous plants. Selection for anti-predation traits will partly depend on the duration and intensity of the interaction [86]. In general, carnivorous plant habitats occupy only a very narrow range of insect habitats, so the interaction frequencies may be low. Consequently, the selection pressure may not be sufficiently high to promote the evolution of anti-predation traits in insects toward carnivorous plants, making it difficult to detect this. However, pollinating insects are an exception to this, as pollinators seek resources such as nectar and pollen and visit flowers of particular species. Therefore, the pollinators of carnivorous plants may have higher interaction frequencies, allowing them to evolve the ability to sense trap leaves and avoid landing on them.

Various *Drosera* spp. are known to have low trapping frequencies of hoverflies (*Syrphidae*) [18, 20], even though these are the major pollinators of these plants (Table 1). Furthermore, Tagawa et al. (unpublished data) found that all hoverfly individuals with a body length of ca. 10 mm were trapped after being placed on *Drosera* leaves, suggesting that they could not free themselves once trapped. Tagawa et al. [87] found that the hoverfly *Sphaerophoria menthastri*, which is a major

pollinator of *Drosera toyoakensis*, exhibited 9 approaches and 2 landings on the trap leaves of *D. toyoakensis*, 60 approaches and 55 landings on the flowers of *D. toyoakensis*, 52 approaches and 49 landings on the flowers of the noncarnivorous plant *Lysimachia fortunei*, and 54 approaches and 49 landings on the leaves of Poaceae and Cyperaceae. Thus, *S. menthastri* had a significantly lower landing rate on the trap leaves of *D. toyoakensis* (22.2%) than on the other organs assessed, suggesting that this hoverfly can sense trap leaves and avoid landing on them. In addition, the same authors considered that the hesitation behavior of hoverflies, whereby they hover forward to backward as if inspecting the safety of a landing site [88], contributes to their recognition of trap leaves, as *S. menthastri* individuals that exhibited one or two hesitation behaviors avoided landing on the trap leaves, whereas individuals that exhibited no hesitation behaviors were trapped [87]. Tagawa et al. [87] did not clarify which cues (e.g., visual traits of the traps or chemical substances emitted from the traps) deter *S. menthastri* from landing on the traps of *D. toyoakensis*, so further research should consider this question.

If predation rates are low in an environment where the prey shares an evolutionary history with the predators, it is likely that counteradaptation of the prey has occurred. *Sarracenia purpurea* does not capture pollinating *Bombus* spp. in its native habitats in North America [7] but frequently traps *Bombus* spp. in Britain, where it has been introduced [50], suggesting that *Bombus* spp. in native *S. purpurea* habitats may have evolved traits that allow them to avoid being trapped by the trap leaves of *S. purpurea*, whereas the same has not occurred in introduced *S. purpurea* habitats. Considering that *S. purpurea* needs pollinator visitation for reproduction [7], the trapping of *Bombus* spp. could reduce the fitness of introduced populations. Alien species of carnivorous plants have been found around the world, and studies on the interactions between these alien populations and insects, including pollinators, may contribute to our understanding of the evolution of pollinators to avoid predation by carnivorous plants. In addition, since the distribution range of prey insects is usually wider than that of carnivorous plants, it may be possible to compare the capture rates of pollinators within and outside the habitats of carnivorous plants to help clarify the evolution of anti-predation traits in pollinators.

6 Conclusions

- Many carnivorous plant species have temporal, spatial, or chemical separation of the trap leaves and flowers, which is effective in preventing or reducing the incidence of pollinator trapping. In addition, it is considered that adhesive traps cannot capture large pollinators due to their limited retention capacity.
- Some carnivorous plants have red trap leaves due to the expression of the secondary metabolite anthocyanin. Many studies have shown that red traps do not attract prey, and some have also found that red traps deter prey, including pollinators, leading to the pollinator–protection hypothesis. However, future studies are required to elucidate the proximate and evolutionary causes of the deterrence of pollinators by red traps.

- Some carnivorous plants emit chemical substances that are usually emitted from the flowers and fruits, such as terpenoids, benzenoids, and aliphatic compounds. Although the secretion of these substances has a positive effect on prey attraction, there have been few studies on their effects on pollinator attraction and trapping. Unlike visual traits, chemical traits tend to vary over a short period of time, so studies on the relationship between pollinator trapping and plastic changes in the quality and quantity of chemicals produced (e.g., in association with the circadian rhythm and changes in nutritional condition) would be informative.
- As occurs in the interspecific relationships between flowering plants, the relationship between the trap leaves of carnivorous plants and the flowers of co-occurring plants may vary from competition through to facilitation. It has been shown that the presence of flowers on co-occurring plants facilitates prey capture by carnivorous plants, but little is currently known about the impacts of pollinator capture on the fitness of co-occurring plants.
- Pollinators of carnivorous plants frequently interact with carnivorous plants and so may have evolved traits that allow them to recognize their traps and avoid landing on them. Further examination of the relationship between alien carnivorous plants and co-occurring insects may be useful for investigating the evolution of these traits.

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Plant Defense and Insect Adaptation with Reference to Secondary Metabolites

31

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Abstract

Insects pose a great threat to plants, and plants, in turn, withstand to insect attack through various morphological and biochemical traits. Among the plant defensive traits, secondary metabolites play a major role against insect herbivory as they are highly dynamic. They either occur constitutively in plants or are induced in response to insect herbivory. These metabolites include sulfur- (terpenes and flavonoids) and nitrogen-containing metabolites (alkaloids, cyanogenic glucosides, and nonprotein amino acids), which are being implicated by plants against insect pests. Plant secondary metabolites either are directly toxic to insect pests or mediate signaling pathways that produce plant toxins. Further, some of the plant secondary metabolites act through antixenosis mode by developing non-preference in host plant to the insect pests. However, some plant secondary metabolites recruit natural enemies of the insect pests, thus indirectly defending plants against insect pests. However, insects have developed adaptations to these plant secondary metabolites. In this chapter, important plant secondary metabolites, their mechanism of action against insect pests, counter-adaptation by insects, and promising advances and challenges are discussed.

Keywords

Plant-insect interactions · Plant secondary metabolites · Insect adaptation · Sequestration · Induced resistance

1 Introduction

Plants and insects constitute the largest diversity with 1 million arthropod taxa (mostly insects) described completely and more than 350,000 plant taxa. Many insects that have been described to date are either pollinators or herbivores. Insects and plants have coevolved for millions of years by continuous adaptation of insects to the plant defensive traits [1–5]. Plants have evolved several morphological and biochemical traits to withstand insect damage; however, insects, in turn, have evolved several defensive and/or adaptive mechanisms, such as behavioral, morphological, physiological, biochemical and genetic traits, to tolerate and/or adapt to plant defensive traits [1–3, 5]. Plants use a number of morphological, chemical, and biochemical defenses against insect herbivores. Morphological traits involved in plant defense include trichomes, spines, cuticles, thorns, and lignified cell walls, are the first line of defense that directly defend plants against insect pests. The biochemical defense is considered as highly dynamic and is mostly mounted only when plants face insect attack. Overall plant defense against insect pests can be categorized into antibiosis and antixenosis resistance [3, 4]. In antibiosis, the plant defensive traits directly affect the insect growth, development and survival; while in antixenosis defense, morphological or chemical factors alter the insect behavior deterring from feeding and egg laying [3, 4]. Plant volatiles play an important role in indirect plant defense against insect herbivory either by deterring the insect pests

and/or by attracting the natural enemies of the pests. Among the plant defensive traits, the chemical barriers, both inducible and constitutive, and the nutritional content in a plant are considered as more important in terms of reducing the insect growth and development [6]. Even though, plants withstand the mechanical barrier by specific behavioral and life cycle adaptations, the adaptation to chemical defense is not so easy as it is highly dynamic.

Plant secondary metabolites are not involved in the normal growth and development of the plant but are involved in the plant defense against a variety of stresses. However, both the primary and secondary metabolites are linked as the primary metabolites serve as precursors for the synthesis of secondary metabolites (Fig. 1). Plant secondary metabolites either occur constitutively in plants or are induced in response to insect herbivory. The constitutive secondary metabolites are known

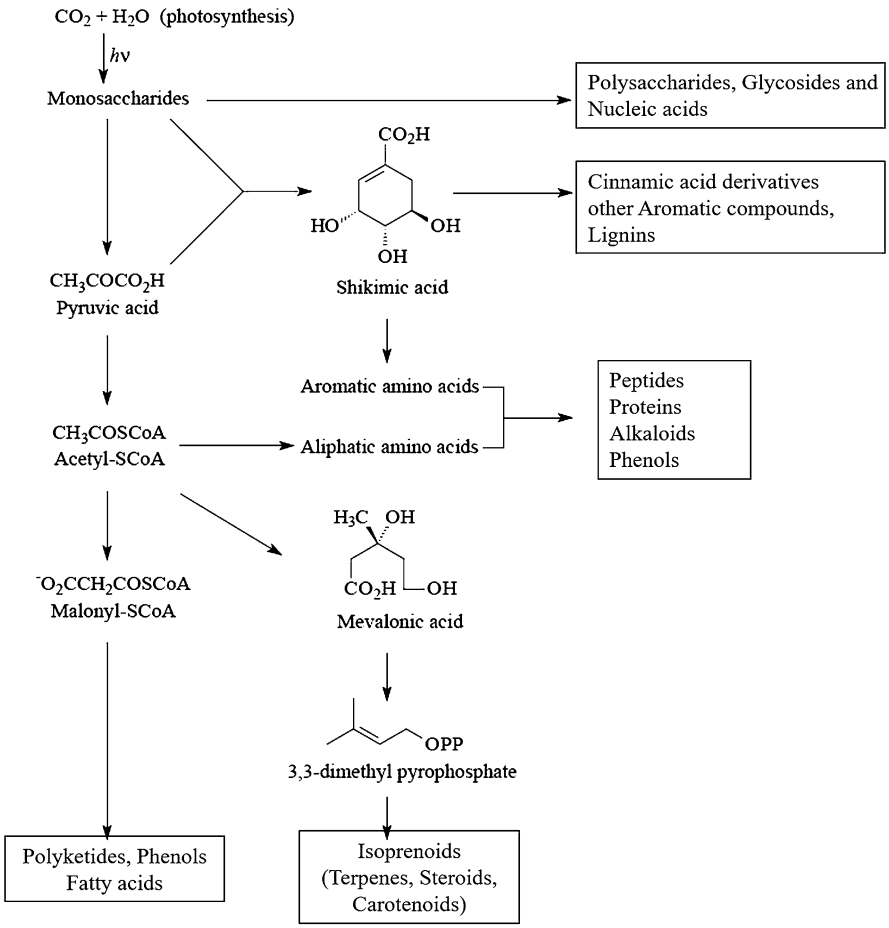

Fig. 1 Linkage between primary and secondary metabolites in plant systems

as phytoanticipins, while the induced ones are known as phytoalexins [3, 4]. Plant secondary metabolites include alkaloids, amines, cyanogenic glucosides, glucosinolates, nonprotein amino acids, organic acids, terpenoids, phenolics, quinones, polyacetylenes, and peptides. It has been reported that plants produce over 100,000 individual structures of these compounds [7–9]. Generally, plants produce a complex of compounds as defense against insect herbivores, which act in combination or synergistically against the stress [8, 10]. The combined effect of these secondary metabolites against insect herbivores has been suggested to reduce the chances of adaption and/or resistance by insect pests to plant chemical defense [3–5]. Though the main role of plant secondary metabolites is defense against biotic stresses, some of these compounds are utilized by plants to attract pollinators. The pollinator insects are attracted to the smell and color of the flowers. The color of flowers is determined by anthocyanins, flavonoids, or carotenoids, and the smell is exhibited by the terpenoids, phenylpropanoids, and amines. However, to prevent plants from eating by pollinators, plant secondary metabolites, which are otherwise attractant to pollinators, deter and are toxic to flower-eating pollinators. Also, flowers contain sugar-rich nectar, which is preferred by insect pollinators to the flower material [11]. Insect-plant interaction shows trophic specialism with about 80% of specialist herbivores that feed on a limited number of plant species belonging to a single genus or family [3–5]. To combat insect attack, plants have evolved a variety of phytochemical defenses. The role of these secondary metabolites against insect herbivores has been studied in detail.

The activation of phytoanticipins during herbivory occurs by β-glucosidase, which then signals the production and release of many biocidal aglycone metabolites [12]. For example, in cruciferous plants, due to the tissue disruption by insect herbivory, glucosinolates are hydrolyzed into cyanogenic compounds by myrosinases (endogenous β-thioglucoside glucohydrolase) during tissue disruption. In Poaceae family, when tissues are ruptured by herbivore damage, the phytoanticipins such as Benzoxazinoids -glucosides are hydrolyzed to biocidal aglycone benzoxazinoids by the plastid-targeted β-glucosidases during tissue by herbivory that are toxic to insect pests [12]. In addition to plant defense against insect herbivores, secondary metabolites increase the fitness of the plants.

2 Insect Herbivory and Sensing by Plants

The sensing of insect attack by plants has been very fascinating. Scientists across the globe have identified several mechanisms by which plants perceive the insect attack and mount defense against them. Plants sense defense elicitors in the insects' oral secretion/saliva and in the ovipositional fluid secreted by insects while egg-laying either to ward off the other insects to lay the eggs at the same place or to glue the eggs to the plant surface [1–3, 13, 14]. Specific oral secretions have been identified from the oral secretions of the insect pests. These include fatty acid conjugates (FACs) that stimulate the plant defense. Oral secretion of *Spodoptera exigua* (Hüb.) contains an elicitor, namely, volicitin (N-(17-hydroxylinolenoyl)-L-glutamine) [15]. This is the

first elicitor identified from the oral secretion of an insect pest. Volicitin application in maize induced the release of plant volatiles that attract the natural enemies of the insect pests [15]. Though many elicitors have been identified in insect oral secretions, only a few of them have been found to induce strong defense against insect pests [1–3, 13, 14]. For example, N-linolenoyl-glu, an elicitor from the oral secretion of the tobacco hornworm, *Manduca sexta* (L.), induced plant defense in tobacco plants by activating various enzymes that mediate the plant defense signaling pathways [16]. These include mitogen-activated protein kinase (MAPK), wound-induced protein kinase (WIPK), and salicylic acid-induced protein kinase (SIPK) (JA-Ile) [2, 17]. Not only against insect pests, but the pathways activated by the elicitors from insect oral secretions also activate plant defense against abiotic stresses including cold and drought [2].

Oral secretion of *Pieris brassicae* (L.) contains β-glucosidase that is perceived by plants to mount defense against the pest. The ovipositional fluid of cowpea weevil contains bruchins that are perceived by legumes, which in turn produce toxic compounds against the pest [14]. Further, the ATPase fragments in plants also elicit defensive response against insect herbivores [1]. The tobacco plants perceived the FACs in insect oral secretion, which induces the synthesis and accumulation of 7-*epi*-jasmonic acid, which, in turn, mediates the octadecanoid pathways involved in plant defense [16]. In addition to FACs, other compounds in insect oral secretions that are perceived by plants for stimulating defense against insect herbivory include inceptins and caeliferins [1, 3, 13]. The oral secretions of *Schistocerca americana* (Drury) contain caeliferins, which are sulfated fatty acids [13]. Though most of the identified compounds in the oral secretions of insect pests activate plant defense, some of the compounds suppress the plant defense against insect herbivory. In *Arabidopsis*, some of the compounds in the oral secretions of *Spodoptera littoralis* (Boisd.) and *P. brassicae* suppress the plant defense, thus causing more plant damage and ultimately increased larval growth and development [18]. Insect oral secretions contain glucose oxidase (GOX) which induced defensive response in some plants but not in others. For example, GOX in the oral secretion of European corn borer, *Ostrinia nubilalis* (Hübner), induces plant defense against the pest by increasing the expression of *LIPOXYGENASE* (LOX) and 12-*OXO-PHYTODIENOIC ACID* (OPR) genes [19–21], which are involved in the JA signaling pathway. The GOX in the oral secretion of *O. nubilalis* also activates defense responses in tomato and not in maize [21]. In tomato, GOX in the saliva of *Helicoverpa zea* (Boddie) triggers the defensive responses [20] but shows a reverse response in tobacco [19]. Additionally, some unidentified compounds in the oral secretions of insect pests induce the expression of *proteinase inhibitor 2* (*PIN2*) in tomato and *maize protease inhibitor* (MPI) in maize [21].

3 Types of Plant Secondary Metabolites

Since plant secondary metabolites are the main plant defensive compounds against a variety of stresses including insect herbivory, it is very important to study the synthesis and application of these compounds to implicate them in successful and

stable pest management programs. The synthesis and the signaling pathways mediated by these compounds are highly complex. These chemical compounds work either in combination or synergistically against particular stress through several modes of action to avoid any resistance and/or adaptation to these chemicals [3, 6–9]. However, some of these plant secondary metabolites are antagonistic as well [9]. A number of techniques have been utilized to identify, purify, separate, synthesize the active compounds, and study their biological activities [8]. The important analytic techniques involved are nuclear magnetic resonance, infrared spectroscopy, mass spectroscopy, and ultraviolet spectroscopy [9, 23]. These techniques have revolutionized the area of analytic chemistry and have made it easier to elucidate the isolated compounds. Plant secondary metabolites are divided into three chemically distinct groups. These include terpenes, phenolics, and nitrogen-containing compounds.

3.1 Terpenes and Defense Against Insect Pests

Terpenes constitute the largest class of secondary metabolites. The name "terpene" has been given after the isolation of the first terpene compound, a monoterpene (C10) from turpentine oil in the 1850s. Though some of the terpenes (gibberellins, brassinosteroids) are primary in function with the role in plant growth and development, most of them are defensive in nature. Terpenes are toxic compounds that act as feeding deterrents to insect pests. Terpenoids consist of about 25,000 compounds [22] having diverse functions. Most of the terpenoids are defensive in nature and are implicated against generalist herbivores as toxins, feeding deterrents, or oviposition deterrents. However, the specialist insect pests that are adapted to plant secondary metabolite herbivores, they use these terpenoids to recognize the host plants as attractants and feeding stimulants. Further, terpenoids can serve as attractants for pollinators and fruit-dispersing agents [22]. The synthesis of terpenoids occurs from the precursor 5-carbon (isoprene) units. Most of the terpenes are thermally decomposed to isoprene gas. However, the 5-carbon isoprenes can be polymerized into diverse terpenoids, also known as isoprenoids [23]. The terpenes are synthesized from acetyl-CoA or its intermediates through mevalonic acid (MVA) pathway or by methylerythritol 4-phosphate (MEP) pathway (Fig. 2) [24]. The C5 units are considered as building blocks in the biosynthesis of terpenes and exist as isopentenyl diphosphate (IPP) or its isomer dimethylallyl pyrophosphate (DMAPP). It is generated from the condensation of three molecules of acetyl-CoA through MVA pathway and is mediated by the enzymes 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA) synthase, HMG-CoA reductase, and MVA kinase. During this synthesis, IPP is repeatedly added to the reaction by the enzyme prenyltransferase enzymes, which then produces the allylic diphosphate esters such as geranyl diphosphate, farnesyl diphosphate, and geranylgeranyl diphosphate. These allylic diphosphate esters serve as immediate precursors for the synthesis of different classes of terpenoids. The prenyl diphosphates then lead to the production of terpenoid skeletons and ultimately produce a variety of terpenoids based on the addition or subtraction of carbon.

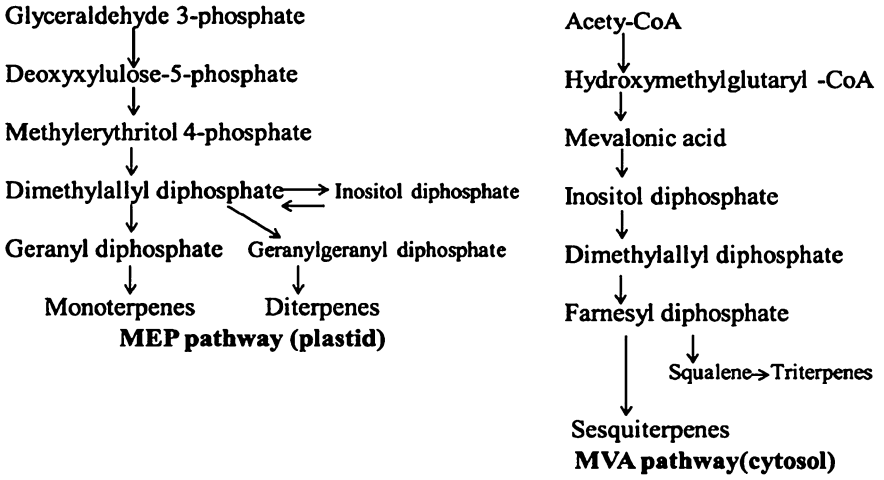

Fig. 2 Terpenoid biosynthesis in plants. (From War et al. [24])

Based on the number of isoprene units in the parent nucleus, the terpenoids are classified into monoterpenes with two isoprene units (C10), sesquiterpenes contain three isoprene units (C15), diterpenes contain four isoprene units (C20), triterpenes contain five isoprene units (C30), tetraterpenes contain eight isoprene units (C40), and polyterpenes contain more than eight isoprene units. Monoterpene-derived compounds also play an important role in defending plants against insect herbivory. One such class is the cyclopentanoid monoterpene-derived compounds known as iridoids. These compounds taste bitter and are considered as a powerful defense against insect pests due to their deterrent effect [25, 26]. Iridoid glycosides form covalent bonds with the nucleophilic side chains via imine formation of the amino acids, proteins, and nucleic acids, leading to their denaturation [26, 27]. This reduces the nutrient quality of the plant tissues rendering insects devoid of protein and nucleic acids. Further, enzymes involved in the synthesis of prostaglandins and leukotrienes are inhibited by iridoids, which has a drastic effect on insect growth and development [27–29]. Under in vitro conditions, insect pests fed on the artificial diets containing iridoids and showed reduced growth, increased larval period, and reduced survival [25, 30]. However, the effect of iridoids on insect growth and development when provided with artificial diets is species specific. For example, when *Lymantria dispar* (L.) larvae are fed on the artificial diet containing asperuloside, they showed reduced growth and development but did not show any difference when fed on diet containing aucubin or catalpol [30]. The aucubin or catalpol affected the growth and development of *Spodoptera eridania* (Stoll); however, no response was observed in this insect in response to asperuloside [25].

Benzoxazinoids are a group of indole-derived plant secondary metabolites involved in defense against insect herbivores. These are present in some important crops such as wheat, maize, and rye [31, 32]. These compounds either have antifeedant, deterrent

effects or are directly toxic to insect pests, thus reducing their growth and development [31, 32]. In addition, these compounds also regulate other defense mechanisms in plants [33]. Their roles in plant defense against insect pests have been studied on the pest species of cereal crops and include insects such as chewing (caterpillars), piercing-sucking (aphids), and root insect pests. In lepidopteran insect pest, the degradation of hydroxamic acids and N-*O*-methylated hydroxamic acids into toxic benzoxazolinones is facilitated by the alkalinity of the gut. In maize, benzoxazinoids toxicity and deterrence toward European corn borer, *O. nubilalis*, have been extensively studied. Larvae of *O. nubilalis*, when fed on artificial diet containing DIMBOA, showed increased mortality and developmental times to pupation in a dose-dependent manner [34]. It has been suggested that the toxicity of benzoxazinoids is positively linked to the degradation products such as benzoxazolinones [35]. These compounds also reduce insect digestibility by inhibiting the activities of trypsin and chymotrypsin [36]. Reduced consumption of leaves of maize by *O. nubilalis* has been reported on account of the higher concentration of these compounds [37]. Benzoxazinoids have also been reported as plant defensive traits against aphids – the piercing and sucking insect pests. These compounds either are directly toxic to aphids or activate the deposition of callose and also signal plant defensive pathways that mediate defense against aphids [33]. Benzoxazinoids such as hydroxamic acid levels in cereals have been reported as positively correlated with resistance toward *Metopolophium dirhodum* (Walk.) [38], *Schizaphis graminum* (Rond.) [39], *Sitobion avenae* (Fab.) [40], and *Rhopalosiphum padi* (L.) [41]. The mixture of volatile monoterpenes and sesquiterpenes in plants is called essential oils. These oils are generally feeding deterrents against insect pests; however, some of them are highly toxic to insect pests. For example, limonene in citrus plants repels the leaf cutter ant *Atta cephalotes* (L.) [42]. The monoterpenes produced in conifers, such as pine and fir, are toxic to several insects, including bark beetles [43]. The phytoecdysones that are steroids isolated from common fern *Polypodium vulgare* L. affect insect growth and development by interfering with molting process due to its similarity to molting hormones [44]. Further, some terpenoids such as amide derivatives act as insect juvenile hormone analogs and interfere with their growth and development [45]. The role of volatile terpenoids in defense against insect herbivores is more thoroughly covered in the later section of this review concerning indirect defenses.

3.2 Phenolic Compounds and Defense Against Insect Pests

A large variety of secondary compounds are produced by plants that contain a hydroxyl functional group (phenyl group) on an aromatic ring. These are the most widely distributed secondary metabolites. Though the terms “phenol” and “polyphenol” have been grouped as substances containing an aromatic ring with one (phenol) or more (polyphenol) hydroxyl substituents, plant phenols constitute a chemically heterogeneous group of nearly 10,000 individual compounds. These compounds are soluble either in organic solvents or in water, and

constitute carboxylic acids and glycosides, however, some are insoluble polymers. The chemical structure of plant phenols is diverse ranging from simple phenols (i.e., catechols and hydroxybenzoic acid derivatives) to catechol melanins (C6)6 long chain polymers with high molecular weight, condensed tannins (C6-C3-C6)n, and lignins (C6-C3)n. Flavonoids (C6-C3-C6) and stilbenes (C6-C2-C6) possessing intermediate molecular weights are also phenolic compounds. The chemical diversity of these phenolic compounds enables them to play a diverse role in plant systems. Most of these phenolic compounds are involved in plant defense against a variety of stresses including insect herbivory, some attract the pollinators, some are involved in mechanical support, and some absorb ultraviolet radiation as well. The phenolic compounds include simple phenols (caffeic and ferulic acid), phenylpropanoid lactones (known as coumarins, psoralen, and umbelliferone), and benzoic acid derivatives (vanillin and salicylic acid). The role of phenolic compounds in plant defense against insect herbivory has been studied in detail. They either are directly toxic to insect pests or oxidized by peroxidases or polyphenol oxidases to toxic compounds that affect insect growth and development [46]. The products of the oxidative reactions include quinones, which either are directly toxic to insect pests or reduce the nutritive value of the plant tissues by cross-linking with the nucleophilic side chains of proteins and free amino acids, thereby reducing their palatability to insect pests [47, 48]. It has been reported that the wheat cultivars with high phenol content are less preferred by cereal aphid *R. padi* [49]. The groundnut plants show higher induction of phenols when attacked by *H. armigera* and by exogenous application of jasmonic acid [50]. In *Salix*, the benzoic acid-derived salicylates reduce growth and development of oak moth *Operophtera brumata* L. larvae [51]. In strawberry *Fragaria*, the spider mites *Tetranychus urticae* do not feed on the plants containing higher amounts of catechol-based phenolics [52]. Gossypol, an important cotton phenolic pigment, is toxic to many insect pests including *Heliothis virescens* [53] and shows repellence against numerous insects [54].

3.2.1 Tannins and Defense Against Insect Pests

Tannins is one of the most important groups of plant secondary metabolites utilized in defense against insect pests. Tannins can be hydrolysable tannins or condensed tannins. The condensed tannins are generally involved in plant defense against insect herbivory. They are bitter compounds, which either are toxic to insects or deter them, thus providing both direct and indirect defense against insect pests. Generally, tannins have an affinity with the midgut proteins and digestive enzymes of insect pests, which affects protein digestion in insects [3, 4, 6, 55]. Binding of tannins to gut proteins through hydrogen or covalent bonds leads to the chelation of metal ions, precipitation of proteins, and the production of gut lesions in insect herbivores [55, 56]. Tannins are constitutively present in plants and are also induced in response to insect herbivory and elicitor application [6, 55, 56]. On insect herbivory, plants show a high accumulation of tannins. For example, in *Pinus sylvestris* L. [57], *Populus* species [6, 58], and some *Quercus* species [59], the application of elicitors or insect infestation activates the synthesis of condensed tannins. Condensed tannins either are directly toxic to insect pests (antibiosis) or deter insect from feeding

(antixenosis). Direct toxicity of tannins has been studied in a number of insect pests [55, 56]. The indirect effect of condensed tannin through feeding deterrence has been reported in *L. dispar*, *Aphis craccivora* Koch, *Euproctis chrysorrhoea* (L.), *O. brumata*, and *Schistocerca gregaria* (Forsk.) Kuhn [60–62].

3.2.2 Flavonoids and Defense Against Insect Pests

Flavonoids and isoflavonoids are toxic to insect pests. They affect the behavior and growth and development of insects [63, 64]. Apart from their effect on insect growth and development, flavonoids in plants scavenge the highly reactive and unstable free radicals, including ROS, and chelate with metals, thus reducing their formation [65]. Plants contain more than 5000 flavonoids, which can be grouped into anthocyanins, proanthocyanidins, flavones, flavonols, dihydroflavonols, flavanones, aurones, flavan, and chalcones [65]. Some of the flavonoids belonging to flavonols, flavones, flavans, flavan-3-ols, proanthocyanidins, flavanones, and isoflavonoids act as feeding deterrents against many insect pests. For example, *Spodoptera exempta* (Walk.) and *S. littoralis* are repelled by the flavones 5-hydroxyisoderricin, 7-methoxy-8- (3-methylbutadienyl)-flavanone, and 5-methoxyisoronchocarpin present in *Tephrosia villosa* (L.), *T. purpurea* (L.), and *T. vogelii* Hook, respectively [66]. In *Arabidopsis*, higher production of flavonoids by overexpressing of transcription factor controlling their production confers high levels of resistance to *Spodoptera frugiperda* (J.E. Smith) [67]. In addition to providing resistance against insect pests, some flavonoids such as Angustone A, licoisoflavone B, angustone B, and angustone C. isoflavones, licoisoflavone A, luteone, licoisoflavone B, and wighteone possess antifungal activity against *Colletotrichum gloeosporioides* (Penz.) and *Cladosporium cladosporioides* (Fres.) [68]. Further, in chickpea, the isoflavonoids judaicin, judaicin-7-*O*-glucoside, 2-methoxyjudaicin, and maackiain showed antifeedant activity against *Helicoverpa armigera* (Hubner) at merely 100 ppm. In addition, judaicin and maackiain have been reported as a deterrent to *S. littoralis* and *S. frugiperda*, respectively [69]. Flavonoids such as cyanopropenyl glycoside, alliarinoside, and isovitexin-6″-D-β-glucopyranoside inhibit feeding activity of the native American butterfly, *Pieris napi oleracea* L. [70].

3.3 Sulfur- and Nitrogen-Containing Plant Secondary Metabolites

One of the important groups of sulfur-containing secondary metabolites present in Brassicaceae and Capparales is glucosides. The glucosides are derived from amino acids, and there are about 120 structures of these glucosides [71]. Amino acid precursor on the side chain determines the type of the glucosides. Four groups of glucosides include the compounds derived from amino acid methionine (aliphatic glucosinolates), glucosinolates derived from tryptophan (indole glucosinolates), glucosinolates derived from phenylalanine or tyrosine (aromatic glucosinolates), and the glucosinolates derived from several different amino acids or the one with unknown biosynthetic origin [71]. These groups constitute 50%, 10%, 10%, and

30% of the total glucosinolates, respectively. The glucosinolates occur in abundance in roots than in shoots. High concentration of indol-3-ylglucosinolate occurs in shoots, but the concentration of its methoxyderivatives and aromatic 2-phenylethyl glucosinolates occurs in roots. Generally, the glucosinolates in roots are constitutive, and the ones in shoots are induced in response to stresses including herbivory [72]. Glucosinolates are present in the cell vacuole [73] and are protected from myrosinases (thioglucosidases). When the cell disrupts due to herbivory, the glucosinolates are hydrolyzed by myrosinases producing toxic isothiocyanates, nitriles, and thiocyanates. These breakdown products are highly toxic to insect herbivore and also act as feeding repellents [74]. The flea beetle feeds on *Phyllotreta cruciferae* Goeze cotyledons of *Sinapis alba* L. (white mustard) with low levels of glucosinolate sinalbin [75]. The insecticidal properties of glucosinolate breakdown products have been reported to be are at par with the synthetic insecticides [76].

4 Modes of Action of Plant Secondary Metabolites

Alkaloids, one of the groups of plant secondary metabolites involved in plant defense against herbivory, is highly toxic to insect pests. These compounds modulate neuronal signal transduction, thus affecting ion channels, neurotransmitter inactivating neurotransmitter receptors, transporters, and the enzymes. The toxic effect of alkaloids on neuronal signal transduction alters the concentrations and expression of neurotransmitters and the activity of neurotransmitter receptors. This leads to the severe changes in insect physiology and behavior of the insect that may eventually lead to the direct insect toxicity, or the insect may develop non-preference for the specific host. Some of the plant secondary metabolites that modulate neuronal signal transduction include erythrina alkaloids, nicotine, tubocurarine, ergot alkaloids, agroclavine, muscarine, caffeine, theobromine, theophylline, etc. [77]. Some serve as protein inhibitors. For example, ricin, abrine, emetine, and lycorine interfere with protein synthesis in ribosomes [7, 77], while some plant secondary metabolites alter protein structure and function. A number of plant secondary metabolites interact with the cytoskeleton of cells, thus interfering with cell division. The specific inhibitors such as colchicine, vinblastine, podophyllotoxin, sanguinarine, maytansine, and rotenone inhibit microtubule assembly, which is required for the assembly of the mitotic spindle during cell division [77]. However, most of the plant toxins such as phenolics interact with protein by forming multiple hydrogen and ionic bonds, thereby altering the 3D structure of proteins [7, 8, 55, 56, 61, 77]. Apart from this, some plant secondary metabolites contain highly reactive but unstable functional groups which interact with amino, sulfhydryl, or hydroxyl groups of amino acid residues of proteins, thereby changing their structure and functional properties. Some of the plant secondary metabolites are lipophilic in nature include mono-, sesqui-, di-, and triterpenes, phenylpropanoids, steroids, and mustards oils. Further, the lipophilic terpenes can modify the 3D structure of the globular proteins by assembling in the inner hydrophobic core of globular proteins. Further, the lipophilic plant secondary metabolites attack the biomembranes surrounding all the living cells and intracellular

compartments. In addition to changing the structure of proteins, lipophilic compounds change the fluidity and permeability of biomembranes by being trapped inside them. Saponins are plant secondary metabolites containing a lipophilic steroid or triterpene moiety with a sugar chain that is hydrophobic. These amphiphilic compounds form complexes with membrane cholesterol. In addition to the role in modulating neuronal signal transduction, inhibiting protein synthesis, altering the protein structures, and interacting with biomembranes, some of the plant secondary metabolites interfere with metabolizing nucleic acid and enzymes [7, 8]. Some of these compounds are involved in intercalating the DNA. The plant secondary metabolites that are usually aromatic, planar, and hydrophobic intercalate between the planar stacks of nucleotide pairs such as GC-pairs. The intercalation of DNA by these compounds stabilizes the DNA during the replication process, thus preventing the activities of helicases and RNA, thereby inhibiting the intermediate steps during DNA replication. Further, the frameshift mutations and deletions by these plant secondary metabolites lead to cell death [78]. The DNA intercalating compounds have been reported in the groups of protoberberine and benzophenanthridine alkaloids, which include berberine and sanguinarine [7, 79]. The alkaloid plant secondary metabolites with intercalating properties have been detected in quinoline alkaloids (such as quinine), emetine, furanoquinoline alkaloids, anthraquinones, furanocoumarins, and beta-carboline alkaloids [7–9]. It has been reported that some plant secondary metabolites with intercalating properties inhibit the activity of DNA topoisomerase I or II that are involved in DNA replication. The DNA alkylating agents directly bind to nucleotide bases and form covalent bonds, which may cause mutations and genotoxicity [7–9, 78].

5 Insect Adaptation to Plant Secondary Metabolites

Insects have developed adaptations to toxic plant secondary metabolites in a number of plant systems. The adaptations to plant secondary metabolites are either by detoxification, degradation, excretion, or sequestration (Fig. 3).

5.1 Plant Secondary Metabolites and Insect Detoxifying Enzymes

Plant secondary metabolites are also detoxified to either less toxic or nontoxic constituents by insect pests using detoxifying enzymes. The involvement of the detoxifying enzymes in insect adaptation/tolerance to plant toxins depends on host diet composition and insect species and can involve glycosylation, glutathionation, sulfation, or deacylation [80–82]. A number of enzymes are involved in the detoxification of plant toxins by insect pests. Some of these enzymes occur in the cytoplasm of midgut cells where they are involved in preventing damage to biological molecules and direct the excretion of toxic compounds; however, some detoxification enzymes are secreted into the midgut lumen where they metabolize plant toxins before they enter cells [83]. The detoxification enzymes involved in plant toxin metabolism include cytochrome P450 monooxygenases (P450s), the

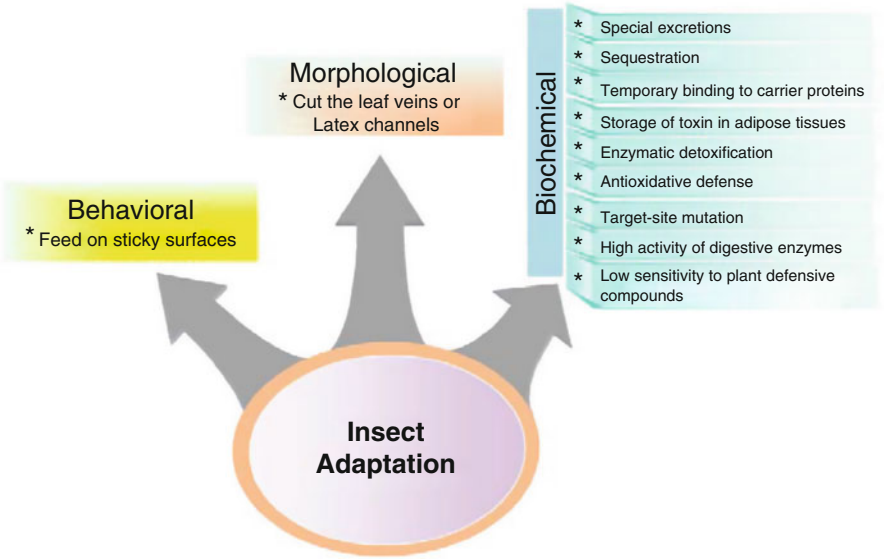

Fig. 3 Adaptation of insect pests to plant defensive traits. (Source [174])

glutathione S-transferases (GSTs), and the carboxylesterases (COEs). These enzymes are generally present in insects in low concentrations but are induced when insects feed on toxic metabolites to convert them into low toxic or nontoxic compounds [64]. Among the detoxifying enzymes, P450s are the main enzymes employed by insects against plant allelochemicals [84].

The complete mechanism of detoxification of furanocoumarins by Papilionidae lepidopterans using P450s has been studied in detail. The role of P450s in detoxification of plant allelochemicals has been studied in many insect pests including parsnip webworm *Depressaria pastinacella* Dup. [85], several *Helicoverpa* species [86], and *M. sexta* [87]. Apart from direct induction of P450s in response to plant toxic secondary metabolites, *H. zea* uses plant signaling molecules such as jasmonate and salicylate, which mediate plant defensive pathways in plants, to produce more P450s that can detoxify plant toxins [88]. The *Drosophila mettleri* Heed that feeds on cactus-containing toxic allelochemicals contains high amounts of P450 that are involved in the detoxification of plant toxins [89]. In *S. frugiperda*, detoxification of highly toxic isothiocyanates such as 2-phenylethyl isothiocyanate, indole-3-carbinol, and indole-3-acetonitrile in insect midgut is mediated by P450 [90]. The *Papilio polyxenes* Fab. that feeds on plants containing furanocoumarins has been shown to tolerate up to 0.1% xanthotoxin in artificial diet that is then metabolized by P450 [91]. The role of P450-detoxifying enzymes in insect-plant interaction has been sequenced in *P. polyxenes*. It has been reported that CYP6B1 gene codes for P450 enzymes, which is responsible for detoxification of plant toxins. Further, the expression of the genes *CYP6B161* and *CYP6B162* that code for P450s is induced in lepidopteran cell lines in response to plant toxins such as furanocoumarins, such as xanthotoxin and

bergapten [92]. In *Arabidopsis*, the toxic dihydrocamalexic acid is converted to less toxic camalexin by P450 PAD3 [93]. Characterization of P450s from insect has led to further understanding of insect adaptation to plant toxic secondary metabolites. It has been reported that in *Musca domestica* L., *CYP6A1* is involved in the detoxification of terpenoids [94]. Gossypol, one of the important plant secondary metabolites in cotton, is metabolized by P450 monooxygenase CYP6AE14 in *H. armigera* [95]. Similarly, in *Anopheles gambiae* Giles, xanthotoxin and bergapten (furanocoumarins), furanochromones, and natural myristicin, safrole, and isosafrole are detoxified by CYP6Z1 [96]; however, xanthotoxin, lignan, piceatannol, and resveratrol are detoxified by CYP6Z2 [97]. In *Diploptera punctata* (Esch.), the sesquiterpenoids are hydrolyzed by CYP4C7 [98]. In bark beetles, *Ips pini* Say and *Ips paraconfusus* Lanier, P450s detoxify monoterpenes, sesquiterpenes, and diterpenoid resin acids [99].

Glutathionation by GST superfamily is an important detoxification mechanism used by insect herbivores against plant chemicals and insecticides. Detoxification of plant allelochemicals by insect pests using GST has been studied in many lepidopteran insects [100–102]. GST is a complex and widespread enzyme family with high levels of variability among insect pests. Generally, insect GSTs catalyze the conjugation of glutathione to electrophilic toxic molecules, leading to the formation of water-soluble glutathione S-conjugates that are easily degraded and eliminated by the insect [82]. Though GSTs are constitutively present in insects, they are often induced in response to plant toxic metabolites [64]. The role of GSTs' detoxification of plant metabolites has been studied in numerous insect pests [100]. In *Myzus persicae* (Sulzer), higher levels of GSTs in response to feeding on brassicaceous host plants have been attributed to the insect adaptation to toxic plant metabolite such as glucosinolates and isothiocyanates [103]. Plant toxic metabolites, when added to an artificial diet fed to *H. armigera*, the insect showed higher levels of GST activity in insect pests, which might be due to the insect trying to adapt to the plant toxins [64]. Predatory hoverfly, *Episyrphus balteatus* (De Geer) feeding on the *Myzus* aphids also showed induction of GST showing that plant toxins in aphid host induced the enzyme activity in the predator [104]. It has been reported that the caterpillars of spruce budworm fed on balsam fir *Abies balsamea* (L.) foliage showed higher expression of *Choristoneura fumiferana* GST mRNA and proteins in whole body extracts of sixth instar larvae than the ones fed on the artificial diet [101]. The detoxification mediated by GST occurs in insect midgut, fat body, and hemolymph [100, 102, 105]. The intracellular GST detoxification of plant toxins occurs by binding of lipophilic compounds to the reduced GSH and is then removed easily from the cells [102]. The GSH is generally present in high concentration in intracellular compartments and has high affinity with GSTs; however, in the midgut, the ratio of reduced to oxidized GSH is used as an oxidative stress indicator from plant toxins [106]. Further, electrophilic sites of lipophilic substrates are neutralized by the attachment of reduced GSH by GST-catalyzed conjugation. Recently, Donkor et al. [107] showed in spruce budworm that GST activity was higher at an alkaline than at neutral pH, suggesting that GST could also function in the midgut lumen of the insect pests. The greater

diversity of GSTs in generalist insect pests than the specialist enables the generalists to adapt to a broader range of plant toxins [103].

Esterases constitute one of the large groups of phase 1 metabolic enzymes involved in the metabolization of insecticides and plant toxins. Carboxylesterases hydrolyze ester bonds in plant chemicals with a carboxylic ester. Though very few studies have shown the role of carboxylesterases in the detoxification of plant allelochemicals, their role in the detoxification of plant-derived insecticides such as pyrethroids has been studied in detail and is thus supposed to metabolize the plant secondary metabolites as well [108, 109]. Esterases detoxify toxic compounds through enzymatic cleavage or sequestration of the toxic compounds. The compounds are hydrolyzed into less toxic or nontoxic polar compounds that are easily excreted from the insect body.

In addition to the above-discussed enzymes, one more family of enzymes in insects involved in resistance to plant secondary metabolites is the UDP-glycosyltransferases (UGTs) [110]. These enzymes catalyze the transfer of a glycosyl group from UDP-glucose to various acceptor molecules. In *M. sexta*, metabolism of plant compounds occurs by UGTs [111]. Further, a gene coding for UGT, BmUGT1 in silkworm *Bombyx mori* (L.), has been reported to degrade the flavonoids and coumarins [112]. Though the detoxification of plant compounds by insects using detoxification enzymes in insect midgut has been studied in detail, the conjugation of plant defensive metabolites in the midgut that renders the nonabsorptive warrants further attention. Understanding the conjugative interaction between plant metabolites and insect midgut that leads to the reduced toxicity of these metabolites in insects will help to target the conjugation process and to overcome the counter adaptation by insect pests.

5.2 Sequestration of Plant Secondary Metabolites by Insect Pests

Plants' defense against insects can be antibiosis or antixenosis. The antibiosis mechanism of defense is mediated through toxic plant secondary metabolites that affect insect growth and development. Further, the biochemical defense in plants is highly dynamic and is at most of the times induced in response to insect herbivory. According to a hypothesis, synergy between direct and indirect plant defensive systems enables the plants to dominate terrestrial ecosystems than the insect herbivores [113, 114]. Direct plant defense limits the food digestibility and negatively affects insect physiological processes, while the indirect defense recruits the natural enemies of insect pests. However, during the coevolution between plants and insects, it has been found that insects have adapted to plant defensive traits. Further, insect herbivores sometimes neutralize both these defenses to protect them against their enemies [115]. Insects discriminate and perceive a wide variety of plant chemicals even in very low concentrations by the chemoreceptors (gustatory and olfactory chemoreceptive systems) present on the antennae and mouthparts. Insects decode the information by the command centers in the central nervous system and decide whether to accept or reject the host plant based on the chemical cues perceived [116]. Insect pests develop a number of adaptations in response to plant defensive

traits. One of the adaptations to which insects withstand plant toxic secondary metabolites is sequestration. Sequestration is defined as the uptake and accumulation of selective and specific toxins of insect pests, which determines their growth and development [117]. A number of studies show the sequestration of plant secondary metabolite by specific tolerance strategies in which the functionality of the toxins is maintained and is tolerated by the insects in their bodies [118, 119].

Insect pests sequester plant toxins as a medium of self-defense to withstand plant toxins. Sequestration of plant toxic metabolites has been reported in many insects including oleander hawkmoth *Daphnis nerii* (L.) and the danaine butterfly *Euploea core* (Cramer) that feed on cardenolide-rich oleander [118–120]. In milkweed bugs (Lygaeinae), cardenolides are tolerated by sequestration [121]. In monarch butterfly *Danaus plexippus* (L.), sequestration of cardenolides occurs by target-site insensitivity, and the cardenolides are tolerated by the substitution of valine and histidine in place of leucine and asparagine, respectively, in a subunit of Na+/K + − ATPase [122, 123]. This substitution results in the reduced sensitivity of Na+/K + − ATPase to the cardenolides; thus, the cardenolides are taken up and accumulated in the insect body. It has been reported that in some monarch butterflies, Na+/K + −ATPase is sensitive to cardenolides, and insects avoid the accumulation of cardenolides in the hemolymph [124]. Further, some monarch butterflies sequester and store calotropin and its configurational isomer calactin from *Asclepias curassavica* (L.) Kuntze or *Asclepias fruticosa* (L.) WT Aiton [125].

Insect pests also tolerate glucosinolates in brassicaceous plants by sequestration. In these plants, a myrosinase enzyme is used to produce isothiocyanates [126]. Insects use sulfatases and nitrile-specifier proteins (NSPs) to reduce the synthesis of isothiocyanates from glucosinolates by covering the latter and avoiding their interaction with myrosinase [127, 128]. In glucosinolate sequestration, the products are not being used by insects for their self-defense; these insects use special mechanisms to withstand these toxins and hydrolysis inhibition of glucosinolates [122]. For example, turnip sawfly, *Athalia rosae* L., sequesters the glucosinolates by converting them to desulfoGS sulfates to avoid the synthesis of toxic isothiocyanates [129]. Furthermore, the larvae of *Athalia liberta* (Klug) and *B. brassicae* aphids also sequester glucosinolates by converting them to desulfoglucosinolate sulfates [129–132]. It has been reported that sequestration of glucosinolates may incur some to be costly to the insect species and may affect their growth and development [133]. Insects uptake certain glucosinolates through the gut membrane and require selective transporters based on the structural differences in the side chains [133, 134].

During tissue disruption, in some plants, the cyanogenic glycosides are hydrolyzed to toxic hydrogen cyanide (HCN) [135]. The HCN is detoxified by insects into less toxic compounds using β-cyanoalanine synthase enzyme [136]. Insects sequester the cyanogenic glycosides by stabilizing these compounds to avoid the synthesis of HCN. For example, the *Zygaena filipendulae* (L.) larvae reduce the activity of plant hydrolases by combining leaf-snipping with the alkaline gut [137]. Though the sequestration is associated with the functionality of the compounds, in some cases, retaining the activity of these secondary metabolites is not associated with sequestration. For example, in non-sequestering *Spodoptera* species, when tissues disrupt,

β-glucosidases stabilize the Benzoxazinoids by reglycosylation [138, 139]. In some milkweed bugs, though there is Na+/K + −ATPase target-site insensitivity, they do not sequester the cardenolides [121].

The sequestration of plant toxins occurs in the insect body, and these toxins need to be moved from the gut to the insect body for effective sequestration. The transportation of plant toxins from insect gut into the hemolymph has been studied in very few insects including *Chrysomela populi* L. and *M. sexta* [140, 141]. Sequestration of pyrrolizidine alkaloids has shown that different mechanisms such as passive absorption versus carrier-mediated transport are involved. Specific transport systems have been identified in insects involved in the transport of plant toxins from insect gut to the body. In *C. populi*, an ATP-binding cassette (ABC) transporter (*CpMRP*) is expressed in the defensive glands. In vitro studies have shown that CpMRP transports the plant-derived phenolglucoside salicin [140]. Further, when *CpMRP* was knocked down by RNA interference (RNAi), reduction of the defensive excretion of the beetles has been reported, thus showing its role in regulating transport and, thus, sequestration of plant toxins in *C. populi*. A model showing the non-selective transport of glucosylated plant secondary metabolites across the gut membrane by ABC transporters and then selectively transferring them into the secretory cells followed by their secretion into the defensive reservoir has been described in many insect pests including leaf beetles [134, 140]. Though some insects show specific pathways for the accumulation in hemolymph, whether the transport of plant toxins from the gut into the insect body for sequestration is selective or nonselective is still a debate. Some insects utilize plant defensive enzymes for the transport of plant toxins to hemolymph. For example, in *M. sexta*, a cytochrome P450 gene (*CYP6B46*) codes the proteins involved in the transportation of plant-derived nicotine from the midgut into the insect body [141]. However, the exact mechanism of CYP6B46 codes for the transport is unknown, whether this gene forms a part of the multicomponent pump involved in the conversion of nicotine into an intermediate compound suitable to cross the gut into the pump and then converts the intermediate compound to nicotine back or if there is some other mechanism facilitated by this gene [141, 142].

Though benzoxazinoids have diverse effects on insect pests, some of the insect pests successfully feed on benzoxazinoid-containing plants. Further, insects have developed resistance to these compounds. Some of the adaptive strategies involved in the adaptation of these compounds in insects include rapid excretion, detoxification, avoidance, sequestration, or target-site mutation [31, 142–144]. It has been reported that *Mythimna separata* Walker larvae excrete DIMBOA-Glc, HMBOA-Glc, and 1-(2-hydroxy-4-methoxyphenylamino)-1-deoxy-b-glucopyranoside-1,2-carbamate in the frass when fed on an artificial diet containing DIMBOA [142, 144]. Further, the incubation of midgut homogenates of insect pests such as Asian corn borer, *Ostrinia furnacalis* (Guenée) with DIMBOA, and UDP-glucose undergo glucosylation and form DIMBOA-Glc by UDP-glucosyltransferases [142–144]. The frass of *S. frugiperda* and *S. littoralis* larvae, when fed on diet containing DIMBOA, contained DIMBOA-Glc, HMBOA-Glc, and MBOA-Glc [138]. In wheat aphid, *S. avenae*, detoxifying enzymes such as cytochrome P450

monooxygenases, NADPH-cytochrome c reductases, glutathione S-transferases, and esterases have been reported to be associated with the adaptation to benzoxazinoids in wheat [145]. A specialist herbivore of maize, Western corn rootworm, *Diabrotica virgifera virgifera* LeConte, when fed on the benzoxazinoid-containing maize showed increased expression gene coding for cytochrome P450 and a cathepsin protease, showing their involvement in detoxification of these compounds [146]. Further, the adaptation of insect pests to benzoxazinoids involves rapid transport of these compounds to hemolymph and excretion with frass [147]. The larvae of specialist herbivore such as *S. frugiperda* performed better on DIMBOA-containing diets than larvae of the generalist *S. littoralis* [138]. This has been attributed to the detoxification of MBOA via N-glucosylation and its excretion into the frass in *S. frugiperda* [37].

Insect pests have developed adaptation of plant tannins. They not only convert tannins to less toxic compounds but also sue them for their growth and development. For example, the tree locust *Anacridium melanorhodon* Walk., when fed on the tannin-containing diet, showed an increased growth by 15% [148]. The adaptation to tannins has been attributed to the higher pH and lower oxygen levels in insect foregut [149, 150]. In caterpillars with higher pH, autoxidation of tannins to toxic compounds is reduced by the low levels of oxygen. In grasshoppers, some of the antioxidative compounds including glutathione, α-tocopherol, and ascorbate reduce the tannin toxicity [56, 151, 152]. Some insects transport tannins through peritrophic membrane into the hemolymph, where they are polymerized to polyphenols and excreted [153, 154]. Ultrafiltration of tannins has been reported in the theca of *S. gregaria* [155], which reduces their toxicity.

Plant volatile compounds play an important role in both direct defense by deterring the insect pests and indirect defense by recruiting natural enemies of the insect herbivores. They are constitutively present in the plants and/or are induced in response to herbivory; then they are known as herbivore-induced plant volatiles (HIPVs). However, insect pests have developed strategies to utilize these volatile compounds for their own benefits. For example, in maize, egg masses deposited by *S. frugiperda* moths suppress the emission of HIPVs [156]. Further, during multiple insect attacks, the HIPVs could deter one insect but may attract the other, thus resulting in increased damage [157].

Glucosinolates are the important plant secondary metabolites whose breakdown products are thiocyanates, which are highly toxic compounds involved in plant defense against insect pests. It is very important for the glucosinolates to come in contact with myrosinase, which are then hydrolyzed to form isothiocyanates. However, the specialist insect pests have developed adaptations to glucosinolates. For example, diamondback moth *P. xylostella* does not allow the glucosinolates and myrosinase enzymes that are present in different compartments of the cell to come together even after tissue disruption on account of insect attack [128]. *P. xylostella* produces sulfatase enzyme to convert isothiocyanates and nitriles into desulfoglucosinolates [128]. Furthermore, mustard greens, *Brassica juncea* (L.) Czern. plants with different myrosinase activity and glucosinolate profiles have been reported to provide defense against both generalist and specialist insect herbivores. Interestingly, the generalist insect pest such as *S. eridania* prefers feeding on

plants with low glucosinolate concentrations, but the lines with low myrosinase activity are preferred by the specialist *P. xylostella* [76]. The cabbage white butterfly *Pieris rapae* (L.) has also adapted to the glucosinolates; however, the mechanism employed is different. This pest redirects toxic isothiocyanate and forms the safe and nontoxic nitrile breakdown products with a specific gut protein [158]. The toxic cyanogenic glucosides in the leaves of *Passiflora auriculata* Kunth (passion vine) are converted to thiols by the specialist butterfly Sara longwing *Heliconius sara* (Fab.) [159]. Further, during this process, the butterfly releases nitrogen that is used in the insect's primary metabolism [159].

6 Ecological Costs of Insect-Plant Interaction

A variety of plant secondary metabolites are produced in plants in response to biotic and abiotic stresses. These secondary metabolites play an important role in plants against insect herbivory. The genetic variability among these metabolites enables them to be deployed against a range of environmental stresses. As the secondary metabolites are not involved in plant growth and development, they are differentiated from the primary metabolism that directly supports growth and development in plants [160]. However, there were assumptions that the production and storage and regulation of plant secondary metabolites may involve resource allocation and may negatively affect plant growth and development [161, 163]. Some authors accept that the production of plant secondary metabolites in response to herbivory incurs resource allocation, however, most of the secondary metabolites are induced only in response to the stress including herbivory, thus, are produced only when in demand [72]. Further, the cost incurred in the synthesis of plant secondary metabolites has been studied only in few cases [72]. In *Arabidopsis thaliana* (L.) Heynh., the use of knockout *Arabidopsis* mutants showed that the glucosinolate production affects the growth of the plant [163] and has been found to require about 15% of photosynthetic energy [164]. Though these reports are not encouraging regarding the role of plant secondary metabolites in plant defense keeping in view the allocation cost, some authors believe that the cost for the secondary metabolites is compensated by the defense against different stresses [162, 164]. Though induced defense is considered as one of the important strategies of plants against insect herbivores, the biochemical traits that are modified during insect-plant interaction may reduce the attraction of pollinators to the plant as the chemicals that are produced to deter the herbivores may also repel the pollinators [162]. Further, induced defense in plants against insect herbivores develops phenotypic plasticity in plants, which reduces the chances of plant adaptation to the defensive traits [165].

Though insects have been successful in mounting an adaptive response against plant secondary metabolites through sequestration and detoxification, this adaptive response may have some ecological costs on the part of the insect pests. The cost that the insect may have to pay for the successful adaptation to plant toxins is manifested by impacting behavior, reproduction, survival, or immunity [166, 167]; however, the actual cost incurred in this process will depend on the type of metabolites

synthesized and the specific method of resistance that insect pests employ [166]. Though the specialist insect herbivores incur costs for adapting to plant toxins, they are benefited more in terms of protection against natural enemies [168]. For example, monarch butterflies, *D. plexippus,* milkweed bugs *Oncopeltus fasciatus* (Dallas) and milkweed aphids *Aphis Nerii* Boyer de Fon., when feed on the cardenolide containing plants are not preferred by the predatory birds [169], mantids [170], and spiders [171], respectively. The types and the concentration of cardenolides differently affect the parasitoid emergence from the cardenolide-adapted aphids [172, 173].

7 Future Outlook

Plants produce highly toxic compounds against insect pests; however, insects in return have developed various adaptations to withstand the toxicity of plant toxins. Though the role of plant secondary metabolites as the components of plant resistance against insect pests has been studied in detail, adaptations by insect pests to them have put a major challenge in utilizing them in pest management program. Further, there is a need to understand the underlying mechanisms of insect adaptations to plant secondary metabolites. To understand the cost incurred by plants in producing toxins and the cost involved in insect adaptation to these toxins is equally important. Furthermore, the identification of genes that encode pest-resistant plant toxins and the genes that encode insect adaptive traits would be highly useful to identify the pathways that could be either activated or blocked through RNAi technology. Further, studying the importance of insect adaptation mechanisms to plant secondary metabolites through molecular manipulation of insect genes would be an exciting future prospect. The availability of insect genome will be very useful to study the insect-plant interaction and identify genes that are involved in the insect resistance to plant toxins.

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How Galling Organisms Manipulate the Secondary Metabolites in the Host Plant Tissues? A Histochemical Overview in Neotropical Gall Systems

32

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Contents

Abstract

The histochemistry approach has been used to understand cell metabolism modulation by galling organisms on their host-plant organs and the relationship of the accumulation of metabolites with the ecological and physiological roles. The main secondary classes of metabolites (i.e., phenolic compounds, terpenes, and alkaloids) have been mainly associated with the protection of galling organisms, scavenging of oxidative stress molecules, and the development of gall tissues. Therefore, this chapter brings together a compilation of the gall researches that

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assessed and discussed the role of secondary metabolites through histochemical approaches. The compartmentalization of secondary metabolites in different gall tissue sites, their related functions, and the detailing of the well-defined histochemical tests adopted in gall researches are the focus of this chapter.

Keywords
Alkaloids · p-dimethylaminocinnamaldehyde · Dragendorff · Ferric chloride III · N, N-dimethyl-p-phenylenediamine · Phenolics · Terpenes

1 Introduction

The interactions between insects and plants are one of the most intriguing and sophisticated relationships in nature. This association boosted the evolution and diversification of insects, the most successful group of organisms on earth, with more than four million estimated species [1]. Taking for granted that some interactions with insects can be harmful to plants, the production of secondary metabolites may provide a defensive role against the attack of those [2] or even indirectly attract their natural enemies [3]. Then, the success of the insects may depend on how they can deviate from both the plant chemical defensive arsenal and their natural enemies. Inside the high diverse group of insects stands out the masters in manipulating plant tissues to their own benefit, the guild of galling insects [4–7]. These are distributed worldwide with special abundance in the neotropics, probably due to the high diversity of potential host plants [8]. This high diversity of plants and associated insects has attracted the attention not only of ecologists but also of plant morphologists, cytologists, and physiologists, who concentrated their studies on highlighting the mechanisms involved on cell redifferentiation for the establishment and development of gall new shapes and functional tissue compartments [9–13]. In addition to understanding the structural changes, lots of studies focus on the metabolism necessary for gall developmental processes, especially using the histochemistry of secondary metabolites (Table 1), and their role in plant defense [38] and in gall tissue homeostasis [39].

Historically, gall shapes have been attributed to the feeding behavior of the galling insects [40], but the specific potential of plant responses linked to the histological development of gall shapes has been just recently assessed [10]. The current histochemical and physiological assays have helped to elucidate the interaction between the requirements of insect's diet and the chemical arsenal of host plants [17, 18, 23, 41–43]. Rather than acting as passive victims in the interactions with the guild of galling herbivores, plants respond to herbivory with the production and accumulation of secondary metabolites (see Table 1). This accumulation has been considered to trigger the whole process of gall induction and development [44]. The first step of this developmental process involves calcium ion fluxes, phosphorylation cascades, and, in particular, the jasmonate pathway, which plays a central and conservative role in promoting resistance to a broad spectrum of insects [45, 46]. Following the step of the recognition of their specific gall-inducing insects, plants

Table 1 Main features extracted from the 44 analyzed cases, based on secondary metabolites in galls. In order of presentation: (i) type of secondary compounds (alkaloids, phenolics, and terpenoids); (ii) the occurrence in gall compartments (outer, inner, or both cortices); (iii) presence in non-galled tissues; (iv) plant species and induced organ; (v) gall morphotype; (vi) gall-inducing species; and (vii) reference

Secondary compound	Occurrence on galled tissue (inner, outer, or both cortices[a])	Non-galled tissue	Host-plant species	Host-plant organ	Morphotype	Galling species/ group	Reference
Alkaloids	Both	+	*Bauhinia ungulata*	Leaf	Leaf fold	Lepidoptera	[14]
	Both	+	*Piper arboreum*	Leaf	Lenticular	Cecidomyiidae	[13]
	Outer	+	*Mikania glomerata*	Leaf petiole	Conic	*Liodiplosis cylindrica*	[15]
	Outer	+	*Mikania glomerata*	Leaf	Globoid	*Liodiplosis spherica*	[15]
	Outer	+	*Mikania glomerata*	Leaf	Fusiform	*Clinodiplosis* sp.	[15]
	Outer	+	*Michelia champaca*	Stem	Amorphous	*Podothrips* sp.	[16]
Phenolics	Inner	+	*Lonchocarpus muehlbergianus*	Leaf	Bivalve shaped	*Euphalerus ostreoides*	[17]
	Outer	+	*Lantana camara*	Leaf	Leaf fold	*Aceria lantanae*	[18]
	Inner	+	*Lantana camara*	Leaf	Globoid	*Schismatodiplosis lantanae*	[18]
	Both	–	*Bauhinia brevipes*	Leaf	Globoid	*Schizomyia macrocapillata*	[19]
	Outer	–	*Aspidosperma spruceanum*	Leaf	Lenticular	Cecidomyiidae	[20]
	Inner	+	*Marcetia taxifolia*	Stem	Fusiform	Lepidoptera	[21]
	Both	+	*Bauhinia ungulata*	Leaf	Leaf fold	Lepidoptera	[14]
	Both	–	*Piptadenia gonoacantha*	Leaf	Lenticular	Not informed	[22]
	Both	–	*Piptadenia gonoacantha*	Leaf	Fusiform	Not informed	[22]

(*continued*)

Table 1 (continued)

Secondary compound	Occurrence on galled tissue (inner, outer, or both cortices[a])	Non-galled tissue	Host-plant species	Host-plant organ	Morphotype	Galling species/ group	Reference
	Both	+	*Marcetia taxifolia*	Floral buds	Pine shaped	Not informed	[23]
	Both	+	*Psidium cattleianum*	Leaf	Globoid	*Nothotrioza cattleianum*	[11]
	Both	+	*Piper arboreum*	Leaf	Lenticular	Not informed	[13]
	Both	–	*Myrcia retorta*	Leaf	Leaf fold	*Holopothrips* sp.	[24]
	Outer	–	*Matayba guianensis*	Leaf	Globoid	*Bystracoccus mataybae*	[25]
	Outer	nt	*Copaifera langsdorffii*	Leaf	Horn shaped	Not informed	[12]
	Both	nt	*Eucalyptus camaldulensis*	Leaf	Globoid	*Leptocybe invasa*	[26]
	Outer	nt	*Piptadenia gonoacantha*	Leaf	Lenticular	Not informed	[27]
	Outer	nt	*Piptadenia gonoacantha*	Leaf	Lenticular	Not informed	[27]
	Outer	nt	*Piptadenia gonoacantha*	Leaf	Lenticular	Not informed	[27]
	Outer	nt	*Piptadenia gonoacantha*	Leaf	Globoid	Not informed	[27]
	Outer	+	*Anogeissus latifolia*	Leaf	Leaf fold	*Lygothrips jambuvasi*	[28]
	Outer	+	*Memecylon lushingtonii*	Leaf	Leaf fold	*Crotonothrips memecylonicus*	[28]
	Both	+	*Mimusops elengi*	Leaf	Leaf fold	*Arrhenothrips ramakrishnae*	[28]

	Both	+	*Pavetta hispidula*	Leaf	Leaf fold	*Teuchothrips longus*	[28]
	Outer	+	*Piper nigrum*	Leaf	Leaf fold	*Liothrips karnyi*	[28]
	Outer	+	*Planchonia valida*	Leaf	Leaf fold	*Cercothrips nigrodentatus*	[28]
	Outer	–	*Schefflera racemosa*	Leaf	Leaf fold	*Liothrips ramakrishnae*	[28]
	Outer	–	*Schefflera racemosa*	Leaf	Leaf fold	*Liothrips associatus*	[28]
	Outer	–	*Ventilago maderaspatana*	Leaf	Leaf fold	*Schedothrips orientalis*	[28]
	Outer	+	*Smilax campestris*	Leaf	Globoid	Not informed	[29]
	Outer	nt	*Piptadenia gonoacanhta*	Leaf	Lenticular	Not informed	[30]
	Outer	nt	*Schinus polygama*	Stem	Fusiform	*Calophya rubra*	[31]
	Outer	–	*Clusia lanceolata*	Leaf	Lenticular	*Clusiamyia nitida*	[32]
	Outer	+	*Michelia champaca*	Stem	Amorphous	*Podothrips* sp.	[16]
	Both	+	*Pistacia terebinthus*	Leaf	Leaf fold	*Paracletus cimiciformis*	[33]
	Both	+	*Pistacia terebinthus*	Leaf	Leaf fold	*Forda marginata*	[33]
	Both	+	*Pistacia terebinthus*	Leaf	Leaf fold	*Forda formicaria*	[33]
	Both	+	*Quercus prinus*	Leaf	Globoid	*Andricus petiolicolus*	[34]
	Both	+	*Byrsonima sericea*	Floral buds	Globoid	*Bruggmanniella byrsonimae*	[35]
	Outer	+	*Schinus polygamus*	Leaf	Lenticular	*Calophya duvauae*	[36]

(*continued*)

Table 1 (continued)

Secondary compound	Occurrence on galled tissue (inner, outer, or both cortices[a])	Non-galled tissue	Host-plant species	Host-plant organ	Morphotype	Galling species/ group	Reference
Terpenoids	Both	nt	*Lonchocarpus muehlbergianus*	Leaf	Bivalve shaped	*Euphalerus ostreoides*	[37]
	Both	+	*Lantana camara*	Leaf	Globoid	*Schismatodiplosis lantanae*	[18]
	Both	–	*Marcetia taxifolia*	Stem	Fusiform	Lepidoptera	[21]
	Inner	+	*Bauhinia ungulata*	Leaf	Leaf fold	Lepidoptera	[14]
	Both	+	*Marcetia taxifolia*	Buds	Pine shaped	Not informed	[23]
	Both	+	*Psidium cattleianum*	Leaf	Globoid	*Nothotrioza cattleianum*	[11]
	Outer	–	*Matayba guianensis*	Leaf	Globoid	*Bystracoccus mataybae*	[25]
	Both	+	*Mikania glomerata*	Leaf	Conic	*Liodiplosis cylindrica*	[15]
	Both	+	*Mikania glomerata*	Leaf	Globoid	*Liodiplosis spherica*	[15]
	Both	+	*Mikania glomerata*	Leaf	Fusiform	*Clinodiplosis* sp.	[15]

Symbols indicate positive (+) and negative (–) results for the presence of metabolites on non-galled tissues (nt refers to studies in which the compound was not tested)

[a]*Both cortices* indicate a homogeneous one or the association between inner and outer ones

reprogram their phenotype into a new structural and functional design, the gall morphotype [sensu 9]. In addition, gall structure can improve the fitness of galling insects by the improvement of nutritional substances, by redifferentiation of mechanical and protective tissues, and by guaranteeing a favorable microenvironment [5, 47, 48].

The biochemical cross-talking between galling insects and host plants guarantee to the herbivore the best ways to deviate from the chemical and defensive counterattack and, consequently, assess their required nutritional resources [5]. In galls, the histolocalization of primary metabolites such as starch, protein, lipids, and reducing sugars are associated with the feeding behavior of the galling organisms, the maintenance of the gall structure, as well as the cellular and tissue metabolism [37]. Naturally, the accumulation of secondary metabolites in galls has been associated with the protection of the galling herbivores against the attack of their natural enemies [e.g., 20, 26, 49]. Recently, it has also been related to scavenging oxidative stress molecules [17, 25] and to gall metabolism toward cell divisions and hypertrophy by auxin action [27].

2 Secondary Metabolites in Galls: Which and Where They Are Detected

The ability of manipulating the host-plant chemical composition prevails in a wide range of galling insect taxa [50]. Gall chemical profile is composed of metabolites compartmentalized in specialized gall tissues related to many functions, such as protection and nutrition [43], as shown in Cecidomyiidae-induced galls on *Piper arboreum* [13]. Many galls have just a homogeneous tissue compartment, also referred as gall cortex, where much metabolic activity takes place [4, 51, 52]. Also, other two or three tissue compartments may occur in different galls. For instance, in globoid galls induced by *Clinodiplosis* sp. (Cecidomyiidae) on *Croton floribundus* occur three tissue compartments: a mechanical layer as the outer cortex; a medium cortex, which stores primary and secondary metabolites; and a nutritive layer as the inner cortex [53]. Many galls have shown cortical tissue compartmentalization, with production and storage of secondary metabolites (see Table 1) related to specific functions. Chemical compounds, especially the secondary metabolites, have been assessed using quantification methods [e.g., 51, 54, 55]. However, histochemical analysis, a qualitative methodology, has also proved to be useful in understanding the structure and metabolism of the gall, revealing the specific sites of production and storage of the secondary metabolites. Moreover, histochemical profiles can also be related to a wide range of the ecological and physiological roles of gall tissue compartments (Table 1).

The overview of the ecological and physiological functionalities of secondary metabolites in galls by histochemical approach follows two main questions: (i) what are the most common secondary metabolites detected in gall tissues? and (ii) what is the functional implications of the secondary metabolites profile in galls? To follow up the answers, a database was built with scientific papers, which were surveyed

through "Google Scholar®" (accessed until March 22, 2018) using "gall secondary metabolism histochemistry" as the search term.

The search resulted in 12,900 outcomes, most of them not related to our questions. After selecting only researches on arthropod-induced galls, we listed 28 papers comprising altogether 44 case studies. Three main classes of compounds have been histochemically tested in galls: (i) phenolic compounds, (ii) terpenes, and (iii) alkaloids (Table 1); however, not all of them were evenly tested across all the studied galls (Fig. 1a). The concomitant investigation of the three main classes of secondary compounds has been performed in only 18.2% of times. Alkaloids along with phenolics were tested in 2.3% of the cases, while phenolics and terpenoids were tested in 11.4%. Most systems had phenolics tested solely (68.2%). The testing for phenolics occurred in all studies, being significantly higher when compared to the other two (chi-square test: $\chi^2 = 33.4$; $p < 0.001$; see "Statistical/Results Notes"). Alkaloids were tested in 20% of cases (9 times) and terpenoids in 30% (13 times).

2.1 Alkaloids and Terpenoids

The alkaloids were found in six out of nine of the investigated galls (66.7%) (Table 1). In four galls, alkaloids were detected only in the outer cortical tissue compartment, while in two galls, alkaloids were detected both in outer and inner cortices. The alkaloids stored in the outer tissue may confer chemical defense to gall against predators or cecidophages, once the external site in gall cortex has been associated with protection in several galls [4], as well as alkaloids present direct implication on the herbivores' tolerance in many plants [56]. Alkaloids are also detected in all the host-plant organs of the study cases addressed herein (four in leaves, one in petiole, and one in the stem), which corroborate the assumption that galling herbivores take advantage of the histochemical profile of their host plants. The conservative aspect of the host plant seems to be constant for the chemical profile of all galls, independent of the herbivore taxa.

The terpenoids were detected in 10 out of 13 galls where these compounds were investigated (80%) (Table 1). Terpenoids were detected in both outer and inner cortices of eight different galls and exclusively in the inner cortex of *Bauhinia ungulata* galls [14], as well as in the outer cortex of *Matayba guianensis* galls [25]. These secondary metabolites commonly occurred in non-galled tissues of leaves (seven), stems (one), and buds (one) of the different host plants. The main function associated with terpenoids in the study cases is similar to those of alkaloids, i.e., gall defense against the attack of natural enemies [15, 57, 58].

2.2 Phenolics

Phenolics occurred in 40 out of 44 investigated galls (90.9%) (Table 1). These compounds were histochemically detected in the inner cortex of three galls and in the outer cortex of 21 galls. They were found in both inner and outer gall cortices

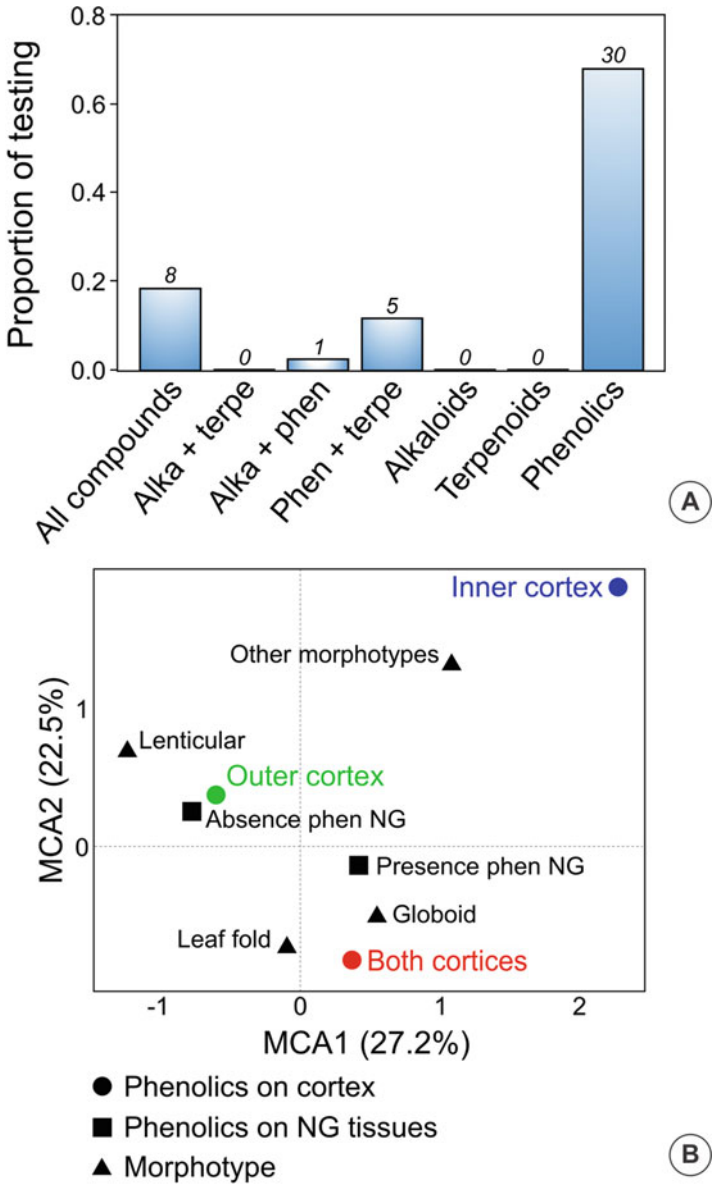

Fig. 1 (**a**) Proportions of times each compound (or combinations) was tested based on the 44 case studies surveyed. Raw numbers are expressed above the respective bar. Alka, alkaloids; terpe, terpenoids; phen, phenolics. (**b**) MCA biplot (axes 1, 2) demonstrates the relationship among variables according to phenolic accumulation. Symbols in closer positions represent higher similarity/relationship

16 times. Concerning systems that histochemically tested phenolics in non-galled tissues, they occurred in 68.8% of times (22 cases). Most of the galls in which phenolics were detected occurred in leaves (35 times; 87.5%), followed by stems (3 times; 7.5%) and floral buds (2 times; 5%).

Due to the pervasive and higher sampling availability of phenolics (Table 1), we analyzed the pattern of their distribution according to (i) their presence on different tissue compartments (outer, inner, or both cortices), (ii) their presence/absence in non-galled tissues, and (iii) gall morphotypes (see "Statistical/Results Notes"). We found that the gall morphotypes may be related to different patterns of storage of phenolics in gall tissue compartments (Fig. 1b). Leaf folding galls and globoid galls generally store phenolics in both outer and inner cortices. Phenolic storage exclusively in the outer cortex was related to lenticular galls. Phenolics in gall inner cortex were associated with other gall morphotypes (grouping of amorphous coalescent, bivalve-shaped, fusiform, horn-shaped, and pine-shaped galls). Nevertheless, such pattern must be interpreted with caution due to the few occurrences of phenolics only in the inner cortex of the galls (6.8%). Furthermore, the accumulation of phenolics in gall outer cortex is associated with the absence of this compound in the related non-galled tissues. Consequently, we can assume that galling organisms may trigger the neo-synthesis of phenolics in gall tissues, even if it is not accumulated in the related non-galled tissues.

The accumulation of phenolics in gall tissues relates to stress dissipation, once the feeding habit of galling organisms and their metabolism inside the nymphal chamber can generate high oxidative stress [25, 39]. Thus, the scarce occurrence of phenolics in gall inner cortex may be justified as it is the feeding site of many insects, as scraping and chewing ones, as phenolics should reduce nutritive tissue palatability [38, 59–63]. Nevertheless, why are there secondary metabolites in both cortices of some galls? The answer is still uncertain, but some authors have discussed the accumulation of phenolics as a consequence of the maintenance of gall metabolism, as antioxidants or their involvement in auxin control as AIA oxidase inhibitors [7, 12, 18, 22, 25, 39, 49, 64].

Gall outer tissue compartment stores metabolites linked to defense, while gall inner cortex develops nutritional roles [13]. Such overall concept relies on the usual accumulation of secondary metabolites in tissues of gall outer cortex [10, 17, 20, 65], while the inner cortex usually accumulates primary metabolites [47, 48, 60, 63]. Like alkaloids and terpenoids, the accumulation of phenolics has also been considered a gall chemical strategy against the attack of natural enemies [11, 13, 19, 23, 24, 43, 60, 61]. In addition to their protective roles, some studies have shown that phenolic compounds can be produced and stored differently among gall morphotypes, even if they are hosted by the same plant species, e.g., *Lantana camara* (Verbenaceae) [18]. In this case, the phenolics occur in the outer cortex of the leaf folding gall morphotype induced by *Aceria lantanae* (Acarina: Eriophyidae) and in the inner cortex of the globoid galls induced by *Schismatodiplosis lantanae* (Diptera: Cecidomyiidae) [18]. Likewise, some specific morphogenetic characteristics may contribute to the accumulation of phenolics in specific sites, such as the vascular bundles [66].

Gall induction and the feeding activity of galling insects besides promoting secondary metabolite storage also generate a cascade of stressor molecules, with particular emphasis on reactive oxygen species (ROS) [36, 41, 42, 44]. The superoxide $\left(O_2^-\right)$, hydrogen peroxide (H_2O_2), hydroxyl radical ($^{\bullet}OH$), and singlet oxygen ($^1O_2$) are examples of ROS molecules [67], which trigger the oxidative stress in galls [44] and damage cell membrane systems [67]. However, recent studies have proposed ROS molecules as the first signals to gall development [39, 44]. Thus, despite redox imbalance provoked by the feeding behavior of galling herbivores, ROS may develop a synergetic effect and trigger the initial process of plant responses to herbivory [45] and gall development. In this sense, polyphenol production is one of the pathways to reduce ROS cellular levels [22, 39, 43, 68–70], once its synthesis depends on ROS generation [39, 71, 72]. The co-occurrence of vacuolar secondary metabolites (e.g., general phenolics and proanthocyanidins) and ROS, as well as cell wall lignification, are evidences of efficient mechanisms of ROS dissipation and maintenance of the homeostasis in gall tissues [25]. Once lignin biosynthesis depends on the generation of ROS, especially radical hydroxyl [71], lignification in gall tissues may represent an important way to stress dissipation and gall metabolic stability.

Although secondary compounds may be defensive molecules against the attack of a range of organisms [25, 27, 38, 55, 65, 73, 74], galls attract diverse direct enemies, such as parasitoids and predators, and indirect enemies, such as the cecidophages [75, 76]. For example, galls induced by *Palaeomystella oligophaga* (Lepidoptera) on *Macairea radula* (Melastomataceae) accumulate secondary metabolites such as phenolics and terpenoids (Fig. 2), but they are attacked by several types of natural enemies causing high mortality [unpublished data]. Accordingly, secondary metabolites may have an adaptive role to the galling herbivores and may occur as relics of past coevolutionary interactions [77].

3 Histochemical Approach in Galls: Tests and Chemical Considerations

Histochemistry is a subarea of histology, which aims to demonstrate the native location of metabolites in tissue and cell compartments by using chemically well-defined methods [78], whose results are based on color development linked to a certain class of compounds and its interaction with a specific reagent. As discussed here, the three general main classes of secondary metabolites (i.e., phenolic compounds, terpenes and alkaloids, as well as more specific substances) have been histochemically tested in galls. Among the diversity of histochemical tests, some have been more usually addressed for studies on galls and for this reason will be discussed here (see works in Table 1). For illustrative purposes, galls induced by *P. oligophaga* (Lepidoptera) on *M. radula* (Melastomataceae) (Fig. 2a–h) were submitted to the most common histochemical tests for secondary metabolites in galls.

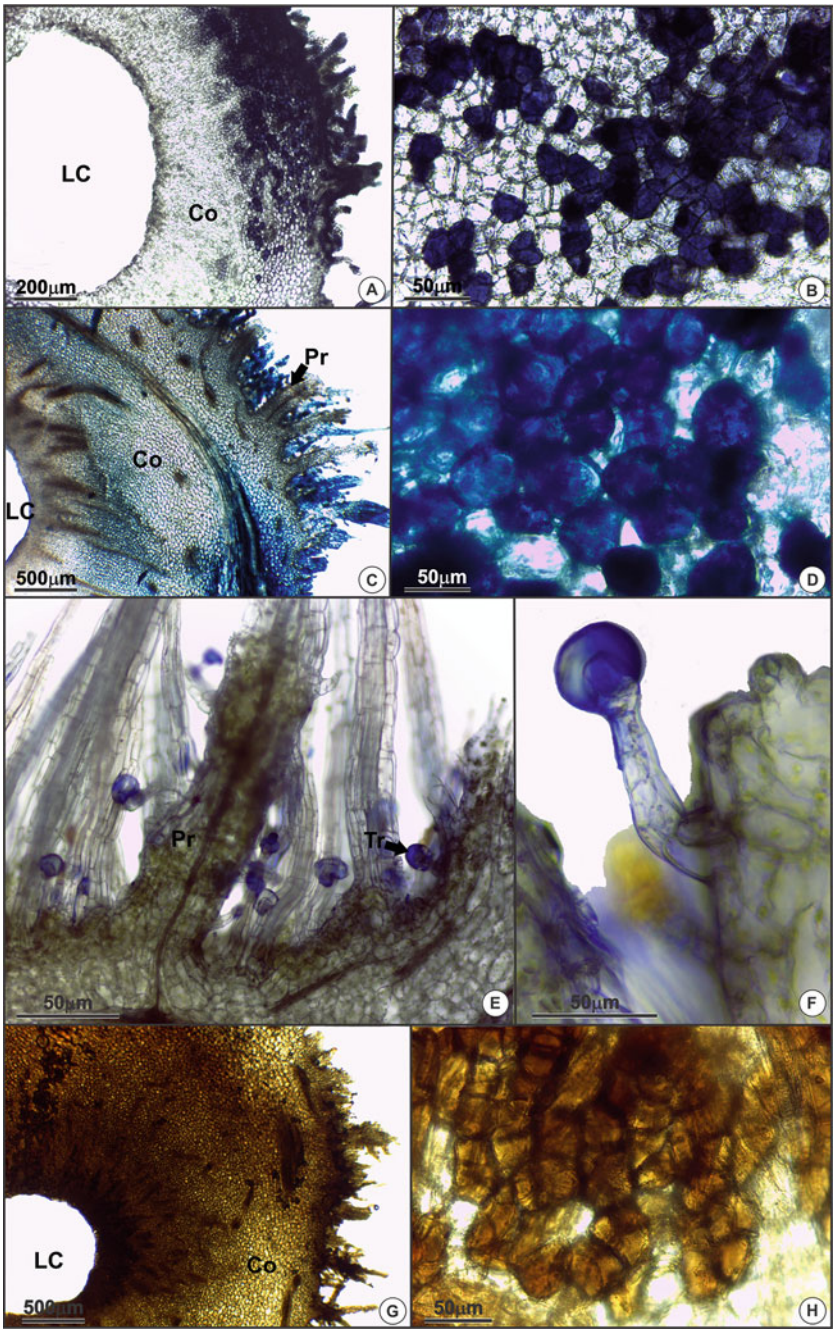

Fig. 2 Histochemical detection of secondary metabolites in young galls induced by *Palaeomystella oligophaga* on *Macairea radula*. (**a**, **b**) Ferric chloride III test generating blue

Plant phenolics are derivative of the shikimate, pentose phosphate (PPP), and phenylpropanoid pathways [79]. Structurally, the phenolic compounds are composed of an aromatic ring, connected with one or more hydroxyl groups [80], and are frequently named as polyphenols. Flavonoids, tannins, and lignins stand out by the widespread occurrence in galls and their host plants, as stored substances or components of cell walls [80, 81]. In galls, general polyphenols and flavonoids are histochemically tested with ferric chloride III [82] and *p*-dimethylaminocinnamaldehyde – DMACA [83], respectively. The ferric chloride III is a solution of sodium carbonate, ferric chloride, and distilled water [82] and can be used to detect simple phenols through the link and complex formation between Fe^{3+} and ortho-dihydroxyphenols, creating precipitates colored in green, blue, black, or purple (Fig. 2a, b) [84]. Distinct metabolites were histochemically detected in two tissue compartments in galls induced by *Leptocybe invasa* (Hymenoptera: Eulophidae) on *Eucalyptus camaldulensis* (Myrtaceae) [26]. In galls induced by *P. oligophaga* on *M. radula*, polyphenols occur just in the outer cortex (Fig. 2a, b).

The DMACA is an aromatic hydrocarbon that has the ability to detect indole derivatives in bacteria [85] and proanthocyanidin/flavonoid compounds in plants [83]. Proanthocyanidins are polymers of flavan-3-ol subunits produced by the flavonoid secondary pathway [86]. For DMACA tests, fixation of biological samples with caffeine and sodium benzoate is required, followed by immersion in *p*-dimethylaminocinnamaldehyde solution [83]. The development of a blue color through the link of DMACA reagent to meta-oriented dihydroxy- or trihydroxy-substituted benzene rings [87, 88] indicates the presence of proanthocyanidins (Fig. 2c, d). Flavonoid derivatives were detected by dark blue stain with DMACA in the meristem-like cells of a Cecidomyiidae gall on *Copaifera langsdorffii* Desf. (Fabaceae) [12] and herein in cortex and outer projections of galls induced by *P. oligophaga* on *M. radula* (Fig. 2c, d).

The terpenes are the most diverse group of plant secondary compounds [89], typically composed of multiple units of five carbon, C_5, C_{10}, C_{15}, C_{20}, C_{25}, C_{30}, and C_{40}, that may be modified, as well [90]. The terpenes are derived from a precursor with five-carbon isoprene units (C_5H_8), which may likely have subsequent rearrangements [91, 92]. The terpenes can be classified by the number of isoprene units, such as hemiterpenes (5C), monoterpenes (10C), sesquiterpenes (15C), diterpenes (20C), and other types [93]. In galls, most researches applied α-naphthol and N,N-dimethyl-*p*-phenylenediamine (NADI) as a method for terpene localization (see terpenoids results in Table 1) [94]. The reaction between these two substances produces the blue of indophenol, which changes its color by the pH alteration and, thus, stain essences in blue (Fig. 2e, f) and resiniferous acid in red

Fig. 2 (continued) color for general phenolics in the outer layers of the cortex; (**c**, **d**) DMACA detection of proanthocyanidin/flavonoid compounds in blue in cortex and outer projections; (**e**, **f**) NADI staining terpenoid oil essences in blue in trichomes; (**g**, **h**) Dragendorff reagent detecting alkaloids in orange-brown in the cortical cells. *LC* larval chamber, *Co* cortex, *Pr* projection, *Tr* trichome

[84]. NADI reagent detected terpenoids in the outer, median, and inner tissue compartments of galls induced by Cecidomyiidae on the axillary buds of *Marcetia taxifolia* (Melastomataceae) [23] and in the trichomes of galls induced by *P. oligophaga* on *M. radula* (Fig. 2e, f).

The alkaloids are cyclic compounds with nitrogen in a negative oxidation state [56], in which the nitrogen comes from amino acids incorporated into a heterocyclic ring [90]. Alkaloids are derived from the biosynthetic pathways, such as terpenoid, amino acid, polyketide, and shikimic acid metabolism [90]. Histochemical detection of alkaloid has been rarely tried in galls, and the knowledge on their relation with tissue compartmentalization or their role in gall protection is scarcely known. The Dragendorff reagent detected alkaloids in the cells of the hypodermis by the development of brown precipitates in Cecidomyiidae galls on *Piper arboreum* Aubl. (Piperaceae) [13], as well as in the cortex of the galls induced by *P. oligophaga* on *M. radula* (Fig. 2g, h). The Dragendorff reagent is a mixture of bismuth nitrate and potassium iodide [82], allowing the detection of tertiary and quaternary nitrogen and rarely primary and secondary amines [84]. Orange-brown, dark-brown, and violet-brown (Fig. 2g, h) may indicate the presence of carbonyl α,β unsaturated or lactones, as well as compounds with hydroxyl group and an isolated double bond [84].

4 Supplementary Material: Statistical/Results Notes

We compared the number of times each of the three main compounds (i.e., phenolics, terpenes, and alkaloids) was tested using a chi-square test expecting equal frequencies for each compound. As phenolics were the only compound with higher sampling availability to further analysis, we investigated the relationship among some variables available in Table 1. We did not consider case studies that did not find

Table 2 Scores (in %) of the two first MCA axes. On each column, the explanations sum 100%

	MCA1	MCA2
Cortex		
Outer	12.0	5.4
Inner	24.7	19.9
Both	3.3	21.1
Phenolics in non-galled tissues		
Absent	13.3	1.8
Present	7.1	1.0
Morphotype		
Globoid	4.3	4.3
Leaf fold	0.2	15.2
Lenticular	21.9	8.0
Other morphotypes	13.1	23.3

phenolics due to their low sample size. We did not include the non-galled tissues types because most of them were on leaves. We analyzed the relationship among (i) phenolics' presence on gall sites (outer, inner, or both cortices), (ii) phenolics' presence/absence on non-galled tissues, and (iii) gall morphotype using a MCA (multiple correspondence analysis) in the R package *FactoMineR* version 1.41 [95]. As some researches did not test the compound in non-galled tissues (Table 1), we performed imputation [sensu 96] on the matrix cells using the R package *missMDA* version 1.13 [97]. As some gall morphotypes had few occurrences (amorphous, bivalve-shaped, fusiform, horn-shaped, and pine-shaped galls), we grouped them as "other morphotypes." This arrangement makes sense because the refereed galls share characteristics such as being extralaminar and non-globoid. All analyses were carried out in R version 3.5.1 [98].

We found that the first MCA axis explained 27.2% of variance and the second 22.5% (Fig. 1b). Together they explained 49.7% of the variance in the dataset. In MCA1, the most explanatory variables were the presence of phenolics in gall inner cortex, followed by lenticular morphotype, absence of phenolics on non-galled tissues, other gall morphotypes, and then presence of phenolics in gall outer cortex (Table 2). In MCA2, other morphotypes, presence of phenolics in both cortices, presence in inner cortex, leaf fold morphotype, and lenticular morphotype were the most explanatory variables, decreasingly.

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Part VI

Bioactive Molecules in Plant Defense

Antimicrobial Compounds (Phytoanticipins and Phytoalexins) and Their Role in Plant Defense

33

Anupama Razdan Tiku

Contents

Abstract

Plants synthesize and accumulate an arsenal of antimicrobial secondary metabolites in order to protect themselves from invasion of foreign elements (microbes, pathogens, and predators). A few of these metabolites act as constitutive chemical barriers against the microbial attack (phytoanticipins) while others as inducible antimicrobials (phytoalexins). Their properties show them as promising plant and human disease-controlling agents. In this chapter, we are discussing the role of both types of antimicrobial compounds involved in plant defense mechanism.

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Phytoanticipins are preformed antimicrobial compounds in plants that are unique in action for their property of being synthesized even before the attack of pathogen or infection, i.e., they exist in healthy plants in their biologically active forms (constitutive). Other forms of phytoanticipins such as cyanogenic glycosides and glucosinolates occur as inactive precursors stored in healthy tissues and get activated only in response to tissue damage. Activation of these compounds involves hydrolases (plant enzymes) which are released only after the breakdown of cells. Still we consider them as constitutive metabolites as they are immediately derived from preexisting constituents. Phytoalexins are LMW antimicrobial compounds produced by plants in response to biotic and abiotic stresses. They are formed from remote precursors only in response to pathogen attack after de novo synthesis of phytoalexin biosynthesizing enzymes. We have discussed the key features of both the types of diverse group of molecules such as chemical structures, biosynthesis, regulatory mechanisms, biological activity against pathogens, and molecular engineering of both the plant secondary metabolites.

Keywords
Secondary metabolites · Antimicrobial compounds · Phytoanticipins · Phytoalexins · Plant defense mechanisms

1 Introduction

Most of the plants are characterized by chemical compounds with large functional and structural diversity and are synthesized in various morphological parts in the form of secondary metabolites. These phytochemicals/secondary metabolites (flavonoids, tannins, alkaloids, tannins, terpenoids, etc.) show antimicrobial properties and perform defensive role against naturally occurring plant pathogens [1].Antimicrobial compounds exhibit organ or tissue specificity in different species and can be identified only at a specific stage of growth and development or may be activated only during the periods of stress, which is either caused due to attack by microorganisms or some other reasons such as nutrient depletion, etc. In order to control the spread of pathogens, plants possess an innate immunity involving various layers of defense responses. Few defense mechanisms are preformed/constitutive while others are activated only after the attack by pathogens (pathogen induced). Based upon this fact, antimicrobial compounds derived from plants are classified into two main groups of phytoanticipins (constitutive) and phytoalexins (induced) [2]. Phytoanticipins are defined as LMW defense-related compounds present in plants even before the attack by pathogens [3] or produced from preexisting precursors. Some of them are located at the plant surface (epidermal portion) while others are concealed as preformed compounds in vacuoles or organelles and are released only by the action of hydrolyzing enzymes after the pathogen attack. Hydrolyzing enzymes involved in liberation of final molecule are not synthesized de novo; therefore these compounds are different from phytoalexins [4]. They mainly include saponins, avenacin, and tomatine, e.g., avenacin A-I present in epidermal cells of *Avena sativa* roots and α-tomatine produced in *Lycopersicon*

esculentum both showing antimicrobial activity against many pathogenic fungi. Phytoalexins are produced by plants only after the attack by pathogenic microorganisms or due to chemical/mechanical injury [5]. They require de novo expression of the enzymes that are involved in their biosynthetic pathways after elicitation and therefore production of phytoalexins requires transcriptional and translational activity in the plant after detection of pathogen. Those induced defense mechanisms also require transportation and secretion of antimicrobial phytochemicals at the site of infection [6]; despite significant efforts of researchers, some confusion still exists and many of these phytochemicals that could be considered as phytoanticipins are reported as phytoalexins or vice versa; for example, resveratrol, a well-known phytoalexin (according to 2132 publications), is considered as a phytoanticipins in few species [7]. Similarly S-methyl-cysteine sulfoxide was reviewed earlier as a phytoalexin but later when its antimicrobial activities were further analyzed the compound appeared more as phytoanticipin [8, 9]. It is important to know that differentiation between a phytoalexin and phytoanticipin is not based upon its chemical structure but on how it is produced in plants. Same phytochemicals can function as a phytoalexin and phytoanticipin in the same plant; for example, maackiain (isoflavonoid derivative), an antimicrobial compound produced in roots of red clover, is classified as a phytoanticipin if it is present as aglycone of a preformed glucoside and is released from injured tissues of plants due to action of a preformed hydrolyzing enzyme (plant glucosidase) during de-compartmentalization of tissue [10]. Same plant can also synthesize maackiain de novo in response to microbial infection or elicitors and is then classified as phytoalexin [11]. When phytoalexins serve as the basis of disease resistance in plants, then there must be an active response from the plant part so that the communication between the plant and microorganism redirects the plants' metabolic activity. In the case of phytoanticipins the affected plant relies on preformed compounds and can be passive during its interaction with a potential pathogen. In the present review both the types of proposed plant antimicrobial compounds will be discussed and their roles in plant defense mechanism studied.

2 Phytoanticipin

During normal growth and development, plants may produce phytochemicals that inhibit the development of pathogens. These antimicrobial compounds (phytoanticipins) can accumulate in dead cells or are excreted into the external environment (e.g., rhizosphere) or maybe stored in vacuoles in an inactive form. For example, quinones, catechol, and protocatechuic acid are present in dead cells of brown-colored skin of onion, inhibiting the germination of *Colletotrichum circinans* (smudge pathogen) and *Botrytis cinerea* (neck rot pathogen) spores. White skin onions do not produce these antimicrobial compounds and therefore are victims of these pathogens [12]. Similarly "borbinol" (antimicrobial phenolic compound) secreted by rootstocks of avocado into the rhizosphere protects the roots from *Phytophthora cinnamomi* causing root rot disease in avocado plants.

Unripe fruits of apples and pears are more resistant to scabs caused by *Venturia pirina* and *V. inaequalis* due to the presence of preformed phenolic compounds such as arbutin, isochlorogenic acid, phloridzin, and chlorogenic acid in the outer layers of fruits. These compounds are only responsible for bitterness of the unripe fruits and during the ripening process these compounds break down and dissolve increasing the sweetness of ripened fruits and making them more susceptible to disease.

Many preformed/constitutive antimicrobial compounds have been reported till date, for example, saponins, cyanogenic glycosides, glucosinolates, phenols, phenolic glycosides, unsaturated lactones, sulfur compounds, etc., which play an important role in plant defense mechanism [1]. However till now only few classes of preformed inhibitors have been studied in detail and in present review the role of possible phytoanticipins in plant defense will be discussed.

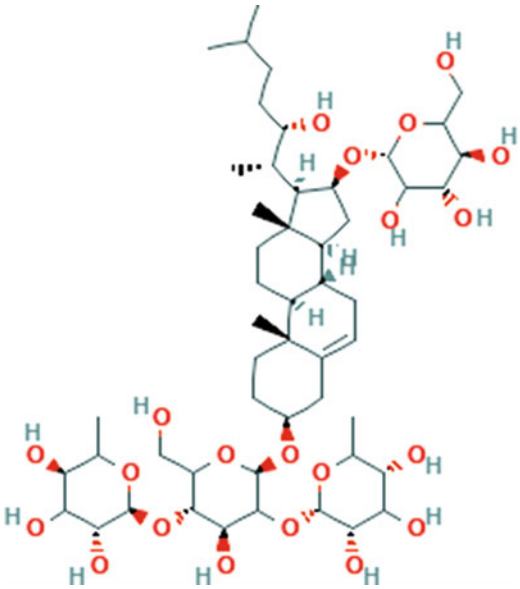
2.1 Role of Saponins

These are plant glycosides with surfactant (wetting agent) properties and are often present at higher levels in healthy plants. They also show other properties like insecticidal, piscicidal, mollusicidal, and allelopathic action and anti-nutritional effects [13]. Saponins are toxic to organisms bearing sterols in their cell membranes as they bind with sterols and destroy integrity and function of membrane. Pathogens having sterols in there plasma membrane will therefore be affected more by saponins produced by host plants. Inactive precursor molecules of saponins are stored mainly in vacuoles of noninfected plant cells. During infection hydrolase enzymes released from ruptured cells convert these precursor molecules to active antimicrobial compounds (saponins). Saponins are glucosides made up of polycyclic aglycone attached to one or more sugar side chains. Aglycone part also known as sapogenin is either a steroid (C27), triterpene (C30), or a steroidal glycoalkaloid. Saponins have bitter taste and are toxic in nature and also known as sapotoxin. In both the types of aglycone units of saponin molecule, carbohydrate side chain is usually attached to the 3 carbon of the sapogenin (Fig. 1). Carbohydrate portion is water soluble whereas sapogenin is fat soluble. Steroidal saponins are mainly present in monocots, for example, Liliaceae, Agavaceae, Poaceae members, etc., and also in few dicots such as digitoxin saponin in foxglove plant [13]. Dicot plants contain mainly triterpenoid saponins along with few monocots. *Avena* species contains both steroid and triterpenoid saponins. Steroidal glycoalkaloids are present mainly in members of family Solanaceae, for example, potato, tomato, etc., and also in Liliaceae family [14]. Saponins produced by oats and tomato show activity against phytopathogenic fungi and are therefore studied in detail.

2.2 "Avenacosides" and "Avenacin" Saponins

Oats contain saponins from both the groups; that is, avenacins (A-1, A-2, B1, and B2) which are triterpenoids and avenacosides (A and B) that are steroidal in structure (Fig. 2) [15]. Avenacins are located in the root part of the plant whereas avenacosides

Fig. 1 Spongioside A



are present in leaves and shoots [16]. Avenacins being monodesmosidic have a single sugar chain attached at C-3 position of aglycone unit whereas avenacosides which are bidesmosidic have an additional D-glucose molecule attached to carbon-26 of aglycone. Avenacosides are biologically inactive and are therefore converted to active forms of monodesmosidic saponins, for example, 26-desglucoavenacosides A and B having antifungal property. This conversion happens during infection period when due to tissue damage oat avenacosidase (glycosyl hydrolase) cleaves the D-glucose molecule at carbon-26 [17, 18]. Root-infecting fungus *Gaeumannomyces graminis* var. *tritici* causes "take-all" disease in wheat and barley plants but is unable to infect oats due to the presence of avenacins.

2.3 α-Tomatine (Tomato Saponin)

α-Tomatine is a steroidal glycoalkaloid and a major saponin of tomato. Like avenacins it is also monodesmosidic and is present in healthy plants in its biologically active form. Sugar moiety attached to 3rd carbon of aglycone consists of two molecules of D-glucose and one molecule of D-xylose and D-galactose (Fig. 3). α-Tomatine level is high in leaves, flowers, and green fruits of tomato plants [19]. Saponin shows specific resistance against vascular wilt fungi *Fusarium oxysporum* f. sp. *lycopersici* and *Verticillium albo-atrum* [20, 21]. A new group of saponins having open-chain steroidal glycosides have been recently studied [22]. They are C27 steroidal skeletons condensed with various glycosides attached at different positions, for example, spongioside A. Toxic action of saponins on fungi occurs when there is complex formation between membrane sterols of the pathogen and saponins causing pore formation in cell membrane [23]. α-Tomatine can cause lysis

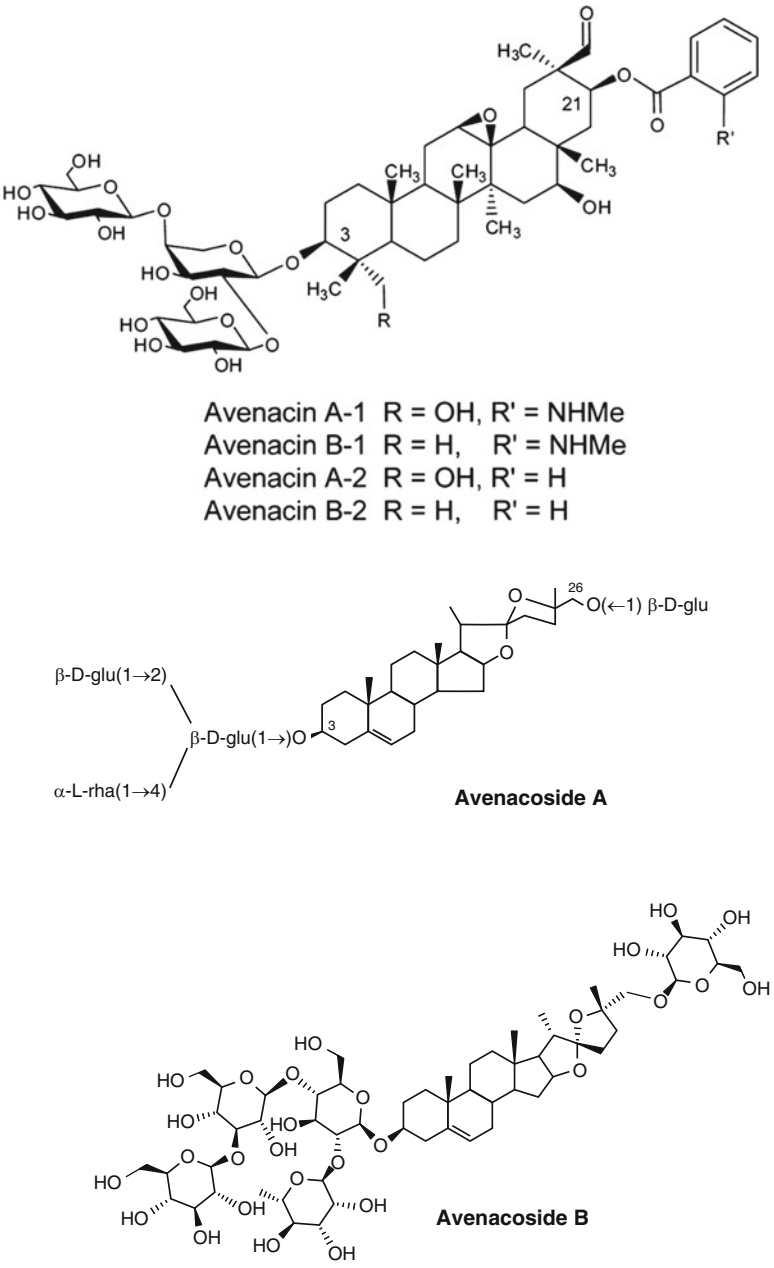
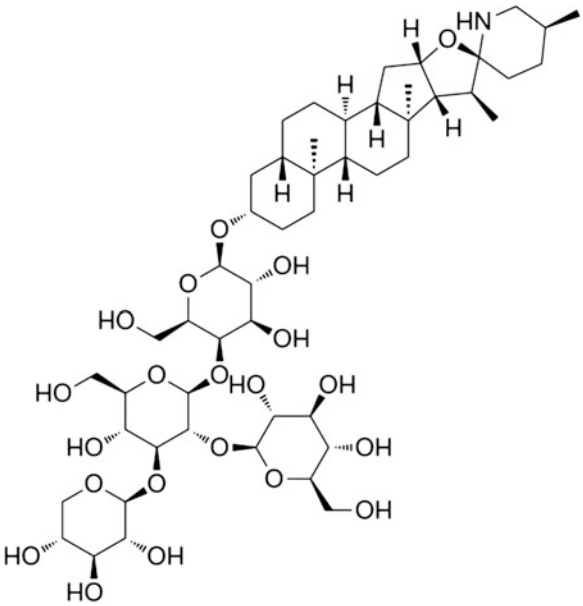


Fig. 2 Structure of avenacin and avenacosides

of pathogen's cell membrane only at specific pH [24]. *Alternaria solani* counters the effect of α-tomatine by lowering the pH at infection site so that the saponin becomes inactive as an antifungal agent. Plants protect their cells from their own saponins by

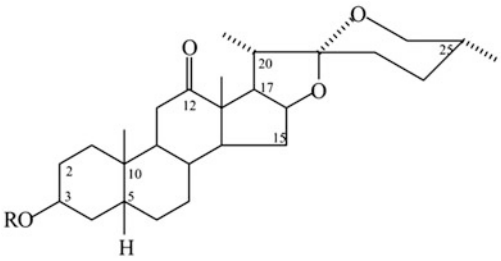
Fig. 3 α-Tomatine



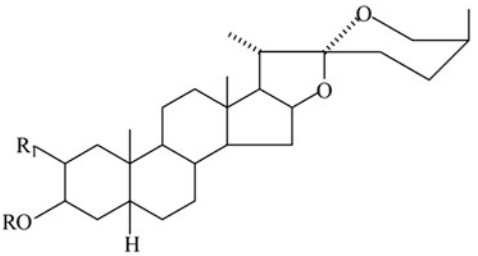
compartmentalizing them either in the vacuoles or in those organelles which can avoid membrane lysis after altering sterol composition of their membranes. Saponins have also been tested against several Gram-negative and Gram-positive bacteria; for example, saponins from *Yucca* (Fig. 4) exhibited antimicrobial activity against positive bacteria [25].

2.4 Cyanogenic Glycosides

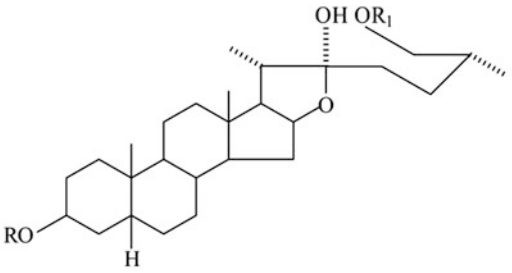
They are nitrogen-containing antimicrobial compounds that can produce highly toxic hydrogen cyanide after degradation by plant enzymes. Approximately 75 different cyanogenic glycosides have been isolated from 2650 plants belonging to 130 different families, for example, Poaceae, Fabaceae, Euphorbiaceae, Rosaceae, Asteraceae, etc. [26]. These glycosidic compounds are present in plant tissues as O-β-glycoside of α-hydroxynitriles (cyanohydrins). Most common cyanogenic glycosides reported in plants are linamarin and lotaustralin and other than that are amygdalin mostly found in Rosaceae family and Dhurrin in Poaceae plants (Fig. 5).Cyanide glycoside is synthesized from amino acid precursors and the monosaccharide (β-D-glucose) unit is directly attached to aglycone (represented by cyanide group). β-D-Glucose sometimes gets modified to a second monosaccharide, a sugar ester [27]. Due to attack by pathogens/herbivores, plant tissues get damaged and β-glycosidases (hydrolyzing enzymes) present in other tissues separated from cyanogenic glycosides come in contact with it to cleave sugars from it due to which cyanohydrins (hydroxynitrile) are formed (Fig. 6). In the second step cyanohydrins



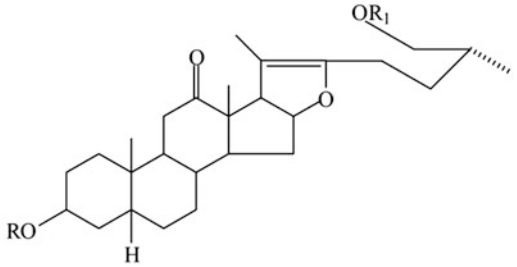
1: R = Glc (1-2)[Xyl (1-3)] Glc
3: R = Glc (1-2)[Glc (1-3)] Glc



2a: R = Glc (1-2) Xyl R_1 = H
2b: R = Glc (1-2)[Xyl (1-3)] Glc R_1 = H
4: R = Glc (1-2) Gal R_1 = OH



5: R = Glc (1-2) Glc R_1 = Glc
6: R = Glc (1-2)[Xyl (1-3)] Glc R_1 = Glc



7: R = Glc (1-2)[Xyl (1-3)] Glc R_1 = Glc

Fig. 4 Chemical structures of saponins identified in *Yucca schidigera* bark. Compounds 1–4 represent monodesmosidic and 6–7 bidesmosidic structures

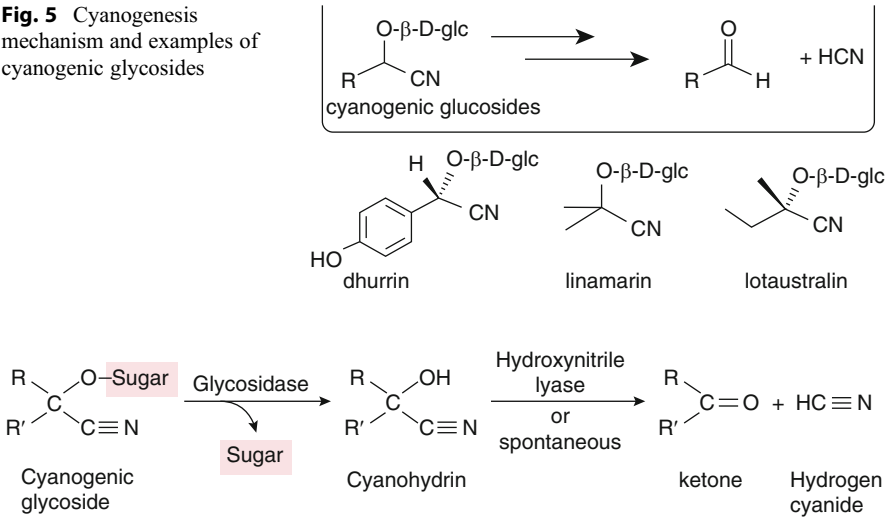


Fig. 5 Cyanogenesis mechanism and examples of cyanogenic glycosides

Fig. 6 General scheme to generate hydrogen cyanide from a cyanogenic glucoside precursor

are degraded by hydroxynitrile lyases to form HCN and ketones/aldehydes (Fig. 6). Cyanogenic glycosides are separated from enzymes catalyzing production of HCN due to compartmentalization at tissue, cellular, or subcellular level [28]. In the case of Sor*ghum bicolor*, Dhurrin is present in vacuoles of epidermal cells, β-glucosidase in the chloroplast of mesophyll cells, and hydroxynitrile lyases in the cytosol of mesophyll cells. Similarly in cassava plant "linamarin" and "lotaustralin" both the glycosides are present in tissues whereas linamarase (hydrolyzing enzyme) in laticifers and latex and hydroxynitrile lyases inside the cell wall of leaf tissues. Cyanide glycoside being the source of HCN (a highly toxic compound for most of animals, insects, and pathogens) acts as effective defense system against predators. HCN binds with electron transport system in mitochondria due to its ability to bind to cytochromes and thus damages the respiratory system of predators. Plants themselves combat the toxic effects of HCN by producing detoxification enzymes, such as rhodanese and β-cyanoalanine synthase [29]. Biosynthesis of cyanogenic glycosides requires synthesis of hydroxynitriles from amino acid precursors and then the nitriles are glycosylated to form cyanoglycoside [30]. Amino acid P ➔ hydroxynitrile➔cyanogenic glycosides. "Amygdalin" is another cyanogenic glycoside responsible for toxicity of bitter almonds and rosaceous seeds. Few fungi such as *Microcyclus ulei* (rubber tree pathogen b) can tolerate HCN due to cyanide-resistant respiration or by detoxifying HCN by producing cyanide-inducible enzyme CHT (cyanide hydratase) which converts HCN to formamide [31, 32]. CHT enzyme is also reported in other fungi and pathogens such as *Stemphylium loti*, *Gloeocercospora sorghi* (sorghum pathogen), and *Fusarium lateritium* (pathogen of sweet potato) [33, 32, 34]. In such pathogens the effect of cyanogenic glucosides is negligible.

2.5 Glucosinolates

They are sulfur-containing glucosides mainly present in members of Brassicaceae (mustard, cabbage, broccoli, *Arabidopsis*, etc.). Like saponins and cyanogenic glycosides these phytoanticipins also play an important role in degrading the plants from fungal attack and pathogens depending upon the nature of their side chains glucosinolates are usually subdivided into three major classes: aliphatic, aralkyl α-amino acids, and indolyl θ amino acid [35]. Distribution of glucosinolates is tissue specific within the plants; for example, in mustard plants indolyl and phenyl ethyl glucosinolates are present mainly in roots and stems whereas aliphatic ones predominate the leaves [36]. Glucosinolates are also activated in response to tissue damage due to activation of plant enzyme myrosinase (a thioglucosidase). In healthy plants a precursor of glucosinolates is separated from myrosinase due to subcellular compartmentalization of tissues. Myrosinase action results into an unstable aglycone (Fig. 7) which then forms highly reactive different products such as nitriles, thiocyanates, and isothiocyanates (mustard oils). The type of product formed depends upon the structure of precursor molecule, form of myrosinase, plant species, and other abiotic factors such as temperature, pH, protein cofactors, and metal ion concentrations [37]. In leaves of *Brassica* allyl-(2-propenyl) and 3-butenyl isothiocyanates are the major breakdown products which are highly toxic to fungi [38]. Many pathogens of *Brassica*, for example, *Peronospora parasitica*, *Alternaria* sp., *Mycosphaerella*

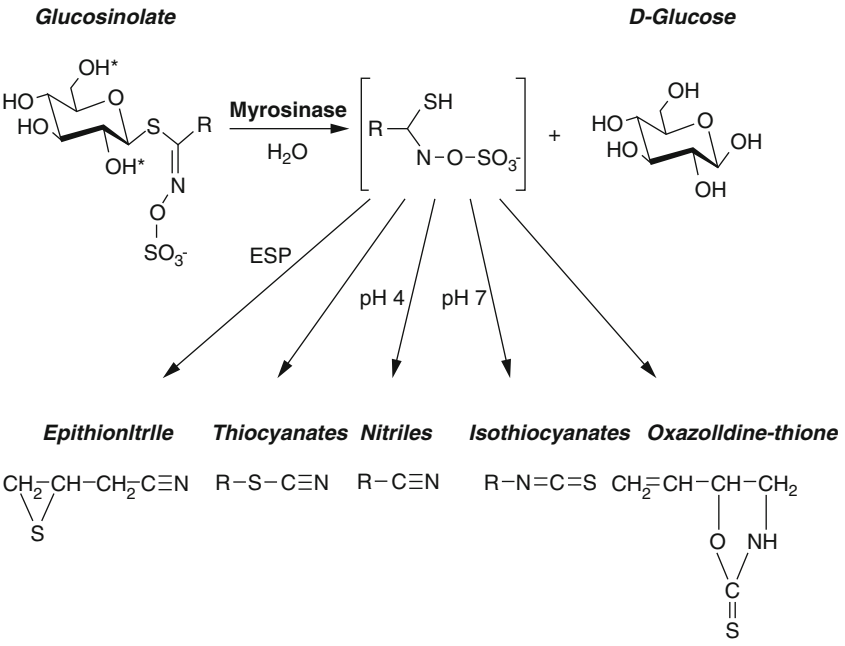


Fig. 7 General scheme for the hydrolysis of aliphatic glucosinolates to produce three active compounds

brassicicola, and *Leptosphaeria maculans*, are affected by these breakdown products. Breakdown products of indolyl glucosinolates may act as precursors to a class of indole phytoalexins that are formed in *Brassica*. These breakdown products are also effective against other pathogens which do not infect *Brassica* and therefore can be used as natural fungicides against cereal pests and postharvest pathogens of vegetables and fruits [39, 40]. GSL biosynthetic pathways, their transport, and regulation have been studied since decades and the knowledge has been used to genetically engineer benzyl-GSL into *Nicotiana benthamiana* Domin and the transformed tobacco plants were very effective in controlling insect predators [41]. Engineered tobacco plants could produce benzyl-GLS, an oviposition attractant of *Plutella xylostella* L. (diamond back moth), and hatched larvae were unable to survive on transformed tobacco which was more attractive for oviposition to female moths than wild-type tobacco plants. [42].

2.6 Benzoxazinoids

They are indole-derived antimicrobial compounds with 2-hydroxy-2H-1,4-benzoxazin-3(4H)-one (HBOA) skeleton and its derivatives synthesized from shikimate indole-3-glycerol phosphate in both monocots and dicots. These phytoanticipins are mainly present in grasses including economically important cereals like wheat, maize, and rye (not rice, oats, and sorghum) along with some dicot species exhibiting various properties such as insecticidal, antimicrobial, antifeedant, and allelopathic [43]. Few species of dicot families like Lamiaceae, Ranunculaceae, Plantaginaceae, and Acanthaceae also produce these phytochemicals [44]. Benzoxazinoids (BxDs) show structural diversity and therefore can be referred as benzoxazinones (glucosides and corresponding aglycones containing a 2-hydroxy- 2H-1,4-benzoxazin-3(4H)-one skeleton) and benzoxazolinones (degradation product). BXDs are stored as glucosides (BX-Glcs) in vacuoles of healthy plant cells [45] and the hydrolyzing enzyme, β-glycosidase, in plastid, cytoplasm, and cell walls. When there is tissue damage due to herbivore/pathogen attack or injury, BX-Glcs are hydrolyzed to release toxic aglycones (Fig. 8); HBOA, DIBOA, and DIMBOA are few common BXDs present in dicots and monocots. Chemical classes of phytoanticipins which have been discussed till know have been considered as traditional examples in many reviews [25]. While some nontraditional classes of phytoanticipins are also discussed here, that are based upon their biosynthetic origins and pathway.

2.7 Fatty Acid Derivatives and Polyketides (Derived from Acetate and Malonate)

Acetylenes or polyacetylenes are the fatty acid derivatives produced by different organisms as well as plants in form of secondary metabolites containing single or multiple triple (acetylenic) bonds. They have been reported in more than 1400 plants

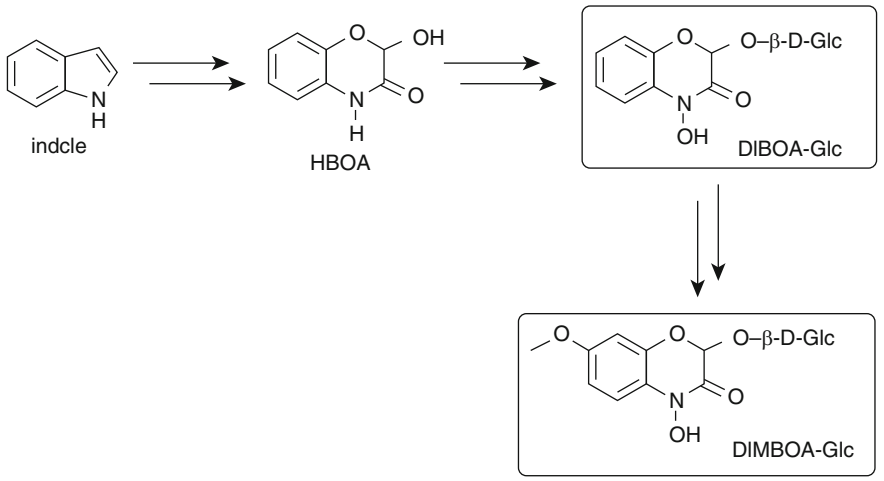


Fig. 8 Example of benzoxazinoids stored in plant cell vacuoles

of Araliaceae, Asteraceae, Apiaceae, and few other families and have been explored for their numerous pharmacological properties such as anti-inflammatory, antibacterial, anticarcinogenic, etc. [46]. Acetylenic metabolites produced by plants mainly provide constitutive defense against microbes and predators; for example, falcarindiol and falcarinol (Fig. 9) (both phytoanticipins) are produced by members of Apiaceae family such as carrots, dill, celery, fennel, and parsley. Falcarindiol has been reported to protect carrot plants from fungal pathogen *Alternaria dauci* causing leaf blight disease [47]. Green leaf volatiles synthesized from C6 aldehydes (2-3 hexanal and E-2 hexanal) via fatty acid pathway [48] can also be considered as phytoanticipins. Similarly oxylipins (product of fatty acid biosynthetic pathway) showing antifungal activity in plants are also included in phytoanticipin group [49]. Polyketide phytoanticipins (product of acetate malonate pathway) are less abundant as compared to polyketide-phenylpropanoids (terpenoids) of mixed biosynthetic origin. Terpenoids are synthesized from dimethylallyl diphosphate and isopentenyl diphosphate (C5 units) via mevalonate or deoxy-xylose pathway and can condense to form different types of carbon scaffolds (C10–C40). These secondary metabolites are abundant in plants and are mainly located in leaves and flowers as volatile phytochemicals, playing defensive roles against insects and pathogens. Tropical leguminous trees, for example, *Hymenaea* sp., contain numerous volatile sesquiterpenes (caryophyllene and caryophyllene oxide) (Fig. 10) in the form of leaf resins showing antifungal activity against different types of fungi sp. [50]. In rice plant phytoanticipins oryzalides A and B, oryzalic acid A (Fig. 10) (both diterpenes) and related compounds are produced that provide defense against *Xanthomonas oryzae* pv. *oryzae* causing bacterial leaf blight disease. Terpenoids are also present in the form of saponins which have been already discussed as a traditional phytoanticipin.

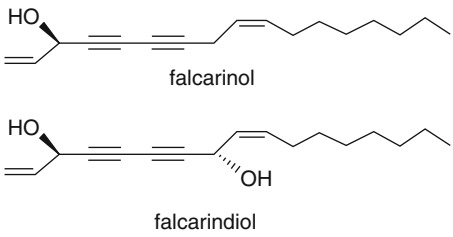


Fig. 9 Structure of falcarinol and falcarindiol phytoanticipins

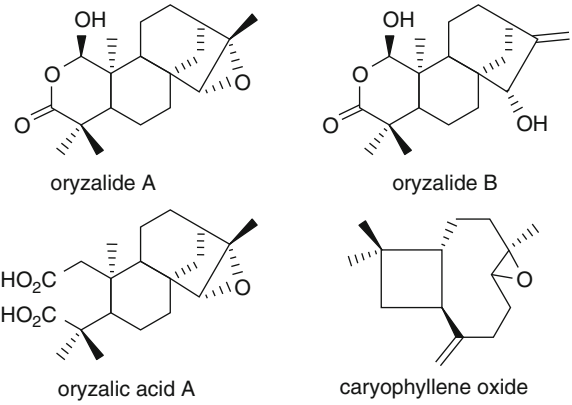


Fig. 10 Structure of nontraditional terpenoid phytoanticipins

2.8 Shikimates, Phenylpropanoids, and Derivatives

Shikimates are important plant secondary metabolites performing diverse roles and are biosynthetic precursor of amino acids tyrosine and phenylalanine (both precursors of phenylpropanoids). Derivatives of phenylpropanoid contain hydroxyl groups on the phenyl ring and are known as phenolics. Phenolic metabolites such as flavonoids and stilbenes have mixed biosynthetic origin and are derived from phenylpropanoids and polyketides. They show antimicrobial activity against diverse plant and human pathogens along with nutritional and therapeutic effects. *Arachis hypogaea* L. is a source of various phenylpropanoids such as caffeic acid, p-coumaric acid, ferulic acid, mucilagin A (Fig. 11), and methoxycinnamic acid when grown axenically. These phytochemicals are antifungal in nature and provide defense from *Aspergillus flavus* Link and *A. parasiticus* Speare and work as phytoanticipins in peanut plant tissues [51]. Similarly there are some more examples of such type of phytoanticipins, in *Citrus* species (*C. sinensis* L., *C. limon* L., *C. unshiu*, etc.). "Hesperidin" is produced which protects the citrus plants from fungi *Penicillium digitatum*. *Dianthus caryophyllus* L. (carnation) is resistant to *Fusarium oxysporum* f.sp. *dianthi* due to synthesis of "kaempferide triglucoside" (kaempferide 3-0-β-D-glucopyranosyl-(1->2)-o-[α-L-rhamnopyranosyl-(1->6)]-β-D-glucopyranoside) (Fig. 11). Hydroxyacetophenone is another phytoanticipin produced by carnation plant against *F. oxysporum*

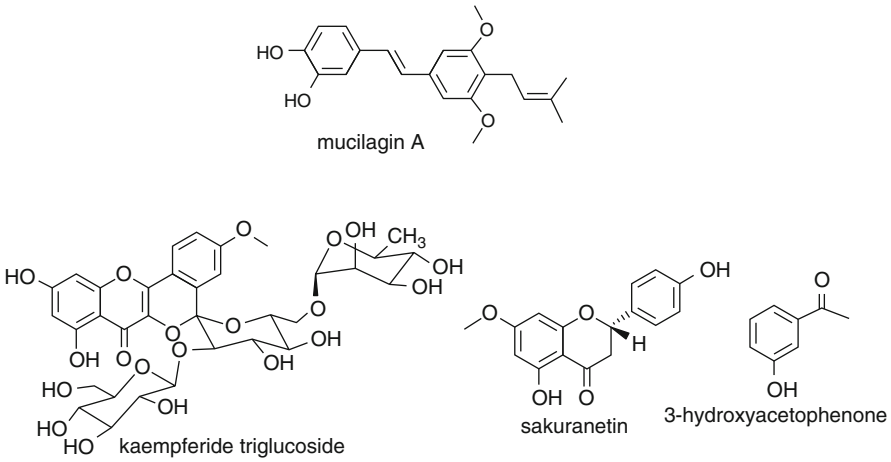


Fig. 11 Chemical structures of phenylpropanoids and derivatives

fungi [52]. Sakuranetin (flavanone) isolated from heartwood of *Prunus avium* L. when induced in rice shows resistance against fungi *Magnaporthe grisea*.

2.9 Benzylisoquinoline and Pyrrolizidine Alkaloids

These alkaloids are huge group of phytochemicals produced in large quantities in plants and other phyla whose chemical structure is derived from various precursors such as amino acids (tryptophan, lysine, tyrosine, anthranilic acids, and nicotinic acid). Few alkaloid groups are also derived from amination reaction of polyketides, shikimates, and isoprenoids [53]. Benzylisoquinoline, chelerythrine, and sanquinarine are some important alkaloids showing antibacterial and antifungal properties. All the three alkaloids have constitutive nature and therefore are considered as phytoanticipins. Retronecine, heliotrine, and senecionine are important pyrrolizidines showing antifungal activity.

3 Phytoalexins

Phytoalexins are defense-related LMW antioxidative phytochemicals (antimicrobial compounds) produced de novo by plants in response to biotic and abiotic stresses and get accumulated hurriedly at sites of pathogen infection. Term phytoalexin was introduced first by Muller and Borger [5] 70 years ago while observing the infection of potato tubers by *Phytophthora infestans* which could inhibit the growth of another strain of *Phytophthora infestans* on potato plants. This inhibition was the effect of hypersensitive reactions taking place due to infection from earlier strain of *P. infestans* and it was also linked to "principle" compound produced by infected

plant cells named as phytoalexin [54]. They act as toxins for the attacking pathogens as they can delay maturation, disrupt metabolism, and prevent reproduction of the pathogen and also puncture the cell walls. After recognizing the particle either from damage cells or from pathogen, plant cell launches two forms of induced resistance. First one is the short-term response in which plants utilize reactive oxygen species like hydrogen peroxide and superoxide to kill invading cells. During pathogen interactions in case of short-term response/hypersensitive response, cells surrounding the site of infection are signaled to undergo apoptosis (programmed cell death) to restrict the spreading of pathogen to the rest of the plant tissues. The other one, that is, long-term resistance/systemic acquired resistance (SAR), will involve communication of damaged plant tissues using plant hormones, for example, ethylene, jasmonic acid, salicylic acid, or abscisic acid with the rest of the plant. After receiving the hormonal signal from the damaged tissues, global changes occur within the plant such as inducing certain genes to translate proteins involved in providing protection against invading pathogens and also synthesis of enzymes involved in biosynthesis of phytoalexins. De novo induction of phytoalexin biosynthetic enzymes and genes is localized to the cells surrounding the infected part of the plant and the synthesis is transient as it reaches a peak after few hours of infection and then declines. Phytoalexin production in plants is restricted to those compounds that are produced from remote precursors via de novo synthesis of enzymes making it a complex process which is further regulated by other factors such as defense-related marker genes, calcium sensors, phosphorylation cascades, and elicitors that include hormone signaling also [55, 56]. Till that hundreds of phytoalexins have been characterized, most of the work is on limited number of families such as Solanaceae and Fabaceae [57]. Fifteen other families are also found to be producing phytoalexins, for example, Euphorbiaceae, Orchidaceae, Chenopodiaceae, Asteraceae, Poaceae, Linaceae, Moraceae, Rutaceae, Rosaceae, Piperaceae, Ginkgoaceae, Amaryllidaceae, Apiaceae, and Convolvulaceae. Few plant species of economically important families such as maize oats, sorghum and rice (Poaceae), cotton (Malvaceae), and Vitaceae species also produce phytoalexins as a mode of inducible defense [58, 59, 60]. "Camalexin" produced by members of family Brassicaceae has been one of the important phytoalexins on which numerous studies focusing on its biosynthetic pathway and regulatory factors involved in its production have been carried out specifically on model plant *Arabidopsis thaliana* [61]. Ubiquity of phytoalexins throughout the plant kingdom still remains unanswered. Knowledge of control mechanisms involved in production, accumulation, and regulation of phytoalexins in different systems could serve as a basis for genetic manipulation of these compounds in genetically engineered plants for enhanced disease resistance [62]. Phytoalexins are chemically diverse compounds; for example, in family Fabaceae six isoflavonoid classes have been reported: isoflavanones, isoflavones, pterocarpenes, pterocarpans, coumestans, and isoflavans (Table 1). Well-known pterocarpans are phaseolin, glyceollin, pisatin, maackiain, and medicarpin (Fig. 12). Pisatin was the first phytoalexin to be isolated characterized from *Pisum sativum* (garden pea) [63]. Legumes also produce nonisoflavonoid phytoalexins such as stilbenes and furanoacetylenes (Table 1). In family Solanaceae

Table 1 Phytoalexins from different plant families

Plant families	Plant species	Types of phytoalexins/examples
Amaryllidaceae	*Allium cepa, A. sativum, Narcissus pseudonarcissus* (daffodil)	Flavans, allixin
Brassicaceae (Cruciferae)	*Camelina sativa* (mustard)	Indole phytoalexins/camalexin Sulfur-containing phytoalexins/ brassinin
Chenopodiaceae	*Beta vulgaris* (sugarbeet)	Flavanones/betagarin Isoflavones/ betavulgarin
Compositae (Asteraceae)	*Carthamus tinctorius* (safflower)	Polyacetylenes/safynol
Convolvulaceae	*Ipomoea batatas* (sweet potato)	Furanosesquiterpenes/ ipomeamarone
Euphorbiaceae	*Ricinus communis*	Diterpenes/casbene
Poaceae	*Oryza sativa* (rice), *Zea mays* (maize), *Sorghum bicolor*	Diterpenoids, momilactones, oryzalexins, zealexins, Phytocassanes, kauralexins Deoxyanthocyanidins/luteolinidin and apigeninidin Flavanones/sakuranetin Phenylamides
Leguminosae (Fabaceae)	*Pisum sativum*	Isoflavones, isoflavanones, isoflavans, coumestans Pterocarpans/pisatin, Phaseolin, Glyceollin, and maackiain Furanoacetylenes/wyerone stilbenes/resveratrol, pterocarpenes
Plant families	**Plant species**	**Types of phytoalexins/examples**
Linaceae	*Linum usitatissimum* (flax)	Phenylpropanoids/coniferyl alcohol
Malvaceae	*Gossypium* sp. (cotton)	Terpenoids naphthaldehydes/ gossypol
Moraceae	*Morus* sp.	Furanopterocarpans/moracins A–H
Orchidaceae		Dihydrophenanthrenes/loroglossol
Rutaceae	*Citrus lemon* (lemon)	Methylated phenolic compounds/ xanthoxylin
Umbelliferae (Apiaceae)	*Daucus carota* (carrot), *Pastinaca sativa*	Polyacetylenes/falcarinol Phenolics, xanthotoxin 6-Methoxymellein
Vitaceae	*Vitis vinifera* (grapes)	Stilbenes/resveratrol
Rosaceae	*Maloideae* (apples and pears)	Biphenyls/auarperin Dibenzofurans/cotonefurans
Solanaceae	*Nicotiana tabacum* (tobacco)	Phenylpropanoid-related compounds Steroid glycoalkaloids Norsequi and sesquiterpenoids Coumarins Polyacetylenic derivatives
Caricaceae	*Carica papaya* (papaya)	Danielone

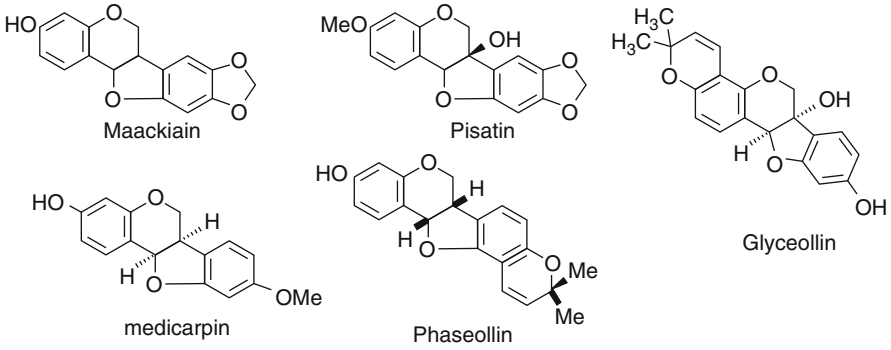


Fig. 12 Examples of pterocarpans (phytoalexins)

five different classes of phytoalexins have been reported: steroid glycoalkaloids, sesquiterpenoids and nonsesquiterpenoids, phenylpropanoid-related compounds, polyacetylene derivatives, and coumarins [55]. Two new phytoalexin classes have also been discovered in members of Brassicaceae and Poaceae family. In rice, maize, and sorghum zealexins, kauralexins, momilactones, phytocassanes, and oryzalexins (members of labdane-related diterpenoid superfamily) phytoalexins (Fig. 13) are produced [64] [65]. Other than that flavones, 3-deoxyanthocyanidins, unusual group of flavonoid phytoalexins, and phenylamides have also been reported [66, 67]. Trans-resveratrol and delta viniferin are the two important phytoalexins produced by *Vitis vinifera* (grapes) to restrict the growth of fungal pathogens *Botrytis cinerea* [68] (Favaron et al., 2009) and *Plasmopara viticola* [69]. Sakuranetin (a flavanone) is produced in rice and *Polymnia fruticosa* against spore germination of *Pyricularia oryzae* [70]. Flavonoid 3′-hydroxylase encoded by SbF3′H2 gene in sorghum expresses in production of phytoalexin 3-deoxyanthocyanidin [71]. In papaya fruit "danielone" phytoalexin provides resistance against fungi *Colletotrichum gloeosporioides* [72]. During the pathogen attack stilbenes (polyphenols) are produced in *Eucalyptus sideroxylon* against rot caused by fungi [73]. The rest of the examples of plants producing phytoalexins as a mode of defense have been presented in Table 1.

Phytoalexin biosynthesis in different plants occurs via different pathways but the three most common and important ones are as follows.

3.1 Phenylpropanoic-Polymalonic Acid Route

This pathway leads to synthesis of different types of flavonoid phytoalexins such as isoflavones, pterocarpans, coumestans, and isoflavonoids along with synthesis of stilbenes and its derivatives (dihydrophenanthrenes). Pathway begins with synthesis of phenylalanine and phenylalanine lyase (PAL) along with tyrosine and tyrosine ammonia lyase (TAL). Reaction between amino acid precursors and hydrolases produces para-coumaric acids, which are then activated in para-coumaroyl-CoA after being ligated to

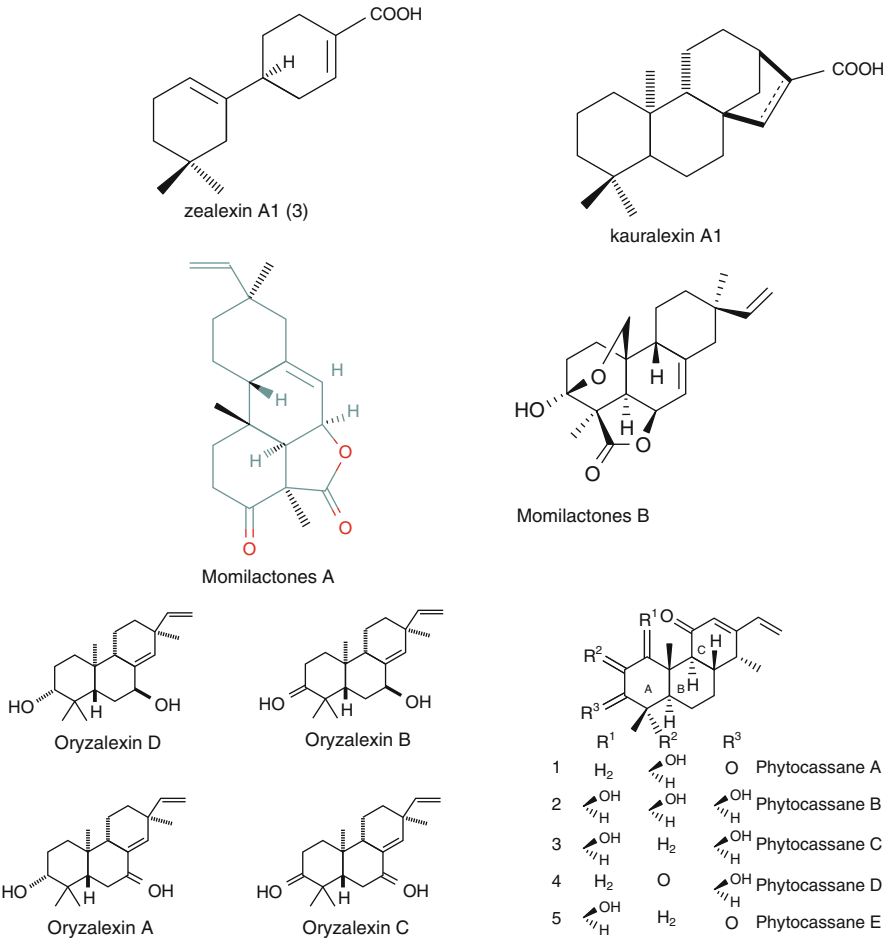


Fig. 13 Examples of phytoalexins from Poaceae family

coenzyme A in the presence of 4-coumaroyl-CoA ligase. Eventually chalcone synthase at one side and stilbene synthase on another side using same substrate and then condensing it with three consecutive units of malonyl-CoA will lead to production of naringenin chalcone (first C15 intermediate of flavonoid pathway) and resveratrol (precursor of stilbenes). This pathway has been studied in the members of family Fabaceae for production of flavonoids and stilbene like phytoalexins [60, 74, 75].

3.2 Phytoalexins Derived from Mevalonoid Pathway

This pathway is important for the production of members of sesquiterpene, carboxylic sesquiterpene, monoterpene, and diterpene families. For synthesis of diterpenoids GGDP is used as a starting material along with action of diverse

enzymes. First enzyme to act on GGDP is copalyl diphosphate synthases (class II diterpene cyclases) leading to cyclization into copalyl diphosphate (CDP). CDP acts as a substrate along with kaurene synthase (class I diterpene synthase enzyme) to produce olefin: main precursor of diterpene phytoalexin families. Kaurene synthase also uses stereochemically differentiated isomers of CDP such as ent-CDP, involved in biosynthesis of phytocassanes A–E and oryzalexins A–F, and syn-CDP is the substrate for momilactone A and B biosynthesis.

3.3 Biosynthesis of Indole Phytoalexins

This pathway mainly synthesizes "camalexin," major phytoalexin of Brassicaceae family. Chorismate produces tryptophan which helps in synthesis of indolic ring of camalexin. Cytochrome P450 homologues CYP79B2 and CYP79B3 regulate the first step (indole-3-acetaldoxime production) of tryptophan to camalexin production. Indole-3-acetaldoxime is transformed into indole-3-acetonitrile (IAN) in the presence of CYP71A13 and P450. Glutathione-S-transferase along with cytochrome P450 conjugates IAN with glutathione. Derivative of IAN glutathione is converted into IAN cysteinyl-glycine in the presence of phytochelatin synthase or into Y-glutamyl-cysteine IAN in the presence of Y-glutamyltranspeptidases 3 and 1 [56]. Both the intermediates of IAN form IAN-cysteine conjugate. In the last step of the biosynthetic pathway dihydrocamalexic acid forms "camalexin" after expression of CYP71B15 (phytoalexin deficient 3, PAD3) gene encoding a multifunctional enzyme. Regulatory mechanisms involved in biosynthesis of phytoalexins are depended upon expression of many endogenous molecules/factors, for example, phytohormones, defense-related genes, transcriptional regulators, cascades, and phosphorylation relays [76] along with nature of infecting pathogen and induced phytoalexin itself. *Arabidopsis* when infected by *Alternaria brassicicola* synthesizes camalexin without the involvement of jasmonic acid; on the other hand, when the same plant is infected by fungal pathogen *Botrytis cinerea*, then JA is involved in the regulatory signaling pathway of camalexin production [77]. Abscisic acid and auxins play a negative role in regulation of phytoalexin production [55]. In *Arabidopsis* suppression of auxin synthesis increases the resistance of plant toward bio-trophic pathogens by redirecting the metabolism of phytoalexins [78]. ABA downregulates the synthesis of phytoalexins; for example, kievitone biosynthesis in bean, glyceollin synthesis in soybean, and rishitin and lubimin production in potato plants are decreased due to ABA production [79, 80]. Overexpression of cytokinins enhances the production of phytoalexins in the same plant systems as tobacco production of capsidiol and scopoletin enhances in the presence of cytokinins developing resistance against *Phytophthora syringae* [81]. Mitogen-activated protein kinases MPK3 and MPK6 are involved in induction and accumulation of camalexin in *Arabidopsis* after treatment with microbe-associated molecular patterns [61]. CYP71B15 gene encoding the multifunctional enzyme required at the end of the pathway is overexpressed in the presence of these two MPKS. Overexpression of Rac protein in rice enhances the production of phytoalexin momilactone A (19,180 folds) inducing resistance for bacterial blight

disease [82]. Sucrose, glucose, and fructose along with glucans, chitosan, glycoprotein, and polysaccharides that could also be the part of fungal cell wall are released by host plant enzymes and act as endogenous signals to regulate biosynthesis and accumulation of some phytoalexins [83]. Gossypol production is enhanced due to overexpression of pathogenesis-related gene 1 playing a crucial role to develop systemic acquired resistance in cotton plants [84].

Information regarding the regulatory mechanisms of phytoalexin biosynthesis will open the way to develop new genetically modified plants resistant to diseases. In grape wine plant phytoalexin (resveratrol) synthesis is controlled by stilbene synthase gene; therefore researchers [85] transferred two grapevine STS genes (Vst1 and Vst2) into tobacco plant increasing the resistance of metabolic engineered variety from *B. cinerea* [85]. Later on other researchers also introduced same STS gene either from grape vine or other sources into different plant systems (rice, barley, alfalfa, wheat, papaya, tomato, and *Arabidopsis*) through genetic transformation technique making them resistant to various pathogens [55]. Soybeans having roots were transformed with peanut resveratrol synthase 3 AhRS3 gene and resveratrol o-methyltransferase ROMT gene [86]. Both the genes are expressed in transformed plants to catalyze the conversion of resveratrol to pterostilbene which increased the resistance of transformed plants from *Rhizoctonia solani*. It is quite difficult to engineer the entire biosynthetic pathway of phytoalexins but the researchers are trying at their level best to choose the right gene for right enzyme that could catalyze the limiting step of this pathway.

4 Conclusion

Plants produce both the types of secondary metabolites (phytoanticipin + phytoalexins) for their protection from various microbial pests and invaders. Novel scientific techniques provide knowledge regarding structure, biosynthesis, regulation, and function of these phytochemicals. From the past decade these secondary metabolites involved in plant defense mechanisms have been an asset to the mankind, due to their applications in plant breeding and engineering crops with better resistance to microbial pathogens and pests. In the present review several examples of both the types of defense-related compounds have been discussed in details but still there are a huge number of secondary metabolites whose functions and biosynthetic pathways need to be investigated. More research in this field and critical reporting of data can only help us in understanding more about the functions of plant secondary metabolites and their applications to breed or engineer more varieties of disease- and pest-resistant crops.

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Brassinosteroids: Molecules with Myriad Roles

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Abstract

Brassinosteroids constitute the sixth class of plant hormones that are implicated in diverse metabolic functions related to plant growth and development. These steroidal phytohormones are widely distributed throughout the plant kingdom and display large structural diversity. Studies on brassinosteroids, aided by the recent developments in technology, have deciphered their role not only in plant growth and developmental processes but also in plant adaptation under changing environmental conditions. Extensive experimental studies have unravelled brassinosteroid biosynthetic pathway and their signalling modules under various environmental conditions. Current trends indicate that brassinosteroids play a pivotal role in plant's tolerance against biotic and abiotic stresses, resulting in efficient stress management under challenging environmental conditions. Due to their distinctive and versatile functions, brassinosteroids are widely used to increase crop quality and productivity. Brassinosteroids are also reported to possess immunomodulatory, anticancerous, and antiviral properties that also find wide potential applications. This chapter focuses on the current status of our understanding about the role of brassinosteroids, their molecular mechanism of action, and their potential applications in agriculture and allied fields.

Keywords

Brassinosteroids · Phytohormones · Molecular mechanism of action · Plant growth regulators · Stress management

1 Introduction

Phytohormones are a class of messenger molecules that are involved in various biochemical, physiological, as well as plant growth and developmental processes that are required at very low concentrations. There are various classes of phytohormones, viz., auxin, gibberellin, cytokinin, abscisic acid, and ethylene, which are crucial for the growth and development of plants at various stages of their life cycle. Phytohormones are also involved in defense mechanisms which include salicylic acid, jasmonic acid, and much later discovered group of brassinosteroids [1]. Certain light sensors like phytochromes such as cryptochrome and phototropin are involved in regulation of plants' growth and differentiation based on quality and intensity of light [2]. Plant growth regulators modify or control growth processes such as stem elongation, leaves formation, flowering, fruit development and fruit ripening, and senescence. There are compelling evidences about the role of these messengers in various stress-responsive processes like providing resistance to various pathogens and defense against herbivores. Auxin is a major phytohormone involved in many physiological processes. Indole acetic acid (IAA) is a major form of auxin derived from its precursor amino acid tryptophan. Important functions of auxin include expansion and elongation of adventitious roots, lateral root development, xylem and phloem differentiation, as well as stimulation of abscission. In tissue culture it is

used along with cytokinin to stimulate cell division. Synthesis of gibberellins occurs in young tissues and in the developing seeds. While the primary precursor for GA is acetate, all the gibberellins are synthesized from ent-gibberellane skeleton. GA3 was the very first GA to be characterized structurally. The major role of GA in plant's physiology includes parthenocarpy, alpha amylase production on germinating seeds, delaying senescence, breaking seed dormancy, etc. Cytokinins are yet another important class of phytohormones that resembles aminopurines and is involved in cell division process. Higher concentrations of cytokinin are found in meristematic tissues and other growing parts of plant. Cytokinins are synthesized via MEP (methylerythritol phosphate) and MVA (mevalonic acid) pathway in roots and transported to shoot via xylem. Apart from its role in cell division, cytokinin is also involved in other processes like induction of apoptosis, stimulation of morphogenesis, growth of lateral buds, leaf expansion, etc. Ethylene is a gaseous compound and is synthesized from its precursor amino acid methionine. Ethylene plays a major role in fruit ripening as well as in induction of femaleness of flowers and leaf senescence; it induces root and shoot growth and differentiation and stimulates fruit and leaf abscission. Abscisic acid is synthesized from its precursor carotenoid via isoprenoid pathway. Abscisic acid is widely known for its role in abiotic stresses such as salinity and drought stress. It stimulates the closure of stomata under abiotic stresses to prevent the water loss and desiccation. Abscisic acid also controls bud dormancy, seed germination and vegetative growth, inhibition of shoot growth, inhibition of alpha-amylase synthesis, and induction of dormancy. Plant hormones like jasmonic acid and salicylic acid play a crucial role in pathogen defense. Both these hormones are involved in signal cascade on pathogen invasion. Salicylic acid plays an important role against biotrophic pathogen attack. Jasmonic acid plays a role in various processes which include root growth, tuber formation, seed germination, tendril coiling, stomatal opening, fruit ripening, and leaf senescence; it is also found to regulate pollen development in some plants. Another important class of phytohormones, brassinosteroids, plays a key role in plant growth and development. Brassinosteroids are reported to stimulate the shoot growth, leaves unfolding, xylem differentiation, and anthocyanin formation and hinder root growth. They are synthesized via isoprenoid biosynthesis pathway [3].

Brassinosteroids are a class of steroidal phytohormones actively involved in various cell developmental processes such as epinasty, germination, growth and differentiation, stem and root elongation, photomorphogenesis of seedlings, floral initiation, fruit and flower development, and male fertility [4]. Identification of brassinosteroid-insensitive and brassinosteroid-deficient mutants has led to the detailed understanding of the role of the brassinosteroids in plants' growth resulting into improved agronomic traits [5]. This class of hormones is also reported to enhance cell division and cell expansion and interacts with other signalling pathways at transcriptional level controlling plant growth [6]. Besides their role in plant growth and developmental processes, they are also involved in various biotic and abiotic stresses and allow plants to survive under adverse environmental conditions [4]. Due to their diverse functionality, these hormones are also used in phytoremediation. Structural determination of brassinolide, the most active

brassinosteroid, from rape pollen showed similarity to the animal steroid hormones. These polyhydroxylated plant steroid hormones bear resemblance to the mammalian steroid hormones which are also involved in cellular dynamics and functions [7].

2 Structure and Distribution of Brassinosteroids

Brassinosteroids are naturally occurring steroidal ketones/lactones that are widespread among the plant kingdom [8]. These are the derivatives of 5α-cholestane, and variability in their structures arises due to the substitutions in carbon side chains. Although they are found in all the plant organs, higher concentration of brassinosteroids is reported in actively growing tissues such as seeds and pollens. Different classes of brassinosteroids have been characterized from at least 79 plant species that include 53 angiosperms, 24 algae, 1 bryophyte, and 1 pteridophyte. Depending upon the carbon numbers in their structures, brassinosteroids are divided into C27, C28, and C29 groups which are found in free or in conjugated forms with fatty acids and sugars [9]. About 40% of total brassinosteroids identified so far belongs to C28 groups followed by C27 and C29 indicating their lower endogenous content than C28 class. Brassinosteroids are present in various parts of a plant ranging from seeds, flowers, roots, stems, leaves, as well as pollen. The maximum concentration of brassinosteroids ranging from 1 to 100 ng/g fresh weight has been reported in seeds and pollen; on the other hand, the lowest concentration has been observed in leaves and shoots which ranged from 0.01 to 0.1 ng/g fresh weight. Typhasterol (TY) and castasterone (CS) are most frequently distributed classes of brassinosteroids in the plant kingdom although the brassinosteroids composition varies depending on the plant species. More than 62 chemical structures have been confirmed so far and still under studies [9, 10] (Table 1).

3 Biosynthesis

Squalene, which is a triterpene, acts as the precursor molecule for brassinosteroid synthesis. Squalene is formed by condensation of two farnesyl-PP molecules using one NADPH as reducing equivalent [2]. Biosynthetic pathway of brassinosteroids can be regulated at multiple steps by feedback regulation maintaining the endogenous brassinosteroid homeostasis. Higher endogenous level of brassinosteroid results into feedback inhibition leading to the activation of genes for brassinosteroid degradation. Various genes involved in biosynthesis of brassinosteroids such as *constitutive photomorphogenesis and dwarfism* (*CPD*), *de-etiolated-2* (*DET2*), and *DWARF4* (*DWF4*) can be modulated to regulate the endogenous level of brassinosteroids in plants. The repression of brassinosteroid biosynthetic genes is due to the action of accumulated brassinazole-resistant 1 (BZR1) and BRI1-EMS-suppressor 1 (BES1)/BZR2. *PhyB activation-tagged suppressor1* (*BAS1*) is the first and most important brassinosteroid inactivating gene in plants. Thus inactivation of

Table 1 Distribution of brassinosteroids in plants

Family/species	Plant parts	Brassinosteroids
Arecaceae		
Phoenix dactylifera **L.**	Pollen	24-*epi*CS
Gramineae		
Lolium perenne **L.**	Pollen	25-*MeCS*
Oryza sativa **L.**	Shoot	CS, DS, BL
	Bram	6-DeoxoCS, 28-HomoTE, 28-HomoTY
	Seeds	CS, TE, 6-DeoxoCS
Phalaris canariensis **L.**	Seeds	CS, TE
	Seeds	CS, TY
Secale cereale **L.**	Seeds	CS, TY, TE, 6-DeoxoCS, 28-NorCS, SE
Triticum aestivum **L.**	Grain	CS, TY, TE, 6-DeoxoCS, 3-DT
Zea mays **L.**		
Dent corn	Pollen	CS, TY, TE
Sweet corn	Pollen	CS, 28-NorCS, DS
Liliaceae		
Erythronium japonicum Decne	Pollen	TY
Lilium elegans Thunb	Pollen	BL, CS, TY, TE
Lilium longiflorum Thunb	Pollen	BL, CS, TY
Tulipa gesneriana **L.**	Anther	3-DT, TE-3-La, TE-3-My, TE-Glu
	Pollen	TY
Typhaceae		
Typha latifolia G.F.W. Mey	Pollen	TY, TE
Betulaceae		
Alnus glutinosa (L.) Gaertn.	Pollen	BL, CS
Cannabaceae		
Cannabis sativa **L.**	Seeds	CS, TE
Caryophyllaceae		
Gypsophila perfoliata **L.**	Seeds	24-*epi*BL
Lychnis viscaria **L.**	Seeds	24-*epi*CS, 24-*epi*SE
Beta vulgaris **L.**	Seeds	CS, 24-*epi*CS
Fagaceae		
Castanea crenata Sieb. et Zucc.	Galls	CS, BL, 6-DeoxoCS
	Shoot	CS
	Leaves	6-DeoxoCS
Polygonaceae		
Fagopyrum esculentum Moench	Pollen	BL, CS
Rheum rhabarbarum **L.**	Panicles	BL, CS, 24-*epi*CS

(*continued*)

Table 1 (continued)

Family/species	Plant parts	Brassinosteroids
Apiaceae		
Apium graveolens **L.**	Seeds	2-DeoxyBL
Daucus carota ssp. *sativus* **L.**	Seeds	BL,CS,24-*epi*CS
Brassicaceae		
Arabidopsis thaliana (**L.**) Heynh.	Shoot	CS
	Ecotype Columbia (wild type)	6-DeoxoCS, TY, 6-DeoxoTY, BL, 28-NorCS, 28-NorTY, TE, 6-DeoxoCT, 6-DeoxoTE, 3-Dehydro-6-DeoxoTE
	Seeds	BL
	Ecotype Columbia (wild type)	24-*epi*BL, CS, 6-DeoxoCS, TY, 6-DeoxoTY, 6-DeoxoTY
	Seeds (ecotype 24)	24-*epi*BL, CS
	Root callus	BL, 3-*epi*BL
Brassica campestris var. *pekinensis* **L.**	Seeds	BL, 28-NorBL, CS, 28-NorCS, 28-HomoCS
Brassica nepus **L.**	Pollen	BL
Raphanus sativus **L.**	Seeds	BL, CS, TE, 28-HomoTE
Fabaceae		
Cassia tora **L.**	Seeds	BL, CS, TY, TE, 28-NorCS
Dolichos lablab **L.**	Seeds	DL, DS, 28-HomoDS, 28-HomoDL, BL, CS, 6-DeoxoCS, 6-DeoxoDS
Robinia pseudoacacia **L.**	Pollen	CS, TY, 6-DeoxoCS
Vicia faba **L.**	Seeds	BL, 24-*epi*BL, CS, 28-NorCS
	Pollen	BL, CS, 28-NorCS, DS
Psophocarpus tetragonolobus (Stickm.) DC.	Seeds	BL, CS, 6-DeoxoCS, 6-DeoxoDS
Ornithopus sativus Brot.	Seeds	CS, 24-*epi*CS
	Shoot	CS, 6-DeoxoCS, 24-*epi*CS, 6-Deoxo-24-*epi*CS, 6-Deoxo-28-NorCS
Phaseolus vulgaris **L.**	Seeds	BL, CS, 2-*epi*CS, 3-*epi*CS, 2,3-Di*epi*CS, 3,24-Di*epi*CS, TY, TE, 6-DeoxoCS, 3-*epi*-6-DeoxoCS, 1β-OH-CS, 3-*epi*-1α-OH-CS, DL, DS, 6-DeoxoDS, 6-Deoxo-28-HomoDS, 25-MeDS, 2-*epi*-25-MeDS, 2,3-Di*epi*-25-MeDS, 2-Deoxy-25-MeDS, 2-*epi*-2-Deoxy-25-MeDS, 2-Deoxy-25-MeDS, 3-*epi*-2-Deoxy-25-MeDS, 6-Deoxo-25-MeDS, 25-MeDS-Glu, 2-*epi*-25-MeDS-Glu
Pisum sativum **L.**	Seeds	BL, CS, TY, 6-DeoxoCS, 2-DeoxyBL
	Shoot	BL, CS, 6-DeoxoCS, TY, 6-DeoxoCT, 6-DeoxoTE, 3-Dehydro-6-DeoxoTE, 6-DeoxoTY

(*continued*)

Table 1 (continued)

Family/species	Plant parts	Brassinosteroids
Myrtaceae		
Eucalyptus calophylla R.Br.	Pollen	BL
Eucalyptus marginata Sn.	Pollen	DS
Rosaceae		
Eriobotrya japonica (Thunb.) Lindl.	Flower buds	CS
Rutaceae		
Citrus unshiu Marcov.	Pollen	BL, CS, TY, TE
Citrus sinensis Osbeck	Pollen	BL,CS
Theaceae		
Thea sinensis	Leaves	28-NorCS, 28-HomoCS, BL, CS, TY, TE
Apocynaceae		
Catharanthus roseus G.Don.	Cultured cell	BL, CS, 6-DeoxoTY, 6-DeoxoTE, 6-DeoxoCS, CT, 6-DeoxoCT, 6-*epi*-6-DeoxoCT, 3-DT, TY, TE
Asteraceae		
Zinnia elegans **L.**	Cultured cell	CS, TY, 6-DeoxoCS, 6-DeoxoTY, 6-DeoxoTE
Helianthus annuus **L.**	Pollen	BL, CS, 28-NorCS, BL
Solidago altissima **L.**	Shoot	BL
Boraginaceae		
Echium plantagineum **L.**	Pollen	BL
Convolvulaceae		
Pharbitis purpurea Voigt	Seeds	CS, 28-NorCS
Cucurbitaceae		
Cucurbita moschata Duch.	Seeds	BL
Lamiaceae		
Perilla frutescens **(L.)** Britt.	Seeds	CS
Solanaceae		
Nicotiana tabacum **L.**	Cultured cell	CS
Lycopersicon esculentum Mill	Shoot	CS, 6-DeoxoCS, 28-NorCS
	Root	6-Deoxo-28-NorCT, 6-Deoxo-28-NorTY, 6-Deoxo-28-NorCY
Cupressaceae		
Cupressus arizonica Greene	Pollen	6-DeoxoTY, 6-DeoxoCS, 3-Dehydro-6-DeoxoTE, CS, TY, TE, BL, 3-DT, 28-HomoCS
Ginkgoaceae		
Ginkgo biloba **L.**	Seeds	TE

(*continued*)

Table 1 (continued)

Family/species	Plant parts	Brassinosteroids
Pinaceae		
Piceae sitchensis Trantv. ex Mey	Shoot	CS, TY
Pinus sylvestris **L.**	Cambial region	BL, CS
Pinus thunbergii Parl.	Pollen	TY
Taxodiaceae		
Cryptomeria japonica D Don.	Pollen	TY
	Anther	DL, 3-DT, 28-HomoBL, 28-HomoDL, 23-DehydroBL (cryptolide), 2-*epi*-23-DehydroBL, 3-*epi*-23-DehydroBL, 2,3-Di*epi*-23-DehydroBL
Equisetaceae		
Equisetum arvense **L.**	Whole plant	CS, DS, 28-NorBL, 28-NorCS
Hydrodictyaceae		
Hydrodictyon reticulatum **(L.)** Lager.	Whole plant	24-*epi*CS, 28-HomoCS
Marchantiaceae		
Marchantia polymorpha **L.**	Cultured cell	TE, 3-DT,TY

Castasterone (CS), typhasterol (TY), dolicholide (DL), brassinolide (BL), teasterone (TE), 3-dehydroteasterone, secasterone (SE), dolichosterone (DS) [11]

brassinosteroids is also crucial to maintain the hormonal homeostasis in plants. Different phenotypic characteristics such as male sterility, curled leaves, and dwarfism have been reported due to the mutations in the genes related to brassinosteroid biosynthetic pathways. Brassinosteroid-mediated signal perception and signal transduction are as crucial as its biosynthesis for plant growth and development. Previous studies on mutants have revealed that application of exogenous brassinosteroid could rescue the mutants in brassinosteroid biosynthetic genes, while the signal-impaired mutants were insensitive to exogenous brassinosteroid application. Brassinosteroid signal transduction and transcriptional regulation have been extensively studied in *Arabidopsis* using mutants. LRR-RLK (leucine-rich repeat receptor protein kinase) and BRI1 (brassinosteroid-insensitive-1) are the primary cell surface receptors that first interact with brassinosteroids which leads to the interaction and transphosphorylation between BRI1 and BAK1 (BRI1-associated kinase1), a brassinosteroids co-receptor, leading to downstream signal transduction [12] (Fig. 1).

4 Brassinosteroid Signalling

Brassinosteroid-insensitive-1 (BRI1) constitute a leucine-rich repeat receptor kinase with extracellular domains that first perceive the brassinosteroids and trans-phosphorylates Somatic Embryogenesis Receptor Kinase (SERK), a co-receptor from smaller LLRK family. BRI1 and SERK forms an active complex which initiates

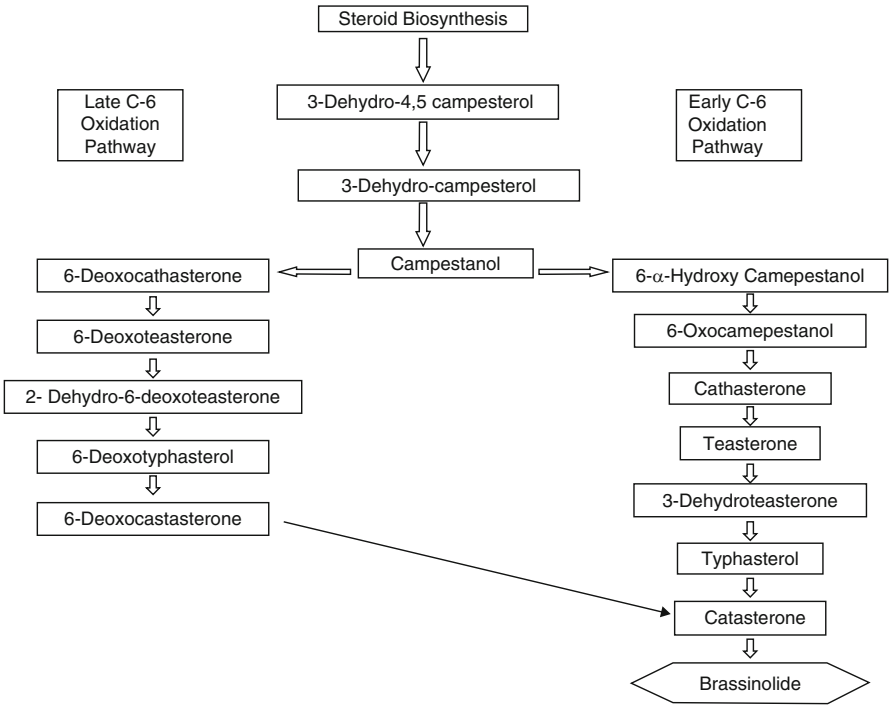

Fig. 1 Biosynthetic pathway of brassinosteroids

downstream signal cascade with the involvement of kinases and phosphatases finally resulting into activation of various transcription factors involved in regulation of specific gene expression [13]. A membrane-bound negative regulator, BKI1 (BRI1 INHIBITIOR KINASE 1), which binds to BRI1 and prevents co-receptors interaction in absence of brassinosteroids, has been well studied in *Arabidopsis thaliana* and rice. The interaction of brassinosteroids leads to the phosphorylation of BRI1 and is released from the membrane forming BRI1/SERK complex initiating downstream brassinosteroid signalling cascade [14]. A protein with similar function as BKI1 has been identified in rice which leads to the disruption of BRI1/SERK complex inhibiting downstream brassinosteroid signalling and is termed as OsREM4.1. It has been found that the gene expression for the same is regulated by ABA where OsREM4.1 is increased with elevated level of ABA involving bZIP transcription factor [15]. Another transcription factor brassinazole-resistant 1 (BZR1), one of the major TFs that regulate brassinosteroid-responsive genes in plant, has been reported to bind to the ABA-responsive promoter ABSCISIC ACID INSENSTIVE 5 (ABI5). Binding of BZR1 leads to the suppression of ABI5 expression resulting into reduced ABA response [16]. Several studies have been reported related to various alterations in physiological processes due to direct brassinosteroid and abscisic acid interactions [17]. Receptor kinases such as BIN2 (GSK3-like kinase) and receptor kinases of ERECTA family have been reported to

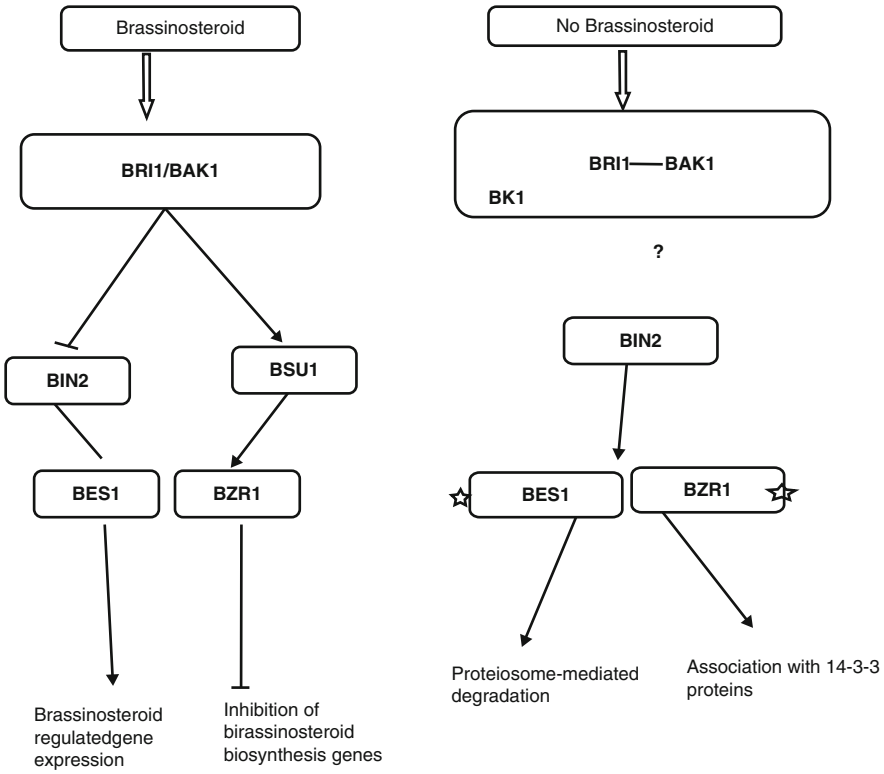

Fig. 2 Brassinosteroids in gene expression

be important components involved in brassinosteroid signalling resulting into inhibition of MAPK module and inhibiting stomatal development [18, 111] (Fig. 2).

5 Role of Brassinosteroids

5.1 Role in Cell Cycle

Brassinosteroids play an important role in the regulation of cell division and cell expansion as reported in numerous previous studies [19–21]. Earlier studies have shown that brassinosteroids cause the induction of various cell cycle-related genes which encode CDKs and cyclophilins involved in cell cycle regulation, cell proliferation, and differentiation [22]. Transcription factor such as BES1 (BRI1-EMS-SUPPRESSOR 1) and GSK kinase has been reported to control the erectness of leaves due to lower abaxial sclerenchyma proliferation [23]. In an another study under abiotic condition, lower CDK expression has been observed along with the higher expression of CDK inhibitors that downregulate their mitotic activity linking cell cycle progression to biotic and abiotic stresses [22, 24]. Under salinity stress

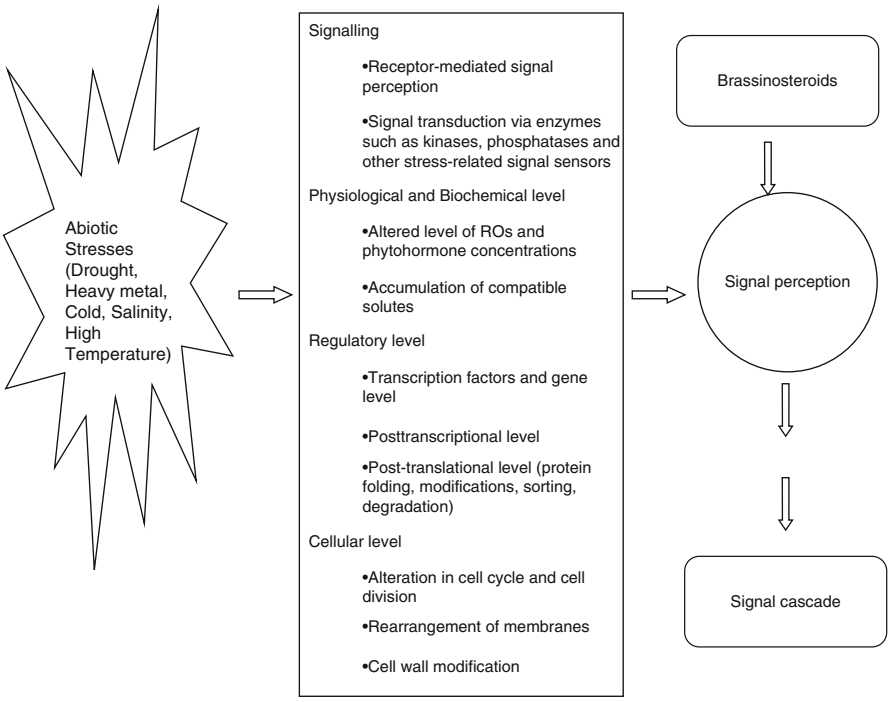

Fig. 3 Brassinosteroid-mediated abiotic stress response

brassinosteroid has shown to regulate the MYB transcription factor and hence regulating plant growth by reducing cell size [25]. Numbers of genes have been identified so far which are involved in plant growth and cell elongation and are directly targeted by BZR1 (brassinazole-resistant 1) [26]. Other brassinosteroid-regulated transcription factors affecting plant growth include BRAVO (brassinosteroids at vascular and organizing center), R2R3-MYB, and BES1 related to division of quiescent center cell (QC) present in roots and stem [21, 27, 28, 112] (Fig. 3).

5.2 Role in Cell Wall Architecture and Membrane Stability

Brassinosteroids provide first line of defense against the environmental stress by modifying and maintaining the cell wall architecture and preventing plant cell functionality from damages caused due to the fluctuating ionic strength under stresses [29, 30]. Expression profiling of various brassinosteroid-induced genes has been studied which included genes responsible for cell wall extension and loosening enzymes such as EXPs (expansins), XTHs (xyloglucan endo-transglucosylase/hydrolase), GLUs (endoglucanases), CESA (cellulase synthase A), and PLLs (pectin lyase-like) [31, 32]. In rice, MYB has been found to be

influenced on interaction with BES1 resulting into the expression of OsEXPs imparting adaptive growth under stresses [25]. BES1 through its downstream signalling regulates the primary and secondary growth in plants under various environmental stresses. Brassinosteroids have also been found to be involved in maintaining and formation of cell wall structure by participating in cellulose synthesis [33]. The role of brassinosteroids in cell differentiation and elongation has been reported by Yamagami et al. in their recent study using the Brz-insensitive-long hypocotyl4 mutant (bil4). The expression of BIL4 gene was observed under early cell elongation and was involved in cell elongation process throughout in *Arabidopsis*. BIL4 was found to interact with BRI1 receptor in endosomes and involved in the activation of brassinosteroid signalling. Increased localization of BRI1 was observed in vacuoles under BIL4 deficiency indicating its role in regulation of brassinosteroid signalling and in cell elongation [34].

5.3 Physiological and Biochemical Functions of Brassinosteroids

Abiotic stresses directly leads to the damage of the photosynthetic apparatus resulting into the imbalance in redox homeostasis and inhibition of PSII repair mechanism and photoinhibition [35]. Application of exogenous brassinosteroids has been reported to increase the photosynthetic efficiency of PSII alleviating photoinhibition under stress [36]. Increased activity of alternative oxidase (AOX) on brassinosteroids application results in decreased ROS accumulation by dissipating excess photosynthetic reductant and creating a balance between chloroplast-to-mitochondria electron transfer [37]. Various studies have been reported on the positive effect of brassinosteroids on enhancing intercellular CO_2 concentration, transpiration rate, stomatal conductance, chlorophyll content, and net photosynthetic rate under environmental stresses [38, 39]. Brassinosteroids have also been found to lower the chlorophyll catabolism under abiotic stresses by reducing the chlorophyllase activity [39]. In a study reported by Xia et al., the exogenous application of brassinosteroids resulted in enhanced activities of RuBisCO and other photosynthetic proteins in *Cucumis sativus*, while its inhibitor brassinazole downregulated the proteins [40]. Brassinosteroids have also been reported to recover the plants from cold stress by enhancing the enzymes of Calvin cycle as well as enzymes involved in antioxidative defense mechanisms inducing the recovery of photosynthetic apparatus as well [41].

5.4 Brassinosteroids and Phytochromes

Phytohormones play a crucial role in various processes associated with plant adaptations under changing environmental conditions [42]. Brassinosteroids on its interactions with other stress-related hormones are involved in plant's stress tolerance [43, 44]. Mutants for ABA, SA, JA, and ET were studied under heat and salinity stress in brassinosteroid-treated and brassinosteroid-untreated plant samples. Exogenous application of brassinosteroids showed salt stress tolerance in ABA-deficient

aba1–1 mutant compared to its wild type. NPR1, a key role player in SA-mediated SAR, was found to be crucial for BR-mediated salt and thermotolerance. Brassinosteroids were found to reduce the oxidative damage caused by oxidative stress by inducing ethylene biosynthesis [45]. These phytohormones, in association with ethylene, increased the H_2O_2 concentration and AOX level resulting in ROS scavenging and provide increased stress tolerance. Similarly, the interaction of brassinosteroids has also been studied with ABA, SA, and polyamines which showed higher stress tolerance in combination rather than the effect of individual hormones [39, 46]. A coordinated regulation of gene expression has been observed in brassinosteroids and abscisic acid, auxin, and jasmonic acid pathway. Enhanced stress tolerance has been observed in mutants for ABA biosynthesis under brassinosteroids application in different plant species [19, 47, 48]. An antagonistic relationship has been observed in between brassinosteroids and auxin in roots in order to maintain balanced cell division and differentiation [27]. Antagonistic association between brassinosteroids and GA has been reported in rice plants under submergence tolerance [49]. Brassinosteroids affect GA signalling by stabilizing its inhibitors such as DELLA protein and SLENDER RICE1 resulting in reduced shoot elongation related to enhanced submergence tolerance in rice. Brassinosteroids in association with cytokinin have also found to confer drought stress tolerance [50]. The genes regulated by these hormones are also regulated by polyamines as both have synergistic effect on conferring heavy metal stress tolerance in plant compared to their individual application. The synergistic effect of brassinosteroids with phytohormones leads to the improved seedling growth, regulation of stomata, embryogenesis, and other physiological functions in plants [46].

5.5 Role in Gene Regulation

Numbers of gene products are reported to be regulated by brassinosteroids such as genes related to redox metabolism (dehydrins, glutathione-S-transferase), cytoskeleton proteins (tubulin and actin), molecular chaperones (HSPs), and genes related to normal metabolism and hormonal biosynthesis [48, 50, 51]. Transcriptomic analysis of brassinosteroids has led to the identification and differential expression of numerous genes involved in abiotic stresses such as WRKY33, acid phosphatase5 (*ACP5*), a BR-responsive-receptor-like kinase (*BRRLK*), and Jacalin-related lectin1-3 (*JAC-LEC1-3*) [48]. Various genes such as *dwarf1* (D1), Taihu dwarf1 (*TUD1*), leaf and tiller angle increased controller (*LIC*), dwarf and low-tillering (DLT), and *CYP90D2*/*D2* involved in maintaining rice cellular architecture under stress have been identified and studied, which are also regulated by brassinosteroids under oxidative stresses [52–54]. In a contradictory study, it was observed that interruption of squalene synthase (*SQS*), which catalyzes the initial step of isoprenoid pathway for synthesis of sterol, enhanced the drought tolerance in rice by reducing the stomatal conductance indicating the role of reduced sterol and brassinosteroid in stress tolerance [55].

Brassinosteroids are reported to regulate various transcription factors involved in stress response through negative regulator like BIN2 and BAR1/BES1. They also

increase the expression of various transcription factors involved in abiotic stresses such as DREB, MYB/MYC, bZIP, and WRKY resulting in enhanced stress tolerance [56, 57]. Their role in inducing posttranslational modification of cold-responsive genes for freezing stress tolerance and acclimation to cold has also been reported in earlier studies [58]. Improved salt, cold, drought, and heat stress tolerance was observed in rice by overexpressing NAC TFs which are also regulated by brassinosteroids. These TFs lead to the drought stress adaptation by enhancing antioxidative enzymes and removing excess ROS, while cold stress tolerance is conferred by regulation of COR genes by TFs [59].

5.6 Role in Growth and Development

Brassinosteroids are well known for their growth-promoting activities and are involved in various physiological processes in a number of plants. In a recent study, various bioactive compounds were measured in carrot at five different growth stages along with the expression profiling of various genes involved in brassinosteroid biosynthesis and signalling pathways. Biosynthetic genes were highly expressed in first developmental stage in roots and petioles. Application of 24-EBL (24-epibrassinolide) resulted in changes in morphological parameters such as higher weight aboveground, increased number of petioles, and increased plant height. Genes involved in brassinosteroid signalling, viz., DcBRI1, DcBZR1, and DcBSU1, showed higher level of expression in petioles in 24-EBL-treated plants compared to the controls. Petiole elongations were also observed on exogenous 24-EBL treatment indicating brassinosteroids potential role in growth and development of carrot [60]. Brassinolide with GA3 has been reported to show higher growth rate of stem and petioles, while application of brassinolide alone only participated in petiole growth and not the stem. It was suggested that a possible positive relation between GA3 and BR1 may have led to the increased GA3 effect on growth rate of *Tabebuia alba* plant. Previous studies have shown that exogenous application of brassinosteroids or over-expressing the rate-limiting genes involved in brassinosteroid synthesis results in better quality and higher yield of various crops. However, extremely dwarf phenotypes of crops were observed under limited brassinosteroid synthesis or alteration in their signalling [61, 62]. A recent study was conducted in an attempt to understand the role of light in brassinosteroid biosynthesis and root growth. DWF4 is an important enzyme responsible for the biosynthesis of brassinosteroids in plants whose accumulation was enhanced in the presence of light. Increased root length was observed when shoots were exposed to the light for a considerable time indicating the role of aerial tissue in accumulation of DWF4 in root tip led to root growth [63].

5.7 Maintenance of Redox Potential

Generation and higher accumulation of ROS in plants is a common outcome under environmental stresses [64, 65]. It has been suggested that higher ROS accumulation

leads to cell death but their lower cellular level is crucial in stress signalling [66]. Exogenous application of brassinosteroids has been found to promote the optimal level of ROS accumulation and induce stress tolerance in plants [67]. H_2O_2 acts as a signalling molecule which is induced by brassinosteroids and also activates MAPK which further induces NADPH oxidase, thus self-propagating cellular H_2O_2 leading to the amplification of the signal. The higher accumulation and subsequent signalling upregulate various stress-related proteins such as dehydrins, HSPs, antioxidative enzymes, and TFs resulting in scavenging and suppression of ROS levels [68]. However, brassinosteroids do not have long-distance transport; thus systemic stress is induced by increasing the production of H_2O_2 [69]. A study has shown that brassinosteroid induced stress tolerance in plant by enhancing H_2O_2 accumulation and higher MAPK1/2. The activity of MAPK1/2 was altered in MPK1/2 and MPK2 silenced plants and not in MPK1 silenced plants showing more crucial role of MPK2 compared to MPK1 in brassinosteroid-mediated signalling [70]. Increased ABA biosynthesis has been observed due to the higher brassinosteroid-induced H_2O_2 cellular level, thus providing prolonged stress tolerance in plants. Various studies have been reported on BR-induced stress tolerance by enhancing various antioxidative enzyme activities and their gene expressions [71]. Another signalling molecule that affects the BR-induced signalling is NO; the inhibition of NO production results in lowered tolerance due to inhibition of antioxidative enzyme activities and their lowered gene expression due to brassinosteroids [72]. Alternative respiratory pathway is also inhibited by inhibiting enzymes nitrate reductase (NR) and nitric oxide synthase (NOS) by gene silencing. However, ROS-dependent NO production is induced by brassinosteroids via nitrite-dependent enzymatic reactions which in turn leads to the induction of expression of various antioxidative genes against stress [73]. The genes of ascorbate glutathione pathway have been found to be influenced by the concentration of brassinosteroids under stress resulting into higher GSH/GSSG ratio. This leads to the understanding of the role of brassinosteroids in maintaining stability of redox-sensitive proteins/enzymes leading to conclusion that brassinosteroids participate in the regulation of ascorbate-glutathione cycle [74].

5.8 Role in Abiotic Stress

Brassinosteroids play an important role in plant defense under abiotic stresses by modulating the activity of various antioxidative enzymes as well as nonenzymatic antioxidants. Exogenous application of BL to water-stressed maize seedling led to the increased level of enzymatic activity of superoxide dismutase, catalase, ascorbate peroxidase, and nonenzymatic antioxidants such as ascorbic acid and carotenoids [75]. Similar results showing higher antioxidative response were observed in rice seedlings under salinity stress when rice seedlings were treated with brassinosteroids. Among defense enzymes, catalase, superoxide dismutase, and glutathione reductase reported significantly higher activity, while APX showed the slightest increase in its activity [76]. However, the activity of catalase, peroxidase,

and ascorbic acid oxidase was reduced in sorghum under osmotic stress [77]. A comparison between a det2 mutant with blocked biosynthesis of brassinosteroid and wild-type *Arabidopsis,* revealed that mesophyll and epidermal cells had thicker cuticle as well as cell wall in mutants. Mutants also showed leaf structure more compact having lesser intercellular space and higher stomatal density compared to the wild-type plants. At lower O_2 concentration, mutants were insensitive to dwarfing effect under stress compared to wild type [61].

5.9 Drought Stress

Drought stress is one of the most deleterious of abiotic stresses. There are numbers of studies on various compounds including brassinosteroids that have been used to enhance the antioxidative properties of plants to combat drought stress. In a study, exogenous application of 24-epibrassinolide on leaves of tomato resulted into lowered lipid peroxidation and H_2O_2 concentration under applied drought stress. Increased antioxidative enzyme activity of peroxidase, superoxide dismutase, catalase, and ascorbate peroxidase was observed in brassinosteroid-treated plants. Higher concentration of carotenoids, ascorbate, and proline was also observed in drought-stressed plants pretreated with brassinosteroids [78]. Similar results have been reported in a study on Chinese medicinal plant *Salvia miltiorrhiza*. Application of brassinosteroid on drought-stressed *S. miltiorrhiza*-sensitive genotype resulted in significantly increased activity of antioxidative enzymes and increased proline content, while MDA content declined on the treatment [79]. Abscisic acid also plays a role in stress tolerance induced by brassinosteroids. Polyethylene glycol-mediated drought stress in maize has been studied to elucidate the role of ABA and brassinosteroid for stress tolerance. Brassinazole (a brassinosteroid inhibitor)-treated plants showed higher level of oxidative damage which was reduced on brassinosteroid and abscisic acid treatment. It has been reported that the treatment of brassinosteroids on stressed plants led to the higher expression of ABA-related genes enhancing its role in stress tolerance. Brassinosteroids are also found to induce NO generation in mesophyll cells in leaves, thus activating ABA biosynthesis, hence increasing drought stress tolerance in maize plants [72]. Brassinosteroids also play a role in maintaining cellular water content by increasing water permeability of aquaporins, membrane water channels. Mutant studies of *Arabidopsis* revealed that brassinosteroid treatment to BR-insensitive (BRI1) mutants resulted in osmotic permeability; however brassinosteroid does not affect plasma membrane directly [80].

5.10 Salinity Stress

Salinity stress decreases the overall plant growth by lowering the water absorption capacity which later leads to the toxic accumulation of ions. High salt stress also results in altered membrane function, change in metabolic/physiological processes, nutrient imbalances, and change in antioxidative status of plant. The role of

brassinosteroids in alleviating salinity stress has been reported in many crops. Increased electrolyte leakage under salinity stress was observed in *Lactuca sativa* variety; however it was lower in case of seedlings treated with 24-epibrassinolide. Foliar application of different concentrations of 24-epibrassinolide on *Lactuca* seedlings showed improved growth parameters such as increased root and shoot dry weight, higher fresh weight of root and shoot, as well as increased nutrient content in leaves and roots [81]. Salt-sensitive "Jinyou 1" and salt-tolerant "Changchun Mici" cucumber cultivars were studied for the effect of brassinosteroids in alleviating salt stress. Foliar application of 28-homobrassinolide improved the negative effects of salinity stress by enhancing antioxidative enzymes such as superoxide dismutase and peroxidase and by lowering MDA content. High biomass compared to the stress-treated plants was observed with increased fresh weight and dry weight of root and shoot as well. The enhanced chlorophyll content along with all the parameters tested indicated the role of 28-homobrassinolide in counteracting negative effects of high salt stress in cucumber varieties [82]. The expression of osmotic stress-related genes such as wall-associated kinase (WAK), plasma membrane intrinsic protein (PIP), and dehydration-responsive element binding (DREB) was determined under salt stress as well as under salinity stress and 28-homobrassinolide treatment in barley. Higher expression of DREB2, HvPIP, and DWARF was observed in salt-stressed plants treated with 28-homobrassinolide [83]. Reduced nutrient and water uptake by roots under high salt concentration resulted in the reduced root growth. Increased Na^+ along with other salts such as Mg^+, K^+, and Ca^+ ions lowered the assimilation and distribution of important mineral nutrients. Lower NO_3^- absorption is one of the major factors due to high salt concentration. Brassinosteroids are believed to increase NO_3^- uptake under stress by acting on membrane and by expressing related genes to alleviate high salt toxicity. Salinity stress lowers the germination percentage and overall biomass of a crop, while treating stressed plants with brassinolide showed higher germination rate, increased root and shoot length, as well as increased nitrate reductase activity compared to stressed plants [84].

5.11 High Temperature Stress

Higher temperature results into the increased rate of root elongation. The level of BRI1 receptor has been found to be influenced by increased growth temperature regulating downstream brassinosteroid signalling and thus root elongation. This study suggests the role of BRI1 receptor combines the effect of temperature and brassinosteroid signalling to enhance root growth under changing environmental conditions [85]. Treatment of heat-stressed *Ficus concinna* seedlings with 24-epibrassinolide led to the higher concentration of ascorbate and glutathione maintaining redox potential under heat stress. Increased activity of various enzymes involved in ROS scavenging, viz., superoxide dismutase, glutathione peroxidase, ascorbate peroxidase, monodehydroascorbate reductase, dehydroascorbate reductase, and glutathione reductase, was observed on 24-epibrassinolide treatment.

The lower accumulation of ROS and MDA content suggests the role of 24-epibrassinolide in maintaining redox homeostasis under heat stress [86]. Application of 24-epibrassinolide on heat-stressed maize plants has been studied to deduce its role in ameliorating heat stress. The treated as well as untreated plants were studied for their stress tolerance potential under high temperature stress. The 24-epibrassinolide-treated plants increased the stress tolerance by increasing the antioxidative response of plants which showed higher activity of peroxidase, superoxide dismutase, and catalase. The treated plants also reported with lower protein degradation under stress maintaining higher stability of cell membrane under stress [87]. Treatment of heat-stressed rice genotypes with 24-epibrassinolide has shown to alleviate the effect of heat stress by significantly lowering the H_2O_2 and MDA content. The elevated antioxidative response was indicated by increased total soluble sugar contents, carotenoid content, and chlorophyll content. Increased values for relative water content, fresh shoot weight, and leaf greenness were observed, while there was reduction in numbers of wilted leaves. The application of 24-epibrassinolide on heat-stressed plants was able to maintain higher level of CO_2 assimilation rate due to increased stomatal conductance [88]. In order to understand the molecular level of brassinosteroid action under heat stress, *Brassica napus* plants under heat stress were applied with 24-epibrassinolide. The results showed the significant increase in thermotolerance of *Brassica* plants as there was accumulation of major heat shock proteins in treated plants compared to the untreated ones due to higher hsp synthesis and increased levels of many initiation and elongation factors involved in translation [89].

5.12 Cold Stress

Brassinosteroids have been found to participate in freezing stress tolerance in plants. The brassinosteroid signalling mutant of *Arabidopsis* showed higher sensitivity under cold stress. Activation of brassinosteroid signalling led to the alleviation of cold stress by enhancing stress tolerance in the *Arabidopsis* mutants. These growth regulators are involved in regulation of transcription factor CESTA (CES), a basic helix loop helix, which then controls C-*repeat/dehydration-responsive element binding factor* further controlling cold-responsive genes. The basal resistance to cold stress is also governed at posttranslational level by modifying CES [58]. Exogenous treatment of brassinosteroids can help plant cope with cold stress only when plants are pretreated. The increased cold stress tolerance was also observed in BRI1 mutants of *Arabidopsis* which however showed defected brassinosteroid signalling and dwarfism. The plants overexpressing BRI1 showed higher degree of sensitivity toward the cold stress compared to the wild-type plants. Higher basal level expression of CBFs/DREB1s was observed in BRI1 mutant and plant overexpressing BRI1 [90]. There are numbers of studies demonstrating the role of brassinosteroids under chilling stress. Brassinosteroids have been reported to reduce the ion outflow in plants under chilling stress. In another study, 24-epiBL was found

to increase the antioxidative enzyme activities in chilling-stressed grapevine plants. Enzymes involved in plant defense mechanisms such as ascorbate peroxidase, catalase, and superoxide dismutase were upregulated in *Brassica juncea* plants on exogenous application of 24-epiBL under chilling stress and lowered the toxic effect of H_2O_2.. Application of 24-epiBL has been found to lower the cold stress by decreasing the accumulation of ROS in cucumber. Higher activities of antioxidative enzymes were observed resulting in the overall protection of photosynthetic machinery. Brassinosteroids have also been reported to have lowered the chilling effect by reducing the injury, controlling ion leakage, maintaining membrane integrity, increasing the level of osmoprotectants, and higher antioxidative enzyme in tomato plants [91].

5.13 Heavy Metal Stress

The effect of brassinosteroids on heavy metal toxicity such as copper, lead, zinc, and cadmium has been studied in various crop plants [39, 92, 93, 94]. 24-Epibrassinolide application reduced the lead content in beetroot by 50% compared to the metal-treated plants possibly due to the effect of hormone on absorption of metal [95]. The effect of brassinosteroids on metal accumulation depends on the developmental stages of the plant. Limited loss of chlorophyll, proteins, and sugars was observed in metal-exposed *C. vulgaris* on brassinosteroid treatment also improving the phytochelatins synthesis [96]. Various studies have been reported showing the effect of brassinosteroids under stress such as higher growth rate of mung bean under aluminum stress on application of brassinolide; EBL has been shown to increase root and shoot fresh weight under aluminum stress; exposure of Indian mustard to nickel stress in the presence of 28-homobrassinolide (HBL) showed improved root and shoot length and improved germination rate [97]. Brassinosteroids have also been found to prevent the damage caused in reaction centers of rape cotyledons as well as O_2 evolving complexes and maintained proper electron transport system under cadmium stress [98]. Reduced metal toxicity has been observed in various plants on application of HBL which influences the activities of various photosynthetic enzymes and stress-responsive enzymes [39]. Considerable reduction in chromium uptake by radish and rice seedlings was reported after the brassinosteroid treatment in chromium-stressed plants reducing chromium toxicity [99]. Effect of three heavy metals, viz., lead, cadmium, and copper, was studied in algae *Chlorella vulgaris*. Increased levels of antioxidative enzymes catalase, ascorbate peroxidase, and glutathione reductase were observed under metal toxicity along with the higher levels of glutathione, ascorbic acid, and carotenoids. The role of brassinosteroids in copper homeostasis under abiotic stress has been poorly understood. Exogenous application of brassinosteroids to *Raphanus sativus* under high Cu concentrations led to the higher Cu stress tolerance by enhanced expressions of polyamine biosynthetic genes as well as genes involved in abscisic acid (ABA) and indole-3-acetic acid (IAA) metabolism [46].

5.14 Role of Brassinosteroids in Biotic Stress

Interaction between brassinosteroids and other stress hormones, like jasmonates and salicylic acid, is important to build up defensive barriers necessary to cope with insects and microbes [100]. Application of brassinosteroid increased plant's tolerance against viruses, whereas GSK-3 inhibitor Bikinin by activating brassinosteroid signalling showed elevated susceptibility toward viral infection. Brassinosteroid-induced response was found to be influenced by the MEK2-SIPK cascade and BES1/BZR1. BES1/BZR1 led to the viral resistance by expressing various plant defensive genes, and inhibiting ROS accumulation also is an important mediator of brassinosteroid signalling participating in plant's immunity against pathogen [101]. The role of brassinosteroid is less studied in plants' defense mechanisms against biotic stress and disease resistance, but recent studies have been reported showing importance of these growth regulators in plants' defense against pathogen [102]. Brassinosteroids have been reported to play an important role in plants local and systemic innate immunity. Exogenous application of BL has been shown to enhance leaf pathogen disease resistance in case of rice and tobacco although the results were variable in each case [103]. Application of brassinosteroid in barley showed protection against *Fusarium* [104]. Despite the studies proving brassinosteroids' positive effect on biotic resistance, there are however various reports showing no significant effect of the same. In a study, the application of brassinosteroid on *Arabidopsis* did not show any alteration in plants resistance to the pathogens *Alternaria brassicicola* and *Pseudomonas syringae* pv. *tomato* (*Pto*) [105]. Some recent studies have also reported the negative effect of brassinosteroids increasing susceptibility of roots toward pathogens *Meloidogyne graminicola* and *Pythium graminicola* in rice. *Pythium graminicola* have been found to exploit brassinosteroids to enhance their virulence by seizing brassinosteroid signalling pathway in rice [102, 106]. Therefore, the role of brassinosteroids in plant-pathogen interaction indicates that they have important role in maintaining homeostasis under biotic stresses by influencing hormone signalling, production of secondary metabolites, pathogen-induced cell death and oxidative metabolism.

6 Application of Brassinosteroids

Recent advancement of our knowledge on brassinosteroid signalling and regulatory functions has led us to exploit their properties for practical application. New discoveries in this area will further expand their uses in agriculture and medicine. Application of pesticide in agriculture has led to the bioaccumulation of various harmful compounds. Toxicity to plants caused by pesticides manifests into necrosis, vein discoloration, and chlorosis which on prolonged use negatively affects the plant growth and development leading to reduced photosynthetic efficiency and hence altered plant metabolism. Brassinosteroids are relatively safer as they are neutral and hence nontoxic with an eco-friendly nature and therefore can be safely used in agriculture for crop improvement [107]. In recent studies brassinosteroids have

shown to have antimicrobial activities and immunomodulatory and neuroprotective activities. Various analogues of this hormone were reported to have antiviral activity against vesicular stomatitis virus, herpes simplex virus type 1, as well as arena viruses. Brassinosteroids and their analogues have also been found to inhibit cell growth in cancer cell lines; they are also considered as potential anticancer drug. They are believed to have tremendous use in therapeutics in the near future [108]. Brassinosteroids are also used in in vitro studies where they exert various effects on growth such as increased chlorophyll content, activities of antioxidant enzymes, shoot multiplication, flowering, etc. Hence they are also used for in vitro establishment of economically important crops such as groundnuts genotypes. They are reported to have a positive effect on physiological functions and growth enhancement of plants [109]. Exogenous application of brassinosteroids has shown growth-promoting functions. Their role in broad-spectrum physiological processes has been reported to enhance crop yield with better quality. Various field trials have been done for using brassinolide, 24-epibrassinolide, and 28-homobrassinolide to determine their effect on crop yield. Higher crop yield and quality were observed under 24-epibrassinolide treatment than the other two. Apart from increasing crop yield and quality, brassinosteroids also confer resistance against various environmental stresses such as high salinity, drought, or nutrient deprivation via various physiological processes [110]. It is reported that the external factors such as light and temperature in association with intracellular brassinosteroids result in regulation of gene expression. For instance, transcription factor BZR1, which is activated by brassinosteroids, and PIF4 (phytochrome-interacting factor), which is regulated by dark and heat, are responsible for regulation of various genes involved in alteration of plant metabolic processes under changing climatic conditions. Brassinosteroids are natural compounds that could be used in agriculture and medicines without any harmful effects. They do not coevolve with pathogen/pests and hence unlike other pesticides and agrochemicals can be in biocontrol of pathogen-borne diseases [59].

7 Conclusions

Brassinosteroids are considered to have versatile functions in plant's development. Abiotic and biotic stresses trigger complex responsive mechanisms in plants. Phytohormones play a great role in escaping or in survival of plants under environmental stresses by various means. The role of brassinosteroids is well studied under such stresses, but still the complete knowledge of this complex mechanism is unclear. Further studies are required to have deeper insight on the brassinosteroid signalling and their role at various regulatory levels during different stages of plants life. The dynamic of brassinosteroid homeostasis mainly depends on its synthesis, signalling, transportation, and degradation which still are not completely understood. Apart from holistic studies of brassinosteroid-mediated physiology, the interplay between brassinosteroids with other phytohormones also needs to be explored to further expand the scope of their commercial use in the future. However, with the available knowledge, analogues of brassinosteroids are being used to improve

agricultural output of various crops. Their wide use is also accounted for their nontoxic properties as they are naturally produced by plants and participate in specific gene expression. Further investigations on the role of brassinosteroids at various cellular and molecular levels would allow to modulate the brassinosteroid-mediated regulation under abiotic and biotic stress as well as to improve the quality of economically important crops. The bioactive components of brassinosteroid are not limited to agriculture but also make these natural steroids important in therapeutics and drug discovery.

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Saponins in Insect Pest Control

35

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Abstract

Insect herbivores are dangerous to all stages of plants, e.g., vegetative as well as reproductive growth, leaves, and shoots. Some of the herbivores feed by sucking plant sap, whereas some insects choose to chew various parts of plants. Thus, all types of herbivores damage plants by feeding directly and cause multiple diseases to plants, leading to plant damage indirectly. However, due to insect attack, plants produce some bioactive compounds (which are known as saponins) to improve their defense mechanism against herbivores. These saponins are further divided into two main categories, i.e., steroidal saponins and terpenoidal saponins. Here, we have highlighted the importance of saponins from multiple plant families against various herbivores. Saponins are present in different wild plants as well as cultivated crops (e.g., soybean, tea, spinach, oat, pepper, capsicum, quinoa, and allium). Some of the saponins play a role as antifeedant while some are insecticidal to different life stages of insect pests. Thus, these saponins play an important role in plant defense against different insect pests. Moreover, different saponins are effective against stored grain pests as well as cosmopolitan insect pests. Therefore, these plant bioactive compounds could be helpful for integrated pest management in different ecosystems.

Keywords

Antifeedant · Biological control · Herbivores · Plant bioactive compounds · Residual toxicity · Saponin purification · Steroidal saponins · Triterpenoid saponins

1 Introduction

The infestation of insects has a direct impact on organic food and stored grains as their damage cause up to 30% loss in production of staple while in case of severe infestation cause up to 90% loss in the agricultural production [1, 2]. The damage is caused by different kinds of insects, e.g., by sap-sucking, chewing, as well as boring into various plant parts of field crops [3]. Therefore, severe damage due to direct feeding and boring of stored product ruins the stored grains and thus accelerates the process of decay. The continuous use of chemical insecticides got control of insect pests worldwide [4–8]. These chemicals are non-biodegradable and highly toxic and have a negative impact on nontarget organisms, e.g., predators and parasites [9–11]. The constant use of these chemicals cause persistent resistance in insect pests and is responsible for resurgence and outbreak of new pests. Their chemical contamination is endangering the sustainable environment

and has adverse effects on arthropods, fauna and flora, amphibians, reptiles, fowls, microorganisms, and humans as well [12]. The indigenous use of persistent chemicals toward nontarget species of agricultural insect pests is a dangerous position and a serious issue of concern for scientists and researchers all over the world. The outcome of these chemicals is hazardous as they produce resistant strains in agricultural insect pests. The accumulation of toxic residues in food grains due to the excessive sprays in agricultural crops as well as in stored grain products cause severe health problems. Human consumption of these cause worldwide mortality due to presence of large amounts of pesticide. Those people who engage with different departments related to production, such as formulators, sprayers, mixers, loaders, and farm laborers, are frequently exposed to harmful chemicals [12]. A number of chemicals including organochlorines, organophosphates, carbamates, and organophthalides have been banned due to their hazardous risks toward nontarget organisms, environment, and human health [13].

The limitations of these chemicals due to their harmful toxic effects cause increased interest toward the use of botanical insecticides to control insect pests. These compounds consist of bioactive ingredients for pest management of field crops and stored products and are known to be safe for health and environment, economically approachable, biodegradable, and easy to use toward alternative pest management products [14]. The presence of secondary metabolites in plants has caused development of many ways to fight against insect pests. These compounds present in plants act as feeding deterrent for various insect pests and caused mortality [15, 16]. The use of these plant-derived compounds resulted in the development of natural biopesticides for a sustainable and healthy cultivation. Saponins extracted from plants are known to be steroidal or triterpenoidal compounds with a diverse range of bioactivities against insect pests (reviewed in [17–19]).

These are nonvolatile compounds consisting of an aglycone (or sapogenin) moiety attached with one, two, or three saccharide chains. They are surface-active compounds due to the presence of polar (sugar chains) and nonpolar (aglycone moiety) group (reviewed in [20]). They are known due to their commercial applications like wetting, emulsifying, and foaming properties (reviewed in [21–23]). These compounds are characterized as having antimicrobial, antioxidant, and insecticidal properties (reviewed in [24–29]). They have been used on security basis to minimize food grain damage in stored grain production as the presence of an active ingredient in saponins influences the stored grains' borers, weevils, beetles, and other microbes that cause infestation [30–32]. Likewise, the saponins extracted from legumes are used for the first time as insecticides against insect pests. Applebaum and colleagues [33] are the first biochemists who explained the nature of saponins as plant defense tools against different insect pests. The plant saponins are used broadly in integrated pest management (IPM) programs, due to their insecticidal activities. In this chapter, our interest is focused on the compilation of information available in the literature on plant-derived saponins and their reported insecticidal activities.

2 What Are Saponins

2.1 Structure and Properties

Saponins are the subdivision of glycosides and well-known due to their soap-like characteristics in the scientific literature. Most of the saponins are terpenoidal or steroidal due to the attachment of hydrophilic saccharide chains (aglycone). Due to carbon (C) skeleton of aglycone, these saponins are further divided into three categories (e.g., triterpenoid, steroidal, and steroidal glycoalkaloid) and have a ring system of 27 steroid carbons with aglycone as well as 30 terpenoidal carbons with sapogenin [34, 35]. Overall, a linear oligoside of two to five lengthy sugar units is the main part of saccharide chain of saponins, and these sugars include the dextrose units of plant carbohydrates, e.g., glucose, mannose, fructose, xylose, galactose, arabinose, rhamnose, and glucuronic acid [36, 37]. Therefore, based on such attachment of aglycone with polysaccharides, saponins are further divided into monodesmosides (due to the presence of single sugar chain at C-3) and bidesmosides (due to the presence of one sugar chain at C-3 and other sugars at C-22, C-26, C-27, or C-28). Moreover, zanhic acid glycoside is a triterpenoid saponin which is known as tridesmosidic due to the attachment of three different sugar chains to aglycone [38, 39]. Biologically monodesmosidic saponins are much effective as compared to bidesmosides or tridesmosides [40, 41], due to the presence of one sugar unit at C-3. A saponin becomes an amphipathic molecule due to strong bonding between a chain of water-soluble oligosaccharides and fat-soluble aglycone, and this amphipathic molecule easily interacts with the cell membrane to enter into the cell. After the saponin enters into a target cell, saponin produces a specific biological activity, e.g., antimicrobial, insecticidal, hemolysis, as well as allelopathic. Thus, a particular saponin molecule affects the living organisms (e.g., insects, animals, microbes) by disturbing their feeding, growth, and reproduction [2, 42, 43].

2.2 Types of Plant Saponins

Wild as well as cultivated plant species have a variety of saponin compounds [44–46], e.g., *Quillaja*, legumes, alfalfa, asparagus, ginseng, oats, and sugar beet [47, 48]. Likewise, different plant parts have various quantities and types of saponins [49, 50]. In the same plant, e.g., a leaf has more types and quantities of saponins as compared to shoots or flowers. Moreover, the types of saponin molecules also depend upon the age of plants as well as plant parts [51–53]. However, the contents of saponin molecules have a huge variation in the quantity and type due to the fluctuation of environmental factors [34, 54]. Functionally, some steroidal saponins become lethal to a lot of harmful soil microbes, because such saponins are abundantly present in the plant roots [44]. Various cultivated crops are supplemented with multiple saponins, which are responsible for plant health [34, 55]. Likewise, triterpenoid saponins are the main part of the defense system of several agricultural

crops, e.g., *Spinacia oleracea*, *Beta vulgaris*, *Glycyrrhiza glabra*, *Helianthus annuus*, *Aesculus hippocastanum*, *Quillaja saponaria*, *Smilax ornata*, and *Camellia sinensis* [20, 34, 56–58]. On the other hand, various steroidal glycosides are also a prominent part of many plants, which are helpful in the plant immune system, e.g., *Allium* spp., *Asparagus officinalis*, *Avena sativa*, *Capsicum* spp., *Dioscorea* spp., *Panax ginseng*, *Solanum melongena*, *S. lycopersicum*, as well as *Trigonella foenum-graecum* [59–63]. Solanaceous plants (like potato and tomato) are also well-enriched with multiple steroidal glycoalkaloids, which play a significant role in the plant defense against herbivores and microbes [34]. Overall, commercially soapbark tree and *Yucca* plant are well-known source of triterpenoid and steroidal saponins, respectively [25, 64].

2.3 Extraction and Purification of Saponins

A plant chemical blend is composed of multiple saponins, which are categorized based on different structures of various identical compounds. Therefore, saponins are categorized into different molecules based on analytical chemistry, chemical characteristics, and biological activities [65–68]. These saponins are isolated through different water-alcohol solvents, although ethanol is considered better than methanol. It is also reported that the combination of both ethanol and methanol with water is more attractive solvent for saponin isolation as compared to the use of individual solvents [69]. It is also documented that the methanol solvent works properly at room temperature, as compared to ethanol that needs a higher temperature for saponin isolation [70, 71]. Due to the high demand of saponins, scientists have improved the isolation method to get maximum yield of plant saponins, e.g., utilization of supercritical CO_2 ($scCO_2$) as a solvent [72]. Saponins are extracted and separated from various parts of plants through different technologies, like microwave- [73, 74] and ultrasonic-assisted extraction [75]. Similarly, saponin quantification is being conducted through spectrophotometric and chromatographic techniques [47]. Spectrophotometry is much feasible for estimation of plant saponin, whereas separation and quantification of a specific saponin molecule are analyzed by chromatography. Moreover, thin-layer (TL) [76] and high-performance liquid chromatography (HPLC) [77–79] were used for saponin purification consistently, but HPLC lacks some prominent ultraviolet chromophores (reviewed in [80]). Therefore, mass spectrometry (MS) and *p*-anisaldehyde-H_2SO_4 were coupled with HPLC and TL systems to improve the estimation of saponin molecules from plant extracts, respectively [81, 82]. For detailed structural studies of saponins, nuclear magnetic resonance (NMR) and Fourier transform infrared (FTIR) spectroscopy are being used. Structural bonding of saponins with sugar unites is determined via NMR, whereas functional grouping and stereochemistry are figured out by FTIR spectrum [83–85]. Thus, the development of such techniques is much helpful for the saponin isolation in the commercial industry, e.g., isolations of saponins from alfalfa, garlic, ginseng, licorice, *Agave attenuata*, *Cestrum parqui*, *Q. saponaria*, and *Saponaria officinalis* (reviewed in [2, 86]).

3 Control of Insect Pests with Plant Saponins

3.1 Mode of Action of Saponins

Insect development as well as reproduction are affected by multiple saponins directly because these bioactive compounds repel the insect herbivores from target host plants. However, if insect pests feed on such defensive host plants, then such herbivores lose their further feeding and movement, which leads them toward lethargy and resultantly into mortality due to high toxicity of saponins [87, 88]. These saponin molecules indirectly affect friendly microbiota within the digestive system of insect pests; also these molecules indirectly affect the insect pest by making different bonding with multiple digestive enzymes. Thus, due to strong binding with specific enzymes, saponins damage the mucous lining of several cells in the digestive system. Similarly, these saponin molecules bind with a complex of cholesterol and cause cellular toxicity; therefore, this complex of saponins and different enzymes results into the insects' ecdysial failure [89], because insects need various ecdysteroids for ecdysis which are least available in the insect body due to improper synthesis of such steroids [90, 91]. So, most of the herbivores avoid feeding on saponin-enriched plants, as we explained here that saponins influence the insect life badly. Thus, the empowerment of the IPM program with different saponins is much effective in the management of various insect pests in different environments (Table 1).

4 Plant Families with Saponins Against Insects

A lot of research work has been conducted to explore the activities of various plant saponins which are insecticidal to different insect pests, e.g., sap-sucking as well as chewing. Saponins from various plant families have an insecticidal effect against insect pests, e.g., *Allium* and *Aster* plants [107].

4.1 Aquifoliaceae

These plants exist in tropics and are often eaten by animals and birds and enriched by a lot of saponins [108]. Various plants of this family contain several secondary metabolites, which play a vital role as antimicrobials, antioxidant, as well as cytotoxic agents [109]. Likewise, it is proved that the plant extracts of *Ilex* genus have lethal impacts against fall armyworm, apple snail, as well as fruit fly [110–112]. The contents of saponin molecules are higher in non-ripe holly fruits (2.43% of dry weight) as compared to red ripe (0.5%) fruits, as the biological activities (e.g., survival and development) of fall armyworms were severely affected on the artificial diet of non-ripe holly fruits.

4.2 Araliaceae

This family covers more than 50 genera and 1,400 species of flowering plants, including perennial herbs, trees, vines, and succulents. The family has large, usually

Table 1 Insecticidal saponins from various plants

Plant species	Source	Effect	Reference
Alfalfa cultivars	Leaf, shoot, root	Insecticidal to pea aphids	[92]
Alfalfa lines		Insecticidal to pea aphid	[93]
Alfalfa plants		Deterrence to pea aphid	[94]
Alfalfa plants	Leaf	Insecticidal to European corn borer	[95]
Berberis vulgaris		Antifeeding to DBM	[96]
B. vulgaris	Leaves	Antifeeding to DBM	[97]
Catunaregam spinosa	Stem bark	Antifeedant to DBM	[98]
C. spinosa		Antifeeding to DBM	[98]
Clematis graveolens	Roots, rhizomes	Insecticidal to aphid, termites	[99]
C. graveolens	Roots, rhizomes	Insecticidal to termite	[99]
Fagaropsis angolensis		Larvicidal to mosquitoes	[100]
Panax notoginseng	Leaves	Antifeedant, anti-oviposition	[101]
Pieris rapae	Leaves	Antifeedant	[102]
Pisum sativum	Flower, seeds	Insecticidal to rice weevil	[89]
Quillaja saponaria	Plant	Insecticidal to aphids	[90]
Quillaja spp.	Bark	Insecticidal to cotton leafworm	[103]
Solanum laxum		Aphid repellent	[104]
Thevetia neriifolia	Leaf extracts	Insecticidal to cotton leafworm	[105]
Trigonella foenum-graecum	Leaves, seeds	Insecticidal to red flour beetle and bean weevil	[106]

alternate leaves, five-petaled flowers arranged in clusters, and berries [113]. Most of the plants are being used as an alternative medicine for several remedies (e.g., cough, arthralgia, fractures, anti-inflammatory, antiallergic) [114, 115], because they are well-enriched with various saponins [116–118].

Previous studies explained the importance of the family members in insect control. For example, Park et al. [119] evaluated the antifeedant and larvicidal activity of total plant extracts from this family against lepidopteran and hymenopteran larvae. Total ginsenosides exhibited increased antifeedant effects against larval and adult stages of insect pests [59, 120] and decreased the oviposition of *Pieris rapae* [102]. The highest nonselective and selective antifeedant activity was observed at a higher concentration where ginsenosides caused antifeedant percentages of 86.09 and 88.90, respectively. The total ginsenosides showed a significantly oviposition-deterring activity of 77.78% against oviposition of different insect pests at low concentration [102, 121]. They further explained that the total ginsenosides had antifeeding activity against *P. rapae* and inhibitory effects on its oviposition. It is assumed that ginsenosides could be used as an agent to prepare botanical new pesticidal formulations.

Static adsorption and desorption experiments were carried out to screen an optimal one from four types of macroporous adsorption resins for the purification of saponin from edible stems of *Aralia continentalis*. The AB-8-type macroporous

resin was shown to be an optimum candidate for saponin purification. Subsequently, dynamic adsorption and desorption experiments were performed to optimize technological conditions for saponin purification using AB-8-type macroporous resin. Results showed that the optimal adsorption conditions were obtained as follows: saponin concentration 0.2 mg mL^{-1} and sample injection amount 20-fold bed volume (BV). Two highly pure saponin fractions having stable superoxide anion radical scavenging capacity and antibacterial potential were obtained from gradient elution with 50% and 70% ethanol, respectively (Purification of Saponin from Edible Stems of *Aralia continentalis* Using Macroporous Adsorption Resin [122]).

4.3 Asparagaceae

The asparagus (Asparagaceae) consists of about 153 genera and some 2,500 species of flowering plants; it is vastly diverse and distributed worldwide. The family members are combined primarily by evolutionary relationships and genetic rather than morphological resemblances. Most of the plants from asparagus family contained various types of saponins, e.g., steroidal saponins and furostanol saponin [123–125]. The extracts from *Agave sisalana* increased the production of nitric oxide and caused cell death in *Aedes aegypti* hemocytes [126]. *Asparagus filicinus* is composed of six steroidal saponins and one ecdysone determined by using reliable high-performance liquid chromatography together with evaporative light scattering detection [127]. Overall, the industry of tequila wasted almost 7.41 million tons of leaves of *Agave tequilana* Weber as a by-product annually, which is used to control major agro-industrial insect pests. The leaves extracts of *A. tequilana* consist of hexane and ethyl acetate that have nematicidal action for *Panagrellus redivivus* and also act as insecticides against *Bemisia tabaci*. The different concentrations of hexane are used to kill the whiteflies as 4% dilution killed 100% and the check Herald®, a pyrethroid, at a dilution of 0.8% killed 100%, whereas undiluted hexane extract killed 31% of whiteflies. The leaves extracts of *A. tequilana* are used as a biological alternative to control the continuous use of insecticide, carbamate, and organophosphate that caused the resistance of pest, pollution to environment, and many health problems [128].

4.4 Asteraceae

Most members of Asteraceae are herbaceous, but some are also shrubs, vines, or trees in polar regions as well as the tropics. It is vastly diverse in the arid and semiarid areas of subtropical and lower temperate latitudes [129]. The Asteraceae may represent as much as 10% of autochthonous flora in many regions of the world. The flower extract of *Helianthus annuus* caused up to 70% mortality of house flies within 2 days [130], which shows that this extract is well-enriched with multiple bioactive compounds. The leaf extracts from *Chromolaena odorata* (siam weed) and *Khaya senegalensis* (mahogany tree) performed toxically and caused mortality of

rice weevil [131]. The toxicity of *C. odorata* assessed against rice weevil, and it acted moderately toxic, while *K. senegalensis* showed to be extremely toxic. The fecundity of rice weevil inhibited due to the high saponin contents from *K. senegalensis* leaf extracts. The mortality of *Periplaneta americana* was higher at the optimum temperature (27 °C) by *C. odorata* extracts that used as insecticides for cockroach [132]. The *P. americana* was treated with leaf extracts (*C. odorata*) at low concentration of the juice, and a short period of exposure (6 h) caused mortality. However, the maximum mortality rate was observed after exposure at a high concentration of leaf extract. The level of confidence for survivals and mortality rate is highly significant at 0.001%. The extraction of saponins using phytochemical analysis isolated alkaloids, flavonoids, saponin, and tannin present in the plant species. The results showed that leaf extracts from *C. odorata* presented some measures of efficacy in the control of *P. americana*.

4.5 Brassicaceae

Brassicaceae is a medium-sized and economically important family of flowering plants, consisting of mustards, crucifers, as well as cabbage [133]. Overall, potent feeding of flea beetle (*Phyllotreta nemorum*) on the cruciferous plants, especially *Berberis vulgaris*, does not influence by the presence of saponin compounds. However, two triterpenoid saponins (hederagenin cellobioside and oleanolic acid cellobioside) caused resistance in the cruciferous plants against flea beetle [134]. The saponins in *Phyllotreta nemorum* produced resistance against *Barbarea vulgaris* [135]. The comparison between hederagenin cellobioside and oleanolic acid cellobioside revealed that hederagenin cellobioside acts as a defensive compound with intense feeding deterrent activity. The chemical composition and structural formula of the saccharide chain and aglycone influence insecticidal activity of saponins. The chemical composition of hederagenin and oleanolic acid contains aglycone, and the removal of carbohydrate moiety from aglycone affects their functions of bioactivity. This pest damages the crops by egg-laying and feeding and having a long range of host plants of Brassicaceae family like broccoli, cauliflower, cabbage, mustard, radish, and turnip. Two types of saponins (glucosinolates and isothiocyanates) are produced by cultivated plants of the Brassicaceae family. This pest quickly recognized the presence of these compounds in the host plants [136]. The survival and infestation level of DBM on cultivated and wild Brassicaceae host plants suggests that availability of wild plants like *Ba. vulgaris* nearby the field reduced the populations [137]. The infestation of DBM controlled by growing trapping plants (*Be. vulgaris*) in or around the cruciferous plants is an eco-friendly approach. Isolation of two kinds of triterpenoid saponins, i.e., oleanolic acid cellobioside and hederagenin cellobioside, from *Be. vulgaris* considered as feeding deterrents, which inhibited the growth of diamondback moth (DBM) (*Plutella xylostella* L.) and caused mortality [96, 97].

Two types of saponin P-type and G-type extracted from *Ba. vulgaris* plants during the growing season caused feeding deterrent toward larvae of DBM [96].

The triterpenoid saponin (3-0-b-cellobiosyloleanolic acid) is reported resistant to G-type plants against DBM. The leaves extracts of *Ba. vulgaris* contained monodesmosidic triterpenoid saponin (3-0-b-cellobiosylhederagenin) acted as feeding deterrent toward this pest [97]. The *Ba. vulgaris* extracts applied to cabbage leaf disks are used as insecticides toward larvae of DMB. This study reported that the active ingredient triterpenoid saponin found in *Ba. vulgaris* inhibits the production and damage of DBM. Two types of saponins contain G-type (3-0-bcellobiosyloleanolic acid and 3-0-b-cellobiosylhederagenin), resistant to DBM, and P-type, not resistant to DBM, used as a "dead-end" trap crop and control, respectively [138]. The results showed that the efficiency of sulfur fertilization increased in G-type *Ba. vulgaris*, as a trap crop for DBM. The younger leaves of *Ba. vulgaris* have higher concentrations of saponins than in older ones. The maximum infestation of DBM is observed among *B. vulgaris* on younger leaves of different size within the same plant. Hence, leaves of *Ba. vulgaris* contained saponins and have potential ability to act as dead-end trap crops for DBM [46]. Some plants in the genus *Barbarea* (Brassicaceae) contain *Ba. rupicola*, *Ba. vulgaris*, and *Ba. verna* consisting of different levels of saponins that act as feeding deterrents for this pest and prevent their survival on the plant. This study shows that *Barbarea* leaves have a high content of saponins not only valuable for the plant but also attracting DBM. The P-type species (*Ba. vulgaris*, *Ba. verna*, and *Ba. rupicola*) have low saponin content that allowed the survival of DBM larvae and caused resistance [139].

4.6 Fabaceae

The Fabaceae is commonly known as the legumes, which covers about 670 genera and 20,000 species, including trees, shrubs, and annual or perennial herbaceous plants [140, 141]. The leaves extracts from alfalfa (*Medicago sativa*) contained a high content of steroidal or triterpenoid saponin that is most effective against the development of aphid. The concentrations of saponin are different in different cultivars, and thus, a total of six cultivars of alfalfa was compared according to the content of saponin and insecticidal effects against aphids [92]. Alfalfa cultivars with high triterpenoid saponin content were found more resistant to pea aphids as they acted as an antifeedant compound by reducing phloem sap ingestion and aphid's performance activities. The feeding behavior of pea aphids is affected by a high content of antifeedant compound saponins, extracted from alfalfa cultivars acting as feeding deterrent by decreasing the ingestion of phloem sap and reduced the growth of aphids. The role of pea aphid fed on high-level saponin from alfalfa cultivars affected aphid's performance activities and caused a reduction in growth, survival, and reproduction rate together with disturbances in colonies growth. In laboratory conditions, the gel combined with saponins showed a significant reduction in number of aphid probes as compared to the control gels (without saponin). Another research found two saponins (zanhic acid tridesmoside and 3-GlcA-28-AraRhaXyl-medicagenic acid glycoside) from alfalfa, which had shown insecticidal effects against aphids [93]. Similarly, extraction of three saponins (zanhic acid tridesmoside,

3-GlcA-28-AraRhaXyl-medicagenic acid glycoside, and 3GlcA-28-AraRha-medicagenic acid glycoside) from alfalfa caused reduction of phloem sap ingestion and pea aphid's performance activities [142]. A mixture of an artificial diet contains these three alfalfa saponins at a concentration of 100 ppm which had directly affected sap ingestion by pea aphids and possessed repellent or deterrent activity. Application of different concentrations of saponins showed mortality on aphid nymphs. The first-instar nymphs of pea aphid after 2 days of feeding on artificial diet, containing more than 0.3% concentration of saponins (from soapbark tree), got aphicidal activity by these saponins. The nymph stages of pea aphid continued smaller as compared to control (without saponins diet), long-lasting and development of adults become restricted by feeding on 0.2% saponin diet caused 70% mortality. The concentration of saponins decreased at 0.1% had no toxic effects against aphid's nymphs [103]. The performance of pea aphids affected by high saponin contents, caused resistance in growth and development of pea aphid. Therefore, pea aphids showed infestation on alfalfa plants containing low contents of saponins [143]. High level of apigenin glycosides in alfalfa plants negatively affects phloem sap ingestion and abundance of pea aphid [41]. The alfalfa plants had high contents of apigenin glycosides which showed inverse effects on phloem sap ingestion and abundance of aphid [41]. The qualitative and quantitative variations of saponins extracted from the foliar tissues of four alfalfa cultivars affect infestation and development of pea aphid [94]. The increased level of saponins in the foliage of alfalfa plants was observed in infested plants by aphid as compared to un-infested plants. The study resulted in a direct relationship among high saponin level of alfalfa plants stimulate defense mechanism that causes feeding deterrent and toxicity to pea aphids. *Q. saponaria* includes steroidal saponins (digitonin and diosgenin) and triterpene saponins (aescin), responsible for feeding deterrents and aphicidal activities against aphids [90]. Rearing of pea aphid [with *Q. saponaria* saponins (3.0 mg mL^{-1}) and aescin (10 mg mL^{-1})] on a synthetic diet strongly affected their survival and caused 100% mortality. The feeding effects of high saponin-rich diet on pea aphid caused raptured epithelial cells of midgut and collapse with no defined cellular structures (nucleus or a plasma membrane) under microscopic examinations. The feeding behavior of aphids indicated that they prefer untreated diet (without saponins) as compared to treated diet (with saponins) that showed deterrent effect of saponins toward aphid. The deterrent effect of saponins suggests triterpene saponins could control pea aphid's infestation. The feeding behavior of aphids controlled by synergistic effects of saponins and apigenin glycosides. Pea aphids exposed to a mixture of saponins (zanhic acid tridesmoside and 3GlcA, 28AraRhaXyl medicagenic acid glycoside) with apigenin glycosides on agarose-sucrose gels showed a fewer number of aphid probes, elongation of passive ingestion, and less salivation into the gels [144]. The isolation and extraction of a toxic saponoside, named Albodorine, done by distilled water or hot ethanol purified with comprising *n*-butanol partition, and precipitated by using acetone-diethyl ether (50/50), Sephadex LH-20 gel chromatography and silica gel chromatography respectively. These techniques were directed by the toxicity tests using mice and homogeneity tests with thin-layer chromatography (TLC). The properties of Albodorine are

as follows: bitter taste, soluble in water or organic solvents, and thermostable. Its acidic hydroxylation produced rhamnose, glucose, and arabinose. Different verification tests were done by using warm- and cold-blooded animals to show its toxicological properties. In mouse, when intraperitoneally administered, it caused acute intoxication mainly presented as hyperpnea, ataxia, and terminal seizures before the animal died. Its LD_{50} was about 9.0 mg kg^{-1} of mouse body weight by intraperitoneal route. In different organs, it caused histopathological lesions characterized by vascular congestions and important hemorrhage in the liver, lungs, and kidneys. In vitro, it reduced the heart rate and force of contraction of isolated rat atria. It had hemolytic activity. Albodorine exhibited toxicological properties that could be exploited under certain conditions for the control of harmful organisms. The role of saponins caused natural feeding deterrent for aphid infestation by decreasing growth and feeding behavior of aphids. The saponins extracted from alfalfa have toxic effects against potato aphids (*Aulacorthum solani* Kaltenbach.) and suppressed the feeding process, survival rate, growth, and fecundity [145]. Extraction of saponins from alfalfa plant showed that it contained soya saponin I and medicoside A that caused reduction in sizes, affected growth and development of nymph, and inhibited fecundity accompanied by mortality of potato aphids (*Macrosiphum euphorbiae*). The sugar beet saponins are found more toxic in its effects on potato aphids. A spirostanic saponin (luciamin) from *Solanum laxum* showed strong feeding deterrent effect acting as repellent for wheat aphids *Schizaphis graminum*. The aphid feeds on an artificial diet with luciamin having toxic effects, which constantly decreased their survival. The first luciamin spirostane saponin is known to have insecticidal properties [104]. This family embeds several flowering plants, including *Yucca* and alfalfa plants, and the isolation of saponins and crude saponin used commercially as insecticides. Leafhoppers (Cicadellidae) on an artificial diet of *Yucca* saponin (1%) or alfalfa crude saponin (5%) died within 3 days. The different concentrations of saponin had different effects on the survival time of each pest as feeding (0.1%) *Yucca* saponin caused survival for 10 days, while a lower concentration (0.01%) had no effect on mortality of leafhoppers. It was observed that leaf hoppers were reluctant to eat food containing saponins, and therefore, these plant-derived metabolites are useful in controlling leafhoppers in agricultural crops [146].

The defensive role of saponins extracted from stored legume seeds of the soybean (*Glycine max* L.) was observed against larvae of bruchid beetles (*Callosobruchus chinensis* L.). Saponins are promising replacements to synthetic insecticides for protection of seed grains during storage [147]. These saponins are the important botanical compounds behind the resistance of legumes to different insect pests [33]. The lucerne saponins inhibit the growth activity of red flour beetle (*Tribolium castaneum*), a well-known pest of stored grains. The growth of beetle affected by feeding a high level of cholesterol diet, like saponins [148]. The saponin isolated from alfalfa plant prevents the growth of red flour beetle [9]. Saponins present in *Trigonella foenum-graecum* (fenugreek) seeds and leaves have insecticidal properties against red flour beetle, producing toxicity to young larvae, reducing fecundity of adults, and decreasing population causing mortality [106]. The potato leaves with

the different dilution of alfalfa saponins significantly affect growth and survival of Colorado potato beetle (*Leptinotarsa decemlineata*). Larvae of Colorado potato beetle died after 4–6 days due to non-preference of leaves treated with 0.5% alfalfa saponins. Treatments with lower concentration (0.01–0.001%) significantly inhibit the growth due to a low feeding rate. The integration of saponin-containing food (potato leaves) consumed by the beetle, caused a reduction in feeding affecting growth and survival rate [149]. The isolated saponins from three alfalfa (e.g., *M. arabica*, *M. hybrida*, and *M. murex*) species act as an insecticide against larvae of Colorado beetle. Less food intake, reduction in body weight, long-lasting larval stage, and the high mortality rate were observed by using 0.5% alfalfa saponins [150]. The alfalfa saponin from roots showed insecticidal activity against Colorado potato beetle. The progeny of second-instar larvae decreased from 51 to 24 per female, and adult emergence rate dropped from 80% (control) to 20% population after using an artificial diet with saponins (750 ppm) for 1 week. The effect of feeding alfalfa saponins showed high mortality, low hatchability, and reduced fecundity in beetle adults [151].

Fenugreek saponins presented insecticidal activity against bean weevil (*Acanthoscelides obtectus*). Topical applications of seed (6 mg per insect) and leaf (30 mg per insect) extracts caused mortality and reduce the fecundity of bean weevil (Bruchinae) within 2 days. A powder of fenugreek leaves (enriched with diosgenin (steroidal saponin)) was mixed in the stored grains of *Phaseolus vulgaris*, and it was observed that there was considerable mortality of cowpea weevil in powder treated peas as compared to untreated cowpeas. This powder also inhibited the larval growth of weevil as well as adult emergence [106]. This insecticidal property of diosgenin was further proved via different concentrations (100%, 10%, and 1%) of root extracts, and all concentrations presented the same trend of insect growth inhibition and mortality [145]. Similarly, triterpenoid saponins are the main chemical part of *Pisum sativum* and *Glycine max* which proved to be antifeedant against *Sitophilus oryzae* (rice weevil), and the prominent saponin molecule was dehydro-soyasaponin I that was responsible for the mortality of rice weevil [89]. Moreover, rice weevil also faced mortality by a seed flour of *Medicago truncatula* which is well-enriched with a saponin molecule (3-GlcA-28-AraRhaXyl-medicagenate) [9]. Triterpene aglycones were proved to be insecticidal by inhibiting the growth of rice weevil because these aglycone molecules affect badly the digestive system and enzymatic activities of rice weevil [87]. Likewise, soyasapogenol A, soyasapogenol B, and hederagenin showed moderate activities. The feeding of cotton leafworm on a semi-artificial diet with alfalfa saponins results in prolonged larval and pupal stages, retardation of growth, increased mortality of larvae and pupae, and decreased fecundity. Moreover, these saponin molecules also mainly affect the digestive system as well as synthesis of specific enzymes and precursors (responsible for ecdysis). Similarly, aglycone molecules (e.g., soyasapogenol A and B and hederagenin) (from various alfalfa cultivars) present insecticidal activities against cotton leafworms. The crops including corn, wheat, pepper, and different weed species are damaged or attacked by the economically important pest European corn worm or corn borer (*Ostrinia nubilalis* Hubner.) [95]. The alfalfa plants contain

natural saponins and do not get infected by pest due to the toxic and inhibitory effect of saponins. The alfalfa saponins extracted from the leaf, shoot, and root tissue have adverse impact on growth and development of the European corn worm [95]. A dried leaf tissue contained up to 0.5% concentration of saponins, and 1.6 mg g^{-1} quantity of saponins caused larval mortality. The growth and development of insect were adversely affected by using root and shoot saponins in an artificial diet having weight of 10 mg g^{-1} fresh saponins. Root saponins appeared more effective than shoot saponins in inhibiting the growth of European corn borer when incorporated at the equal concentrations. Alfalfa saponins extracted from roots have glycosides of medicagenic acid that showed high bioactivity incorporated with ground and non-ripe green [110].

4.7 Passifloraceae

It consists of flowering plants, including about 750 species categorized in almost 27 genera, including lianas, shrubs, trees, and climbing plants in tropical regions [152, 153]. The name of this family originated from edible passion fruit (*Passiflora edulis*); the passionflower genus (*Passiflora*) takes its name along with garden plants, consisting of running pop and maypop. The *P. alata* (winged-stem passion-flower) was found to have saponins which caused reduction in the population of armyworm during different developmental stages [154]. Hence it is proved that the saponins present lethal and sublethal effects, and around 70% deformation of the structure was observed. The high population of deformed insects (treated with saponins) was due to the high concentration of saponins, demonstrating the potential effects of passionflower saponins, isolated from winged stem, which was being acting as a controlling agent for fall armyworm. The plants of *Allium* cultivar (contain onion, garlic, and leek) infested with *Acrolepiopsis assectella*, known as onion leaf miner or leek moth. The damage caused by feeding onion bulbs and within the tissue of leaves via mining [155].

4.8 Quillajaceae

It consists of one genus *Quillaja* along with two or three known species [156]. Soapbark tree (*Quillaja saponaria*) saponins restrain feeding and disturb the development of several insect pests and produced a toxic effect to larvae of beetle and nymph of aphid. Larvae fed on leaves treated with saponins showed less food consumption and high mortality [90, 91, 157]. The *Q. saponaria* (soapbark tree) extracts saponin from the inner bark, which is a natural soap and used as an insecticide against potato beetle. Colorado potato beetle is a commonly important pest of potato crops; their damage caused a reduction in yield due to resistance to chemical pesticides [158]. The development of different efficient techniques to inhibit the destruction of this pest by exploring insecticidal activities of different plant-derived metabolites such as glucosinolates, alkaloids, and saponins is useful

for their control [159]. The oleanane-type saponins isolated from the soapbark tree at different concentrations (1–7%) showed insecticidal activities against cotton leafworm caterpillars (*Spodoptera littoralis*). The feeding of third-instar larvae at low concentration of saponins (1–2%) in diets had not shown detrimental effect, whereas the increased level of saponins (3–7%) in diets caused 40–50% pupal mortality, and adult stage showed 70% mortality compared to control [103]. The in vivo effects of extracted saponins from soapbark tree were tested against third-instar larvae of cotton leafworm by providing an artificial diet containing saponins (30–70 mg g^{-1}). The result showed significant drop in larval weight and 70–84% pupal mortality of cotton leafworm. In cotton leafworm, the midgut epithelium cells raptured, and wholly collapsed because of a high level of saponins in diet. The potential effects of saponins have insecticidal action against cotton leafworm due to deterrent feeding and cytotoxicity by rapid cell membrane permeation of midgut epithelium. Hence, the destruction of the midgut epithelium wall proved strong insecticidal activity of saponins [91].

4.9 Rubiaceae

It contains about 13,500 species in 611 genera and covers madder, coffee, as well as bedstraw plants. It includes flowering plants, terrestrial trees, shrubs, lianas, or herbs [160]. The members of this family are quickly recognized due to their morphological structures having opposite leaves with interpetiolar stipules. These plants (having toxic metabolites) [161] are deterrent to several insect pests, e.g., *Spodoptera litura* and *L. decemlineata* [162]. In tropical regions, *S. litura* (leafworm moth) caused an infestation in herbal plants and field crops. The extraction and separation of saponins from *Catunaregam spinosa* resulted in seven triterpenoid saponins, consisting catunaroside A–D, swartziatrioside, aralia-saponin IV, and aralia-saponin V, which act as feeding deterrent toward DBM. The study confirmed that triterpenoid saponins have intense repellent action to second-instar larvae of DBM. Monodesmosidic saponins (catunaroside A, catunaroside B, and swartziatrioside) exhibit strong insecticidal effects than bidesmosidic saponins (catunaroside C, catunaroside D, aralia-saponin V, and aralia-saponin IV) [98].

4.10 Sapindaceae

It is known as the well-known soapberry family, which includes 138 genera, with most abundant genera *Serjania*, *Paullinia*, *Acer*, as well as *Allophylus*, and almost 1,858 species [163, 164]. The Sapindaceae is found in tropical and temperate areas throughout the world, and many occur in laurel forest habitat. Many are laticiferous that contain latex, a milky sap, and mildly toxic saponins with soap-like qualities in the foliage, seeds, and roots. The isolated saponins from well-known plants of this family have pesticidal activities, and the hydrolyzed products of these saponins from *Sapindus mukorossi* and *Diploknema butyracea* are used against leafworm moth

[165]. The saponin 16-a-hydroxyprotobassic acid and hederagenin extracted from *D. butyracea* and *S. mukorossi*, respectively, are recognized as repellent or feeding deterrence and caused toxicity against third-instar larvae of leafworm moth. Hence, for the control of this pest, a concentration of 3.4 g L^{-1} alkaline and 1.2 g L^{-1} acid hydrolyzed of diploknema saponins was used [165]. The stem bark of *Elattostachys apetala* and root bark of *Haplocoelum congolanum* have triterpenoid saponins, respectively [166, 167]. The aqueous extraction and separation of total triterpenoid saponins by using foam fractionation from the pericarps of *S. mukorossi* showed biological activities against *Thysanoplusia orichalcea* used as biological activity [168–170].

4.11 Solanaceae

It covers annual and perennial herbs, shrubs, lianas, epiphytes, trees, crops, medicinal plants, spices, and ornamentals, from 98 genera and about 2,700 species [171–173]. Various plants are commonly being used with their action as potent alkaloids and very toxic in nature. The extraction of steroidal glycosides from the foliage of ten *Solanum* species (resistant variety) their effects were studied based on survival and feeding behavior of the *Empoasca fabae* (potato leafhopper). A positive correlation was observed between the saponin level of the potato leaves and resistance of leafhopper. It results due to the presence of steroidal glycosides present in potato have a role in potato leafhopper resistance [174]. The potato leaves foliage developed resistance due to high content of steroidal glycosides and decreased infestation level by 57% after seven generations of selection of potato leafhopper [175]. The high level of steroidal glycosides or glycoalkaloid content in leaves of potato caused the resistance of potato leafhopper under field conditions [176]. The insecticidal effects of steroidal glycosides or glycoalkaloid extract from potato leaves after concentrations of 0.03% and 0.09% caused mortality of leafhopper up to 50%. The highest concentration of 0.27% caused the highest mortality rate in the range of 69–100% against potato leafhopper adults [177]. The furostanol saponin extracts from the seeds of *Capsicum annuum* have antimicrobial activities [178]. The cotton leafworm (*S. littoralis*) is distributed in different areas of the sphere and a well-known pest of many cultivated crops along with a broad host range including 40 different families of dicotyledon plants [103]. The ursolic acid saponins at a level of 5000 ppm present in corkwood (*Duboisia myoporoides*) tested by the leaf-disk method produced 91.96% inhibition in cotton leafworm and showed feeding deterrence and insecticidal activities against cotton leafworm [179]. The isolated saponins have insecticidal activities against this cotton leafworm [180].

4.12 Theaceae

This family contains shrubs and trees from over 40 genera [181–183]. The *Camellia oleifera* is a primary source of edible oil, and this oil is enriched with different

saponin-rich active ingredients, tea seed pellets (TSPs). TSPs are eco-friendly for beneficial soil insects, and they did not control black cutworms or white grubs in the treated field. Hui et al. [184] investigated the insecticide resistance and the significantly different actions of various compounds [carboxylesterase (CarE) and acetylcholinesterase (AChE)] on DBM populations that have a wide range of host feeding plants. The extraction and isolation of *Camellia* plant seed produced an important saponin called tea saponin (TS) [88]. The DBM fed on various host plant species strongly influenced by tea saponin regarding nutritional indicators, hormone titers, development, and reproduction [185]. The multiple effects of different concentrations of tea saponin (TS) on DBM rearing on three host plants caused mortality of third-instar larvae [186]. In addition, they evaluated growth and development parameters, nutritional indicators, and juvenile hormone (JH) and molting hormone (MH) titers in second-instar larvae exposed to the various dose of TS having concentration (LC_{20} and LC_{50}) used in second-instar larvae for the determination of growth regulator hormones including juvenile hormone (JH) and molting hormone (MH) titers, nutritional indicators, and some parameters of growth, development, and survival rate [88]. The results indicate the influence of LC_{20} and LC_{50} doses of TS on DBM caused prolonged development periods of larvae and pupae, and deterrence of feeding; reduction in growth rates, pupal weights, and frass production; and slower pupation, as well as adult emergence along with diminished fecundity, were observed in treated DBM larvae. There was no significant difference in approximate digestibility among treatments, and controls were determined, but the efficiency of conversion of ingested and digested food increased. The host specificity and dose of TS influenced the JH and MH titers as these hormones were higher after TS treatments. It indicates that tea saponin, a new alternative insecticide based on its natural origin, cheap cost, and environment-friendly property, is used. Moreover, TS acts as an antifeeding agent against various insect pests, e.g., cabbage worm (*Pieris rapae* L.) [187].

4.12.1 Residual Toxicity of Saponins

In residual toxicity assay, tea saponins were used as alternative insecticides against larvae of DBM and aphid, *Aphis craccivora* Koch. In residual toxicity study, the different concentrations of tea saponins against second-instar larvae of DBM were used with a dose of LC_{50} 21.06 g L^{-1}, and for the aphid, TS dose at LC_{50} 5.41 g L^{-1} was used which was observed more effective after 4 days of application as compared to control. In repellent activity assay, tea saponins showed 48.57% higher repellence at 4.0 g L^{-1} dose against third-instar larvae of DBM, but feeding preference index (PI) of saponin against third-instar larvae of DBM decreased as concentration (0.63) increased. It showed that TS was more effective against second-instar larvae of DBM, after the application for 3 and 4 days (LC_{50} 25.79 g L^{-1} and 21.06 g L^{-1}, respectively) as compared to standard check, azadirachtin (LC_{50} 72.55 g L^{-1}), after 96 h [185]. TS negatively influences the growth rate, feed consumption, frass production, pupal weight, percentage pupation, adult emergence, and fecundity as well as prolonged larval and pupal period of DBM. Repellent and feeding preference activity of tea saponin against DBM Different concentrations of tea saponins

evaluated for their repellent and feeding preference activity against DBM [88]. Therefore, saponins from other plants were discussed against Lepidoptera and Hemiptera. Based on the field efficacy data, TS could be recommended for the management of target pests. The biopesticide formulation contains TS used as an insecticide for the management of DBM and other pests of fruits/vegetables [185, 188]. Similarly, Adebisi et al. [189] also described the effects of saponin from *Eupatorium adenophorum* Spreng against DBM as well as aphids, where saponin extract showed toxicity (with $LC_{50} = 31.76$ g L^{-1}) and repellent activity (with $RC_{50} = 20.71$ g L^{-1}) to larvae of DBM within 24 h.

The growth and development of *Ostrinia nubilalis* were affected by alfalfa saponins along with a high dose of saponins that caused mortality of more than half the population [95]. Similarly, application of saponins extracted from alfalfa caused prolonged growth or development and reduction in fecundity as well as high mortality rate against *S. littoralis* [87]. The toxicity of tea saponins in terms of LC_{50} values and other regression parameters was found more effective against *Aphis craccivora*, in a treated group as compared to control. Residual toxicity study, application of tea saponins against *A. craccivora*, after the application of 3 and 4 days (LC_{50} 6.21 and 5.41 g L^{-1}, respectively) as compared to standard check, azadirachtin (LC_{50} 36.69 g L^{-1}) after 96 h. In mortality assay, tea saponins take less time to kill aphids and showed 50% mortality of *A. craccivora* population at a dose of 3.0 g L^{-1} and 4.0 g L^{-1} (LT_{50} 21.07 and 19.19 h), respectively. The application of saponin isolated from *Q. saponaria* was found more effective against pea aphid; *Acyrthosiphon pisum* caused toxicity (LC_{50} 0.55 mg mL^{-1}) and feeding deterrent activity (0.97) [91]. The alfalfa saponins showed maximum mortality rate (100%) within a short period (2 days) against *Empoasca fabae* [190]. The bioactivities and the field control properties of tea saponin against *Ectropis obliqua*. (i) A leaf-dip bioassay was used to evaluate the toxicity of TS to third-instar *E. obliqua* larvae and effects of TS on the activities of enzymes glutathione-*S*-transferase (GST), acetylcholinesterase (AChE), carboxylesterase (CES), and peroxidase (POD) of third-instar *E. obliqua* larvae in the laboratory. (ii) Topical application was used to measure the toxicity of 30% TS (w/v) and two chemical insecticides (10% bifenthrin EC and 50% diafenthiuron SC) to two species of spider, *Ebrechtella tricuspidata* and *Evarcha albaria*. (iii) Field trials were used to investigate the controlling efficacy of 30% TS against *E. obliqua* larvae and to classify the effect of TS to spiders in the tea plantation [191]. The toxicity of TS to third-instar larvae of *E. obliqua* occurred in a dose-dependent manner, and the LC_{50} was 1.64 g mL^{-1}. Activities of the detoxifying-related enzymes, GST and POD, increased in third-instar *E. obliqua* larvae, whereas AChE and CES were inhibited with time by treatment with TS. Mortalities of *E. tricuspidata* and *E. albaria* within 2 days with 30% TS treatment (16.67% and 20%, respectively) were significantly lower than those with 10% bifenthrin EC (80% and 73.33%, respectively) and 50% diafenthiuron EC (43.33% and 36.67%, respectively). The highest controlling efficacy of 30% TS was 77.02% at 5 days after treatment, which showed no difference to 10% bifenthrin EC or 50% diafenthiuron SC. Thirty percent TS was placed in the class N (harmless or slightly harmful) of IOBC

(International Organization for Biological Control) categories for natural enemies, namely, spiders.

5 Conclusion

Saponins possess explicit insecticidal activities as they exert a rapid-working and robust action against a broad range of insect pests that are different from neurotoxicity. The most observed effects are increased mortality, lowered food intake, weight reduction, retardation in development, and decreased reproduction. Previously, several approaches have been employed to involve maximum eco-friendly molecules from various sources (e.g., plants, microbes) for the management of insect pests of field crops as well as stored grain products. For example, the application of different entomopathogens (e.g., fungal, bacterial) was explored with multiple combinations of insecticides against insect pests, and they presented excellent results. Moreover, plant extracts (from leaf, shoot, flower) are also consistently being used for the management of herbivores and stored grain insect pests. Therefore, the focus was enhanced on the characterization and isolation of plant metabolites from different plant parts. After that, these metabolites were categorized into different classes according to their potential and activities. Some of the characterized metabolites gave insecticidal impacts against different life stages of insect pests, whereas some molecules presented antimicrobial activities. The main insecticidal component of these metabolites was saponin molecules, and these saponins are characterized as steroidal or triterpenoid saponins. These saponins play a significant role in the growth inhibition of insect pests, by disturbing various enzymatic activities of field and stored grain insect pests, including sap-sucking as well as chewing. Therefore, there is dire need to improve the commercial techniques for saponin isolation and purification and to explore deeply the interaction of saponin molecules and various insect enzymes as well as susceptible insect cells. Such explorations could also help to find out the molecular pathways of the insect immune system. Then, these pathways could be exploited via different molecular approaches (e.g., RNAi, CRISPR-Cas9), by which genetic makeup of insect pests could be altered, and it could be much helpful in the management of insect pests.

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Perspectives of Microbial Metabolites as Pesticides in Agricultural Pest Management

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Contents

Abstract

In the present-day agriculture, crop protection has become an inevitable event to sustain production. Chemical pesticides are considered to be an excellent strategy to any given pest problem, but overreliance on them raised different

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environmental concerns besides being ineffective due to resistance development. At this juncture, microbial pesticides had emerged as an alternative strategy due to high target specificity and ecological safety. Although a variety of microbes (bacteria, fungi, and nematodes) are commercially available and in the process of development as well, the actual pathogenicity and host killing are achieved by the metabolites they produce. So, it is obvious that the selection of a strain of any given microbes for pest management is a function of pesticidal metabolites it produces and their bioactivity against target pest. With the advances in applied microbiology and genetic engineering, isolation and characterization of bioactive genes and their products of microbial origin had become one of the fast-growing wing of pesticide chemistry. These efforts lead to commercialization of avermectins and spinosad, the biopesticides with metabolites of microbial origin as active ingredients with wider application in pest management. This chapter includes pesticidal (insecticidal, antifungal, antibacterial, and nematicidal) activities (target pests, modes of action, chemical structures, etc.) of different metabolites produced by diverse pathogenic microorganisms of agricultural importance. The molecular modifications for improving bioactivity, biotechnological approaches, and commercial implications of these microbial origin metabolites are also discussed in view of the existing literature.

Keywords

Secondary metabolites · Microbes · Biopesticides · Insecticidal · Antifungal · Nematicidal · Formulations · Genetic improvements

Abbreviations

ATP	Adenosine triphosphate
Bt	*Bacillus thuringiensis*
CAGR	Compound annual growth rate
Cry	Crystal
EPB	Entomopathogenic bacteria
EPF	Entomopathogenic fungi
EPN	Entomopathogenic nematodes
GABA	Gamma-aminobutyric acid
GlcNAc	*N*-Acetylglucosamine
HSP	Host-specific phytotoxins
kDa	Kilodaltons
Mcf	Makes caterpillars floppy
NHSP	Non-host-specific phytotoxins
ORF	Open reading frame
Pir	*Photorhabdus* insect related
RNA	Ribonucleic acid
Tc	Toxin complex
VIP	Vegetative insecticidal proteins

1 Introduction

Microbial diversity is one of the rich resources for a variety of products and processes having vast applications in industrial, pharmaceutical, and agricultural sectors. In particular, predominant use of microbials in agriculture is targeted against insect pests and diseases as biocontrol agents. Although the microbial biocontrol of pests is reported during the mid-1990s, their action and potential are over-masked by the chemical pesticides. After "silent spring" scientists and society realized that the chemical intensive pest management is lethal to the environment and did not support safe food security for growing population. So, the recent approaches of sustainable agriculture reoriented the therapeutic pesticidal control toward preventive pest management practices with different economical, ecological, and human concerns.

The pest problems in present-day intensive agriculture make the plant protection an inevitable event. Besides, development of pesticide resistance aggravated the pest problems, and the global trade increased the problems of nonnative invasive pest species. At this juncture, restricting the use of chemical pesticides and ecofriendly protection of crop plants is possible with realizing the importance of microbial pathogens or microorganism and their products. Probably, the basic reasons behind the poor adoption of microbial pesticides by farming community are unavailability of quality commercial products, poor visualization of action under field conditions, inconsistent performance, short shelf life, lack of awareness, etc. [1]. Laborious processes involved in isolation, identification, suitable formulation, and ecotoxicity establishment of microbial pathogen are some of the backstopping issues of scientific community [2]. However, with the advent of different molecular tools, identification and characterization of microbial pathogens became easy, and the biological control intensified with microbial pathogens became reality. At present, microbial pesticides are considered as imperative alternatives to chemical pesticides with high host specificity, biodegradability, and environmental safety.

In 2013, the global biopesticide market was estimated to be approximately $3 billion which accounts to 5% of total pesticide market. This is expected to grow to more than $4.5 billion by 2023 [3], among which microbial products are the fastest-growing segment [4]. Over the years, strains of *Bacillus thuringiensis* occupied prime position in biopesticide market followed by entomopathogenic fungi (*Beauveria bassiana*, *B. brongniartii*, *Metarhizium anisopliae*, *Lecanicillium lecanii*, and *Hirsutella thompsonii*) targeting wide range of arthropod pests. Different strains of *Bacillus*, *Pseudomonas*, and *Trichoderma* are being applied against a variety of plant pathogens [5]. In the United States, 356 biopesticides are registered with a total of 57 species of microbes [1]. Whereas in developing country like India, 970 formulations with 15 species are registered by 2017 [6]. Majority of these products contain the fermented cultures of species or strain of microbial agent, and some contain their by-products or synthetic chemical analogues as active ingredient.

The toxicity or pathogenicity of any given microbial biocontrol agent against target pest is manifested by microbial origin metabolites. These metabolites either

have direct toxicity to invading cells or weaken the system there by facilitating the microbial invasion. This pesticidal activity has received greater attention in recent years due to their versatile structures and novel modes of action with fascinating target sites. The discovery of avermectins and spinosyns proved that microbial metabolites are interesting targets for identification of environmentally safe, biodegradable, target-specific, and effective pesticidal compounds [7]. There are over 23,000 known secondary metabolites [8] including fungicides (blasticidin S, polyoxin, kasugamycin, validamycin, mildiomycin, etc.), insecticides (avermectin, milbemycin, bialaphos, etc.), and miticides (tetranactin) with excellent activity against target pests (structures of some prominent pesticidal metabolites are detailed in Figs 1 and 2) indicating many other pesticidal metabolites to be uncovered [7]. However, the discovery of new metabolites is a function of keen interest for novel pesticides which depends on improvements in screening technologies, exploration in novel ecological niches, applications of genetic techniques, progress in biochemistry of pesticide sciences, and ultimately the synthetic chemistry with commercial product facet.

There is an uncountable list of pesticidal microbes and metabolites. *Streptomyces* species have a special mention in production of pesticidal secondary metabolites. In the late 1990s, about 60% of insecticidal and herbicidal compounds reported are of *Streptomyces* origin [7]. Similarly, the metabolites of *Bacillus thuringiensis* (Cry, VIP proteins, and chitinases) are also considered to be an ever-growing list of insecticidal, nematicidal, and antifungal pesticides [9, 10]. In recent past, metabolites of entomopathogenic fungi and other novel groups of microbes have gained importance due to great biodiversity and possible identification of competent pesticides [11]. Besides, the advancements in cost-effective high-throughput whole genome sequencing techniques also facilitated the exact identification of pathogenic metabolites and prediction of modes of action. In this chapter, we chiefly focused on details of important pesticidal toxins reported against economically important agricultural pests that are derived from microorganisms PN). Further improvements and applications of these metabolites as pesticides are also discussed in view of the existing literature for successful pest management strategies.

2 Secondary Metabolites of Microbial Origin with Insecticidal Properties

2.1 Insecticidal Metabolites of EPB Origin

2.1.1 Cry Toxins

Cry toxins are the most prominent and commercially used insecticidal proteins against a wide range of insect pests and nematodes also. They are constitutively expressed as water-soluble parasporal crystals during sporulation of *Bacillus thuringiensis* (Bt), a soil bacterium with more than a century of history in agricultural pest management and nearly two decades of viable application in production of pest-resistant transgenic crops [12]. Studies also reported the production of Cry toxins

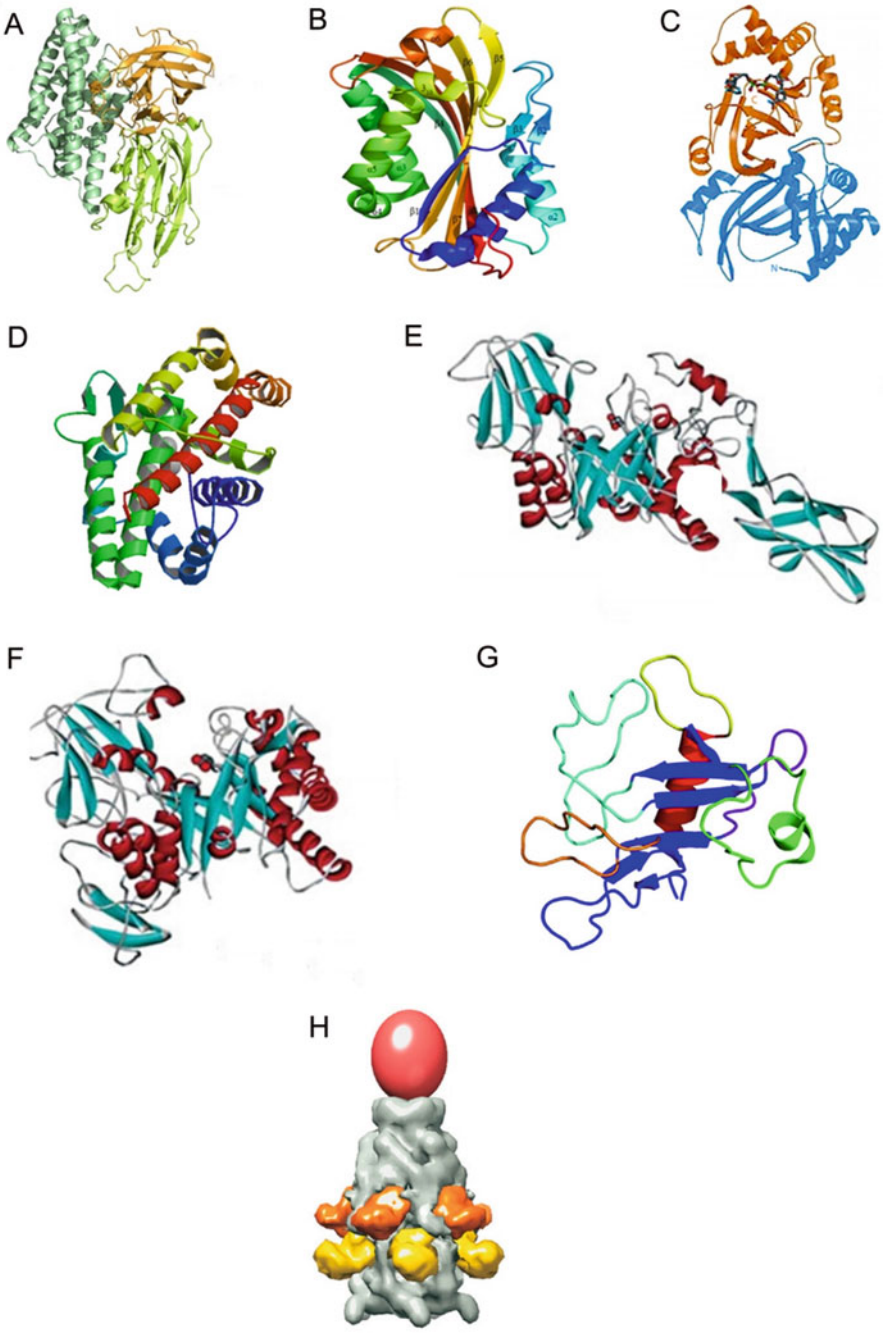

Fig. 1 3D structures of (**A**) an activated cry toxin, (**B**) Cyt toxin (adopted from Xu et al. [107]), (**C**) Vip2 toxin (adopted from Chakroun et al. [19]), (**D**) phospholipase C (adopted from Hough et

in other bacteria like *Bacillus popilliae* and *Clostridium bifermentans* [13]. Single gene-derived toxins, target specificity, risk-free against humans, nontargets and beneficials, biodegradability, etc. are the major characteristic features governing its wide usage.

Based on the primary structure (amino acid sequence), Cry toxins are classified into 67 families (Cry1 to Cry67) with more than 500 genes [14]. Structurally they are a three domain components and, after conformational alterations, interact with several pest-specific midgut proteins (cadherin, aminopeptidase-*N*, and alkaline phosphatase in lepidopteran, dipteran, and coleopteran insects, respectively) of susceptible insects [15] with sequential formation of pre-pore oligomers, membrane insertion, and pore in plasma membrane of midgut epithelial cells resulting in osmotic imbalance [13, 16]. These disruptions in midgut cells lead to immediate cessation of feeding and ultimately death (reviewed by Bravo et al. [16]) suggesting a conserved bio-toxicity. In recent past, a different mechanism of Cry toxin action by necrotic death of target cells due to disturbances in intracellular signaling is also proposed [9].

2.1.2 Cytolysins

They are reported from *B. thuringiensis* extracellular proteins produced during vegetative growth of the bacterium. Cytolysins cause cell lysis and can synergize Cry and other insecticidal proteins [17]. They chiefly interact with phospholipid receptors or phosphatidylethanolamine of cell membrane either specifically or non-specifically in a detergent-like manner, where the structural hydrophobic patches bind with amphipathic phospholipids leading to pore formation as that of Cry toxins and subsequently leading to cell death by a process called colloidal osmotic lysis [18].

2.1.3 Vegetative Insecticidal Proteins

Studies on the cultural supernatant proteins of *B. thuringiensis* and *B. cereus* led to identification of insecticidal protein called vegetative insecticidal proteins (VIPs) that are produced during vegetative growth stage of bacteria. They are completely unrelated insecticidal toxins and share no homology with Cry proteins. Till date, four families of VIP genes are identified, viz., Vip1, Vip2 specific to coleoptera and hemiptera, VIP3 specific to lepidopteran pests, and VIP4 with unknown toxicity [19]. Structurally VIP1 and VIP2 toxins contain N-terminal signal sequence, while VIP3 lacks it. Although individually toxic, in some instances, both VIP1 and VIP2 are located in single operon and are required together for bioactivity against some insects thus are considered as binary toxins [20]. As a binary toxin, VIP1 component binds with specific receptors and forms an oligomer that allows translocation of the

Fig. 1 (continued) al. [108]), (**E**) ChiA from *Serritia marcescens*, (**F**) ChiB from *Serritia marcescens* (adopted from Horn et al. [109]), (**G**) hirsutellin (adopted from Olombrada et al. [51]), (**H**) *Yersinia entomophaga* toxin complex. (Adopted from Landsberg et al. [110])

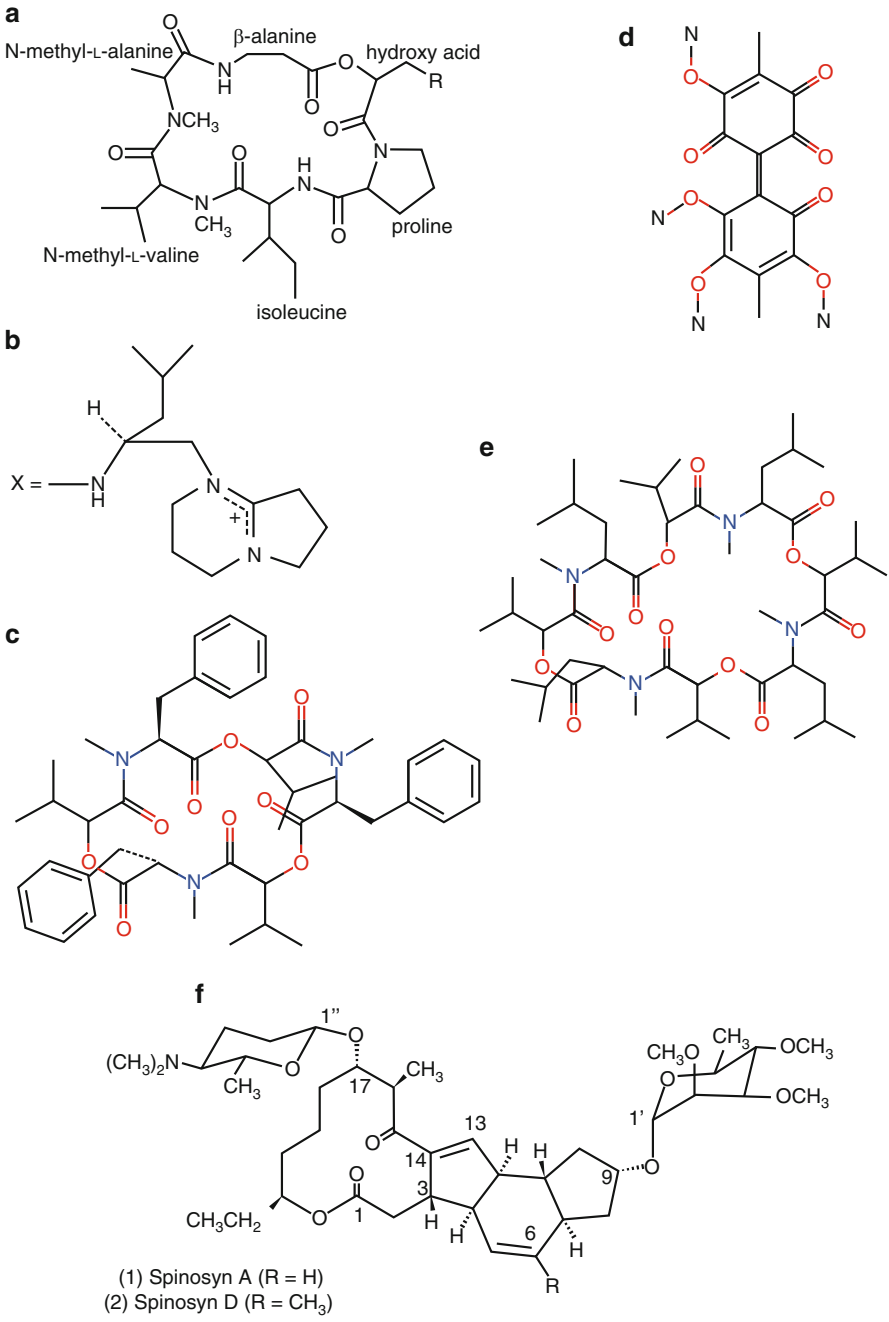

Fig. 2 (continued)

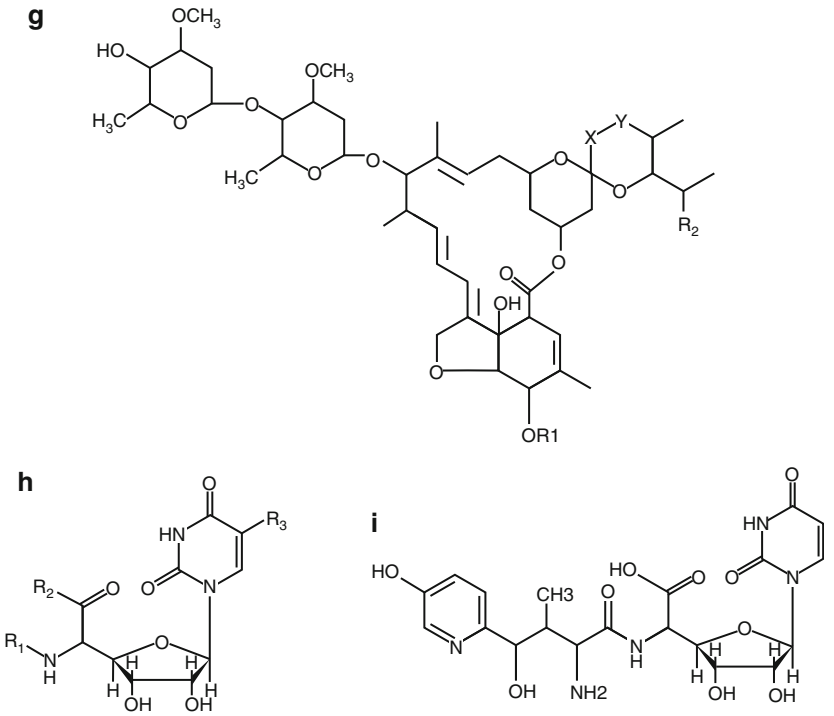

Fig. 2 Chemical structures of **(a)** destruxins (adopted from Donzelli et al. [111]), (**b**) efrapeptins (adopted from Krasnoff and Gupta [43]), (**c**) beauvericin, (**d**) oosporein, (**e**) bassianolide (adopted from Ortiz-Urquiza and Keyhani [112]), (**f**) spinosyns (adopted from Kirst [113]), (**g**) avermectins (adopted from Qiu et al. [114]), (**h**) polyoxins, (**i**) nikkomycin. (Adopted from Li et al. [115])

enzyme domain, the VIP2 component which acts as ADP-ribosyltransferase against actin, thereby preventing formation of microfilaments. The proteolytically activated Vip3 proteins upon receptor binding in midgut epithelial cells of susceptible insects cause apoptotic cell death. Although it shares similar mode of action as that of Cry toxins, binding sites are unique. Due to this differential binding sites, both the toxin genes can be used in gene pyramiding for increased target pest spectrum and delayed resistance. Interestingly, in case of insensitive insects, VIP3 proteins didn't bind with epithelial cells [21] giving its target specificity.

2.1.4 Thuringiensin

It is also known as β-exotoxin and is a thermostable metabolite of oligosaccharide nature with insecticidal activity against insect pests of Diptera, Lepidoptera, Coleoptera, Orthoptera, Hymenoptera, and Isoptera and even some nematode pests. The insecticidal activity is manifested by interfering the RNA polymerase activity by competing with ATP binding sites. The toxicity of thuringiensis is visualized only at the time of moulting and pupation [22].

2.1.5 Phospholipase C

It is a heterogenous group of esterase which is usually associated as surface protein and sometimes secreted into medium. Although produced by a large variety of Gram-positive and Gram-negative bacteria, phospholipase C is directly involved in pathogenicity of pesticidal *B. cereus* strains against coleopteran pests. These enzymes are involved in hydrolysis of glycerophospholipids which directly influence membrane dynamics and cellular signaling in particular. It is important to note that phopholipases from other than *B. cereus* showed human toxicity [23].

2.1.6 Other Metabolites from EPB in Support of Pathogenicity

In addition to Cry and VIP toxins, recent discoveries of novel insecticidal metabolites from *B. thuringiensis* like secretary insecticidal proteins, thuringiensin with oligosaccharide nature, insecticidal lipoproteins and PS201T6 strain toxicity against some hymenopteran pests (reviewed in Mnif and Ghribi [10]), and antimicrobial secondary metabolites like zwittermicin, thuricin, kurstakins, etc. show the bacterium is an eternal source of pesticidal toxins. Some unfamous EPBs like *Yersinia entomophaga*, *Chromobacterium subtsugae*, *Brevibacterium frigoritolerans*, *Pseudomonas entomophila*, etc. may also harbor novel range of pesticidal compounds.

2.2 Insecticidal Metabolites of EPN Origin

2.2.1 Toxin Complex (Tc) Proteins

The toxin complex (Tc) proteins are multiple-subunit, high molecular weight (more than 100 kDa) insecticidal toxins identified in both Gram-negative and Gram-positive bacteria [24]. They were initially identified from supernatant protein of a Gram-negative bacterium *Photorhabdus luminescens* strain W14 [25] and *Xenorhabdus nematophila* [26] the symbionts of entomopathogenic nematodes. Although the individual subunits exert toxicity, their complex showed multiple pathogenicity against variety of insect pests and so potentiates each other, an evolutionary adaptation to invade different hosts. Recent studies also reported existence of Tc complexes in some other entomopathogenic bacteria like *Serratia entomophila* [24], the causal agent of "amber" disease in New Zealand grass-grub [27]. The Tc complexes are highly conserved with respect to amino acid sequences as they are encoded by multiple copies of single Tc loci which have different open reading frames [25, 28].

The insecticidal potential of these toxins include both coleopteran and lepidopteran pests. Oral toxicity is most prominent and reported to cause progressive deformations and deteriorations in midgut epithelial cells [29]. Pathogenic *Pseudomonas* species are also reported to be efficient producers of Tcs [30, 31] and induce apoptic cell death in host through antioxidative stress and activity against macrophagosis. So, Tcs can be used as alternatives to *B. thuringiensis* Cry toxins in transgenic production [28, 32] with an additional advantage of multiple and diversified pest resistance. Additionally, cross-potentiation between different toxin complexes of varied origin is also reported which helps in the production of "stacked"

transgenic plants. However, Tcs consist of number of protein subunits which might be difficult to express together transgenically to realize full potential.

2.2.2 *Photorhabdus* Insect-Related (Pir) Binary Toxins

This is a two-component insecticidal toxin derived from *PirAB* gene with two genetic loci from *P. luminescens* and *P. asymbiotica* [33]. They are orally active against *Plutella xylostella* and different mosquito larvae. The mode of action is similar to Cry toxin pore formation and leptinotarsin as well which has amino acid similarity with juvenile hormone esterase-like protein. This also suggests neurotoxicity by promoting Ca^{2+} influx and release of neurotransmitters from presynaptic nerve.

2.2.3 Makes Caterpillars Floppy Toxins

These toxin genes (Mcf) are identified during screening of a cosmid library of *P. luminescens* strain W14 as a single 8.8 kb open reading frame (ORF). Injection of transformed *E. coli* with these toxin resulted in loss of turgor pressure in larvae of *Manduca sexta* leading to death thus the name Mcf [34]. Another ORF of Mcf gene with similar symptomology is also identified during further screening of same cosmid library with differences in N-terminal region. Further progress in genome sequencing also showed the presence of Mcf genes in *P. fluorescens*, *Providencia* sp., and *Vibrio* spp [32]. These toxins cause apoptosis in insect midgut epithelial cells and hemocytes which may cause disturbance in osmoregulation leading to typical floppy phenology.

2.2.4 Other Metabolites from EPN in Support of Pathogenicity

The insect immune system directly responds to any foreign entities by various humoral and cellular responses. Other than direct toxic metabolites, evasion of these responses of host immune system is a challenge to invade host for which a variety of antimicrobials such as proteases, lysozyme, cecropins, hemolysins, etc. are reported [35, 36–38]. In addition, an indirect pathway of inhibiting melanization, phagocytosis, and nodule formation (usually associated with cellular response of immune system) through effecting phenoloxidase cascade is also a response against cellular immunity. *Photorhabditus* has a dedicated type III secretion system for these activities, whereas *Xenorhabdus* species had several cytotoxic strategies. Indeed, the whole genome sequence of *P. luminescens* strain TT01 revealed that it encodes a huge number of insecticidal genes than any other bacteria known [38], indicating an extensive resourceful nature of these symbiotic bacteria for pesticidal toxins.

2.3 Insecticidal Metabolites of EPF Origin

2.3.1 Destruxins

They were first discovered in *Metarhizium anisopliae* later reported in majority of entomopathogenic fungi, viz., *Aschersonia* sp., *Nigrosabulum globosum*, and *Beauveria feline*, as well as some plant pathogenic fungi [39, 40]. They are classified

into families, destruxins A, B, C, D, and E, which occur as isomers or congeners with a basic structural backbone of five amino acids and an α-hydroxyl acid. They are structurally cyclic depsipeptides with insecticidal, antiviral, and phytotoxic properties. Till date, a total of 39 destruxins were identified mostly from *M. anisopliae* [41]. Some natural pathogenic analogues like roseotoxin and bursephalocids (A and B) are also identified from different sources [39].

Lepidopteran insects are reported to be highly susceptible to destruxins with typical tetanus followed by flaccid paralysis upon injection. They are mainly responsible in weakening the host immune defense and damaging muscular and digestive systems [42]. They are also known to inhibit nucleic acid and protein synthesis [23, 43]. Upon feeding they are reported to cause growth reduction and influence pupal weight and adult emergence. However, the contact toxicity of destruxins is controversial, and mode of action is still unclear. It is also important to note that destruxins E have systemic toxicity against aphid pests like *Brevicoryne brassicae* and *Myzus persicae*.

2.3.2 Efrapeptins

These are a linear peptide molecules with 15 amino acids isolated from an entomopathogenic fungus, *Tolypocladium* sp. [43], a soil hyphomycetes. They are reported to be inhibitors of intracellular protein transport system and ATPase of mitochondria [44] by acting as catalytic site competitive inhibitors with insecticidal and miticidal properties and limited antimicrobial activity. Low doses results in antifeedant and growth inhibitory activities.

2.3.3 Oosporein

Oosporeins are chiefly produced by *Beauveria* sp. and are known to be produced during infection process on cuticle [45]. They are red pigmented dibenzoquinone antimicrobial substances against Gram-positive bacteria. They result in malfunctioning of different enzymes by disorienting tertiary structures through SH group redox reaction of amino acids and also showed inhibitory effect on ATPases [46].

2.3.4 Beauvericin

Beauvericins are isolated from *Beauveria* and *Paecilomyces* species. They structurally represent hexadepsipeptide with cyclic repeats of phenylalanine and hydroisovaleric acid and are reported to be similar to membrane damaging antibiotic, enniatin [47], with adequate antibacterial property. They are cationophoric and usually increase the permeability of cell membranes by forming Na^+ and K^+ complexes. In addition, Ojcious et al. [48] reported beauvericins can act as cholesterol acyltransferase inhibitors and are also capable of fragmenting DNA.

2.3.5 Bassianolide

They are cyclo-octadepsipeptide of four molecules each of L-*N*-methyl leucine and D-α-hydroxyisovaleric acid produced by *B. bassiana* and act like ionophore antibiotic with differential reaction to cations. At high doses bassianolides are lethal, but at low doses they simply caused atonic symptoms [49].

2.3.6 Bassiacridin

It is a monomeric 60 kDa protein fraction isolated from a locust infesting strain of *B. bassiana* with β-glucosidase, β-galactosidase, and *N*-acetylglucosaminidase activities. The toxicity of bassiacridin is distinct from other *Beauveria*-originated macromolecular toxins, and at cellular level, it causes melanized spots and structural deformities on tracheal system.

2.3.7 Hirsutellin

They are non-glycosylated and thermostable proteins produced by *Hirsutella thompsonii* by a unique gene. They showed both ingestion and injection toxicity against variety of aphids, mites, and fruit flies. In some instances contact toxicity is also observed. They are cytolytic and inhibit protein synthesis by specific cleavage of rRNA and so-called ribotoxins [50, 51].

2.3.8 Organic Acids

Different EPF origin organic acids like oxlic, kojic, cylcopyazonic, fusaric, 4-hydroxymethylazoxybenzene-4-carboxylic acids, etc. have been reported to be toxic to various lepidopteran and dipteran pests. They are important in solubilizing specific cuticular proteins and can synergize proteases and chitinases.

2.3.9 Other Metabolites from EPF in Support of Pathogenicity

Some genes and their products are specifically designed to cater specific needs of EPF. For example, adhesins in *M. anisopliae* (Mad1 and Mad2) are involved in holding of spores to insect cuticle and plant cells; immune evasion genes (Mcl1) are involved in weakening the host immune system, etc. [52]. Viridoxins are nonprotein metabolites identified in *M. anisopliae* var. *flavoviride* having insecticidal activity against *Leptinotarsa decemlineata* [53]. In majority of cases and especially in species-specific pathogens, such compounds are interaction specific. In addition, some unfamous EPF species like *Agerata*, *Sphaerostilbe*, *Podonectria*, *Myriangium*, *Aschersonia*, etc. and nematicidal fungi like *Purpureocillium lilacinum* and *Pochonia chlamydosporia* are still needed to be explored for their novel pesticidal metabolites.

2.4 Insecticidal Metabolites of Actinomycetes Origin

2.4.1 Spinosyns

They are derived from actinomycetes, *Saccharopolyspora spinosa*, with two major families, viz., A and D, that are most active and unique insecticidal compounds with specific activity against Lepidoptera, Diptera, Thysanoptera, and some species of Coleoptera and Orthoptera [54]. Structurally spinosyns are macrolides containing a backbone of 21-carbon tetracyclic lactone attached with two deoxysugars (an amino sugar, tri-*O*-methylated rhamnose, and a neutral sugar, forosamine) that are essential for insecticidal activity. Except for rhamnose, a cluster of biosynthetic and bioconversion genes for spinosyn components spans 74 kb of the *S. spinosa* genome

that includes five genes for type I polyketide synthase and 14 genes for sugar biosynthesis and their attachment to the polyketide [55].

Spinosad exhibits rapid contact and ingestion toxicity. Like organophosphates and carbamates pesticides, it shows excitation of the nervous system, involuntary muscle contractions, tremors, and paralysis which are unusual for a biological product. All these effects are consistently expressed through activation of nicotinic acetylcholine receptor and GABA receptors which are unique to spinosad. The site of action of spinosad is also different from the other nerve acting neonicotinoids and avermectins with no known cross resistance. Low toxicity to nontarget organisms including humans and relatively fast degradation by photolysis make it a relatively safe insecticide [54].

2.4.2 Avermectins

Avermectins (abamectin, ivermectin, and emamectin benzoate) are a novel class of macrocyclic lactones produced by soil actinomycetes, *Streptomyces avermitilis* [56]. Although highly toxic to bees and fish, rapid photodegradation and no apparent bioaccumulation result in environmental acceptance of avermectins. They showed bioactivity against around 84 species of insect pests belonging to ten insect orders and also have nematicidal and acaricidal activity. However, its worldwide commercial use is as acaricide against variety of mite species (Tarsonemidae, Tetranychidae, and Eriophyidae) associated with horticultural, food, commercial crops, and even livestock.

At cellular level, avermectins affect neural and neuromuscular transmissions in central nervous system by disrupting the receptors for γ-aminobutyric acid and glutamic acid (GABA-gated chloride channels) resulting in chloride ion influx at neuromuscular junction. Disturbances in water balance, moulting, metamorphosis, reproductive developments, etc. are the major symptoms associated with poisoning by avermectins. Most importantly, they also have translaminar activity which provides prolonged residual pest management.

2.4.3 Polyoxins and Nikkomycins

These are a group of peptidyl nucleoside antibiotics produced by *Streptomyces* species. They inhibit the enzyme chitin synthetase by acting as competitive inhibitors thereby inhibiting chitin formation. Thus both polyoxins and nikkomycins act as insect growth regulators and have potential in controlling of various insect, mite, and fungal pests. Due to the absence of chitin in higher organisms and humans, these compounds show substantial target specificity. Structurally they are pyrimidine rings with attached dipeptide uridyl-ribose moiety and are produced in complex pathway [23].

2.4.4 Chitinases

Chitinases (EC 3.2.1.14) are the catalytic enzymes belonging to glycoside hydrolases involved in degradation of chitin, the second most abundant natural homopolymer after cellulose. They hydrolyze the β-1,4-linkage between the monomeric units of chitin chains, *N*-acetyl-D-glucosamine. These enzymes are produced by a variety

of organisms including bacteria, fungi, viruses, insects, plants, and even humans [57] either constitutively or inductively, mostly the latter by substrate. However, the release of free *N*-acetyl glucosamine from chitin chains is undertaken by a complex combination of enzymes, i.e., chitototetriose, chitotriose, and diacetylochitobiose and chitobiases and β-*N*-acetylglucosaminidases. Based on the cleavage site, these enzymes were categorized as exochitinases (cleaves chitin chains from reducing or nonreducing end of the chitin chain) and endochitinases (cleaves chitin chains at random locations) [58]. Based on amino acid similarity, chitinases are classified into three different families, viz., families 18, 19, and 20. Majority of family 18 chitinases are produced by microorganisms like bacteria, fungi, viruses, and some insects and plants. Family 19 chitinases are especially plant derived and some from bacterial like *Streptomyces griseus* [59]. Family 20 are the newly identified chitinases from *Vibrio harveyi*, *Dictyostelium discoideum*, and humans.

In any given insect pest, chitin is the major structural component of vital organs (exoskeleton, appendages, peritrophic membrane, etc.), and in the case of PPF, mycelia are made up of chitin, so chitin metabolism is one of the essential biological activity. Many studies reported that an extraneous application of microbial-derived chitinases results in damage to midgut peritrophic membrane and epithelial cells [60] effecting feeding, digestion, nutrient utilization, and ultimately growth [61]. Although no direct toxicity is observed, antifeeding effects, growth reduction, and developmental deformities are prominent against a variety of insect pests [62–67]. It is important to note that chitinases are produced by a variety of entomopathogens as a part of pathogenicity and in some cases chitinolytic strains exhibited greater toxicity over non-chitinolytic strains [60]. Studies also reported that they can be considered as biological synergists for toxicity with a variety of chemical and biological insecticides (reviewed by Subbanna et al. [61]).

2.4.5 Other Pesticidal Metabolites

Newly identified heat-stable low molecular weight proteins, Cry protein homologues binary toxins [62, 63], species-specific toxins like *Photorhabdus* virulence cassettes [27], variety of enzymes (collagenases, proteinase, proteases), and proteinaceous metabolites (surface proteins, GlcNAc-binding protein, antigen proteins, bacillolysins) are also reported to have either direct insecticidal activity or assist in pathogenicity of respective bioagents [62, 63]. One day all these compounds may have their say in pest management.

3 Secondary Metabolites of Microbial Origin with Antimicrobial Properties

3.1 Antifungal Metabolites Produced by Fungi

Secondary metabolites are small organic compounds (molecular masses generally less than 3000 Da); secondary metabolites are interesting for various reasons, e.g., their structural diversity and their potential as drug candidates or as natural

Table 1 Microbial metabolites produced by different microorganisms with fungicidal and antibiotic action against different diseases

Pesticidal metabolite	Origin microorganism	Target pest	References
Macrolactin A	*Bacillus* sp. *sunhua*	Potato scab pathogen (*Streptomyces scabies*)	Han et al. [74]
Syringomycin E	*Pseudomonas syringae*	Citrus green mold (*Penicillium digitatum*)	Bull et al. [75]
Blasticidin S	*Streptomyces griseochromogenes*	Rice blast caused by *Pyricularia oryzae*	Fukunaga [116]
Kasugamycin	*Streptomyces kasugaensis*	Rice blast (*Pyricularia oryzae)*, leaf spot in sugar beet and celery (*Cercospora* sp.), and scab in pears and apples (*Venturia* sp.)	Umezawa et al. [117]
Cryptocin	*Cryptosporiopsis quercina*	Rice blast (*Pyricularia oryzae)*	Strobel et al. [118]
Cytochalsins	*Phomopsis* sp.	*Sclerotinia sclerotiorum*, *Fusarium oxysporum*, and *Botrytis cinerea*	Fu et al. [119]
Colletotric acid	*Colletotrichum gloesporioides*	Brown spot of rice (*Helminthosporium sativum*)	Zou et al. [120]
Rufuslactone	*Lactarius rufus*	*Alternaria brassicae*, *Botrytis cinerea*	Luo et al. [121]
Oxytetracycline	*Streptomyces rimosus*	Fire blight of apple (*Erwinia amylovora*)	Finlay et al. [122]
Streptomycin	*Streptomyces griseus*	*Xanthomonas oryzae*, *X. citri*, *Pseudomonas tabaci*	Saxena [8]

pesticides. Secondary metabolites are low molecular weight compounds and well known for their ability to restrict the growth of other microorganisms. Microbes are ubiquitous and display various interactions with other living organisms mediated by a myriad of chemical interactions that exhibit diverse biological activities. There are over 23,000 known secondary metabolites, of which 42% are produced by different fungi, 42% by actinomycetes, and 16% by other bacterial species. (Details of some prominent ones are given in Table 1.) A wide range of antimicrobial compounds have been isolated from microbes and developed into drugs [8] like streptomycin (*Streptomyces griseus*), penicillin (*Penicillium chrysogenum*), and bacitracin (*Bacillus subtilis*). The ascomycetes filamentous fungus *Aspergillus fumigates* secretes more than 226 secondary metabolites including commonly studied polyketides, such as cyclic peptides, alkaloids, and sesquiterpenoids [64, 65]. Members of another class of secondary metabolites produced by *A. fumigatus*, termed the epipolythiodioxopiperazines (ETPs), are characterized by an internal disulfide bridge across a diketopiperazine ring, where the first and best characterized member being gliotoxin [66, 67]. Among different *Aspergillus* species, only those associated with aspergillosis, such as *A. fumigatus*, *A. terreus*, *A. flavus*, and *A. niger*, produce gliotoxin [68, 69].

A phytotoxin is a microbial metabolite excreted (exotoxin) or released by lysed cells (endotoxin), which, in very low concentration, is directly toxic to cells of the susceptible host. Plant-pathogenic fungi mediate their pathogenesis by virtue of biochemicals which overcome the defense mechanisms of plants and induce wilting, suppression of growth, chlorosis, necrosis, and leaf spots. The partial success of fungal biological control agents is attributed to the production of phytotoxins. These have been categorized as host-specific phytotoxins (HSPs) and non-host-specific phytotoxins (NHSPs). HSPs are active toward the plants which are host of the toxin-producing fungus and essential determinant for pathogenicity [70], while NHSPs are not primary determinants of pathogenicity and may contribute to the virulence of the fungus. The fungi which produce HSPs are of the genera *Alternaria*, *Cochliobolus*, *Leptosphaeria*, *Venturia*, *Ascochyta*, and *Pyrenophora*. AK-toxin and AM-toxin host-specific phytotoxins produced by *Alternaria kikuchiana* and *Alternaria mali* are the causative agents of black spot disease and necrotic spots on leaves of pears and apples, respectively [71]. Nonselective phytotoxins include tentoxin, a cyclic tetrapeptide produced by *Alternaria alternata*.

3.2 Antibacterial Metabolites Produced by Bacteria

Many bacteria produce antimicrobial substances such as non-ribosomally synthesized antibiotics and ribosomally synthesized proteinaceous compounds referred to as bacteriocins. Bacteriocins most often act on closely related species only and are thus of interest for application as targeted narrow-spectrum antimicrobials with few side effects. Bacteriocins that exert their antimicrobial action by self-assembling into cytotoxic phage tail-like fibers have also been observed in Gram-negative plant pathogens [72]. Bacteriocins are classified as protein bacteriocins and colicin/S-type pyocins produced by *Pseudomonas syringae* pv. *syringae* [73] effective against other *Pseudomonas* sp. Other bacteriocins include peptide bacteriocins; trifolitoxins are peptide bacteriocins produced by Gram-negative species such as *Agrobacterium tumefaciens* and *Rhizobium leguminosarum*. Macrolactin A, macrolactin A (IV), and iturin A produced by *Bacillus* sp. *sunhua* inhibited the potato scab pathogen *Streptomyces scabies* and are also fungicidal to *Fusarium oxysporum* causing dry rot disease [74]. Similarly, s Syringomycin E from *Pseudomonas syringae* ESC 10/11 controls the citrus green mold, *Penicillium digitatum* [75].

3.3 Nematicidal Metabolites Produced by Fungi

Fungi also parasitize the nematodes directly or indirectly and play major role in their biological control. Thus secondary metabolites produced by nematode-predating fungi may be exploited to develop biorational nematicides. Omphalotin A, cyclic dodecapeptide produced by *Omphalotus olearius*, is known to produce ivermectin with high selectivity [76, 77]. *Caryospora callicarpa* produces caryospomycins A, B, and C with potential nematicidal activity to pinewood nematode *Bursaphelenchus xylophilus* [78]. *Paecilomyces* sp. produces a unique nematicidal compound 4-(4′-

carboxy-2′-ethyl-hydroxypentyl)-5,6,-dihydro-6-methylcyclobuta[b]-pyridine-3,6-dicarboxylic acid which is effective against root-knot nematode, *Meloidogyne incognita* [79].

4 Genetic Improvements in Pesticidal Metabolites

Genetic improvement in pesticidal microbes is by manipulating and improving the strains for enhanced metabolite production and also for improving the efficacy of the metabolites and also for the exclusion of unwanted cometabolites. Microbial strain improvement can be done by classical genetic methods (including genetic recombination) and by molecular genetic methods [80]. Classical genetic methods for improvement in microbial metabolites rely mostly on mutation (both using physical and chemical mutagens) followed by rational screening. Rational screening is made for a particular characteristic which is different from that of final interest but easier to detect. Microorganisms possess regulatory mechanisms that regulate metabolite production and thus prevent overproduction. So the mutants are to be selected for over production of desired metabolites. Genetic recombination methods for improvement are by sexual or parasexual cross in fungi and conjugation in actinomycetes and protoplast fusion in both [81].

Molecular methods of genetic improvements require biochemical and molecular genetic tools apart from knowledge on the biosynthetic pathway and effective transformation protocols [80]. There are many methods used for molecular genetic improvement for secondary metabolite production. It is reported that the genes responsible for metabolite biosynthesis are found in clusters in the organisms and which are amplified for higher copies. Molecular improvements can also be made by targeted duplication or amplification of secondary metabolite production genes and amplification of whole pathway. The negative process of inactivating the competing pathways, silencing the regulatory genes, etc. can be also used in the genetic improvement process. Genetic improvements in different Cry toxins are discussed by different authors by using variety of molecular techniques [12, 15, 16, 82].

4.1 Genetic Improvement in Cytolysins

The *Enterococcus faecalis* cytolysin is related to antibacterial peptides termed lantibiotics which can be engineered to desired levels of cytotoxicity [83]. The lantibiotics are ribosomally synthesized, posttranslationally modified peptide containing unusual amino acids, such as dehydrated and lanthionine residues [84].

4.2 Genetic Improvement in Vegetative Insecticidal Proteins

Genetic improvement of vegetative insecticidal proteins aims in broadening the target pest spectrum along with higher toxicity to the pests. The gene vip3Aa7, its native promoter and cry3A promoter, was subcloned into *B. thuringiensis*

acrystalliferous BMB171 to generate BMB8901 and BMBvip, respectively, and the latter produced 3.2-fold Vip3Aa7 protein. Therefore, the vip3Aa7 gene under the control of cry3A promoter was transformed into strain YBT152, which was tenfold more toxic to *Spodoptera exigua* without meddling the toxicity of against *Helicoverpa armigera*. This will widen the spectrum of effect of *B. thuringiensis* against *S. exigua* apart from *H. armigera* and *P. xylostella* [85]. The vip3A(a) gene product has already demonstrated its activity against *Agrotis ipsilon*, *Spodoptera frugiperda*, *Spodoptera exigua*, *Heliothis virescens*, and *Helicoverpa zea* [21]. The deduced amino acid sequence of the vip3Aa14 gene from *B. thuringiensis tolworthi* was reported effective against *S. litura* and *P. xylostella* [86]. In a study, two genes encoding the corresponding proteins of the binary toxin, designated as vip2Ae and vip1Ae, were cloned, sequenced, and expressed in *E. coli.* Bioassays on aphids with the recombinant proteins confirmed toxicity of both the toxins in combination. The use of this gene for developing transgenic crop plants against sap-sucking insect pests is warranted [87]. Genes encoding inhibitors of insect proteases and vegetative insecticidal proteins (VIP) were considered for introduction into transgenic tomatoes in conjunction with cry1Ac gene [88].

4.3 Genetic Improvement in Chitinases

Entomopathogens can produce a series of chitinases which cause pathogenicity to insects and also fungi by degrading the cuticle/cell wall. Genetic improvement in chitinases aims in enhanced production of chitinase and its efficacy against target pests, e.g., overproduction of Bbchit1 by molecular means enhanced the virulence of *B. bassiana* on aphids. Transgenic rice plants with rice chitinase *chi11* gene were reportedly conferring resistance to sheath blight [89].

4.4 Genetic Improvement in Avermectins

Avermectins possessed activity against nemathelminthes and arthropods. Ivermectins and selamectins are semisynthetic derivative of avermectin. Ivermectins have effect against human onchocerciasis and strongyloidiasis, whereas selamectin is effective against heartworms and fleas. Genetic improvement in avermectin producing *S. avermitilis* has aimed in higher production of avermectins especially by cloning multiple copies of genes and eliminating unwanted cometabolites. *Streptomyces avermitilis* has gene *afsR2*, and incorporation of multiple copies of *afsR2* from *S. lividans* into *S. avermitilis* increased avermectin production by 2.3-fold [90]. Novel erythromycins were produced using *Saccharopolyspora erythraea* with the loading domain of the erythromycin PKS replaced by *Streptomyces avermitilis*, producer of avermectin [91]. Another regulatory gene of *S. avermitilis* which stimulates actinorhodin and undecylprodigiosin has also found stimulating avermectin production in *S. avermitilis* by 2.5-fold [92]. A troublesome toxic oligomycin in *S. avermitilis* was eliminated by transposon mutagenesis [93].

Ivermectins are synthesized by hydrogenation of avermectins B1a and B1b in the presence of catalyst rhodium chloride [94]. It was demonstrated that the ivermectins can be directly produced by replacing PKS genes of *S. avermitilis* with that of *S. venezuelae* by genetic engineering [95].

4.5 Genetic Improvement in Spinosyns

Saccharopolyspora spinosa produced secondary metabolite, spinosad which is widely used as insecticide with exceptional nontarget safety. Improved strains of *S. spinosa* were obtained by four rounds of genome shuffling of ten strains using nitrosoguanidine and UV irradiation [96]. Most of the genes involved in the biosynthesis of spinosyn are found in a contiguous 74 kb region of *S. spinosa* genome [55]. Gene duplication was also been done to increase the spinosyn yield significantly [97] which is about 288 times higher than the parent [98]. Metabolic engineering of the spinosyn gene cluster yielded 21-cyclobutyl-spinosyn A and 21-cyclobutyl-spinosyn D, which have enhanced insecticidal action against cotton aphid and tobacco budworm than that of spinosyns A and D [99].

5 Biotechnological and Commercial Implications of Pesticidal Metabolites of Microbial Origin

Plant genetic engineering paves a way for insect-resistant plants by insertion and expression of entomopathogenic proteins in the plants itself. The most successful and widely used biotechnological approach for pest management is the transgenic plants expressing insecticidal Cry proteins derived from *Bacillus thuringiensis*. The crystal (Cry) and cytolitic (Cyt) proteins of Bt are reported to be active against insects of different orders, Lepidoptera, Coleoptera, and Diptera, and also against other invertebrates such as nematodes. *Bacillus thuringiensis* produces insecticidal crystalline inclusions which are made of protoxins while sporulation. Genetic engineering helps in the transfer and expression of *B. thuringiensis* genes in the plants to protect them from any kind of target insect infestation. Since the inception of transfer of Bt genes in tobacco and tomato during 1987, it is been transferred into cotton, rice, maize, etc. with Lepidoptera as the main targets. Later, desired genes were synthesized partially or totally, in which the nucleotide sequence was modified with no alteration in amino acid sequence. Transgenic tobacco plants expressing *B. thuringiensis* toxins were experimented in the fields. In 1995, the first transgenic plant of corn (Maximizer™) with CryIA(b) toxin, cotton (Bollgard™) with CryIA(c) toxin, and potato (Newleaf™) with CryIIIA toxin was approved for commercial purpose [100]. Vegetative insecticidal protein (VIP) from Bt was also put into the genetically engineered plants to confer broader resistance against insect pests, viz., rice plants [82], thus serving as a potential alternative to Cry proteins. Corn plants with insecticidal protein isolated from *Pseudomonas chlororaphis* were found

effective against western corn rootworm which are reported resistant to cry toxins [101].

Development of insecticide resistance and public awareness are the major concerns about the continuation of traditional chemical pesticides. This necessitates the use of novel pesticidal compounds of which tackle both these issues. The commercial formulations of microbial origin metabolites, avermectins [56], and spinosad [54] have been established as potential protective pesticides with diverse group of target pests. Both these pesticides are recommended by the World Health Organization as safe and even recommended for pest management in organic agriculture. The commercial formulations of other actinomycete metabolites like milbemycin (Matsuguard, Koromite, Milbeknock, etc.) and polynactins are available in market as potential miticides [102]. This alleviated the interests on screening of pesticidal metabolites from microorganisms. Besides, due to their novel modes of action, they are recommended against resistant populations also [54]. This story comprehends the potential of pesticidal metabolites of microbial origin, and their putative chemical structures can be developed into a biorational pesticide to complement and even substitute the conventional chemical pesticides [8]. In addition, the novel site of action may become a target for further improvements in traditional pesticide developments.

Being living agents, microbials may alter native microflora. Sometimes, complete replacement of native pathogenic species may be possible. But these issues may not arise with the metabolites as pesticides, as they are the inert chemical compounds with respect to nontarget species including microflora. Due to their high target specificity and biodegradability, no bioaccumulation is possible.

6 Future Prospects and Conclusions

The positive public perception and acceptance of microbial pesticides due to their safety to nontargets made them an ecofriendly and sustainable strategy against a variety of agricultural pest problems. In the United States, the growth rate of commercial biopesticides is projected to be 17% of CAGR from 2016 to 2022 as against 3% CAGR of conventional chemical pesticides [103]. As a whole, the bacterial biopesticides claim major share (74%) followed by fungal (10%) and viral (5%) pesticides [104]. Although the bacterial pesticides contribute major share, majority of the products are based on *B. thuringiensis* only. In addition to these commercialized products, there exists a vast literature reporting new species of entomopathogens and strains of existing species with improved bioactivity. We suppose that unavailability of commercial products of these isolates might be due to lack of environmental competency data, problems in mass multiplication and formulations, etc. In addition, a true transformational technology interventions are not yet achieved to realize the full potential of biopesticides and the associated bioactive metabolites [105]. These problems can be conquered by utilization of pesticidal secondary metabolites either directly or by their chemical analogues which are easy to handle. These molecules have been evolutionarily selected over

millennia to enable microbes to interact with their environments. Besides, these compounds might have good environmental fitness due to their immediate pesticidal activity and ready biodegradability. However, the identification, characterization, further improvements, formulation, pesticidal evaluation, and safety analysis of these secondary metabolites from diverse entomopathogens require an interdisciplinary approach involving pest scientists, biotechnologist, organic chemist, environmental scientist, etc. In view of the importance of pesticidal microbes and metabolites, a centralized facility might tackle all these issues and facilitate the availability of microbial biopesticides to cater the needs of diverse pest problems.

A survey of recent literature revealed identification and characterization of many new bioactive genes and metabolites. But majority of the studies failed to understand the actual biophysical and biochemical changes they incur in host tissues. Understanding these induced changes and pathogenicity mechanisms may help in the interpretation of novel target sites for pesticidal activity. Some metabolites of pesticidal pathogens, although not directly involved in pathogenicity, synergize the pesticidal activity. For example, chitinase-producing strains of *B. thuringiensis* have greater toxicity than nonproducers [61]. Identification of these compounds may open new avenues in bio-synergism with greater efficacy. Similarly, adhesion, penetration, and nutrient uptake by contact pathogens are some of the poorly understood issues which may yield bioactive enzymes. Above all, patenting of novel pesticidal toxins and secrecy of industry have made some potent pesticidal metabolites unavailable and under progressed.

Omura [106] edited a book that describes different strategies and methodologies for screening of bioactive microbial metabolites after which many molecular and biological screening techniques came into picture. In general, majority of the pesticidal metabolites identified are based on conventional, biological, and sometimes chemical screening of pesticidal activity [7]. Differences in screening procedures may overlook some important metabolites, as production is a function of existing nutrient environment. In general, majority of secondary metabolites are repressed during logarithmic growth and is depressed during the suboptimal or stationary growth phases. Sometimes they are inducible as well. In such situations, generalized screening procedures may not portray all the capable bioactive compounds of a given bioagent. So a set of diversified screening techniques may be adopted to understand the complete set of metabolites involved in pathogenicity and mortality.

Besides pathogenic microorganisms, different pest-associated microorganisms like gut symbionts are in direct relation with host biology and physiology. Any induced disturbances to this relationship are lethal to the pest. Similarly, plant growth-promoting endophytes influence the pest invasion and biology by different classes of secondary metabolites (aliphatic compounds, peptides, phenylpropanoids, alkaloids, polyketides, and terpenoids). Understanding the biology, genetics, and biochemistry of these compounds may open novel possibility of pest management. Some of the microbial metabolites are claimed as "biostimulants" which induce or aggravate plant defense, thereby offering protection against diverse pest species. This multifaceted pest management strategy may also contribute to consistent production systems.

Insecticide resistance development, withdrawal or de-registration of many synthetic pesticides, public awareness about environmental issues, etc. direct crop protection toward sensible pest management practices. In view of this, interest and importance of pesticidal metabolites of microorganism are growing significantly. In general, the present-day pesticide chemistry is progressed by unexpected discoveries but it now needs an enhanced interest. Increasing number of publications on genome-wide analysis of microbial pesticides may answer the long-standing questions about pathogenesis besides revealing metabolic complexes involved. So a concrete knowledge of organic chemistry and basic sciences coupled with interdisciplinary approach on pesticidal metabolites of microbial origin with commercial product facet may overcome issues related with good quality market products of biopesticides.

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