Progress in Biological Control

Jean-Michel Mérillon
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Plant Defence: Biological Control

Second Edition

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Biological control of pests, weeds, and plant and animal diseases utilising their natural antagonists is a well-established but rapidly evolving field of applied ecology. Despite its documented applications and systematic development efforts for longer than a century, biological control still remains a grossly underexploited method of pest management. Its untapped potential represents the best hope to providing lasting, environmentally sound, and socially acceptable control of most problem pests in agriculture, and of invasive alien organisms threatening global biodiversity. Based on the overwhelmingly positive features of biological control, it is the prime candidate in the search for reducing dependency on chemical pesticides. Public demand for finding solutions based on biological control is the main driving force in the rapid developments in the various strategies of utilising natural enemies for controlling noxious organisms. This book series is intended to accelerate these developments through exploring the progress made within the various aspects of biological control, and via documenting these advances to the benefit of fellow scientists, students, public officials, and the public at large. Each of the books in this series is expected to provide a comprehensive, authoritative synthesis of the topic, likely to stand the test of time.

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Preface to Second Edition

We are pleased to present the second revised edition of *Plant Defence: Biological Control*. Production of a second edition in itself is evidence that the first one was a great success. The work on biological control agents is preferred due to their environmental friendly role. Firstly, the use of biodegradable and eco-friendly control agents is a necessity as continuous long-term use of chemical pesticides has detrimental effect on soil health and consequently residue present in plants has long-term effect on human health. Secondly, search for safe alternative agents like biological control of diseases still continues. This endeavour this is a timely compilation of state-of-the-art information dealing with various aspects of biological control agents. The present book is organised into four parts: I. Biology of Plant Defence, II. Use of Natural Compounds, III. Use of Biological Agents, and IV. Market and Commercialization. Most of the chapters are new and a few are updated.

The book will be useful to upper students studying crop protection, agricultural sciences, applied entomology, plant pathology and plant sciences. Biological and agricultural research scientists in biotechnology, forestry, plant pathology and post-harvest technology, crop management and environmental sciences, agrochemical and crop protection industries, and in academia will find this book very useful. The editors wish to thank all contributors as well as the staff at Springer for their cooperation in completion of this book.

Bordeaux, France — Jean-Michel Mérillon

Udaipur, India — Kishan Gopal Ramawat
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Preface to First Edition

Approximately 6.6 billion humans inhabit the Earth. Notably, the human population has grown nearly tenfold over the past three centuries and has increased by a factor of four in the last. Therefore, demand for food, feed and fodder is ever increasing.

Plant diseases worldwide are responsible for billions of dollars' worth of crop losses every year. Productivity of crops is at risk due to the incidence of pests, pathogens and animal pests. Crop losses to pests can be substantial and may be reduced by various control activities. Estimates of crop loss are available for major food and cash crops at the world level. Among crops, the total loss potential of pests worldwide varies from 25 to 40%. Globally, enormous crop losses are caused by plant diseases, which can occur from the time of seed sowing to harvesting and storage. Important historical evidences of plant disease epidemics are Irish Famine due to late blight of potato (Ireland, 1845), Bengal famine due to brown spot of rice (India, 1942) and Coffee rust (Sri Lanka, 1967). Such epidemics have left their effect on the economy of the affected countries and deep scars in the memory of human civilization.

Plant diseases, caused primarily by fungal and bacterial pathogens, cause losses to agricultural and horticultural crops every year. These losses can result in reduced food supplies, poorer quality of agricultural products, economic hardship for growers and processors, and ultimately higher prices for consumers. For many diseases, traditional chemical control methods are not always economical nor are they effective, and fumigation as well as other chemical control methods may have unwanted health, safety and environmental risks.

Biological control involves use of beneficial microorganisms, such as specialised fungi and bacteria, to attack and control plant pathogens and diseases they cause. Biological control offers an environmental friendly approach to the management of plant diseases and can be incorporated into cultural and physical controls and limited chemical uses for an effective integrated pest management system. Due to the high cost of synthetic pesticides and concerns over environmental pollution associated with the continuous use of these chemicals, there is a renewed interest in the use of botanicals and biological control agents for crop protection. Benefits and risks are always associated with new technologies and their utilization. These types

of considerations have encouraged microbiologists and plant pathologists to gain a better knowledge of biocontrol agents to understand their mechanism of control and to explore new biotechnological approaches to induce natural resistance. This book provides a comprehensive account of interaction of host and its abiotic stress factors and biotic pathogens and development of biological control agents for practical applications in crops and tree species from temperate to subtropical regions. The contents are organised into the following parts:

- General Biology of Parasitism
- Applications of Biological and Natural Agents for Disease Resistance
- Host Parasite Interaction
- Mechanism of Defence

The chapters have been written by well-known researchers in their field.

The book is primarily designed for use by upper undergraduates and postgraduates studying crop protection, agricultural sciences, applied entomology, plant pathology and plant sciences. Biological and agricultural research scientists in biotechnology, forestry, plant pathology and post-harvest technology, crop management and environmental sciences, agrochemical and crop protection industries, and in academia will find this book very useful. Libraries in all universities and research establishments where agricultural and biological sciences are taught should have multiple copies of this very valuable book on their shelves. The editors wish to thank all contributors as well as the staff at Springer for their cooperation in completion of this book.

Bordeaux, France — Jean-Michel Mérillon

Udaipur, India — Kishan Gopal Ramawat
April, 2011

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Biology of Plant Defence

Chapter 1
Biological and Molecular Control Tools in Plant Defense

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1.1 Introduction

A major challenge of humankind is to feed the increasing human population in a sustainable manner. If left uncontrolled, herbivorous pests and pathogens can be highly destructive to crops causing significant yield losses, often above 30% [1, 2]. Pesticide application, an important component of the so-called Green Revolution, remains currently the most common method to control key pests and pathogens of

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crops, despite being incompatible with current regulations(e.g. Directive 2009/128/EC) that promote the reduced input of pesticides and the use of non-chemical methods in crop production, a global trend driven by a strong demand for agricultural products with reduced load of chemicals that also contribute to the increasing levels of pesticide resistance in populations of crop pests.

To limit environmental impacts of harmful pesticides and improve agricultural sustainability, a conversion to a new green movement is required [3] taking into account the complexity of the ecological nature of the problem. Novel strategies, complementary and/or alternative to the existing ones are required to control pests and pathogens in the most efficient and environmental-friendly manner. A growing emphasis on biological control tools such as the use of beneficial organisms and/or environmentally friendly (non-GMO) molecular tools is necessary to overcome technical challenges that are crucial in food production and pest/disease control. This has to be achieved with an approach to minimize environmental risks.

To this end, we herein focus on biological control and the theoretical framework underlying plant defense responses against biotic stressors such as herbivorous arthropods and pathogenic microorganisms with the aim to identify biological and relevant molecular tools that could be used to combat harmful key pests and diseases of crops. We further focus on beneficial soil microbes and zoophytophagous predators and present solid evidence about their potential in plant defense induction and in sustainable crop protection. Molecular tools that could be exploited in agriculture are addressed in light of the mechanisms involved in positive interactions among beneficial organisms and plants, resulting in the production/activation of chemicals such as peptides, toxins, anti-digestive compounds and secondary metabolites (e.g. volatiles). In addition, we refer to the development of molecular biopesticides based on RNA molecules designed to selectively downregulate genes involved in pathogenicity of pests and pathogens through RNA interference (RNAi). This chapter ends with a special section on endophytic fungi as a case study of beneficial microbes that display both plant growth promoting and plant protection capabilities.

1.2 Basal Plant Defenses Against Arthropods and Pathogens

To cope with pathogens and herbivorous pests, plants have evolved sophisticated defense mechanisms broadly classified as passive or constitutive and active or inducible (Fig. 1.1). Passive or constitutive defense mechanisms are constitutively expressed and provide protection from initial invasion or attack [4–6]. Against pathogens, these may include physical barriers, such as wax layers [7], cuticle [8] and cell wall [9], as well as preformed chemical compounds with antimicrobial (generically called phytoanticipins) and lytic effects [4, 10]. If these preformed barriers are overcome, pathogens can still be confronted by inducible host plant defense mechanisms, which prevent further colonization or pathogen spread [4]. Similarly, arthropods are confronted with an array of constitutive and/or inducible plant

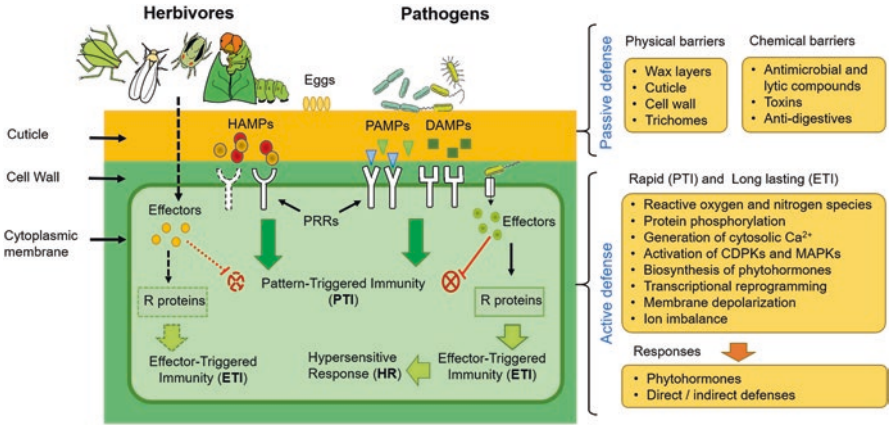

Fig. 1.1 Global overview of plant defense responses against herbivores and pathogens. Herbivore-, pathogen- or damage-associated molecular patterns (HAMPs, PAMPs and DAMPs, respectively) are recognized by pattern recognition receptors (PRRs) and lead to pattern-triggered immunity (PTI). Effector-like molecules from herbivores and pathogens can suppress PTI and result to effector-triggered susceptibility (ETS). The recognition of these molecules by plant resistance proteins (R proteins) lead to effector-triggered immunity (ETI) that, in the case of pathogens, often culminates in hypersensitive response (HR). Uncharacterized elements are indicated by dashed lines. Defense mechanisms (passive and active defense) operating during herbivore attack and pathogen infestation are indicated on the right

defenses such as physical traits (trichomes, wax layers, etc.) and chemicals (toxins, anti-digestive compounds, secondary metabolites) that aim at killing, deterring or retarding the population growth of pests [5]. Plants can also defend themselves indirectly by emitting volatile compounds that attract the natural enemies of herbivores [11, 12]. As with pathogens, inducible plant defenses against herbivores are initiated upon recognition of the attacker and downstream activation of defense signalling [13, 14]. Compared to constitutive defenses, induced plant responses are considered to be cost-saving, preventing auto-intoxication and more advantageous as they can be tailored to the attacker after specific cues recognition by the plant [15–17].

1.2.1 Pathogen Perception by Plants and Defense Induction

The first defensive line of plant immunity relies on the perception of pathogen- or damage-associated molecular patterns (PAMPs and DAMPs, respectively) by receptors called pattern recognition receptors (PRRs) localized on the plant plasma membrane [18] (Fig. 1.1). All plant PRRs identified to date belong to receptor-like kinases (RLKs) or receptor-like proteins (RLPs) [19]. RLKs are proteins with an extracellular domain involved in the perception of signal molecules (*i.e.*, PAMPs/DAMPs), and additionally of a transmembrane domain and an intracellular kinase

domain, which amplify or transduce these signals into the cell, respectively [20]. RLPs have a similar structural organization but lack the intracellular kinase domain [20]. Recent studies suggest that sensing of PAMPs/DAMPs could be also happening through membrane lipids [21]. PAMPs comprise a diverse array of structural components of the pathogen, such as bacterial flagellin, fungal cell wall-derived chitins and glucans, as well as pathogen-specific lipopolysaccharides, proteins, peptidoglycan, elongation factors (*e.g.*, EF-Tu) or microbial nucleic acids [19, 20, 22]. DAMPs are molecules of plant origin released upon pathogen-induced cell damage, and include mainly cell wall or cytosolic proteins, peptides, nucleotides, and amino acids [23].

The recognition of PAMPs/DAMPs by PPRs can activate the immune plant response, a process collectively termed 'pattern-triggered immunity' (PTI) [24]. In this process, a complex network of signalling events is activated, leading to a series of cellular and physiological responses. Such signalling events include, for instance, the rapid generation of cytosolic Ca^{2+} and reactive oxygen species (ROS) or reactive nitrogen species, ion efflux, protein phosphorylation, activation of Ca^{2+}-dependent protein kinases (CDPKs) and mitogen-activated protein kinases (MAPKs), increased biosynthesis of phytohormones, and transcriptional reprogramming [20, 25]. This complex signaling network leads to the establishment of a number of plant defense responses, such as plasmodesmata closure to inhibit molecular exchanges among cells, stomatal closure to limit pathogen entry, production of antimicrobial compounds (*e.g.*, phytoalexins) and generation of ROS either to signal downstream defenses or inhibit growth of pathogens, callose deposition to provide a physical barrier for pathogen attacks, and accumulation of pathogenesis-related proteins such as lytic enzymes (chitinases, glucanases, and proteases) [20].

In general, PTI is sufficient to fight off most pathogens, in particular host non-adapted pathogens [18]. However, some pathogens have developed strategies to evade PTI and for these, plant initiates a second layer of inducible defense, termed as Effector-Triggered Immunity (ETI), resulting in an incompatible reaction [26] (Fig. 1.1). In general, ETI activation results from the intracellular recognition of pathogen effector molecules by plant resistance proteins (R proteins) [26]. These effectors, synthetized by the pathogen and injected into the host cell cytosol, have an important function in pathogenesis [27]; some enhance pathogen virulence and suppress PTI, while others aid pathogens to propagate on their host by reprogramming host cell metabolism and physiology, causing effector-triggered susceptibility (ETS) [27]. Plants, in turn, recognize these effectors by receptor R proteins in a specific manner [28]. Recognition by R proteins can be mediated either through direct physical interaction with the effector (ligand-receptor model) or indirectly by detecting modifications on other host proteins caused by effector activity (guard model) [29]. Most of the R proteins identified so far belong to the nucleotide binding leucine-rich repeat (NB-LRR) type [26]. In comparison with PTI, ETI is a stronger and more efficient response, and often culminates in hypersensitive response (HR), a type of programmed cell death that limits the spread of the pathogen from infection sites [24]. Several studies suggest that ETI utilizes the same defense signalling network as PTI, but in distinct ways, emitting stronger and longer-lasting

responses [29]. In general, ETI restores and amplifies PTI basal transcriptional programs and antimicrobial defences [24]. Both PTI and ETI can induce immune responses against pathogens on uninfected distal tissues [30]. Among the diverse chemical signals identified so far, the plant hormone salicylic acid (SA), has been found to play an important role in systemic resistance that provides broad spectrum and long-lasting protection to future infections [30]. Establishment of systemic resistance involves the generation of signals in the damaged tissue, and their further transport via vascular system to sites further from the injury location.

1.2.2 Herbivore Perception by Plants and Defense Induction

Herbivore-associated molecular patterns (HAMPs) include all herbivore-derived signalling molecules that, when in contact with the host plant, are capable of eliciting defense responses [31, 32]. HAMPs can be elicitors deriving from the herbivores found in their saliva, regurgitant or other secretions such as honeydew and those used for eggs attachment to the plant surface [33–35]. Furthermore, plant-derived DAMPs such as cell wall fragments, or endogenous compounds released upon the disruption of plant tissue during herbivory can be responsible for the elicitation of non-specific plant defense responses [14].

Plants can detect herbivorous arthropods based on their HAMPs. These are presumed to be recognized by receptors leading to PTI [14, 36] (Fig. 1.1). Despite our vast knowledge on different types of PRRs involved in pathogen recognition by plants, to date only a few examples exist for PRRs involved in plant-herbivore interactions [32, 37]. As with the R-gene mediated recognition of effectors in plant-pathogen interactions, indications exist about the evolvement of similar recognition mechanisms underlying plant-herbivore interactions that may lead to ETI (Fig. 1.1); however, much less is known about such effectors and respective plant receptors [36, 37]. Polyphosphoinositides generated at the plasma membrane are believed to act as second messengers just as they do during pathogenesis [38]. Changes in the plasma membrane potential follow ion fluxes across the plasma membrane and afterwards, protein kinase cascades can activate ROS production such as hydrogen peroxide that can have direct effects on herbivores or change cell's redox status. The increase in cytosolic Ca^{2+} can also activate nitric oxide-mediated processes that precede phytohormone (JA) upregulation [39]. These responses occur not only locally but also in distal undamaged tissues. As with pathogens, a complex signalling network modulates the expression of defense-related genes and the production of defensive compounds that are active against herbivores [13]. The phytohormones jasmonic acid (JA) and SA, ethylene (ET) and abscisic acid (ABA) are key regulators in plant defense against herbivores, modulating the expression of defense-related genes and the production of defensive compounds [14, 40]. Cross-talk among the phytohormonal pathways (e.g. JA and SA antagonism) is considered to be fine-tuning plant defenses against specific attackers [41–43].

As with pathogens efficiently evading PTI, many arthropods have evolved a variety of strategies to cope with plant defenses including behavioural adaptations and mechanisms to decrease exposure (e.g. via detoxification or sequestration) or sensitivity (e.g. via target-site sensitivity) to defense compounds [34]. Furthermore, certain herbivores are known to be able to manipulate sink source flows or to suppress plant defenses [14, 34–36, 44]. Similar to pathogen effectors, effector-like molecules from herbivores, specifically those secreted via their saliva into the host plant are presumed to also interfere with PTI and lead to ETS [36, 37]. However, as with HAMPs and PRRs, our knowledge on herbivore effectors is still limited.

1.3 Plant Defense Priming

Plants are surrounded by multiple threats that they must face by responding effectively to survive. After specific attacker's recognition, plants need to re-organize all immune machinery to counteract the attack. The speed and intensity of the response will determine the final output. As described above, at first, plants may use constitutive defense barriers, and if those are not efficient enough, inducible defenses are activated to defeat pathogens and pests. To mount an efficient response, plants need to sense "the non-self". Different stimuli can prepare plants to gain these inducible defenses and set plants' immunity in a manner that they can respond in a shorter time and more efficiently to pathogen/pest attack [45, 46] (Fig. 1.2). Upon perception of appropriate stimuli ('sense of danger') different physiological and molecular changes, timely and quantitatively, prepare defenses for future attacks, resulting in incompatible interactions. Those changes taking place between the sensing of the stimuli and the presence of the challenge are known as the 'priming state' [46] (Fig. 1.2). During this phase, the plant adapts its immune responses by learning from experience.

Distinct stimuli may trigger the priming state, like beneficial organisms, arthropods, pathogens, and avirulent bacteria, as well as chemical compounds or even abiotic cues that may stimulate the production of active compounds. A silent timeframe comes until the challenge shows up (the 'priming phase'). Hence, when the plant is exposed to a subsequent stress, it is sensitized to respond faster and with higher intensity, and this is the so-called 'post-challenge primed state'. In this phase, there is an enhancement in the response following perception of danger and signal transduction. For example, sour orange citrus displays constitutive priming against the two-spotted spider mite *Tetranychus urticae* due to a high level of flavonoids and a faster activation of the oxylipin pathway [47].

Among the different stimuli, there are genes that confer constitutive priming. For instance, a mutation in the gene *NRT2.1* that functions as a transceptor in *Arabidopsis* confers constitutive priming against the pathogen *Pseudomonas syringae* pv tomato DC3000 [48]. The knockdown of *NRT2.1* allows a lower sensitivity to the toxin coronatine, preventing the plant from the effector manipulation. Another example of constitutive priming in *Arabidopsis* is generated by the mutant *edr1* (*ENHANCE*

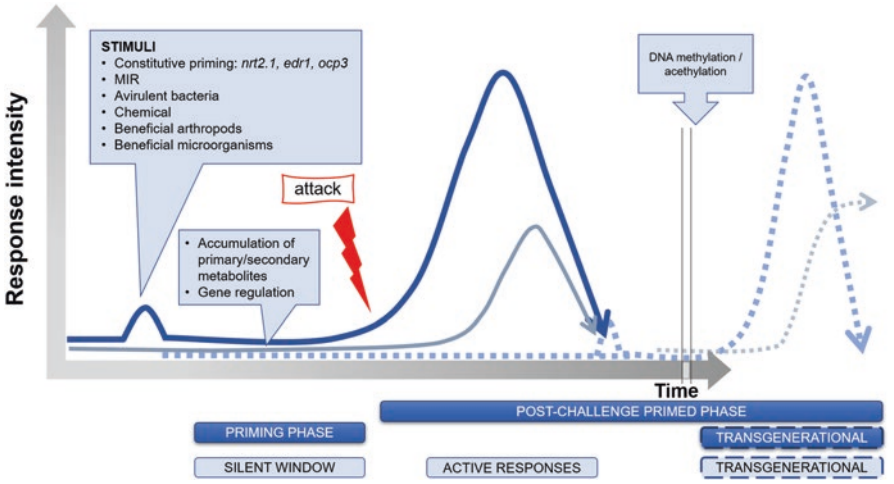

Fig. 1.2 Intervals of action in priming defenses. Different stimuli in plants can produce a transient and small response that tend to equilibrate afterwards. Priming inducers may range from biological (MIR, beneficial microorganisms and arthropods, avirulent bacteria) to chemical (BABA, I3CA) or genetic inputs (for example, downregulation of NRT2.1, OPC3 or EDR1). When plant defenses go to basal levels, a memory window lasts until the threat appears. This period is the so-called 'priming phase'. Along this phase, different players have been described, such as changes in primary and secondary metabolism, although this is dependent on the interaction between the priming inducer and the plant species. Then, after the attack of a pathogen/pest, the post-challenge primed phase starts. At this stage, primed plants (dark continuous blue line) respond faster and stronger to the challenge than non-primed plants (grey continuous line). Different mechanisms may orchestrate and coordinate a horizontal response to overcome the infection/attack. The intensity of the response in the long term depends on the interaction between plant -pathogen/pest- priming inducer, and may be associated with changes in the chromatin and histone modifications. Stressful memories can be transmitted to the offspring (transgenerational phase) through epigenetic modifications if the presence of the stress persists along time (blue dashed line corresponds to response intensity of plants that are still primed and grey dashed line, to the ones that have not been primed before). The dark blue squares show the names of the priming periods of priming and light blue squares show the type of defense responses ("silent", active responses or transgenerational)

DISEASE RESISTANCE1), also displaying priming of ROS and callose accumulation in response to PAMPs [45], and thus being more resistant to *P. syringae* and *Hyaloperonospora arabidopsidis* [49]. Additionally, the mutant *edr1* can also express constitutively two MAPK kinases MPK3–MPK6 that have been associated to priming [50].

Lack of activity of other genes may also confer constitutive priming. This is the case of the *OVEREXPRESSOR OF CATIONIC PEROXIDASE 3 (OCP3),* which mediates the response to necrotrophic pathogens and tolerance to abiotic stress [51, 52]. Mechanisms behind *OCP3* constitutive priming are the accumulation of ROS and the activation of the kinase cascade in a controlled manner, in which a positive interplay between ABA-JA and callose are key elements to mount defense priming. Interestingly, the *Arabidopsis* mutant *vtc1*, which is impaired in the production of

ascorbic acid, also shows constitutive priming of *PR1* and SA [53]. Thus, these genes may function as nodes that balance plant decisions relative to growth, abiotic stress tolerance or resistance to biotic insults. Loss of function mutants of these genes may be constitutively prepared for hyperactivation of defense responses without costs in plant fitness.

1.3.1 Mechanisms Regulating the Priming Phase

Despite the pre-challenge phase has been described in the past as uneventful and without fitness cost, now it is known to be associated with several molecular changes. Subtle changes during that phase may be translated into fitness cost, that it can be compensated by the final result when a threat appears [54]. A plant strategy during this "silent" phase (Fig. 1.2) is the accumulation of hormone and metabolite conjugates that will be hydrolysed to their active form upon a challenge. Following certain priming stimuli such as β-aminobutyric acid (BABA) and avirulent bacteria, the two main glycosylated forms of SA (SAG and SGE) are accumulated [55]. Other glucose conjugates of phytoanticipins also accumulate at this stage, such as the aliphatic and indolic glucosinolates [56] or benzoxazinoids [45], which are sequestered in the vacuole allowing their faster release upon pathogen/herbivore attack.

An open debate is whether changes and induced resistance by beneficial organisms may be mediated by defense priming [54] (TIPS). Among them, Arbuscular Mycorrhizal Fungi (AMF) were shown to protect a wide range spectrum of plant species against pathogen insults [57]. Reasonably, since AMF symbiosis and interactions with beneficial microorganisms take place before the challenge, there are obvious metabolic changes in the symbiont. Mycorrhiza-Induced Resistance (MIR) is a particular defense priming since in the priming phase, there is a whole molecular and metabolic dialogue between the plant and AMF leading to the symbiosis. In fact, priming during MIR is under consideration since it may be tissue dependent. MIR is effective against several root and foliar pathogens and current studies aim to elucidate the changes in the priming phase related to MIR.

Since carbon–rich compounds, amino acids and lipids are the main metabolites exchanged between AMF and the host plant, AM symbiosis is expected to impact primary metabolism. Several metabolites related to carbon metabolism were accumulated in AM-*Lotus japonicus*plants before challenge [58]. Tomato plants colonized by *Rhizoglomus irregularis* (formerly *Glomus intraradices)* showed enhanced OPDA content and up-regulation of *LOX-D* gene expression level in the priming phase [59]. Changes in the pre-challenge priming state usually targets the primary metabolism, such as sugar and amino acid pathways, not only in AM priming but also with other priming stimuli. Using qPCR and mutant approaches, an ABA-dependent regulation of starch degradation after BABA and I3CA priming was shown [60], and the sugar-derivative glycerol-3-phosphate has been reported as a key signal in the azelaic acid-induced systemic immunity and priming [61].

Amino acids are the precursors of many secondary metabolites that can participate in the subsequent defense responses. Pastor et al. [62] reported changes in *Arabidopsis* primary metabolism, mainly in tricarboxylic acid (TCA) metabolites such as citrate, fumarate, malate and 2-oxoglutarate as well as an enhanced biosynthesis of phenylpropanoid pathway following BABA priming before challenge. In the same study, authors compared changes occurring after BABA and *P. syringae* pv tomato (*PstAVRpt2*) priming treatment and found that pathways that were up-regulated after BABA priming were repressed after *PstAVRpt2* treatment. BABA is a water-soluble chemical compound that is rapidly distributed throughout the plant while the bacteria use the plant sensing mechanisms to coordinate the interaction between themselves and the plant. The different responses to these two priming stimuli recorded by the authors, highlighted that not only plant species but also the nature of the stimulus is important for the priming response. Hence, priming is a horizontal phenomenon that triggers multiple metabolic pathways shortly after infection/attack, resulting in enhanced defensive responses.

1.3.2 Mechanisms Regulating Post-Challenge Primed State: Internal and External Strategies

The spatiotemporal input of priming has been recently revisited as the 'internal' and 'external' strategies of plant defense [63]. As part of the internal plant defense responses, priming is a mechanism regulating the boosted defense reaction upon challenge along with systemic acquired resistance [46]. This internal response in primed plants, the so called 'post-challenge primed state', ranges from hours after challenge to longer period, which may also be extended to the progeny [46, 64, 65] (Fig.1.2). This transgenerational, epigenetically regulated defense priming may be fixed along evolution terms by genetic adaptations, leading to ETI. Conversely, defense priming regulates boosted responses during the external strategies that are based, on the one hand, on interactions with microbes at the root or shoot level that trigger the well-known induced systemic resistance [46, 66] (ISR) and, on the other hand, on recruitment of natural enemies, the so-called 'induced indirect defense'. During herbivory, VOCs are released within the first few hours after attack and attraction of natural enemies takes place at shorter term [67]. In a longer term, priming by beneficial microbes leads to the formation of disease-suppressive microbiomes [68, 69] that may protect plants through antibiosis, competition and induced resistance [70–72].

As regards internal strategies, several mechanisms were shown to be involved during the post-challenge priming state (Fig. 1.2). One of the first responses of primed plants after PAMPs perception is stronger production of H_2O_2, preceding an earlier and stronger callose accumulation [45]. Surprisingly, primed plants that are effectively protected by this battery of early responses do not trigger, or even down regulate, subsequent immune responses [73]. When the activation of subsequent

defensive layers is required, in addition to the biosynthesis of phytohormones that is costly and takes longer time, primed plants were also shown to target signaling cascades in a non-costly manner as a fast and strong immune response. For example, priming activates a subset of glycosyl hydrolases releasing active forms from inactive glycosylated hormones [48, 55, 74] while, Beckers et al. [75] defined an enhanced accumulation of non-active MPK3 and MPK6 in primed plants that were rapidly phosphorylated once the challenge was present triggering much faster *PR1*, *PAL* gene transcription and other SA-dependent responses. The accumulation of a specific set of secondary metabolites defined as the 'priming fingerprint' is described as one of the latest short-term responses of primed plants [76]. Primed defenses are defined as a horizontal plant response that is dependent on the plant-stress interaction. The range of mechanisms implicated in the long-lasting defense response entails an effort from the scientific community, and different laboratories are tackling the basis of mechanisms behind epigenetic changes and transmission of priming defenses to the offspring, against biotic and abiotic stress. Nevertheless, still further research is needed to gain knowledge in this area from the molecular level to higher scale for practical use in agriculture.

1.3.3 Transgenerational Priming State

As time following the 'post-challenge priming state' progresses, the direct, hormonal-regulated immune responses decay in intensity and epigenetic mechanisms start being more relevant [45, 46] (Fig. 1.2). One of the first reports describing chromatin remodeling as a long-term priming and SAR was proposed by Jaskiewicz et al. [77]. SAR-related priming was associated with relaxed density of the chromatin that increased methylation and acetylation of histones packing WRKY promoters. This histone modification leads to a faster gene transcription following a pathogen or herbivore attack and a subsequent faster and more efficient defense response. Following this pioneer publication, shortly after, increasing evidence of DNA methylation associated with heterochromatin [78] was shown to be involved in long-term priming [79]. In this latter work, the primed expression of WRKY and SA-dependent genes was regulated via the RNA-directed DNA methylation pathway. Later, transgenerational priming and SAR-associated priming were shown to be regulated in the progeny of primed plants by epigenetic changes [80, 81]. Noteworthy, transgenerational priming is not only functional in SA-dependent immune responses but also in JA-dependent defenses against insect attacks [82].

1.3.4 Induced Indirect Resistance

The so-called 'external strategies' of plants are long been known. The study of beneficial insects that are attracted by plants following herbivory can be useful in Integrated Pest Management (IPM) programs. Plants in response to HAMPs release HIPVs that improve the recruitment of beneficial arthropods [67, 83]. Importantly, external strategies of plants can be enhanced via priming, for example, when plants are exposed to appropriate stimuli. In fact, several interesting studies in phylogenetically distant plant species such as maize and citrus show similar outputs when susceptible plants are exposed to VOCs [84, 85]. Maize plants exposed to VOCs released by plants treated by caterpillar regurgitant were more efficient to mount effective defenses against *Spodoptera littoralis* [84]. In addition, maize plants primed with VOCs were more attractive to the parasitic wasp *Cotesia marginiventris* while control plants and plants only treated with VOCs did not result in a significant attraction. Similarly, mite-susceptible citrus genotypes can express resistance after priming by VOCs released by resistant citrus attacked by the spider mite *T. urticae*. VOCs-mediated priming results in enhanced resistance against spider mites and priming of JA-dependent responses [85]. Thus, priming against herbivores, either by stimulating direct (internal) or indirect (external) defenses, is another example of adaptive immune responses of plants [86, 87]. Notably, plants are not only able to be attractive to aboveground beneficial arthropods but also to beneficial microbes present in the rhizosphere [88]. It is well-known that plants exposed to phosphorous deficiency are more attractive to mycorrhizal fungi by the release of strigolactones at the very early stages of the mycorrhizal symbiosis, which at a later stage ends up in MIR that is also mediated via priming [57, 59].

1.4 RNA Interference in Plant Defense

In addition to the aforementioned strategies, plants have developed a powerful nucleotide sequence-specific defense mechanism based on RNA interference (RNAi). RNAi is triggered by double stranded RNA (dsRNA) molecules that are cleaved by DICER-LIKE (DCL) endonucleases into by 20–25-nt small RNA (sRNA) duplexes [89, 90]. One of the two strands of the occurring sRNA duplex associate with ARGONAUTE (AGO) effectors proteins and recognize (1) complementary mRNA for degradation or translational inhibition and (2) cognate DNA for methylation and heterochromatinization [91, 92]. In plants, a plethora of sRNAs regulate development, control genome stability, fine-tune epigenome plasticity, tame transposon activity and mediate pathogen defense [93–96]. Concerning the latter aspect, plant viruses having RNA or DNA genome generate through replication or transcription dsRNA intermediates which are processed by plant DCLs into sRNAs that target the viral RNA genome for degradation and viral DNA genome for methylation [97, 98]. Indeed, it has been proposed that RNAi mechanism in plants

has evolved as a major antiviral defense mechanism [93, 99]. Recently, it has been suggested that RNAi is also involved in antifungal defense, since plants send sRNAs into fungal pathogens in order to target essential fungal genes, as cotton does against *Verticillium dahliae*, Arabidopsis against *Botrytis cinerea* and wheat against *Fusarium graminearum* [100–102].

The tremendous gene silencing potential of RNAi has not skipped the attention of plant biotechnologists. During the last two decades, plant scientists have transformed a plethora of plants expressing dsRNAs against various viruses, fungi, oomycetes, insects, mites and nematodes, all resulting in very high levels of plant defense against each corresponding target [98, 103–108]. Common denominator in all these approaches was the use of a transgene consisting of an invertedly repeated cDNA that, upon transcription, would generate dsRNA molecules that would trigger RNAi against the selected target. However, since the use of transgenes, transgenic plants and genetically modified organisms (GMOs) in general have been met with considerable public and scientific concern, plant biologists have lately resorted to GMO-free RNAi approaches by simply exogenously applying dsRNAs and sRNAs inplants against various pests and pathogens using methods such as high-pressure spraying and trunk injection [109–111] (Fig. 1.3). RNAi-based biopesticides, consisting solely of dsRNA and/or sRNA molecules, could exhibit an extremely specific mode of action since they require only 21 nt homology with their target, thus aiming specific regions of specific genes in specific species, practically eliminating undesired off-target effects. Importantly, according to the 40th annual meeting of the Toxicology Forum, the exogenous application of RNA molecules pose no threat to human health even when present in diet [107]. Not surprisingly, the non-GMO, non-toxic and highly specific character of RNA-based tools has rendered them a vital importance in modern crop protection platforms [112, 113].

1.5 Exploiting Biological and Molecular Tools in Plant Defense

1.5.1 RNA-based Strategies Against Viruses, Viroids, Fungi and Insects

Viruses cause epidemics on almost all agronomical important crops, posing a serious threat to global food security and being responsible for yield losses roughly estimated to cost worldwide more than 30 billion USD annually [114]. Most plant viruses exhibit a single stranded RNA genome and replicate in plant cell cytoplasm through dsRNA intermediates, thus serving as targets for host RNAi machinery. Hence, a well-established strategy involves pre-treating of plants with dsRNAs/sRNAs designed to target specific viral regions (e.g. coat or movement protein) in order to resist imminent viral infection (Fig. 1.3). Indeed, leaf spraying and/or mechanical inoculation of RNAi molecules targeting viral sequences resulted in

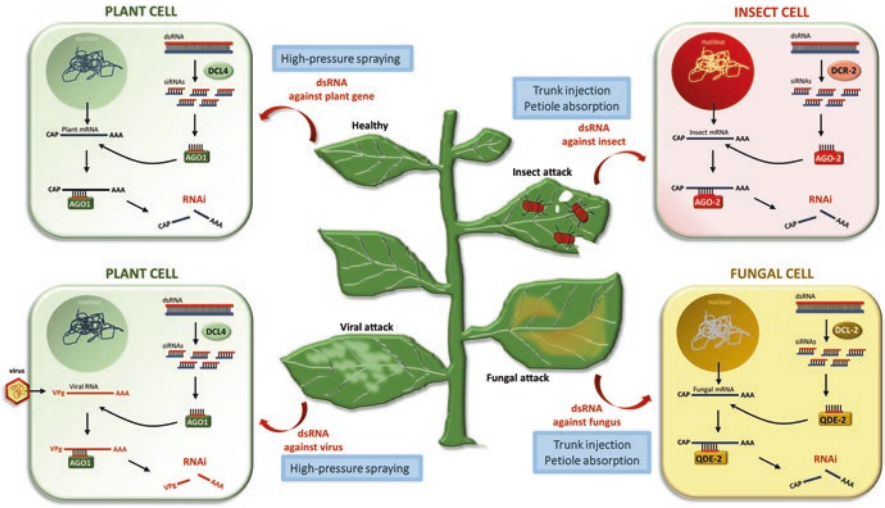

Fig. 1.3 Transgene-free RNA-based molecular control tools in plant defense involve the exogenous application of in vitro and/or in vivo transcribed dsRNA molecules in plants with the objective to trigger RNAi against (1) plant/weed genes, (2) viruses/viroids, (3) fungi/oomycetes and (4) insects/mites. In cases (1) and (2), the exogenously applied dsRNA needs to be efficiently taken up by the plant cell in order to be processed by plant DCLs into siRNAs that will target for degradation the corresponding transcripts in the cytoplasm. To achieve efficient delivery inside the plant cell, the dsRNA needs to be applied by high-pressure spraying which allows the mechanical disruption of the plant cell wall. In cases (3) and (4), the exogenously applied dsRNA is supposed to trigger RNAi not inside the plant cell but inside the fungal and/or insect cell. To increase RNAi efficiency inside the fungal and insect cells, the applied dsRNA needs to avoid processing by plant DCLs and, instead, be processed solely by the fungal or insect Dicers into siRNAs which will target the corresponding fungal or insect mRNAs for degradation. To achieve this, the exogenous dsRNA needs to be applied by trunk injection and/or petiole absorption, since by these two methods the dsRNA is transported exclusively through the plant xylem and apoplast (where no plant DCLs are present) to distant tissues and are thus accessible to be taken up by the plant tissue-penetrating fungi and by the chewing and/or xylem sap-feeding insects. However, trunk injection and petiole uptake are not suitable in the case of phloem-sap feeding insects (e.g. aphids) since in that case the xylem-residing dsRNA would be inaccessible to them. In the latter case, high pressure spraying of dsRNA would be more advisable, since it allows the symplastic delivery of RNA molecules to systemic tissues. Image adopted by permission from Dalakouras et al. [110]. Copyright American Society of Plant Biologists

significant viral resistance (1) in *N. benthamiana* (against Pepper Mild Mottle Virus, Tobacco Etch Virus, Alfalfa Mosaic Virus, Tobacco Mosaic Virus), (2) in *N. tabacum* (against Tobacco Mosaic Virus, Potato Virus Y, Cucumber Mosaic Virus), (3) in *Cucumis sativus* (against Zucchini Yellow Mosaic Virus), (4) in *Vigna unguiculate* (against Bean Common Mosaic Virus), (5) in *Zea mays* (against *Sugarcane Mosaic Virus*), (6) in *Carica papaya* (against Papaya Ringspot Virus) and (7) in *Pisum sativum* (against Pea Seed-borne Mosaic Virus) [115–124]. Closely related to viruses are viroids which are non-encapsidated, non-coding, circular, single stranded RNA pathogens [125]. Similar to antiviral applications, mechanical inoculation in

Solanum lycopersicum, *Gynura aurantiaca* and *Dendranthema grandiflora* leaves of dsRNAs targeting regions of potato spindle tuber viroid, citrus exocortis viroid and chrysanthemum chlorotic mottle viroid, respectively, resulted in considerable resistance of these plants to the corresponding viroids [126].

Fungal pathogens are responsible for devastating crop diseases worldwide. According to a *Molecular Plant Pathology* survey, the 'top 10' fungal plant pathogens list includes, in rank order, *Magnaporthe oryzae*, *Botrytis cinerea*, *Puccinia* spp., *Fusarium graminearum*, *Fusarium oxysporum*, *Blumeria graminis*, *Mycosphaerella graminicola*, *Colletotrichum* spp., *Ustilago maydis* and *Melampsora lini* [127]. It is thus of utmost importance that novel, sustainable-but-effective tools are developed against these pathogens. RNA-based approaches could play here a foremost role as well (Fig.1.3). However, as precondition, it needs to be ascertained that the target-fungus under consideration contains an active RNAi machinery; notably, *Saccharomyces cerevisiae* and *Ustilago maydis* lack RNAi components and thus cannot serve as targets for RNA-based approaches [128]. Nevertheless, most fungi do encode DCLs and AGOs and even RNA-dependent RNA polymerases and are thus susceptible to RNAi. Indeed, exogenous application of RNAi molecules in (1) *Hordeum vulgare* (against *Fusarium graminearum*), (2) *Triticum aestivum* (against *Fusarium asiaticum*), (3) *S. lycopersicum* (against *B. cinerea*) and (4) *Brassica napus* (against *Sclerotinia sclerotum*) compromised fungal infection in these plants [129–132].

But perhaps the most important implications of exogenous RNAi reside in insect management (Fig. 1.3). Similar to antifungal approaches, the applied RNA needs to be delivered inside the insect cell. Yet, this is not as straightforward as it may seem. The uptaken (by the insect) RNA needs to survive the salivary nucleases in the midgut and haemolymph, absorbed by epithelial cells and systemically spread in order to trigger homogeneous RNAi of an essential gene throughout the insect body. Yet, despite these negative prospects, such a task is indeed feasible. Thus, (1) when dsRNA designed to target arginine kinase of *Diaphorina citri*, *Bactericera cockerelli* and *Homalodisca vitripennis* was injected in the trunk of *Citrus aurantifolia* and *Vitis vinifera*, it suppressed the corresponding pest populations [133]. Similarly, pest mortality was observed when (2) sRNAs targeting the *Plutella xylostella acetylcholine esterase* were sprayed in *Brassica oleracea*; (3) dsRNA targeting the *Diabrotica virgifera vacuolar ATPase* was applied *in S. lycopersicum*; (4) dsRNA targeting *Nilaparyata lugens P450* was root-absorbed by *Oryza sativa* roots; and (5) dsRNA targeting the *Tuta absoluta vacuolar ATPase* was absorbed by *S. lycopersicum* petioles [126, 134–137]. The prevailing assumption is that coleopterans are the most susceptible to exogenously applied RNAi, while lepidopterans and hemipterans are significantly resistant to it, seemingly because lepidopterans restrict the absorbed dsRNA to endocytic compartments, and hemipterans inject nucleases into the plant tissue before feeding [138]. However, the use of liposomes, chitosan nanoparticles, cationic core-shell nanoparticles, and guanylated polymers promise to significantly increase dsRNA stability in such applications [139, 140]. Overall, RNA-based plant defense approaches are highly promising pest and pathogen

control methods, complementary to plant resistance strategies, such as induced defense and priming.

1.5.2 Priming-based Biological Control and Induced Resistance: Applied Aspects

Knowledge on priming during the last 5–6 years has grown exponentially and many published studies have paid attention to the mechanisms underlying this adaptive immune response [46, 63, 141]. Most studies focus on model plant species covering fundamental aspects of priming and, research in applied aspects of priming in common crops has received much less attention. Reasonably, since the availability of molecular tools in common crops is less abundant, most research data of priming in crops such as potato, wheat, barley, cowpea or citrus refer to yield improvement, disease phenotypes or pest resistance and sometimes, hormonal or metabolic imbalances during post-challenge primed state [46, 87, 142, 143].

Accordingly, our knowledge on the mechanisms underlying biocontrol priming in crops is scarce. In many cases, the application of the triggering priming agent, either a chemical or a beneficial organism, is reported not to display a benefit on crop growth, until a disease infestation or insect attack. In barley, it was shown that saccharin treatments did not increase plant growth, although primed plants increased grain yield in the presence of the fungus *Rhynchosporium secalis* [144]. Seemingly, plant colonization by AMF has rather variable outputs in terms of growth [145]. Despite these limitations, the low or non-existent benefits of priming sensing during the priming phase counterweights the benefits following disease or insect attack.

In semi-field experiments, priming triggered by mycorrhizal symbiosis was shown to be functional in potato against the herbivore *Trichoplusia ni* [142]. Although mycorrhization had no effects on potato growth, it effectively reduced larval weight that may be explained by enhanced JA-dependent responses. In studies on priming in citrus trees, sour orange rootstock was found to display constitutive priming against spider mites [47, 85]. Interestingly, rootstock resistance is transmitted to the scion, therefore these findings can be applied to commercial varieties to stimulate plant immunity in the field. Another unexplored field aspect is the improvement of IPM strategies by using citrus plants that are more attractive to natural enemies. Recently, it was shown that sour orange recruits more efficiently the generalist predatory mite *Euseius stipulatus* that may improve the efficiency of pest control in agriculture [146]. Priming has also been shown in a context of treatments with natural extracts such as mint volatiles that were proven to confer enhanced defenses in field trials on soybean against both the herbivore *Spodoptera litura* and the fungus *Phakopsora pachyrhizi* [147]. Therefore, defense priming known as ‘green vaccination’ has been proposed as the perfect match to IPM strategies which, following appropriate field experimentation, could be transferred to applied science [148].

1.5.3 Priming Induced by Beneficial Organisms

Beneficial microbes belonging to the rhizosphere are known to induce resistance against a broad spectrum of pathogens and pests. Root-associated microorganisms that colonize root surfaces, or those that may enter the host tissue, can also sensitize plants against aboveground pathogens or pests systemically, via ISR [66, 149–151]. The rhizosphere contains the major part of the microbiota of plants, and part of the microbial community is involved in plant growth stimulation via plant growth-promoting microorganisms (PGPM) and in boosting the plant immune system thus, impacting plant health [152–154]. Best known beneficial microorganisms include different phyla of the bacteria *Actinobacteria, Proteobacteria* and *Firmicutes* [152, 155] and fungi, such as Ascomycota (*Trichoderma* sp.) and Glomeromycota (AMF) [156–158].

The interaction of microorganisms in the rhizosphere with plant roots is plant-microbe dependent [152]. The establishment of mutualistic symbiosis with mycorrhizal fungi is fine-tuned by the plant, which controls the recruitment and the entrance of the fungi [159]. On the contrary, *Trichoderma* fungi exert nutrient competition, or mycoparasitism in the rhizosphere [160]. Also, *Trichoderma* induce ISR through volatile compounds in the shoots against pathogenic fungi, priming JA responses [161]. The mechanisms behind this sort of induced resistance are SA-independent. Instead, they use the JA/ET dependent signaling to combat aerial attacks, with the overaccumulation of the AP2/ERF family of transcription factors (TF), which has been demonstrated to participate in the regulation of ET/JA-dependent defences [162]. The TF *MYC2* also plays an important role in ISR, since it was discovered to bind in a common site found in ISR-primed genes in *Arabidopsis* [163]. Experiments with *myc2* mutants showed that *Pseudomonas fluorescens* WCS417r and *Piriformospora indica*, two beneficial root-associated microorganisms inducing ISR, were unable to induce resistance against *P. syringae* and *H. parasitica*, pointing to this TF as an essential element in ISR.

Additionally, certain *Fusarium* fungi may be useful for the biocontrol of soil-borne microorganisms and herbivorous pests. For example, *Fusarium solani* strain K (FsK) is a root-restricted endophytic fungal isolate that colonizes tomato roots [164]. In tomato, FsK can confer ethylene-dependent resistance against fungal root and foliar pathogens [164]. FsK-colonized plants were recently shown to be more resistant to plant damage caused by the zoophytophagous predator *Nesidiocoris tenuis*, possibly via the JA and/or ethylene signaling pathways [165] and to the two-spotted spider mite, *T. urticae* [166]. FsK-colonization of tomato plants was shown to result in differential expression of defense-related genes as well as volatile emission in response to spider mite feeding. Notably, FsK colonized plants were more attractive to *Macrolophus pygmaeus*, a natural enemy of spider mites [166]. In addition, certain strains of the soil-borne *F. oxysporum* were shown to be efficient in controlling *V. dahliae* in eggplant through SA-dependent responses increasing the expression of *PR1* [167]. The efficacy in protecting plants by this fungus has been also shown in olive and pepper plants against *V. dahliae* and *Phytophthora capsici,*

by the induction of *PR1* gene among others [168, 169]. Interestingly, the strain *F. oxysporum* 47 (Fo47) could not protect these plants from foliar infection by *B. cinerea*. Perhaps the induction of SA in plants colonized by Fo47 blocks other defenses that influence other diseases. This fungus may act at several levels like the production of VOCs, plant growth promotion, antibiosis and mycoparasitism *in vitro*, induced resistance, also by competition at the root site [170].

Other beneficial microorganisms that are emerging as potential biocontrol agents, are strains belonging to the *Rhizobia* genus. Traditionally, this genus has been considered an essential player in nitrogen fixation and uptake by the plant. Nevertheless, evidence suggests additional roles in plant defense regarding root diseases. *Rhizobium* bacteria can produce and release proteolytic enzymes and parasite fungi in the rhizosphere such as pathogenic strains of *F. oxysporum* [171]. Also, *Rhizobium leguminosarum* strain Rl was able to protect chickpea against the pathogen *F. oxysporum* f. sp. *ciceris* (Foc) [172]. This protection is also present against other microorganisms (bacteria, viruses) and nematodes, via ISR [173]. Additional responses like emissions of antimicrobial VOCs, siderophore production, competition and changes in volatile plant compounds are also contributing to plant defense by *Rhizobium* [173].

Besides beneficial soil microbes, zoophytophagous predators such as the mirids *M. pygmaeus, N. tenuis* and *Orius laevigatus* have been shown to induce plant defenses against herbivorous pests via their phytophagy [83, 174–178]. Exposing plants to *M. pygmaeus* negatively affected the performance of the two spotted spider mite *T. urticae* in tomato and the western flower thrips *Frankliniella occidentalis* in pepper [174, 175, 178]. These negative effects against pests were attributed to the increased accumulation of transcripts and the activity of proteinase inhibitors (PI) in the mirid-exposed tomato plants [175], and to the activation of the JA-related responses in pepper plants [178]. Furthermore, tomato and pepper plants exposed to *N. tenuis*werefound to be more attractive to predator conspecifics [179] and to the parasitoid *Encarsia formosa,* a biological control agent of whiteflies [176]. This indirect plant defense response was related to changes in the volatile blend released by the mirid-exposed plants, via the activation of ABA and JA signaling pathways [176]. Notably, mirid-induced plants were shown to be less attractive to key pests such as the tomato leaf miner *T. absoluta*, the whitefly *Bemisia tabaci*, the western flower thrips *F. occidentalis*and the two-spotted spider mite *T. urticae* [83, 176, 177, 180, 181]. Overall, the above studies suggest that zoophytophagous predators may serve as ‘plant vaccination agents’ at the early stages of the establishment of a crop directly affecting herbivores via predation and indirectly, via the induction of direct and indirect plant defense responses, eventually enhancing their overall biocontrol efficiency [174, 182].

Interestingly, zoophytophagous predators have been recently shown to positively interact with beneficial soil microbes to the benefit of their host plant. The colonization of tomato plants with a root restricted endophyte, the non-pathogenic stain FsK was shown to result in reduced feeding symptoms (necrotic rings on leaves and stems) by the zoophytophagous predator *N. tenuis* possibly via the upregulation of the ethylene and JA pathways [165], and to alter volatile blend emission by tomato

plants and enhance their attractiveness to *M. pygmaeus* [166]. Similarly, *M. pygmaeus* population growth was enhanced on tomato plants colonized by *Trichoderma longibrachiatum* that were also more attractive to conspecifics [183], and similar results were obtained for the AMF *Rhizophagus irregularis* [184]. Finally, inoculation of tomato plants with *Fusarium oxysporum* Fo162 was shown to enhance the efficiency of *M. pygmaeus* to control *T. vaporariorum*, possibly due to a shift in the feeding preference of the predator from plant- towards prey consumption [185]. Taken together, zoophytophagous predators engage in complex interactions with plants also involving beneficial soil microbes and the manipulation of innate plant defense responses. The outcomes of such interactions are currently shown to be positive in terms of plant protection. Further studies are required to understand underlying mechanisms and estimate field efficiency to be able to propose biocontrol strategies and management schemes involving zoophytophagous predators and microbe-inoculation in agricultural settings.

1.5.4 Chemical Priming

Most of chemical priming inducers are natural compounds isolated from challenged plants, or compounds mimicking the structures of natural immune inducers. They do not have *in vitro* antimicrobial activity, and target the main defense-related phytohormone pathways. The first chemical inducers of priming studied were SA and synthetic SA analogues such as 2,6-dichloroisonicotinic acid (INA) and thiadiazole-7-carbothioic acid (BTH). Both were shown to prime parsley cells to resist *Phytophtora sojae* [186]. Accumulation of SA is a common trait in SAR and mediates the activation of a set of pathogenesis-related (PR) genes. Mono- and dichloro substituted SA and fluoro-SA derivatives were found to induce PR proteins in tobacco against TMV infection [187, 188]. While SA regulates defense against biotrophic pathogens, JA and MeJA control mainly the immune responses against necrotrophic pathogens and herbivores. JA and several synthetic JA mimics have been shown to induce priming by activating JA signaling and defense responses in different plant species (reviewed by Zhou and Wang [189]). In most cases, when phytohormone analogues are used as priming agents, it is concentration that determines whether priming or direct defenses are displayed by the plant [49].

Besides the main phytohormones and their analogues, several chemical compounds such as BABA and Indol-3-carboxylic acid (I3CA) are known to prime the plants to cope with environmental and biotic stresses [190, 191]. Among these chemical inducers, BABA-IR has the widest protection spectrum; it has been shown to protect about forty plant species including mono- and dicotyledonous against several pathogens and pests, including viruses, Protista, bacteria, oomycetes, fungi and arthropods being effective in a wide range of applications (foliar spray, soil

drench etc.) [192]. Importantly, there are indications that BABA-mediated priming can reduce herbivores (aphids) growth without displaying direct negative effects on their parasitoids [193]. BABA-IR acts by potentiating defense mechanisms depending on the pathosystem [194]. Defense against *Plectosphaerella cucumerina* is mounted through an ABA-dependent signaling that contributes to callose accumulation, whilst defense priming against *P. syringae* pv *tomato* (*Pst*) is mediated by SA-dependent responses. Despite BABA-IR is known for almost 60 years now, it was only a few years ago when the receptor and the perception mechanism for BABA-IR was identified, being the *Impaired in BABA-induced Immunity 1* (*IBI1*) gene which encodes for an aspartyl-tRNA synthetase [195, 196]. A recent study has also identified BABA as an endogenous metabolite present in several plant species [197]. Studying the BABA-IR in *Arabidopsis* against *P. cucumerina,* Gamir *et al.* [191] described for the first time a common fingerprint of various priming stimulus within specific plant-pathogen interactions. In this study, I3CA was identified as one of the metabolites mediating BABA-IR. Further studies showed that I3CA was also capable to act as priming stimulus in *Arabidopsis* upon *P. cucumerina* by increasing ABA levels in the pre-challenge stage and enhancing callose deposition upon infection [60]. In addition, a series of secondary metabolites that were shown to mediate priming, can trigger defense priming on themselves, as is the case for pipecolic acid, dehydroabietal, imprimatins, azelaic acid and glycerol-3-phosphate among others [46].

Another class of chemical inducers are those that prime cells without targeting metabolism or a specific signaling pathway; this is the case of silicon as priming agent. Silicon does not react within the cell and its action is mainly based on its deposition within or between the cells, in the cell wall or as phytoliths [198]. Plants obtain Si as silicic acid ($Si(OH)_4$) from the soil and deposit it as silica which helps to construct mechanical barriers (phytoliths) and abrasive structures (Si-fortified leaf trichomes) to prevent insect feeding [199]. When *Spodoptera exempta* was fed on Si-treated grass they showed reduced insect growth rates and irreversible wear down of their mouthparts [200]; however, the exact mechanisms for Si-IR remain controversial. In addition to the physical benefits of silicon, systemic defense responses were recently shown to be stimulated following Si treatments (reviewed by Coskun et al. [201]). Perennial ryegrass grown in Si-amended soil showed increased papillae deposition and lignin-associated phenolic compounds against *M. oryzae* leading to a reduction of disease incidence and severity [202]. In addition, certain defense-related enzymes such as peroxidases (POX), phenylalanine ammonia-lyase (PAL) and polyphenol oxidase (POD) were increased in Si-treated rice (*O. sativa*) upon *Cnaphalocrocis medinalis* attack [203]. Hence, the current understanding of fundamental and mechanistic aspects of priming generate enough knowledge to design new sustainable technological tools that may be complementary to IPM improving the efficiency of crop protection.

1.6 Endophytic Fungi in Plant Defense

1.6.1 Endophytic Fungi Mediating ISR

Endophytic microorganisms occur ubiquitously in plants, where they spend part of (facultative endophytes) or all (obligate endophytes) their life-cycle, without causing any signs of disease [204]. Endophytes colonize all plant organs, generally the inter- and intracellular spaces of their inner tissues [204]. They may derive from the surrounding environment, such as the rhizosphere and phyllosphere, but also from vegetative planting material or from seeds [205]. Endophytic communities are very diverse and their composition is influenced by a broad spectrum of factors, such as host genetics [206], geographic location [207], local environmental conditions [208], pathogen infections [209] and anthropogenic influence [210]. The capacity of endophytes to confer resistance or tolerance to the host plant is largely attributed to endophytic production of bioactive metabolites in colonized plants. These compounds may suppress biotic stressors either directly, by antibiosis, parasitism and competition, or indirectly via the induction of plant defenses [211]. These mechanisms frequently operate simultaneously. Some of the compounds that inhibit biotic stressors directly include defense metabolites (*e.g.,* terpenoids, alkaloids and polypeptides), volatile organic compounds (*e.g.,* acids, alcohols, alkyl pyrones, ammonia, esters, hydrogen cyanide, and ketones), iron-chelating compounds (*e.g.*, siderophore), quorum sensing inhibitors and hydrolytic enzymes [212–215].

Elicitation of ISR by endophytes has been reported to be important to fight an array of pathogens, including fungi [216–219], bacteria [220, 221], oomycetes [222] and recently, arthropods [149–151]. Some of the most important endophytes found to induce ISR in crop plants include fungi belonging to the genera *Trichoderma*, *Penicillium*, *Fusarium* and *Phoma*, and bacteria of the genera *Pseudomonas* and *Bacillus* [223–225].

Knowledge on cellular, molecular, and biochemical defense responses activated by endophytes against pathogens or pests is so far limited. The few studies performed suggest that endophytes activate ISR response via their contact with the plant receptor (*i.e.*, PRRs) in the same manner as pathogens, but differ in the induction of defense responses [226, 227]. Elicitors involved in ISR triggered by endophytes are not so well characterized as compared to pathogens. Endophytic elicitors identified so far are common among all microbes and include endophytic-cell components, such as chitin or chitin derivative (*e.g.*, chitosan), β-glucans, ergosterol and flagellin, as well as proteins (*e.g.*, cerato-platanins), peptides (*e.g.*, peptaibols, elicitins), lipopolysaccharides and enzymes (*e.g.*, xylanases, proteinases and cellulases) secreted by endophytes [224, 228–230].

Increasing evidence suggests that endophytes defend themselves from plant defense mechanisms. Endophytes can, for instance, prevent themselves from being recognized by plant receptors [231] or succeed in being perceived in a different way as compared to pathogens [232], and can also protect themselves from ROS generated by the plant as a defense response [233]. Upon recognition of the endophyte by

the plant, a set of signal molecules are generated to induce and amplify out the defense response at long distance. JA and ET are known to be the major signal molecules involved in systemic defense responses of plants mediated by endophytes [162, 234, 235]. Despite the common association of SA with SAR, this plant hormone was also shown to induce systemic responses, activated by endophytes [66, 236]. However, in a pathosystem involving *F. oxysporum* Fo47, against *Fusarium* wilt disease in tomato, induced resistance triggered upon endophytic colonization was demonstrated to be independent of the SA/JA/ET pathways [237]. These contradictory results open several questions related with the necessity of phytohormones to induce endophyte-mediated resistance and the classification of induced resistance response as ISR or SAR. Defense responses can include strengthening of structural barriers by callose accumulation, generation of ROS, synthesis of pathogenesis-related (PR) proteins (which have a recognition role in defense and stress as well as antimicrobial activity), production of defense-related enzymes (*e.g.*, peroxidases, polyphenol oxidases, phenylalanine ammonia-lyase), anti-microbial metabolites (*e.g.* phenolic and flavonoid compounds) and proteins that inhibit pathogen growth, along with the increased anti-oxidant capacity of the host [217–220, 236].

1.6.2 Endophytic Entomopathogenic Fungi as Biocontrol Agents

Endophytic entomopathogenic fungi (EEPF) are naturally occurring soil microbes [238] which show similar characteristics to the non-clavicipitaceous (class III) endophytic fungi [239]. Among these traits are their occurrence primarily or exclusively on foliar tissues, their horizontal transmission (via airborne spores) and high diversity of host range [239–241]. EEPF are classified in two groups, the generalist facultative insect pathogens (mainly Hypocreales species) that inspire a broad research interest, and the host-specific obligate pathogens (Entomophthorales and a small number of Hypocreales species) with a narrow host spectrum [242].

The dual ability of EEPF to establish themselves as both endophytes and entomopathogens [243] provide a successful crop protection method in a sustainable agriculture context. Studies on EEPF carried out some decades ago report *Beauveria bassiana* [244], *Metarhizium anisopliae* [245], *Verticillium* (=*Lecanicillium*) *lecanii* [246], *Paecilomyces farinosus* (Holmsk.) (=*Isaria farinosa*) [247], *Paecilomyces* sp. [248], *Paecilomyces varioti* [249], *Cladosporium* [250], and *Purpureocillium lilacinum* (formerly*Paecilomyces lilacinus*) [251] as pathogenic agents against thrips, aphids, whiteflies, mosquitoes, fruit flies, mites and other arthropods and plant parasitic nematodes [252]. Because of their peculiar life-style (i.e. symptomless endophytes of plants and infectious to herbivorous insects), EEPF have received much attention recently as promising biological control agents [253–255]. However, the mechanisms underlying their interactions with plants and pests remain poorly understood while their insect-killing capabilities have prompted many studies on

the basis of their biotechnological potential [240]. Notably, latest research has focused on the role of EEPF in secondary metabolites production as well as their ability to promote plant growth and enhance resistance [256].

ISR has been demonstrated for diverse EEPF. Perhaps, the most striking example is the genus *Beauveria* (particularly the species *bassiana*), which accounts for the 67% of EEPF studied [240]. Inoculation of cultivated cotton seeds with *B. bassiana* conidia resulted in lower survival and development of the corn earworm *Helicoverpa zea* [257]. Likewise, *Rachiplusia nu* larvae consumption on colonized corn plants with *B. bassiana* was reduced [258]. Against *Aphis gossypii*, inoculation of cotton seeds had a negative effect on reproduction with an increased mortality after successful establishment of *B. bassiana* [259]. *Beauveria bassiana* was also used as an endophyte against the leaf miner *Liriomyza huidobrensis* resulting in reduced oviposition, mortality, longevity and adult emergence [260]. These authors further confirmed that colonization is species-specific, depending on the host plant, the fungal isolate and plant part. Assessing the effect of *B. bassiana* on the growth of *Arabidopsis thaliana* and its resistance against two herbivorous species (*Myzus persicae* and *P. xylostella*) and a facultative parasitic Ascomycete fungus (*Sclerotinia sclerotiorum*), Raad et al. [261] found a decreased leaf lesion area caused by the pathogen. Nonetheless, population growth of *M. persicae* or *P. xylostella* was not affected. A deeper transcriptomic, phytohormone and glucosinolate analysis showed that the expression of genes involved in plant defense varied; conversely, JA and SA levels as well as those of leaf glucosinolates remained unchanged. This was again a confirmation for the species-specificity of the induced defense mechanism. *Beauveria bassiana* can also act against bacteria by lowering the severity of *Xanthomonas* bacterial blight [262], and also confer resistance against the *Zucchini Yellow Mosaic Virus* (ZYMV) in colonized squash plants [263]. The latter was the first report on *B. bassiana* being involved in plant defense against viruses followed by other reports such as ISR against melon viruses [264]. Several other studies were carried out with other EEPF such as *Metarhizium* species (*acridum, robertsii, anisopliae, brunneum, pingshaense*), *Purpureocilium lilacinum, Isaria fumosorosea, Clonostachys rosea* and *Lecanicillium lecanil* that showed successful endophytism in different host plants and plant parts, with induction of systemic resistance [240].

Direct effects of EEPF on plant biotic stressors are attributed to mycoparasitism, competition with other endophytes or the production of secondary metabolites. Mycoparasitism is defined as an antagonistic interaction between two fungal organisms by the production of extracellular enzymes such as chitinases, cellulases and glucanases by the parasite to digest the host cell wall [265]. It has been described in depth for *Trichoderma* spp. [266, 267] and *Lecanicillium* spp. [213, 268] under laboratory conditions. Likewise, Griffin [269] showed an ability of *B. bassiana* to parasite the fungus *Pythium myriotylum*, a serious pathogen of many crops. Competition for space and resources can occur between EEPF and pathogens thus conferring protection and reducing the probability of colonization by pathogens [243]. In the case of initial colonization by EEPF, resources are expected to be exhausted, limiting nutrient availability for the pathogen. Consequently, the disease is expected to be suppressed [270] as for example, with *B. bassiana* inoculated to

grapevine that was shown to control the infection by the pathogen *Plasmopara viticola*via an antagonistic effect.

Antibiosis and feeding deterrence are non-entomopathogenic mechanisms of EEPF [240, 260, 271, 272]. They are well known to be sources of secondary metabolites that exert inhibitory effects on pests and pathogens. Beauvericine [273, 274], oosporein [275, 276] and bassianolide [277] are toxic secondary metabolites produced by *Beauveria* spp. These metabolites pose insecticidal, antibacterial, antifungal and antiviral activities [278]. Most of the work carried out has demonstrated the secretion of these metabolites *in vitro*; however, their production *in planta* is not evidenced since production may be temporary or degrading rapidly [272]. Our knowledge of the mechanisms of action of EEPF as entomopathogens and as plant growth promoters is well advanced during this last decade. However, there is still a lot to investigate in particular the relationship between EEPF and their plant hosts with the community of symbionts they harbour.

1.7 Aspects of Commercial Application

Compared to conventional agrochemicals, RNA molecules (dsRNAs, sRNAs) seem to win the race in terms of environmental considerations and risk assessment. RNAi molecules are not toxic to humans even when present in their diet [107]. Moreover, their mode of action is extremely specific, since it is based on a nucleotidic complementarity of 20–25 bases with their target. Thus, off-target effects are practically minimized. Concerning cost issues, a rough estimation has suggested that for field-scale application of RNA molecules against pests and pathogens 10 g of dsRNA per hectare is required [279]. For laboratory experiments limited amounts of dsRNA may be generated by the commercially available *in vitro* transcription kits (average cost 100 USD per 1 g of dsRNA). Yet, for field-scale applications alternative dsRNA production systems need to be sought for, such as the one provided by RNAagri (https://www.rnagri.com/) and AgroRNA (www.agrorna.com) wherein bacteria engineered to produce the desired dsRNA multiply in large fermentators and huge quantities of encapsidated dsRNA are isolated with low-cost methods (average cost 2 USD per 1 g of dsRNA). Yet, the degradation rate of the applied RNA in field conditions due to nucleases and/or hydrolysis is an issue that needs to be taken into consideration. To this end, lipid double hydroxide clay nanosheets ('BioClay') have been developed, wherein the dsRNA is bound to clay nanosheets and is significantly resistant from degradation [117]. Similarly, for enhanced biopesticide efficacy, Nanosur (www.nanosur.com) offers formulated RNAs for improved translocation across cellular membranes and reduced degradation. Moreover, chemical enhancers such as Sortin1 and Isoxazolone have recently been developed whose mere application in plants seems to boost host RNAi machinery [280]. Cumulatively, the above discussed advances have facilitated the development of commercial RNA-based plant defense products that are soon to emerge in the market, such as 'BioDirect' (Bayer/Monsanto), which is designed for pest (Colorado potato beetle, brassicas

flea beetle, varroa mites), virus (Tospovirus) and weed (glyphosate resistance) control.

Current applied and fundamental research has offered a plethora of potential chemical stimulants of the plant immune system that have the potential to protect crops in a more sustainable way. Most chemicals discovered by means of induced resistance and priming are natural compounds that contribute to signal subsequent plant defenses. Hence, their potential use as active matters in the future design of agrochemicals offers an opportunity of a new generation of sustainable products. However, despite the enormous interest for these compounds to fit in a new generation agriculture, the legislation needs to be redefined in parallel to adapt new discoveries to the applied field. Indeed, although a low environmental impact of these naturally occurring metabolites is expected, knowledge of their impact on non-target crops and organisms is important. Furthermore, an adapted legislation, out of the phytosanitary frame but supervised, is needed for their wide acceptance and use in the field.

Considered as a relevant tool to unfold and sustain agriculture, EEPF have already been used as potent tools in empowering sustainable agriculture. Nevertheless, more research is required to invest in technical challenges. As stated above, EEPF can be exploited in crop protection for both their entomopathogenic and non-entomopathogenic roles. Their endophytism offers an advantage compared to entomopathogenic fungi used as contact biocontrol agents that are limited by their susceptibility to biotic and abiotic factors. EEPF action as entomopathogens on the other hand, has been extensively studied for plant-surface inhabiting pests. However, their effects against endophytic insects, whose larvae feed internally in stems, flowers, seeds, parenchyma leaves and fruits are not proven yet. Moreover, colonization of plant parts by EEPF differs among plants and fungal species and its persistence is not completely prevailed [270]. A recent transcriptomic analysis of *Beauveria*-colonised plants showed a reprogramming of plant defense pathways [261]. Hence, further studies are required for a better understanding of mechanisms regulating plant responses to EEPF and those governing EEPF-mediated tritrophic interactions. Finally, current EEPF formulations (e.g. BotaniGard ES/WP, Mycotrol, Naturalis L, BioCeres WP, Velifer, balance, XPulse, PFR-97 WDG, Semaspore Bai and MeloCon WG) rely on a single microorganism and are commercialised to serve exclusively as insecticides [252]. Endophyte consortium formulations would be of interest, possibly offering synergistic efficiency. Nonetheless, the validation and commercialisation of economically viable EEPF constitutes laborious challenges and potential risks i.e., introducing organisms into new ecosystems, toxicosis risks [281] and field efficiency. EEPF were proved harmless for beneficial insects (e.g. bees) [260, 282, 283]. Yet, they may interfere with nest-mate recognition and also promote inter-colony transmission of diseases in honey bees [284]. Future studies are crucial to cast light on EEPF side-effects on beneficial organisms.

The same holds for all pest control strategies presented in this chapter. Biological pest control with the use of natural enemies (predators, parasitoids) is an important alternative to chemical control, commonly employed in sustainable crop production to suppress pest populations, especially in greenhouse crops. Despite the vast

fundamental knowledge on mechanisms of plant defense and RNAi and their effects on plant pathogens and pests, our understanding of their impact on beneficial organisms (natural enemies, pollinators) is largely poor. Plant defense and RNAi tools may negatively affect natural enemies directly, by causing mortality or slowing down their development, or via interfering with their herbivorous prey.

1.8 Conclusions & Future Considerations

Increasing population on Earth makes uncertain the future regulation of food security and supply. The United Nations have gathered these needs up and push for reaching certain goals of sustainability until 2030 [285], and fulfilling by the end of the millennium other objectives, like the end of hunger and poverty. All these new policies, strongly supported by higher education and research institutions, prompt us to revisit current agricultural practices [286], also by considering environmental sustainability. Future agro-technological considerations may include the concept of defense priming as well as RNAi, as new strategies in crop protection by key pests and pathogens [110, 287]. Moreover, the long lasting and transgenerational aspects of priming should be considered. They can add value to this sustainable concept, by providing defense to plants without requiring additional treatments [80–82]. The mechanisms described in this chapter, tackle the cross-kingdom (including bacteria, plants and pests) and -scale (from molecular to applied in fields) relevance of this type of adaptive immunity, highlighting ecological implications in plant defense [288].

Furthermore, soil-borne beneficial microorganisms are of particular interest as vaccination agents of crops, capable of enhancing plant resistance to biotic stressors. An important prerequisite for the development and application of effective beneficial inocula is a solid knowledge of the mode of action of these organisms, the mechanisms and regulatory pathways involved in microbe-induced resistance and, how context dependency influences beneficial interactions among crops and pests/pathogens. The ability of beneficial microorganisms to activate phytohormone-mediated plant defense responses is well-established. However, most studies on the molecular mechanisms that govern the complex multi-partite interactions of plants are limited to a few model plants and also refer to certain pathosystems. Hence, little is known of the universality of these mechanisms in crop plants and their pathosystems. An untargeted approach to identify microbe-induced defense mechanisms is needed and the molecular tools are now applicable to economically important plants, too.

The advancements in Next Generation Sequencing (NGS) technologies provide an unprecedented insight into the genetic patrimony of different living organisms. These technologies revolutionized the methods of deciphering DNA sequences as well as the exchange, storage and analysis of enormous quantities of resulting sequence data. The generation of sequencers belonging to the third generation (e.g. PacBio, Oxford Nanopore Technologies), that enable long length sequences read

and accuracy [289, 290], allow for genome assemblies of organisms identified as suitable for biological control, such as the beneficial microbes dealt with in this chapter. In the last decades, several genomes of biocontrol agents have been sequenced and assembled leading the way for understanding their biology and functional characteristics that are beneficial to plant defense and biocontrol activities against plant pathogens and pests. The development of NGS has also facilitated comparative and functional genomics in these organisms that allow for a better description of genes responsible for the main beneficial properties of biocontrol agents in different ecosystems. In addition, important reservoirs of genes that could play key roles in ecosystem functioning may be accessed. For example, it was recently shown that two bacterial genes in biocontrol *Pseudomonas* strains are necessary for the acidification of the rhizosphere, which in turn modulates plant immunity to facilitate normal plant growth [291]. In yet another case, genome reconstruction at strain-level derived from a metagenomics analysis of the endophytic community in sugar-beet, a novel gene cluster encoding nonribosomal peptide synthetases (NRPSs) and polyketide synthases (PKSs) was identified as essential for disease suppression by the endophytic community [68].

In conclusion, it becomes more and more obvious that disease/pest suppression is the final outcome of complex and multipartite plant-microbe interactions leading to either coevolution or physiological adaptation in a context-specific manner. The challenge is to take a holistic perspective in future studies to assess the suppressive function of microbial assemblages at a community level and apply molecular tools not only on harmful organisms but also on the ecosystem. Such a community-level approach is crucial to determine the feasibility of novel biocontrol molecular tools in sustainable crop production.

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Chapter 2
Specialized Metabolites and Plant Defence

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2.1 Introduction

In nature, plants protect themselves against pathogen attack mainly by mechanical and chemical defences. Mechanical defences include structures such as spines, trichomes, thick cuticle, and hard, sticky, or smooth surfaces which prevent pathogens from picking for food or laying eggs. Chemical defences include a variety of substances that are toxic, repellent, or that render plant tissues indigestible to animals [1]. Chemical defence due to secondary metabolites is prominently developed in plants, often providing protection to the plant [2]. Plants can present either independent mechanical and chemical defences [3] or a combination of each type of these defences such as in the case of glandular trichomes and secretory canals. The synergic interaction triggers the entrapment of the pathogen in sticky and toxic secretions [4]. Many plants produce resins, gum, lattices and mucilage, which are stored under pressure in networks of canals throughout the cortex of the stems and in the leaves, where they follow the vascular bundles. These secretions are rich in several secondary metabolites: for example, oleoresin present in *Abies grandis* is a complex mixture of monoterpenes, sesquiterpenes and diterpenoid acids, used to deter insect

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pests and their symbiotic fungal pathogens [5]. Some *Bursera* species resins, rich in mostly monoterpenes and sesquiterpenes [6] are under considerable pressure, and so when a leaf is damaged, resin may be released in a spectacular syringe-like squirt. This squirt may travel up to two meters and lasts a few seconds, so it represents a good example of mechanical and chemical defence interaction [7]. Some species of *Asclepias* (milkweeds) latex contains cardenolides and cardiac glucosides which help the plant in defence response [8]. These toxic steroids have an interesting use in monarch butterflies. Adult monarch butterflies store the cardenolides they have built-up during their larval stage, feeding mostly on *Asclepias*. This stored cardenolide content in butterflies deters them from their vertebrate predators.

The chemical defence can be constitutive and/or inducible. The term constitutive means that the defence is present in the plant whether the predator attacks or not. Many constitutive defence chemicals are produced by epidermal hairs that can trap and kill insect larvae. Inducible systems are those that are absent before a pathogen or predator attack, but are induced upon attack. Defence related responses can occur in the plant organ originally attacked (local response) or in distant unaffected parts (systemic response). Examples of chemical defences can be found in various reviews: Field and co-workers [9], Mithöfer and Boland [10], and Soledade and co-workers [11].

Plants can present a compatible response towards its pathogen, when their contact leads to a successful infection, or plants can have a non-compatible response when the plant and pathogen contact leads to a non-successful infection. In incompatible interactions, infection by pathogens inducts a set of local responses in and around the infected host cell which can lead to cell death [12]. Thus, the pathogen may be 'trapped" in dead cells and this prevents the infection from being spread. Local responses in the cells include oxidative burst, changes in cell wall composition that can inhibit penetration by the pathogen, and de novo synthesis of antimicrobial compounds such as phytoalexins and pathogenesis related (PR) proteins. Phytoalexins are mainly characteristics of the local response, PR proteins occur both locally and systemically [13].

The aim of this review is to reveal new avenues of research in the area of elicitor imparted secondary metabolites production in plants which play important role in providing them resistance against diseases.

2.2 Secondary Metabolites and Defence

Plants (>300,000 species) and insects (likely >1,000,000 species) have co-evolved, still plants dominate the landscape [14]. This is partly due to the presence of secondary metabolites that make the plants both repellent and toxic to most pathogens, insects and other grazing animals. Secondary metabolites are the molecules that appear to be dispensable for normal growth, or are required only under particular conditions, whereas primary metabolites are involved in the physiological functions as vegetative growth or reproduction. These secondary products are the key components of active and potent defence mechanisms in plants [15, 16]. They are the active part of the chemical war between plants and their pathogens [17].

About 1–10% of the dry mass of some plants is made up of chemicals designed for defence against predators. Plants synthesize a huge array (around several tens of thousands) of different secondary metabolites [16]. Synthesizing a particular chemical so that it accumulates in the plant to a significant level has an associated cost. Various biosynthetic pathways are involved in secondary metabolites production and there is requirement of substantial amount of ATP. Besides their synthesis during the time of attack by pathogen, their storage in the vacuole requires energy as well. The energy for uphill transport and often for trapping the metabolite in the vacuole is provided by H^+ - ATPase. In addition, some metabolites are transported into the vacuole with the help of ATP-binding cassette transporters (ABC-transporter) which depend on ATP [18, 19]. It can be seen that if the cost of producing a defence compound is minimal and allows the plant that produces it to leave more offspring, then that plant has a greater evolutionary fitness than its non-defended colleagues. This can readily be demonstrated by partially defoliating plants or giving them a mild bacterial, fungal, or viral infection. Such plants grow much less vigorously and produce fewer seeds. However, if secondary metabolisms have not been very important in the biology of different organisms, evolution would not have selected and maintained the complex pathways leading to secondary metabolism [16].

Most of the secondary metabolites are derived from the isoprenoid, phenylpropanoid, alkaloid or fatty acid/polyketide pathways [16, 20]. It is observed that related plant families generally make use of related chemical structures for defence, e.g. sesquiterpenes in the Solanaceae, stilbenes in the Vitaceae, isoflavones in the Leguminosae, sulfur-based glucosinolate–myrosinase in the Brassicaceae and limonoids among members of the families Meliaceae and Rutaceae. In plants, the best understood secondary metabolites are implicated in pathogen defence, sensing and signaling [21]. This list is continuously growing by the extensive use of biochemical and genetic approaches to reveal the undiscovered metabolites and their complex signaling pathways that mediate plant disease resistance.

Pathogens, insects, and other parasites initially establish physical interactions with hosts via surface contact, and the plant surface initiates chemical signaling in response to it. The secondary metabolites content of the surface exempt of disease is also an important aspect to be studied in order to discover new defence molecules. For that, accurate methods of detection and quantification are required. Nowadays desorption electrospray ionization mass spectrometry (DESI-MS) has made possible fine scale evaluation of compounds on native surfaces of the plants. Lane and co-workers [22] reported presence of bromophycolides, antimicrobial compounds, on the surface of macroalga *Callophycus serratus* in sufficient quantity for inhibition of *Lindra thalassiae*, a marine fungal pathogen. Hamm and co-workers [23] have used laser desorption/ionisation time-of-flight mass spectrometry (LDI-ToFMS) to analyze phytoalexins at the surface of *V. vinifera* leaves. They have found that the amounts of resveratrol and pterostilbene are directly related to the degree of *P. viticola* contamination.

Several large groups, such as phenolics, alkaloids, terpenoids, iridoid glycosides, cardenolides, and cyanogenic glycosides have been implicated in plant defence systems. The literature on secondary metabolites is extensive. Here we summarized phenolics related to plant defence system.

2.3 Phenolics and Disease Resistance

Phenolics are represented by having at least one aromatic ring with one or more hydroxyl groups attached, and are widely present throughout the plant kingdom [24]. They are known to contribute to pigmentation of different organs along with their role against different biotic and abiotic stresses [25]. Phenolics occurring naturally in plant tissue can be classified into two groups, the flavonoids and the non-flavonoids.

Depending on the structural complexity of flavonoids (with an estimated 10,000 structurally different members), particularly on the oxidation state of the central ring C, flavonoids are themselves subclassified as flavonols, flavones, flavan-3-ols (catechins and their oligomers: proanthocyanidins), anthocyanins, flavanones and isoflavones and those that are present in less quantity in diet are dihydroflavonols, flavan-3,4-diols, chalcones, dihydrochalcones, and aurones [26] (Fig. 2.1). Majority of flavonoids exist naturally as glycosides. Both, the hydroxyl groups and sugars, increase water solubility of flavonoids [27]. Flavonoids in general are polyphenolic compounds comprising of 15 carbons, with 2 aromatic rings connected by a 3-carbon bridge (C_6-C_3-C_6). They consist mainly of 2-phenylchromans and also 3-phenylchromans for isoflavonoids. The key enzyme for the formation of the flavonoid

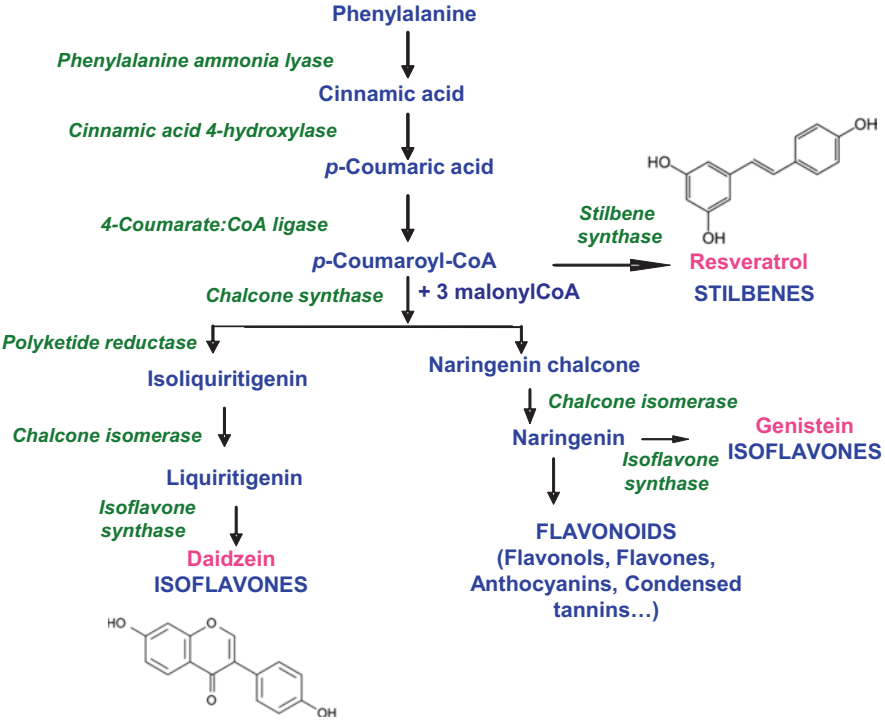

Fig. 2.1 Biosynthetic route of isoflavone and stilbene production

skeleton is chalcone synthase, which catalyses the stepwise condensation of three acetate units from malonyl-CoA with 4-coumaryl-CoA to the intermediate chalcone. Flavonoids play important role in defence against microorganisms and pests [28].

There are many instances which describe their potent role in disease resistance. A recent investigation by Koskimaki and co-workers [29] observed that accumulation of individual phenolic compounds could be specific for a particular infection. They demonstrated biosynthesis of different phenolic compounds in bilberry (*Vaccinium myrtillus*) after infection by a fungal endophyte (*Paraphaeosphaeria* sp.) and a pathogen (*Botrytis cinerea*). A study of barley mutants showed that proanthocyanidins and even small amounts of dihydroquercetin are involved in the defence against *Fusarium* species [30]. The wild species of groundnut, *Arachis kempff-mercadoi* is resistant to tobacco armyworm *Spodoptera liture* due to its flavonols quercetin and its glycoside rutin [31]. Similarly, nematode resistance in banana is due to flavan-3,4-diols and condensed tannins [32]. Flavonoids also play a major role in postharvest resistance of fruits and vegetables [33]. High concentrations of flavonoids mainly in unripe fruits prevent them from pathogens; thus, ripe fruits are usually more sensible to fungal decay.

There are also several classes of non-flavonoids, dominated by phenylpropanoids containing only the C_6-C_3 phenylpropane skeleton and these compounds are directly linked to lignin (polymer phenylpropanoid) biosynthesis in vascular plants. The most important examples are cinnamic acids and their derivatives such as chlorogenic acid, *p*-coumaric, ferulic and sinapic acids. Another class of non-flavonoid polyphenols which are less frequently found in diets (except for the grapes and peanuts) is constituted by the stilbenes with C_6-C_2-C_6 skeletons [34–36]. Hydroxycinnamic acids and flavonoid classes are widely present in higher plants whereas classes like isoflavones (e.g., Fabaceae) and stilbenes (e.g., twenty-three families only: Vitaceae, Cyperaceae, Dipterocarpaceae, Iridaceae, Fabaceae, Moraceae, Orchidaceae and Polygonaceae) are limited to particular families.

2.3.1 Isoflavones

Isoflavones are characterized by having the B-ring attached at C_3 rather than the C_2 position [37] (Fig. 2.1). Till 2011 about 1,600 isoflavones have been identified and the list is continuously growing [38]. Indeed, 391 new natural isoflavonoids have been isolated and identified between 2012 and 2017 [39]. The isoflavones like daidzein, genistein, and glycitein are synthesized via the phenylpropanoid pathway and stored in the vacuole as glucosyl- and malonyl glucose conjugates. The pathway to daidzein branches from the phenylpropanoid pathway, that is common to most plants, following the chalcone synthase reaction (Fig. 2.1) through a legume specific enzyme, chalcone reductase. Glycitein synthesis is likely to be derived from isoliquiritigenin. Genistein synthesis shares the naringenin intermediate with the flavonoid/anthocyanin branch of the phenylpropanoid pathway. In all cases, the

unique aryl migration reaction to create the isoflavones is mediated by isoflavone synthase [40, 41]. Oxidative rearrangement of naringenin (flavanone) with a 2.3-aryl shift yields the isoflavone. The initiating step in isoflavone formation may be an epoxidation catalyzed by a cytochrome P450-dependent mono-oxygenase. After structural rearrangement, aryl shift and addition of a hydroxyl ion to C-2, elimination of water by a dehydratase gives the isoflavone structure. Details of isoflavones structure can be found in the article of Veitch, 2007 [38].

These compounds were initially recognized for their roles in plant disease resistance and as signal molecules to promote *Rhizobium* nodulation [20]. They also serve as precursors for the production of major phytoalexins during plant–microbe interactions [42] and inhibit pathogen attack [43–46]. Isoflavones have demonstrated efficient antimicrobial and antifungal activities. The isoflavones like daidzein inhibits the growth of *Fusarium culmorum*, while glycitein and formononetin can reduce mycelial development in *Aspergillus ochraceus* [47]. Biochanin A and genistein exhibit antifungal activity against *Rhizoctonia solani* and *Sclerotium rolfsii* [48]. Antimicrobial and antifungal properties of various isoflavones have been well demonstrated. Isoflavones from stem bark of *Flemingia paniculata* [49] and from *F. stropbilifera* [50] showed significant antibacterial activity. Extract of *Tamarix gallica* containing quercetin [51], *Prunus americana* containing isoflavones [52] and *Glycyrrhiza glabra* containing glabridin [53] showed promising antimicrobial activity. On elicitation by *Aspergillus sojae*, soybean produced antifungal glyceollins [54] effective against *Fusarium oxysporum*, *Phytophthora capsici*, *Sclerotina sclerotiorum* and *B. cinerea*, while lactofen induced isoflavones were correlated with defence responses [55].

2.3.2 Stilbenes

Stilbenes are a group of phenylpropanoid-derived compounds characterized by a 1,2-diphenylethylene backbone (C_6-C_2-C_6). Stilbenes exist in the stereo isomeric forms (*E* and *Z* forms) depending on the position of where the functional groups are attached in relation to one another on either side of the double bond. Stilbenes constitute an important group of natural products that are of particular interest owing to their wide range of biological activities [56]. Combretastatins, piceatannol, pinosylvin, rhapontigenin, pterostilbene and resveratrol are some of the naturally occurring stilbenes. Of these, combretastatins and resveratrol have been extensively studied. Most plant stilbenes are derivatives of the basic unit *trans*-resveratrol (3,5,4′-trihydroxy-stilbene). From this relatively simple structure, over a thousand stilbenoid compounds have been characterized, resulting from different chemical substitutions patterns like methylation, glycosylation or isoprenylation, in addition to oxidative condensations of monomers into dimers (for example viniferins) and subsequent condensations of these [36, 57]. All higher plants seem to be able to synthesize malonyl-CoA and CoA-esters of cinnamic acid derivatives, but only few plant species are able to produce stilbenes, as the stilbene synthase (STS), the

fundamental enzyme of stilbene synthesis, is present in a limited number of plant species, for example *Vitis* spp., *Arachis hypogea, Pinus* spp., *Rheum* spp. and *Fallopia* spp. *STS* genes exist as a family of related genes in these plants [58]. Stilbene synthase catalyzes, in a single reaction, the biosynthesis of the stilbene backbone from three malonyl-CoA and one CoA-ester of a cinnamic acid derivative (Fig. 2.1). Grapevine genome contains more than 20 *STS* genes [59] and nearly all of them are expressed in grape following infection with *P. viticola* [60].

Some plant species, such as *Fallopia japonica* (formely *Polygonum cuspidatum*), pine (*Pinus* spp.) and grapevine (*Vitis* spp.) constitutively accumulate large amounts of stilbenes [58]. However, most studies concerning stilbene biosynthesis have been conducted on peanut, grapevine and pine. Induction of stilbenes synthesis is well known in response to a wide range of abiotic and biotic stresses. As example in grapevine, upon infection with different microbial pathogens, including powdery mildew (*Erysiphe necator*) [61], downy mildew (*P. viticola*) [62, 63], or gray mold (*B. cinerea*) [64, 65], coordinated activation of STS and upstream enzymes in this pathway occurred.

Stilbenes can accumulate in plant tissues to concentrations necessary to inhibit fungal growth [66, 67]. Stilbenes like pinosylvin and pinosylvin 3-*O*-methyl ether, which occur naturally in conifers, have strong antifungal activity in in vitro assays. These compounds are active in vitro against *Coriolus versicolor* and *Gloeophyllum trabeum*, two wood-destroying fungi [68]. Moreover, these two compounds present at a high level in a knot extract from *Pinus pinaster* show a strong antimildew activity by inhibiting *P. viticola* zoospore mobility and mildew development on grapevine leaves [69]. Other stilbenes like resveratrol inhibit conidial germination of *B. cinerea* (the gray mold agent on grapes) [62] and also reduce the germination of sporangia of *P. viticola* (the downy mildew agent) whereas its glucoside piceid reduces spore germination of *Venturia inaequalis* (the causal agent of apple scab) [70] at concentrations compatible with the activity range of other phytoalexins. It is interesting to note that *trans*-resveratrol, piceids, viniferins and pterostilbene concentrations reach up to 50–400 μg/g DW in infected grapevine leaves [58, 71]. Pterostilbene, the dimethylated form of resveratrol, has a fivefold higher activity than resveratrol in inhibiting fungal growth in vitro, indicating that methylation of hydroxyphenyl groups could lead to increased biocidal activity of phenolics [72]. Pterostilbene, piceatannol and ε-viniferin were also effective against some fungal agents responsible of Esca, a grapevine trunk disease, mainly against the ones belonging to Botryosphaeriaceae family, as for example an IC50 of 163, 299 and 260 μM, respectively toward *Diplodia seriata* [73]. More complex stilbenes, such as tetramers (r-viniferin, r2-viniferin and hopeaphenol), found at high levels in wood and roots from grapevine, can have greater antifungal activity than the monomeric and dimeric forms, with IC_{50} values of 12–20 μM against *P. viticola* [74, 75].

To demonstrate the role of these phytoalexins in plant disease resistance, the stilbene synthase *VST1* gene fused to an alfalfa pathogen-inducible promoter was introduced in 41B grapevine rootstock. Resulting transgenic plants produced more resveratrol under biotic and abiotic stress conditions and showed reduced symptoms after infection with *B. cinerea* [76]. Two stilbene synthase genes *VST1* and *VST2*

from grapevine (*V. vinifera* L.) and the pinosylvin synthase gene (*PSS*) from pine (*Pinus sylvestris* L.) were stably transferred into bread wheat. Upon inoculation with the biotrophic pathogen *Puccinia recondita* f. sp. *tritici* several VST transgenic wheat lines showed a significant reduction of disease symptoms compared to wild-type plants. The reduction of disease symptoms was even more obvious after inoculation with the facultative biotrophic pathogen *Septoria nodorum* Berk [77]. Similarly, the transfer of stilbene synthase genes to tobacco, tomato, barley and rice leads to the accumulation of resveratrol and the resistance of the resulting transgenic plants to fungal pathogens [78–80]. In some cases, such as in kiwifruits and poplars, the heterologous expression of stilbene synthase did not result in an improved pathogen resistance, but resveratrol glucosides (less active on fungi than the aglycone form) were accumulated [81, 82].

Resveratrol is one of the most extensively studied natural products, even in human health field. Indeed, plethora of studies have demonstrated that resveratrol has preventive effect against a wide variety of diseases including cancer, cardiovascular diseases, as well as AIDS [83, 84].

2.3.3 Mechanism of Action of Secondary Metabolites

Flavonoids and phenylpropanoids are widely distributed in plants and exhibit different modes of action against pathogens. It is interesting to know that hundreds of clinical antifungal drugs in use, target only six different processes. Mostly they act as analogues of cellular signal compounds or substrates. They affect various physiological processes and some parts of the pathogens like biomembranes, enzyme inhibition, estrogenic properties and DNA alkylation [85]. These molecules usually have several phenolic hydroxyl groups in common, which can dissociate in negatively charged phenolate ions. Phenolic hydroxyl groups form hydrogen and ionic bonds with proteins and peptides. The higher the number of hydroxyl groups, the stronger the astringent and denaturing effect [86].

Proteins can only work properly if they have the correct three-dimensional structure, called conformation. Conformational changes alter their properties and can prevent effective crosstalk between proteins, and between proteins and DNA or RNA. Most secondary metabolites interact with proteins in one or another way by binding, complexing, denaturing, thereby changing protein conformations. Most secondary metabolites form covalent bond with protein, often by binding to free amino-, SH- or OH- groups, e.g., phenylpropanoids binds to amino groups, SH reagents and epoxides couple to free SH groups. The covalent modification can lead to a conformational change and thus loss of activity; or protein turnover is altered because proteases can no longer break down the alkylated protein. Polyphenols (phenylpropanoids, flavonoids, catechins, tannins, lignans, quinines, anthraquinones) interact with proteins by forming hydrogen bonds and the much stronger ionic bonds with electronegative atoms of the peptide bonds and/or the positively charged side chains of basic amino acids (lysine, histidine, arginine). A single of

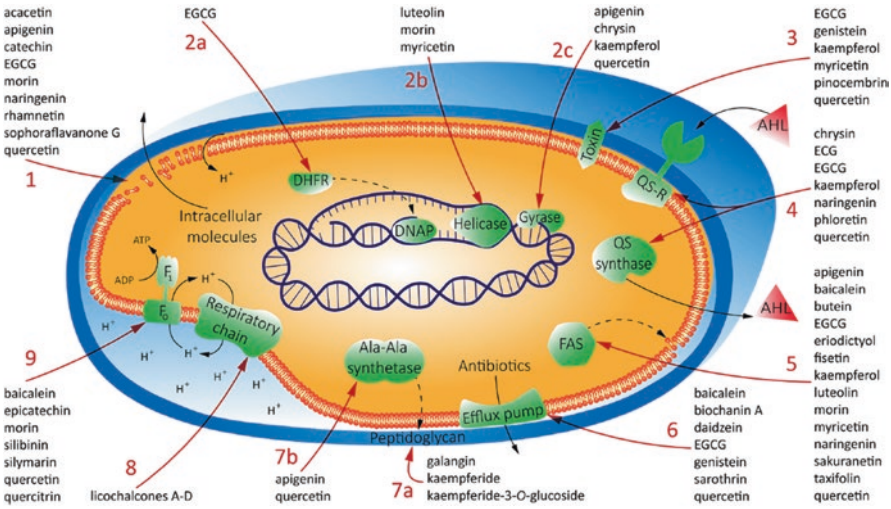

Fig. 2.2 Antimicrobial activity of flavonoids: mechanisms of action. (From Gorniak et al. [28]) This antimicrobial action can be performed by several ways: (1) membrane disruption, (2) inhibition of nucleic acid synthesis (2a-dihydrofolate reductase inhibition, 2b-helicase inhibition, 2c-gyrase/topoisomerase inhibition), (3) bacterial virulence inhibition (toxins), (4) quorum sensing inhibition, (5) inhibition of cell envelope synthesis (FAS = fatty acid synthase), (6) efflux pump inhibition, (7a,b) inhibition of peptidoglycan synthesis, (8) inhibition of the respiratory chain (NADH cytochrome C reductase), (9) inhibition of ATP synthase

these non-covalent bonds is quite weak. But because several of them are formed concomitantly when a polyphenol encounters a protein, a change in protein conformation or a loss in protein flexibility is likely to occur that commonly leads to protein inactivation. Regarding human health, since most polyphenols are quite polar and therefore, hardly absorbed after oral intake, they are usually not regarded as serious toxins [87]. Recently, Gorniak et al. [28] published a review on the action mechanism of flavonoids focusing on their antimicrobial activities (Fig. 2.2). Concerning the antifungal activity of stilbenes, a variety of ways are possible to explain their mechanism of action: cell wall and membrane disruption, increase of membrane permeability, disorganization of organelles and coagulation of cytoplasm, inhibition of ergosterol biosynthesis, ROS generation and apoptosis [88–91].

The effect of two stilbene compounds, pinosylvin and resveratrol, on the growth of several fungi was evaluated in plate tests. Wood decay tests were carried out with birch and aspen samples impregnated with one of these two stilbenes. In plate experiments, resveratrol had an enhancing effect on growth at concentrations where pinosylvin was already able to prevent the growth of most fungi studied [92]. Looking at the efficient mode of action of plant secondary metabolites against insect pests and pathogens they are gaining increased attention and interest among those concerned with environment friendly, safe, and integrated crop management approaches [93]. For example, stilbenes from grapevine roots and canes are a promising source of bioactive substances for the development of natural insecticides [94,

95]. Indeed, they cause mainly chronic mortality to the larvae of *Spodoptera littoralis* and *Leptinotarsa decemlineata*. The stilbenoid tetramers, r-viniferin and r2-viniferin, appear as the most active compounds. Regarding the mechanism of insecticidal action of stilbenes, several targets have been found: inhibition of tyrosinase (very important enzyme involved in the insect moulting process), as ligand on the ecdysteroid receptor (affecting ecdysis), and induction of an oxidative stress and detoxifying enzymes in insect larvae [95 and references inside].

2.4 Elicitation

Infected or elicited plants accumulate an array of plant defencive compounds. Nowadays, it is well accepted that plant secondary metabolites are involved in this plant defence system [96]. In 1982, Wolters and Eilert [97] reported for the first time that in rue callus cultures the acridone alkaloid content increased when it was co-cultivated with fungi. Through the years, fungal cell wall components, microbial preparations, various heavy metals, UV irradiation or ultrasound treatment are able to enhance secondary metabolites accumulation in plants. Plants treated with non-specific elicitors develop a general defence mechanism. This induced defence is a phenotypic trait. The process of inducing resistance using elicitors is environmental friendly and is advantageous over the chemical based pesticides. It is based on induction of the native "immune" potential of the host plant rather than on suppression of phytopathogens. This strategy could be an alternative solution to reduce the use of pesticides.

Elicitors are physical or chemical factors which when comes in contact to a living plant cell system triggers morphological and physiological responses such as phytoalexins accumulation. Elicitation is the process in which elicitor induces a sequence of reactions in the living cell, particularly related to biosynthesis of metabolites such as phytoalexins.

On the basis of their nature, elicitors can be divided into two types namely biotic and abiotic ones. Abiotic elicitors are the substances of non-biological origin, like organic salts and physical factors acting as elicitors (Table 2.1, [98, 99]). Biotic elicitors are substances with biological origin, like polysaccharides, derived from plant cell walls (pectin or cellulose), plant gums and microorganisms (chitins or glucans) and glycoproteins or intracellular proteins whose functions are coupled to receptors and act by activating or inactivating a number of enzymes or ion channels [98, 100]. Biotic elicitors can be further divided on the basis of their source into exogenous and endogenous groups. Exogenous elicitors are considered as the primary signals in plant pathogen interactions. They originate in the pathogen itself, mostly have a limited mobility within plant tissues, and evoke a response in cells in the immediate vicinity to the pathogen. Endogenous elicitors are of plant origin and arise as a result of the interaction with the pathogen. Most appear to be apoplastic and their function may be to modulate the extent of the response in the surrounding

Table 2.1 List of commonly used biotic and abiotic elicitors

Abiotic elicitors
Heavy metals (salts): Cu, Cd, Ag, Al, Zn, Co, Ni, Cr, V, La…
Physical stressors
Temperature: Freezing and thawing cycles, low and high temperature
Light: light intensity, UV radiation
Osmotic and oxidative stress: mannitol, sorbitol, potassium & sodium chloride…
Salinity and drought
Electromagnetic waves, electric field, ultrasound, pH…
Signal molecules
Jasmonic acid, methyl jasmonate
Salicylic acid, acetyl salicylic acid
Ethylene/Ethephon
Nitric oxide, systemin
Biotic elicitors
Yeast & fungal extracts
S. cerevisiae, *Phytopthora* sp., *Trichoderma* sp., *T. viride, A. niger, Fusarium* sp., *B. cinerea, Pythium* sp., *Verticillium* sp., *Colletotrichum* sp., *R. arrhizus, G. lucidum, Rhizoctonia* sp.…
Bacterial extracts
Pseudomonas sp., *Bacillus* sp., *Enterobacter* sp., *Azotobacter* sp., *Azospirillum brasilense, Streptomyces* sp., *Rhizobium* sp., *A. tumefaciens* & *A. rhizogenes*…
Plant extracts
Polysaccharides
Alginate, pectin, xanthan, glucan, chitin, chitosan, oligogalacturonic acid, oligosaccharides, laminarin, mannane, cyclodextrins…
Proteins/enzymes
Cellulase, hemicellulase, pectinase, glycoproteins, harpin, flagellin, lectins…

tissue. This modulation can be exerted independently of the presence of exogenous elicitors or in a synergistic manner [101].

In order to initiate defence, elicitors must be recognized by plant receptors localized to the plasma membrane or the cytoplasm. Elicitors subsequently or indirectly activate the corresponding effectors such as G-proteins, lipases and kinases, which then transduce the elicitor signal to downstream defence responses. The defence reaction involves synthesis of pathogenesis related proteins or defence secondary metabolites [102].

The influx of Ca^{2+} is a critica1 event in elicitor induced signal transduction that leads to accumulation of plant secondary metabolites. For example, treatment of grapevine cells with various elicitors rapidly triggers Ca^{2+} influx, alkalinization of extracellular medium, oxidative burst, activation of MAP kinases and protein phosphorylation/dephosphorylation events. These early events are followed by the induction of defence gene expression (including PAL and STS), resulting in the production of resveratrol, piceid and ϵ-viniferin [18, 103, 104].

2.5 In Vitro and In Vivo Studies

2.5.1 Abiotic Elicitors

2.5.1.1 Jasmonic Acid

Jasmonic acid (JA), an oxylipin-like hormone with its more active derivative methyljasmonate (MeJA), is derived from oxidized linolenic acid. Intensive investigations by many laboratories about the signal cascade of elicitation process resulted in the identification of JA and its derivatives as important elicitors [105]. It plays a key role in the elicitation of defence signaling pathways involved in resistance to pathogens, especially necrotrophs. JA is also used in a large number of cell and organ culture systems to increase the secondary metabolite yields [106].

Fungal diseases are a major problem in grapevine cultivation around the world. In order to limit these infections some alternative eco-friendly strategies like elicitation of plants with plant defense stimulators (PDS) have been adapted. Several studies report the stimulation of stilbene production by exogenous application of MeJA in grapevine cell cultures (Table 2.2). Some in vivo studies demonstrated that MeJA-treated leaves showed increased transcript levels of genes coding pathogenesis related proteins and coding enzymes involved in phytoalexin biosynthesis (phenylalanine ammonia-lyase and stilbene synthase) (Table 2.3). This was correlated with the accumulation of stilbenes (antimicrobial compounds). The eliciting activity of MeJA was confirmed by enhanced tolerance of grapevine foliar cuttings and vineyard against powdery mildew (75% and 73%, respectively) [143]. On the basis of these original results, MeJA could therefore act as an efficient elicitor in an alternative strategy of grapevine protection. Moreover, MeJA as well as benzothiadiazole (a synthetic analog of salicylic acid) application improves grape stilbene content in many varieties [149, 150].

Similarly, MeJA treated in vitro cultures of plants like *Pueraria montana*, *P. candollei, P. tuberosa, Astragalus membranaceus, Trifolium pratense, Medicago truncatula* accumulated significantly increased amount of isoflavones in comparison to control cultures. The isoflavone like daidzein is the precursor to the major phytoalexins including medicarpin which are produced in *Medicago* and *Glycine*, respectively [41]. There are reports for the increased production of these metabolites by using MeJA and other biotic elicitors (Table 2.4). There are studies on *Medicago truncatula* which demonstrated that on infection with the fungal pathogen *Macrophomina phaseolina,* genes involved in flavonoid and isoflavonoid biosynthesis were strongly up-regulated in the shoot. In addition, some genes in jasmonates (JAs) or ethylene (ET) pathways were not strongly induced in infected root tissue. Treating plants with methyl jasmonate (MJ) induced partial resistance in *M. truncatula* plants [175].MeJA can also increase activities of phenylalanine ammonia lyase (PAL) thus leading to the enhancement of flavonoid production in cell suspension culture of *Hypericum perforatum* [176].

Table 2.2 Effect of elicitors on the stilbenes content of *in-vitro* grown plants

S. No.	Plant species	Culture types	Elicitors used	Products	References
1	*Arachis hypogaea*	Hairy root	Methyl jasmonate, cyclodextrin	Piceatannol, resveratrol, arachidin-1 and -3	[106, 107]
2	*Cayratia trifolia*	Root culture	Yeast extract, salicylic acid, methyl jasmonate, ethrel	Piceid, resveratrol, viniferin, ampelopsin	[108]
3	*Cayratia trifolia*	Cell culture	Salicylic acid, methyl jasmonate, ethrel and yeast extract, salicylic acid and angiosperm parasite *Cuscuta*	Piceid, resveratrol, viniferin, ampelopsin	[109, 110]
4	*Morus alba*	Callus culture	2-hydroxypropyl-β-cyclodextrin	Resveratrol, oxyresveratrol (mulberroside A)	[111]
5	*Polygonum multiflorum*	Cell culture	Methyl jasmonate, salicylic acid	2, 3, 5, 4′-tetrahydroxystilbene-2-O-β-D-glycoside	[112]
6	*Silybum marianum*	Transformed cell culture	methyl jasmonate, cyclodextrin	Resveratrol	[113]
7	*Vitis rupestris* and *Vitis vinifera* cvs	*In vitro* plants	UV irradiation, aluminum chloride, and *Botrytis cinerea*	Resveratrol	[114]
8	*Vitis* spp.	Non-embryogenic callus	UV-C irradiation	Resveratrols and piceids	[115]
9	*Vitis vinifera*	Cell culture	Methyl jasmonate	*Trans*-resveratrol and piceids	[105, 116]
10	*Vitis vinifera*	Cell culture	Cyclodextrins	Resveratrol	[117]
11	*Vitis vinifera*	Cell culture	Chitosan	Resveratrol	[118, 119]
12	*Vitis vinifera*	Cell culture	Methyljasmonate, cyclodextrins	Resveratrol	[103, 120]
13	*Vitis vinifera*	Cell culture	Dimethyl β-cyclodextrin	Resveratrol	[121, 122]
14	*Vitis vinifera*	Cell culture	Salicylic acid, Na-orthovanadate, jasmonates, chitosan and the monomers D-glucosamine and N-acetyl-D-glucosamine, ampicillin and rifampicin	Resveratrol	[123]

(continued)

Table 2.2 (continued)

S. No.	Plant species	Culture types	Elicitors used	Products	References
15	*Vitis vinifera*	Callus culture	UV irradiation	*Trans*-resveratrol	[124]
16	*Vitis vinifera* cv. *Barbera*	Cell culture	Jasmonic acid, methyljasmonate and Na-orthovanadate	*Trans*- and *cis*-resveratrols; *trans*-resveratrol and piceids	[125, 126]
17	*Vitis vinifera* cvs Michele Palieri and Red Globe	Cell culture	Methyl jasmonate	*Trans*-piceid and ε-viniferin	[127]
18	*Vitis vinifera*	Cell culture	Methyl jasmonate, dark	Resveratrol, piceid, other phenolics	[128]
19	*Vitis vinifera* *Vitis rupestris*	Cell culture	Methyl jasmonate, light	Resveratrol, piceid, ε- and δ-viniferins	[129]
20	*Vitis vinifera*	Cell culture	Methyl jasmonate, UVC, salicylic acid, chitosan	*Trans*- and *cis*-piceids, resveratrol, ε-viniferin	[130, 131]
21	*Vitis rotundifolia*	Hairy root	Methyl jasmonate	*Trans*-piceid, *trans*-resveratrol, ε-viniferin	[132]
22	*Vitis vinifera*	Cell culture	Methyl jasmonate, ultrasound	ε- and δ-viniferins	[133]
23	*Vitis vinifera*	Cell culture	Methyl jasmonate, red light	*Trans*- and *cis*-resveratrols, *trans*- and *cis*-piceids, *trans*- and *cis*-resveratrolosides	[134]
24	*Vitis vinifera*	Cell culture	Methyl jasmonate, cyclodextrins	*Trans*-resveratrol	[135]
25	*Vitis vinifera*	Cell culture	Oligogalacturonide, laminarin	Resveratrol, piceid	[136]

2.5.1.2 Ethephon

Ethephon is an ethylene-releasing compound. It has been known for a long time as an inducer of phenylpropanoid biosynthesis which may be related to a general wound and/or stress response. An ethephon related increase of PAL activity had been described long back [177–179]. There are some in vivo and in vitro studies which demonstrate increased stilbene and isoflavone accumulation in the plants by ethephon/ethrel treatment (Tables 2.2, 2.3, and 2.4). In a study, Belhadj and co-workers [145] treated grapevine foliar cuttings (*V. vinifera* cv Cabernet Sauvignon) with ethylene-releasing ethephon. This resulted in an increase in the number of pathogenesis-related protein gene copies (CHIT4c, PIN, PGIP, and GLU) and in an enhancement of phytoalexin biosynthesis by inducing the *PAL* and *STS* genes that

Table 2.3 Effect of elicitors on the stilbenes content of *in-vivo* grown plants

S. No.	Plant species	Plant parts elicited	Elicitors used	Products	References
1	*Vitis vinifera*	Whole plants	Laminarin	Resveratrol and ε-viniferin	[137]
2	*Vitis vinifera sylvestris*, *Vitis vinifera sativa*	Postharvest grapes	Ultraviolet C	Total stilbenes	[138]
3	*Vitis vinifera*	Plants	*Plasmopara viticola* infection, ultraviolet light, and $AlCl_3$	Pterostilbene	[139, 140]
4	*Vitis vinifera* L. cv. *Barbera*	Berries	*Aspergilli japonicus, A. ochraceus, A. fumigatus* and isolates of *A. carbonarius*	*Trans*-resveratrol	[141, 142]
5	*Vitis vinifera*	Plants; berries	Methyl jasmonate	Stilbenes	[143, 144]
6	*Vitis vinifera*	Grapevine detached leaves and grapevine foliar cuttings	Ethephon	Resveratrol, piceid, viniferins pterostilbene	[145]
7	*Vitis vinifera* cv. *Barbera*	Berries	*Aspergillus carbonarius*	*Trans*-resveratrol and piceatannol	[146]
8	*Vitis vinifera*	Flowers, berries	UV radiation	Resveratrol	[147]
9	*Vitis vinifera*	Plants, berries	Methyl jasmonate, chitosan, yeast extract	*Trans*- and *cis*-resveratrols, *trans*- and *cis*-piceids, viniferin, other phenolics	[148, 149]
10	*Vitis vinifera*	Plants, berries	Methyl jasmonate, benzothiadiazole	*Trans*-resveratrol, *trans*- and *cis*-piceids, other phenolics	[150]
11	*Vitis vinifera*	Plants, berries	Chitosan	resveratrol, piceid, ε- and δ-viniferins	[151]

correlated with the accumulation of stilbenes (antimicrobial compounds). Moreover, ethephon treatment triggered the protection of grapevine detached leaves and grapevine foliar cuttings against *E. necator*, the causal agent of powdery mildew (64% and 70%, respectively). These studies emphasize the major role of ethylene in grapevine defence.

Production of isoflavones like puerarin, daidzein, genistin and genistein were also increased in ethephon treated cell cultures of *Pueraria tuberosa* [172]. Effects of ethephon on isoflavones production in different plants still need attention.

Table 2.4 Effect of elicitors on the isoflavones content of *in-vitro* grown plants

S. No.	Plant species	Culture types	Elicitors	Products	References
1	*Albizzia kalkora*	Root cultures	Strains of *Rhizobium* sp	Daidzein and genistein	[152]
2	*Astragalus membranaceus*	Hairy root	Methyl jasmonate, UVA, UVB, UVC, chitosan	Calycosin-7-*O*-β-D-glucoside, ononin, astraisoflavan-7-*O*-β-D-glucoside, calycosin, formononetin	[153–155]
3	*Astragalus membranaceus*	Adventitious roots	Chilling, salicylic acid	Calycosin-7-*O*-β-D-glucoside	[156]
4	*Cicer arietinum*	Callus/tissues	*Hypnea musciformis* (red algae)	Formononetin, maackiain, naringin and naringin melonate	[157]
5	*Glycine max*	Hairy root	*Fusarium solani*	Genistin, daidzin, glycitin and their malonyl conjugates and aglycones, coumestrol and glyceollin	[158]
6	*Glycine max*	Cell culture	Methyl jasmonate	Daidzin, malonyldaidzin, malonylgenistin	[159]
7	*Glycyrrhiza echinata*	Cell culture	Yeast extract	Formononetin and daidzein	[160]
8	*Lupinus albus*	Seedlings	Purified yeast cell wall	Prenylated isoflavone aglycones	[161]
9	*Manihot esculenta*	Cell culture	Yeast extract	Phenylpropanoids and scopoletin	[162]
10	*Medicago truncatula*	Cell culture	Yeast extract and methyl jasmonate	Formononetin and biochanin-A medicarpin and daidzin	[163]
11	*Phaseolus vulgaris*	Seedlings	$CuCl_2$, chitosan, gentamycin, saccharosamine, galactosamine and glucosamine	Phaseollin, coumestrol, genistein and daidzein	[164]
12	*Psoralea corylifolia*	Hairy root, cell culture	Yeast extract, chitosan, salicylic acid	Daidzein and genistein	[165, 166]
13	*Psoralea corylifolia*	Hairy root	Jasmonic acid, acetyl salicylic acid	Daidzin	[167]

(continued)

Table 2.4 (continued)

S. No.	Plant species	Culture types	Elicitors	Products	References
14	*Pueraria candollei* var. *candollei* and *P. candollei* var. *mirifica*	Cell culture	Copper sulfate, methyl jasmonate (MeJA), and yeast extract, chitosan, laminarin	Isoflavones	[168]
15	*Pueraria candollei*	Hairy root	Methyl jasmonate, chitosan, salicylic acid, *Agrobacterium*, and yeast extract	Isoflavones	[169]
16	*Pueraria montana*	Hydroponically grown seedlings	Cork pieces, XAD-4, and methyl jasmonate	Daidzein, genistein, daidzin, genistin, and puerarin	[170]
17	*Pueraria tuberosa*	Cell culture	Yeast extract	Puerain, genistin, daidzein and genistin	[171, 172]
			Salicylic acid		
			Methyl jasmonate		
			Ethrel		
18	*Trifolium pratense*	Seedlings	Chitohexose	Formononetine-7-*O*-glucosyl-6″-malonate and maackiain-3-*O*-glucosyl-6″-malonate.	[173]
			Copper chloride		
19	*Trifolium pratense*	Cell culture	Jasmonic acid	Isoflavonoids	[174]

2.5.1.3 Salicylic Acid

In addition to jasmonic acid and ethylene, a third major signal molecule, salicylic acid, plays an important role in plant defence responses against mainly biotrophic pathogens by the induction of local and systemic acquired resistance (SAR). The existence of a crosstalk between salicylic acid and ethylene/jasmonic acid signaling pathways has been found [180]. Jiao et al. [181] showed that the resistance of grapevine (*Vitis pseudoreticulata*) to powdery mildew is linked to a better recruitment of salicylic acid signaling for the induction of stilbene synthase gene expression and stilbene accumulation. Concerning the isoflavonoids, Durango et al. [182] found a correlation between the phytoalexin content in *Phaseolus vulgaris* cotyledons increased by salicylic acid treatment, and the resistance against *Colletotrichum lindemuthianum*.

There are some in vitro and in vivo studies showing an increased accumulation of stilbenes and isoflavones by using salicylic acid or functional analogues (Tables 2.2, 2.3, 2.4, and 2.5).

Benzothiadiazole, a salicylic acid analogue, induces defences in a variety of plant species against different pathogens [198, 199]. For example, it provides a

good protection against *B. cinerea* in grapevine bunches in the vineyard, and also against downy and powdery mildews in grapevine leaves in a greenhouse. In this last case, an up-regulation of pathogenesis-related protein gene expression and of pterostilbene accumulation was observed.

2.5.1.4 UV Light

Resveratrol production and expression of the genes related to resveratrol biosynthesis were investigated in the skins of three *V. vinifera* cultivars after exposure to ultraviolet (UV). Resveratrol concentration in the skins of all the grapes increased significantly when exposed to UV-C (254 nm) irradiation [200]. In another study, it was demonstrated that in *V. rupestris* UV irradiation induced a high, constant level of *STS* mRNA production which was correlated to resveratrol accumulation [115]. Another group of workers evaluated the response to UV radiation of grape flowers and green berries of 72 grape genotypes for their ability to produce resveratrol. This was used to establish a selection criterion for screening genotypes for resistance to gray mold and powdery mildew. There was a strong negative correlation between UV-induced resveratrol production and susceptibility to *Botrytis* infection [202]. Callus cultures of *V. vinifera* were exposed to 254 nm UV light. About 15 min of UV irradiation period was found to be effective for induction of *trans*-resveratrol production (62 μg/g callus fresh weight) [125]. Thus, UV light can be used as an efficient elicitation source for stilbene production (Tables 2.2 and 2.3). Similarly, UV treated hairy root cultures of *Astragalus membranaceus* and UV treated in vivo grown plants of *Lotus japonicus*, *A. membranaceus* and *Glycine max* exhibit an increase of the isoflavone accumulation in comparison to control cultures (Tables 2.4 and 2.5).

2.5.1.5 Cyclodextrins

Cyclodextrins (CD) are cyclic oligosaccharides of 6, 7 or 8 α –D-glucopyranoside residues linked by α 1→4 glucosidic bonds, which are called α-, β-, and γ-CD. They have a hydrophilic external surface and hydrophobic central cavity that can trap a polar compound [201]. The production of cyclodextrins is relatively simple and involves treatment of ordinary starch with a set of easily available enzymes [202]. Dimethyl -β-cyclodextrin (DIMEB), an oligosaccharide consisting of 2,6-methylated cyclic α (1→4)- linked glucopyranose moieties, has shown to be capable of inducing stilbene biosynthesis in liquid *V. vinifera* cell cultures, also in the absence of pathogenic organisms [122] (Table 2.2). This molecule seems to mimic a defence elicitor which enhances the physical barriers of the cell, stops cell division and induces phytoalexin synthesis [123].

A grapevine liquid cell culture system was used to examine the properties of CDs as inducers of defence responses. This work shows that the chemically pure heptakis (2,6-di-*O*-methyl)-β CD caused a dramatic extracellular accumulation of the

Table 2.5 Effect of elicitors on the isoflavones content of in vivo grown plants

S. No.	Plant species	Plant part elicited	Elicitor	Products	References
1	*Astragalus membranaceus*	Plants	UV	Calycosin-7-*O*-β-D-glucoside, calycosin, formononetin, daidzein, isoliquiritigenin, liquiritigenin	[183]
2	*Cicer arietinum*	Seedlings	Reduced glutathione	Biochanin A, formononetin and medicarpin and maackiain, homoferreirin and cicerin	[184]
3	*Glycine max*	Seeds	Lipo-chitooligosaccharides, chitosan, *Streptomyces melanosporofaciens* strain EF-76 and yeast extract	Daidzein, genistein, glycitein	[185]
4	*Glycine max*	Seeds and seedlings	*Aspergillus sojae* cell wall extract	Glyceollins	[186]
5	*Glycine max*	Cotyledons	*Diaporthe phaseolorum* f. sp. *meridionalis*	Daidzein, genistein and glyceollins, apigenin	[187]
6	*Glycine max*	Seeds	β-glucan from *Phytophthora sojae*	Daidzein, coumestrol, genistein, luteolin and apigenin	[188]
7	*Glycine max*	Cotyledon tissue	β-glucan from *Phytophthora sojae*	Glyceollin	[189]
8	*Glycine max*	Sprouts	UV B	Malonylglycitin, malonyldaidzin, malonylgenistin (glucosides and aglycones)	[190, 191]
9	*Lotus japonicus*	Seedlings	Reduced glutathione	Vestitol	[192]
10	*Lotus japonicus*	Leaves	UV B	Vestitol, isoliquiritigenin, sativan, medicarpin, formononetin, daidzein, biochanin A	[193]
11	*Lupinus angustifolius*	Seedlings	*Pleiochaeta setosa*	Genistein, 2′-hydroxygenistein	[194]

(continued)

Table 2.5 (continued)

S. No.	Plant species	Plant part elicited	Elicitor	Products	References
12	*Medicago sativa*	Plantlets	*Colletotrichum trifolii*	Medicarpin	[195]
13	*Medicago truncatula*	Plantlets	*Phoma medicaginis*	Formononetin 7-*O*-glucoside and malonylated formononetin 7-*O*-glucoside	[196]
14	*Phaseolus vulgaris*	Cotyledons	Salicylic acid and structurally related compounds	Genistein, dalbergioidin, phaseollinisoflavan, phaseollidin, daidzein, 2′-hydroxygenistein, kievitone, coumestrol, phaseollin	[182]
15	*Trifolium pratense*	Plantlets	Yeast extract and chitosan	Genistein, daidzein, formononetin and biochanin A	[197]

phytoalexin resveratrol and changes in peroxidase activity and isoenzymatic pattern. Other modified CDs tested on several grapevine cell lines resulted in different eliciting capacities of CDs and different sensibilities of the cell lines. The spent medium of elicited cultures containing polyphenolic compounds released by plant cells was shown to disturb *B. cinerea* growth in a plate assay [118].

Moreover, co-treatment with cyclodextrin and methyl jasmonate can lead to sustained high levels of stilbenes in *V. vinifera* cell cultures and *Arachis hypogaea* hairy root cultures [Table 2.2]. Komaikul et al. [112] showed that the addition of 2-hydroxypropyl-β-cyclodextrin in *Morus alba* callus cultures improves resveratrol and oxyresveratrol production, but does not increase mulberroside A content.

2.5.1.6 Phosphites

Phosphite is a neutralized solution of the phosphonate anion [203]. Phosphite contains one less oxygen (O) than phosphate, making its chemistry and behavior quite different. Phosphite is less chemically stable and more soluble than phosphate, when applied to plants, it is quickly absorbed by leaves, roots and branches, thus high concentrations can be toxic for plants. It is able to move in both xylem and phloem. Unlike phosphates, phosphites stimulate the pathogen defence mechanisms in plants and have antifungal activity by inducing production of phytoalexins [204, 205]. It is effective against fungi like *P. viticola, E. necator*, *Pythium* sp. and *Phytophthora nicotianae*.

The relatively limited fungicidal effect—combined with its ability to stimulate plants to make a broad spectrum of biologically active metabolites— makes phosphite relatively benign to the environment and safe to use [206]. Nowadays there are many reports on phosphite induced cellular responses to pathogen challenge and suppressed pathogen ingress in both in vitro and in vivo cultures [207, 208]. Different mechanism of phosphite actions were postulated by Grant and co-workers [209]. With the indirect actions, phosphite is hypothesized to cause the pathogen to produce elicitors or inhibit its production of suppressors, allowing plant defence responses to halt invasion by the pathogen [210].

After the treatment of grapevine foliar cuttings with three elicitors, methyl jasmonate, acibenzolar-S-methyl or phosphites, an extensive reprogramming of primary metabolic pathways was observed in leaves, using a metabolomics approach (NMR technique). Thus the induction of resistance to phytopathogenic agents correlated with these metabolic changes could have a negative impact on the physiology of plants [211].

2.5.1.7 Pulsed Electric Field and Ultrasound

Pulsed electric field (PEF) is considered as an external stimulus or stress, and it is proposed as a promising new abiotic elicitor for stimulating secondary metabolite biosynthesis in plant cell cultures. The effects of PEF on growth and secondary metabolite production by plant cell culture were investigated by using suspension cultures of *Taxus chinensis* as a model system. A significant increase in intracellular accumulation of taxuyunnanine C (Tc), a bioactive secondary metabolite, was observed by exposing the cells in the early exponential growth phase to a 30-min PEF [212]. The effects of PEF and ethephon on growth and secondary metabolites accumulation were also investigated in suspension culture of *V. vinifera* L. cv. Gamay Fréaux as a model system. After the treatments, production levels of extracellular phenolic acids, 3-*O*-glucosyl-resveratrol was increased in the cultures [213]. In another recent study, isoflavones production in the Glycine cell culture increased with PEF application at 1.6 kV, and aglycone forms were influenced to a greater extent [214]. These results show that PEF induces a defence response of plant cells and may alter the cell/membrane's dielectric properties.

Low-energy ultrasound used alone or associated with methyl jasmonate leads also to the stimulation of δ- and ε-viniferins accumulation in *V. vinifera* cell cultures [133].

2.5.2 Biotic Elicitors

2.5.2.1 Chitosan

Chitosan is a polysaccharide called poly [β-(1→4)-2-amino-2-deoxy-*D*-glucopyranose]. It is a plant defence booster derived from deacetylation of chitin which is extracted from the exoskeleton of crustaceans such as shrimps and crabs,

as well as from the cell walls of some fungi [215, 216]. The primary unit in the chitin polymer is poly[β-(1→4)-2-acetamido-2-deoxy-*D*-glucopyranose]. The units are combined by 1,4 glycosidic linkages, forming a long chain linear polymer. Removal of most of the acetyl groups of chitin by treatment with strong alkalis yields chitosan [217]. Agricultural applications of chitosan are for stimulation of plant defence. The chitosan molecule triggers a defence response within the plant, leading to the formation of physical and chemical barriers against invading pathogens [218].

Chitosan conferred a high protection of grapevine leaves against grey mould caused by *B. cinerea*. Treatment of grapevine leaves by chitosan led to marked induction of lipoxygenase (LOX), phenylalanine ammonia-lyase (PAL) and chitinase activities, three markers of plant defence responses. Strong reduction of *B. cinerea* infection was achieved with 75–150 mg/l chitosan [219]. In some studies it was observed that grapevines with higher assays of chitinase or ß-1,3-glucanase had greater resistance to powdery mildew, and when combined had even greater field resistance against powdery mildew (*E. necator*) [220].

In an investigation, chitosan increased the amounts of genistein and 2-hydroxygenistein monoprenyls in roots of white lupin and in the exudates [221]. Further studies indicated that chitosan triggers either the de-novo synthesis of phenolic compounds as the first defensive line designed to inhibit growth of the fungus and β-1,3-glucans act as a second mechanical barrier for blocking potential invasion by fungal cells and protecting the tissue against phytotoxic substances [222, 223]. Chitosan coating of litchi fruits increased their content of flavonoids and resistance to browning and postharvest decay [224]. Chitosan has also been used in in vitro cultures of *V. vinifera, Psoralea corylifolia, Pueraria candollei* and *Astragalus membranaceus* to enhance the stilbene and isoflavone production, respectively (Tables 2.2 and 2.4). Besides its use in in vitro studies, chitosan is also used in in vivo studies to increase the isoflavone production and also the stilbene content (resveratrol, piceid, viniferin) in grape bunches (*V. vinifera*) (Tables 2.3 and 2.5). Chitosan is a nontoxic biodegradable material, acting as an elicitor. Thus, it has the potential to become a new class of plant protecting agent, assisting towards the goal of sustainable agriculture.

2.5.2.2 Laminarin

The molecule laminarin (also known as laminaran) is a linear glucan found in brown algae. Laminarin is clearly a high-energy carbohydrate. It is used as a food reserve in the same way that chrysolaminarin is used by phytoplankton. It is made up of β(1→3)-glucan with β(1→6)-linkages and a β(1→3):β(1→6) ratio of 3:1 [225]. In a study, β-1,3-glucan laminarin derived from the brown algae *Laminaria digitata* was shown to be an efficient elicitor of defence responses in grapevine cells and plants. It also effectively reduced *B. cinerea* and *P. viticola* development on infected grapevine plants. Defence reactions elicited by laminarin in grapevine cells include calcium influx, alkalinization of the extracellular medium, oxidative burst, activation

of two mitogen-activated protein kinases, expression of 10 defence-related genes with different kinetics and intensities, increase in chitinase and β-1,3-glucanase activities, and production of two phytoalexins (resveratrol and ε-viniferin). When applied to grapevine plants, laminarin reduced infection of *B. cinerea* and *P. viticola* by approximately 55% and 75%, respectively [137]. In another study with grapevine cells, a significant action of laminarin on the stilbene accumulation was not found, but the treatment with an oligogalacturonide led to a high induction of stilbene synthase activity and stilbene accumulation (resveratrol, piceid) with an important impact on primary metabolism [136].

Laminarin increased also the isoflavone accumulation in cell cultures of *Pueraria candollei* (Table 2.4).

2.5.2.3 Yeast Extract

Yeast extract is the common name for various forms of processed yeast products made by extracting the yeast cell contents (removing the cell walls). Yeast has been proved to be an efficient elicitor for the increased accumulation of isoflavone and stilbene in different plants (Tables 2.2, 2.4, and 2.5). Yeast extract-treated suspension cultures of a new cell line, AK-1, of *Glycyrrhiza echinata* were induced to produce an isoflavonoid phytoalexin (medicarpin). From these cells, putative full-length cDNAs encoding cytochrome P450s, (2S)-flavanone 2-hydroxylase and isoflavone 2′-hydroxylase were cloned [226]. A cDNA encoding UDP-glucose: formononetin 7-*O*-glucosyltransferase, designated UGT73F1, was also cloned from yeast extract-treated *Glycyrrhiza echinata* L. cell-suspension cultures. Recombinant UGT73F1 was expressed as a histidine-tag fusion protein in *Escherichia coli.* The purified recombinant enzyme was selective for isoflavone, formononetin and daidzein as substrates [160]. Besides this, there are various reports where yeast extract was the most efficient elicitor in comparison to other elicitors [169].

2.6 Conclusions

The application of biotic and abiotic elicitors in developing plant resistance is still in the early stages of use. Currently, our knowledge is mainly based on the experimental trials. There are many reports which prove their efficacy as potent natural pesticides. It is well established that these elicitors impart disease resistance by elevating or developing some secondary metabolites in the plants. Effect of several elicitors on the production of stilbenes in different *Vitis* species and other stilbenes containing plants has been summarized in Tables 2.2 and 2.3. Similarly, the recent studies on the effect of different biotic and abiotic elicitors on the isoflavones production have been summarized in Tables 2.4 and 2.5.

Numbers of studies, especially concerning grapevine inoculated with various pathogens, have established a positive correlation between stilbene levels and

pathogen defence. Evidence supporting the role of stilbenes (resveratrol) in resistance to pathogen infection was supplied by the transfer of stilbene synthase genes in plants that do not produce stilbenes, such as tobacco and alfalfa [78, 80].

Isoflavones function in both, the symbiotic relationship with rhizobial bacteria and the plant defence response. The importance of isoflavones can be judged by different reports where in order to increase the disease resistance in plant, production of certain types of isoflavones was enhanced by genetic manipulations [227]. The non-legume plants like *Arabidopsis*, *Nicotiana tabacum* and *Zea mays* (for human consumption) were metabolic engineered for isoflavones production. Due to complexities in regulation of inter-related biochemical pathways, metabolic engineering to affect the isoflavones biosynthetic capacity of a target plant tissue, presents a challenge [228]. However, by using elicitors this process can be simplified and can become more practically feasible in fields.

Use of elicitors has an added advantage over the chemicals used to prevent plants from diseases. There are increasing evidences, that elicitors could be used in the future as alternatives to traditional pesticides for managing pathogens and pests in agriculture and nursery production of forest trees. Moreover, the use of elicitors could lead to a better nutraceutical quality of crops [229].

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Chapter 3
Sources of Variation in Defensive Traits in *Quercus* Species: Insights Gained from Research Spanning Individuals to Communities and Local- to Broad-Scale Factors

Xoaquín Moreira and Luis Abdala-Roberts

3.1 Introduction

Quercus (Fagaceae) is a species-rich genus that includes close to 600 species of shrubs and trees distributed throughout the Northern Hemisphere [1–4], and as such represents one of the most diverse and broadly distributed tree genera with species occurring from the tropics to northern latitudes and from the Americas to Europe and Asia. Paleoecological data indicate that the genus originated in America where highest levels of species richness are currently found at middle latitudes (Mexico), and subsequently spread to the Old World [2, 5]. Extant oaks are a dominant component of terrestrial vegetation in many temperate regions and are considered foundation species that support a high diversity of associated consumers and shape ecosystem functions in different types of temperate forests [6].

Throughout their distribution, oaks are attacked by a diverse fauna of vertebrate and invertebrate herbivores. Notorious examples in the New World include white-tail deer *Odocoileus virginianus*, the leaf-tying caterpillar *Psilocorsis quercicella*, the western tussock moth *Orgyia vetusta*, and the gold-spotted oak borer *Agrilus coxalis* [7–10], whereas in the Old World common herbivores are the European roe deer *Capreolus capreolus*, the gypsy moth *Lymantria dispar*, the oak processionary *Thaumetopoea processionea,* the sycamore *Acronicta aceris*, the pale tussock *Elkneria pudibunda*, the leaf miner *Ectoedemia albifasciella*, and the purple hairstreak *Favonius quercus* [11–13] (Fig. 3.1). There is strong evidence linking several of these herbivores, particularly in the case insects, and putative defensive traits for a

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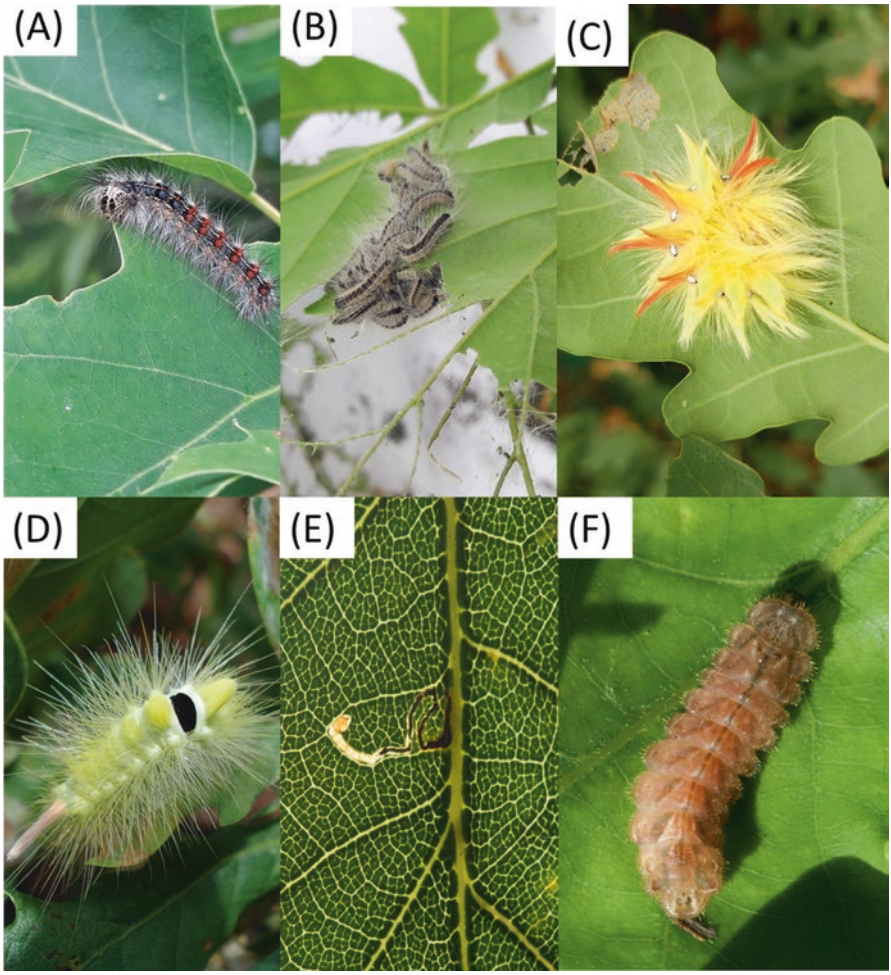

Fig. 3.1 (**a**) The gypsy moth *Lymantria dispar* (Photo credit: Thomas Damestoy), (**b**) the oak processionary *Thaumetopoea processionea* (Photo credit: Thomas Damestoy), (**c**) the sycamore *Acronicta aceris* (Photo credit: Bastien Castagneyrol), (**d**) the pale tussock *Elkneria pudibunda* (Photo credit: Bastien Castagneyrol), (**e**) the leaf miner *Ectoedemia albifasciella* (Photo credit: Ayco Tack), and (**f**) the purple hairstreak *Favonius quercus* (Photo credit: Ayco Tack)

number of oak species [14–18]. These include physical traits such as toughness, fiber content, and trichomes [8, 19, 20], chemical traits such as phenolic compounds (e.g. tannins; [10, 15, 17, 18]), and phenological traits such as leaf longevity and timing of leaf senescence [21].

Research on oak anti-herbivore defences over the last half century spans more than 60 species, ranging from perennial shrubs to deciduous trees found in plant communities that range from montane tropical forests to temperate deciduous forests or Mediterranean-type vegetation [8, 18]. Much of this work has focused on

correlations between insect attack and several of the above-mentioned oak traits. For example, studies have looked at associations between chemical defences and herbivore abundance or performance [14, 16, 22], leaf phenological escape from herbivory [21, 23], as well as landscape-scale (e.g. patch-level or fragment) spatial variation in chemical defences [24, 25]. In most of these cases, studies have usually focused on one or two co-occurring oak species at the local scale, though recent efforts have been made to address broader-scale (biogeographic) patterns in defences within-species as well as across a number of species or clades using phylogenetic methods. Examples of the latter include work on oak defences along climatic or latitudinal gradients [17, 19, 26], elevational gradients [27, 28], as well as continental-scale comparisons [18].

Knowledge gained thus far from these studies has shed light into the importance of diverse biotic and abiotic contributors to intra- and inter-specific variation in oak putative defensive traits. We hereby seek to summarize this rich body of research by describing the most relevant findings on patterns of variation in oak defences, ranging from individual- to community-level variation and spanning local-scale to broad-scale patterns. In each case, we analyse and synthesize the most important underlying biotic and abiotic factors shaping these patterns, and, in doing so, provide an integrative view of sources of variation shaping intra- and inter-specific variation in oak anti-herbivore defences. In addition, we also point at potential linkages across scales to be addressed in future work.

3.2 Local and Landscape-Level Sources of Variation in Oak Defences

3.2.1 Individual- and Population-Level Variation

3.2.1.1 Plant Genetic Variation

Intra-specific variation in plant defences occurring at the level of individual plants can be genetically- or environmental-based [29–31]. To date, however, relatively few studies have assessed patterns of genetically-based intra-specific variation in oak defensive traits. Despite this, there are a few recent studies reporting on intra-specific variation in chemical defences for a handful of species, most of which have measured phenolic compounds. For example, Damestoy et al. [32] found that the concentration of leaf total phenolics in *Q. robur* exhibited significant (three-fold variation) across a pool of 30 genotypes sourced from a single site. In addition, Solla et al. [33] found significant genotypic variation in leaf phenolic concentration within populations *Q. ilex*, but variation among several populations was low and not statistically significant. Although in combination these studies report on intra-specific variation for merely two oak species, results suggest genetically-based variation in oak chemical defences in both cases, either within or among populations. These results are comparable to levels of intra-specific genetic variation in

chemical defences (including phenolics) reported for other better studied taxa of temperate trees such as *Pinus* [31, 34, 35], *Salix* [30, 36, 37], and *Populus* [29, 38, 39]. Accordingly, further investigations spanning other oak species are necessary to gain a better understanding of patterns of intra- and inter-specific variation in *Quercus* defences, its underlying biotic and abiotic correlates, and its ecological implications [40].

3.2.1.2 Environmental-Based Variation and Plant Plasticity

Induced defensive responses to biotic or abiotic factors have been evaluated for several oaks species, among which the effects of resource availability and herbivory have received the most attention (see studies in Hunter [41]). In the case of resource availability, a study by Forkner and Hunter [42] evaluated the effects of soil nutrients and found that addition of fertilizer caused a reduction in leaf tannin concentration and an increase in leaf chewer, phloem feeder, and leaf miner abundance for *Q. rubra* and *Q. prinus*. In addition, a previous study reported that soil fertilization weakened induced resistance to herbivory in these species [43], which in combination with the latter study indicates soil resource controls over both constitutive and induced defences in these species. These results agree with findings of studies with other tree species [29, 31] and are consistent with plant defence theory in that plants growing under resource-rich environments grow faster and allocate less resources to defences ("Resource Availability Hypothesis", [44]). Other studies with oaks have tested for effects of additional sources of abiotic variation, in some cases showing inconsistent patterns. For example, with respect to water availability, Castagneyrol et al. [25] found that leaf physical defences in *Q. robur* were not significantly influenced by artificial irrigation, whereas Schwanz et al. [45] reported for the same species that drought stress reduced the activities of antioxidative enzymes in leaves. In addition, Barber and Marquis [46] found that leaves on *Q. alba* trees exposed to high light availability were better defended (higher toughness and content of phenolics) than leaves on trees under shaded conditions. These findings are consistent with findings for other temperate and tropical tree species suggesting that photo-induction of these secondary metabolites is relatively common [47, 48]. Overall, findings concerning effects of nutrient availability are in agreement with plant defence theory, whereas effects of water and light availability are less consistent and may vary as a function of the type of secondary metabolites and their propensity for photo-induction (e.g. phenolics). Provided the importance of phenolic compounds as putative defensive traits in oaks, investigating the concurrent and interactive effects of multiple sources of resource availability (e.g. light, water and nutrients) for a larger number of species will yield insight on how the abiotic environment shapes oak defence investment.

3.2.1.3 Plant Ontogenetic Variation

Ontogenetic variation combines genetically-determined effects as well as plasticity responses to changing biotic and abiotic pressures over plant development. Although there is little work on ontogenetic variation in oak defences, a couple of recent studies by our group point at interesting results that merit further attention. Specifically, we found that levels of leaf physical and chemical defences in *Q. robur* were in most cases significantly higher in saplings than in adult reproductive trees, whereas insect leaf herbivory was correspondingly lower in saplings than in adult trees, a pattern that was possibly explained by differences in defence levels [24]. Similarly, in a recent study including eight oak species sampled throughout the Iberian Peninsula we also found that the concentration of chemical defences was higher for saplings than for adult trees [49]. However, in this case we found no significant effect of plant ontogeny on insect leaf herbivory [49]. Another study by Wang et al. [50] similarly found that saplings of *Q. variabilis* had a higher concentration of leaf chemical defences compared to mature trees across populations. These results could be seen as consistent with theory, as defence levels for long-lived plants are expected to decline from saplings to more advanced stages of adult life [51]. Overall, these findings point at ontogeny as an important source of variation in oak defences deserving further attention in other species to unveil ontogenetic trajectories in oak defensive investment. Multi-species experiments explicitly testing for plant ontogeny including multiple developmental stages as well as a more detailed control of early life stages (seedlings to saplings) under natural field conditions would provide a good step in this direction.

3.2.2 Plant Community-Level Variation

3.2.2.1 Plant Neighbourhood Effects

Plants are embedded in diverse communities where the presence of conspecific and heterospecific neighbours around a focal plant can greatly affect growth, survival and reproduction of individual plants [52, 53], as well as the strength of plant-herbivore interactions [54–56]. For example, the fact that the relative frequency of a particular host plant species is lower in heterospecific relative to conspecific neighbourhoods results in a lower likelihood of herbivore attack on this focal species (Resource Concentration Hypothesis; [57]). Similarly, herbivore attack on focal plants may decrease in species mixtures due to the presence of one or more heterospecific neighbours, which either attract herbivores or somehow interfere with herbivore location of the preferred host plant (i.e. associational resistance; [58]). Studies have also reported that the diversity or species composition of plant neighbourhoods can indirectly affect herbivory on focal plants by modifying plant nutritional quality (e.g. physical traits and secondary metabolites; [59–62]). For

example, competition for resources or facilitation among heterospecific plants may alter plant growth or the nutritional value of plant tissues to herbivores [63, 64].

A number of studies have evaluated the effects of population- and community-based habitat heterogeneity on oak defences and herbivory, most focusing on pedunculate oak *Q. robur* due to its broad distribution and dominant status in temperate forests of Western Europe. For example, in a recent observational study, we found that leaf physical and chemical defences in *Q. robur* did not vary between stands with a lower vs. high frequency of this species [24]. In contrast, leaf herbivory on this species was (as expected) lower in low-frequency stands and this pattern was apparently not associated with the measured leaf traits [24]. In a similar study, Castagneyrol et al. [65] found that leaf physical defences in *Q. robur* were greater in pure stands of this species relative to mixed stands, but defence levels varied depending on the canopy stratum, suggesting an influence of microhabitat abiotic conditions. Although some leaf traits were correlated with herbivory in this study, tree diversity effects on insect herbivory were only partially driven by variability in oak leaf traits [65]. In another study, Castagneyrol et al. [25] conducted a manipulative experiment with varying levels of tree species richness and found that leaf physical defences in saplings of *Q. robur* trees planted in mixtures varied depending on the tree species neighbour identity though in most cases defences were higher in oak monoculture stands. Counter to expectations, leaf trait variation again was independent of herbivory. Together, these studies provide evidence of emergent neighbourhood effects on English oak defences and herbivory, though patterns of variation in defence and herbivory appear to be uncoupled in some cases suggesting that other (unmeasured) defensive traits could be playing a role. Further tests of this type are needed for other oak species found in different community types in order to start unveiling relevant community-level predictors of variation in oak defences and herbivory.

3.2.2.2 Landscape-Level Variation

To date, most oak studies have centred on arthropod community variation at the landscape level [11, 66, 67] whereas less attention has been given to oak defensive traits, a key component needed to explain spatial herbivory or arthropod community structure. Initial work by M. Hunter, although conducted at a local (rather than landscape) scale, pointed at the importance of associating spatial variation in arthropod community structure and oak defences. For example, Hunter et al. [68] found within-stand correlations of opposing sign between oak (*Q. velutina* and *Q. rubra*) leaf secondary chemistry and densities of leaf-chewing and leaf-mining insects, but no correlation with sap-sucking insects. Leaf-chewers were negatively associated with secondary metabolite concentrations, whereas leaf-miners were positive associated with these traits suggesting that this guild obtains benefits against natural enemies from a diet high in secondary metabolites [69]. A couple of recent studies have explicitly addressed landscape-level variation in oak defences. In one of these studies, Maldonado-López et al. [70] found that the concentration of secondary

metabolites in *Q. castanea* did not vary as a function of fragment size. Given that leaf damage by insect leaf-chewers did depend on fragment size, these findings suggest that differences in herbivory were underlain by other unmeasured leaf traits or abiotic factors [70]. In contrast, Cuevas-Reyes et al. [71] found higher levels of secondary metabolites *Q. deserticola* in large than in small fragments, and that spatial variation in some groups of phenolic compounds was correlated with mistletoe infection. Insect herbivory and mistletoe infection were in turn negatively and positively correlated (respectively) with the concentration of secondary metabolites in this oak species [71], suggesting causal linkages between oak defences and these consumers across sites.

Overall, there have been few studies over the last two decades addressing landscape level variation in oak secondary chemistry and this appears to holds true for other tree species [41]. Thus, whereas early work pointed at the importance of investigating the relationships between arthropod community structure and oak (and other tree taxa) secondary chemistry in a spatially-explicit manner [72], since then there has been little progress in addressing this challenge. Nonetheless, the few studies conducted to date involving non-oak species have found patterns that suggest a link between spatial variation in herbivory, tree (secondary and primary) chemistry, and soil nutrient levels [41, 73]. Investigating these links at the landscape level will allow to assess the feedbacks between consumer effects, oak chemistry, and ecosystem processes (nutrient dynamics; [41]).

3.3 Broader-Scale Sources of Variation in Oak Defences

3.3.1 Regional to Global Patterns

3.3.1.1 Latitudinal and Elevational Gradients

Environmental gradients have played an important part in the study of spatial variation in species interactions, particularly plant-herbivore interactions [74, 75]. Classical hypotheses hold that stronger herbivore pressure under more stable climates towards the equator and sea level has resulted in stronger biotic selection on plant defensive traits [75, 76]. Although a number of empirical studies have found support for this pattern, recent syntheses on latitudinal (reviewed by Moles et al. [76] and Ansttet et al. [77]) or elevational (reviewed by Rasmann et al. [78] and Moreira et al. [79]) studies show mixed evidence for the predicted patterns.

Pertaining latitudinal gradients, Pearse and Hipp [17] found that constitutive leaf defences in 56 oak species were higher at lower latitudes, and that these latitudinal gradients were strongly predicted by climate. However, a closer look at studies addressing intra-specific clines reveals substantial variation among species. For example, Wang et al. [50] similarly reported that constitutive chemical defences (and insect herbivory) in *Q. variabilis* trees increased with decreasing latitude, a pattern that was strongly associated with latitudinal variation in climatic conditions.

In contrast, we reported that levels of constitutive leaf defences in *Q. robur* populations decreased with decreasing latitude and that this pattern was associated with higher insect herbivory at lower latitudes [26]. Importantly, our results also indicated climatic and soil variables predicted latitudinal variation in oak defences, and that this cline in defences in turn shaped the latitudinal gradient in herbivory from the "bottom-up" [26]. Finally, Loughnan and Williams [80] recently reported that constitutive physical defences and herbivory in leaves of *Q. garryana* were best explained by climatic variation (spring precipitation), but did not vary significantly with latitude.

With respect to elevational gradients, we recently found that constitutive chemical defences in leaves of eight oak species in the Iberian Peninsula increased towards higher elevations [49]. In another study, we found that inducibility of chemical defences (but not constitutive defences) in leaves of 18 oak species increased towards higher elevations, and that this gradient was not explained by climatic predictors [28]. Finally, in another recent study [27] we found that constitutive chemical defences and insect herbivory on leaves of *Q. robur* trees increased towards higher elevations, and abiotic factors explained elevational variation in leaf defences and herbivory, but in an independent manner (i.e. abiotically-determined clines in herbivory and defences are not related to each other).

Overall, latitudinal variation in oak defences fits the prediction of increasing defences towards the equator, although there is considerable variation among species in the strength and sign of latitudinal gradients. Elevational work, on the other hand, similarly shows considerable variation among species, in some cases being supportive but in other not of theory predicting increasing defences at lower elevations. Abiotic factors (climate, soil conditions) appear to be important third-party factors affecting clinal variation in herbivory, oak defences or both, and should therefore be included in future work to better explain underlying forces shaping pattern variability.

3.3.1.2 Continental-Scale Patterns

Plant intra- and inter-specific patterns of defence investment can be strongly shaped by historical processes [81–83]. For example, biogeographical studies involving large-scale comparisons of Nearctic (i.e. North America) and Palearctic (i.e. northern Eurasia) for several plant taxa have shed light into how historical processes have shaped current patterns of defensive investment, including extinction rates, demographic history, and the history of colonization of plant and herbivore species [84–87].

In the case of oaks, phylogenetic and paleobotanical data suggest that the center of diversification of *Quercus* spp. is at middle latitudes of America [2, 5]. Some of these species subsequently migrated to the Old World prior to the break-up of land bridges linking the northern continents, whereas others (e.g. red oaks) evolved too late to cross [2, 5]. Research holds that extinction rates of temperate tree species (including oaks) have been lower and plant and herbivore rates of recolonization

have been higher for the Nearctic compared to the Palearctic since the last glacial period [84, 85]. Following from this, a stronger recent history of herbivore pressure in the Nearctic would result in oak species from this region being more heavily defended than their Palearctic counterparts. However, a recent study of ours showed that Nearctic oak species exhibited higher levels of lignins, but lower levels of condensed tannins, compared with Palearctic oak species [18], the latter compounds typically correlating more strongly with herbivore resistance. The lack of information on the history of herbivore pressure for different regions makes it difficult to establish predictions. Still, with increasing oak paleoecological and phylogenetic data progress can be made in understanding how historical factors operating at broad scales have shaped macro-evolutionary patterns of oak defences across different biogeographical regions.

3.3.1.3 Effects of Insularity

Insular systems have proven highly useful for elucidating the ecological and evolutionary mechanisms shaping biodiversity [88, 89], species interactions [90, 91], and trait evolution [92]. With respect to plant-herbivore interactions, islands frequently have low abundances and diversity of vertebrate and invertebrate herbivores relative to their mainland counterparts because of dispersal limitation and habitat filtering, which presumably results in lower levels of herbivory relative to mainland regions [88, 93, 94]. As a result, insular plants are expected to show lower levels of physical (e.g., spines, thorns) and chemical (e.g., secondary metabolites) defences than their mainland counterparts [93, 95–97].

Despite these predictions, recent studies have been unsupportive of these expectations with insular plants having higher (rather than lower) levels of physical and chemical traits putatively associated with herbivore resistance (e.g. [98–101]). For example, we found that insular populations of holm oak (*Q. ilex*) exhibited expectedly lower insect leaf herbivory, but instead higher chemical defences (condensed tannins) than their mainland counterparts [101]. This suggests that insularity effects on herbivory and the measured leaf traits appear to be uncoupled. Furthermore, abiotic factors did not explain differences in either leaf traits or herbivory between mainland and island populations, pointing to other unmeasured drivers of oak defensive investment. Expanding this work to include other oak species would allow for better generalization and understanding of insularity effects on oak defences, possibly revealing contrasting evolutionary histories depending on the underlying drivers and historical processes driving plant-herbivore interactions for each of the studied oak taxa.

3.3.1.4 Effects of Urbanization

Urbanization usually leads to significant changes in local environmental conditions (e.g. increasing temperatures and levels of CO_2) which can dramatically alter the quantity, availability, as well as temporal and spatial distribution of biotic and abiotic conditions needed to support plant and animal communities [102, 103]. For plant-herbivore interactions, a number of studies conducted over the last two decades have reported on patterns of insect herbivory and plant defensive traits on trees and herbaceous plants along urban-rural gradients [104, 105]. The overall patterns have been inconclusive; some studies have reported increased insect herbivory and defences in urban areas relative to rural habitats [106, 107], whereas other studies have found the opposite pattern [108–110].

There have been a few recent studies looking at effects of urbanization on oak defences. For example, in a recent study we found that *Q. robur* trees found in urban locations had lower levels of chemical defences and leaf chewer herbivory [110]. In contrast, urban trees had increased levels of leaf nutrients (nitrogen and phosphorus) compared to trees in natural forest locations [110]. Urbanization effects on chemical defences (but not herbivory) were in turn associated with urban to rural changes in CO_2 concentrations [110]. Although there have been other studies measuring urban-rural gradients in oak herbivory [111], one of these reporting on 10 temperate tree species (including *Q. robur*; [108]) these have not measured plant defensive traits. Pending further studies with additional oak species, evidence thus far for *Q. robur* suggests that oak defences are relaxed in urban environments whereas leaf nutrients increase, presumably leading to an overall increase in plant quality. More work is needed, however, to gain a clearer understanding of which are the most important biotic or abiotic correlates of urbanization effects on oak defence investment.

3.3.2 Phylogenetic Patterns

In recent decades, due to technological advances in molecular biology, ecologists have begun to consider the influence of phylogenetic relatedness among taxa on current patterns of inter-specific variation in plant defence allocation [82, 112, 113]. Ehrlich and Raven's classic work [114] predicted that (1) more closely related plant species should share similar defensive chemistry, (2) more derived species should be more highly defended, and (3) closely related herbivores are expected to feed on closely related plants as a result of co-evolutionary arms races between trophic levels [115]. In this sense, insightful studies by Pearse and Hipp with 56 oak American species found a strong phylogenetic signal in plant physical and chemical defences, as well as in leaf herbivory [8, 17]. However, a recent study by our group including 18 oak species from America and Eurasia found no phylogenetic signal in constitutive chemical defences (phenolic compounds) and their inducibility [18], suggesting that phylogenetic patterns of variation in oak defences do not follow predictions

by classic co-evolution models, at least for the studied species. While these findings should be taken with caution given the limited number of species studied, they also highlight the need to develop an expanded view of macro-evolutionary patterns of plant defences. Such views should consider alternative scenarios that depart from classic plant-herbivore coevolutionary theory (e.g. herbivore resource tracking, see Endara et al. [116]), as well as other ecological forces (e.g. abiotic factors) which might influence in the evolution of plant defences, independently of herbivore pressure [8].

3.4 Linkages Across Scales: Oaks as Model System

Oaks, together with other taxa of widespread, dominant trees, represent a good ecological model for integrating research across levels of study. Their high taxonomical and functional diversity allows for intra- and inter-specific comparisons to test for different mechanisms of biotic and abiotic control over tree defensive investment, whereas their status as foundational and dominant species implies strong local and landscape-level associations between oak defences, arthropod community structure, and ecosystem processes. In addition, this genus contains numerous species with contrasting distributions (e.g. latitudinally or elevationally) which serves the goal of assessing both local (e.g. using sympatric species) and broad-scale (e.g. comparisons across species with contrasting distributions) factors. For example, future work can assess patterns of both intra-specific (genetic) and inter-specific variation in defences, herbivory, and herbivore community structure for groups of co-existing oak species found in similar habitat types. Results can yield insight into the linkages between and relative contributions of intra- vs. inter-specific variation in oak defences and their implications for arthropod community structure [40]. Studies could involve both observational and experimental studies manipulating oak species (as well as that of other common co-occurring tree species) composition or phylogenetic diversity to connect these ecological patterns to evolutionary history. Likewise, expanding on this to address the ecosystem consequences of oak defence variation would involve measurements of nutrient cycling and decomposition to understand the linkages between oak intra- and inter-specific variation in tissue chemistry and ecosystem processes [41, 117]. Finally, also expanding from the previous examples, studies assessing these intra- and inter-specific sources of oak defence variation under different habitats or community types (e.g. along gradients or contrasting habitat types) are needed to understand how abiotic factors shape oak defences [118], as well as its associations with herbivory and herbivore community structure [118, 119]. These types of studies could also involve manipulations of abiotic factors and herbivory to assess their relative importance in shaping oak defence investment [120]. Work could make use of either natural and human-impacted or transformed (e.g. urban) habitats to test for anthropogenic controls over biotic and abiotic factors shaping oak defensive traits.

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Chapter 4
Glycans as Plant Defense Priming Agents Against Filamentous Pathogens

Chayanika Chaliha, Robert A. Field, and Eeshan Kalita

4.1 Introduction

Plant cell walls are complex arrangements, primarily composed of polysaccharides and carbohydrate-derived composite material, such as glycoproteins and glycolipids,in addition to polyphenolics (lignin) and polypeptides (protein) [1]. These materials act as the first line of defense against microbial invasion. Many microbial pathogens located in the plant microenvironment possess hydrolytic enzymes that can rupture the plant cell wall by breaking down the polymerics polysaccharides, proteins, and lignins [2]. The breakdown products accumulate in the apoplastic zone, which can be regarded as the initiation of molecular interaction between microbes and plant responses [3].

The conserved molecular signatures, which are either the effectors secreted by microbes or the resulting breakdown products during plant-microbe interactions, are referred to as PAMPs/MAMPs or DAMP (Pathogen or Microbe Associated Molecular Patterns or Damage-Associated Molecular Patterns) (Fig. 4.1) [4]. The plant innate defense reactions known as PAMP-triggered immunity (PTI) is activated upon perceiving these conserved signature by the host cell surface localized Pattern Recognition Receptors (PRRs) [5]. Thus, the host apoplast represents the zone where the contact between microbial invaders and plants is often established wherein the apoplast is responsible for shelter and nutrient provisions to the pathogens (Fig. 4.1) [6]. PAMPs and DAMPs constitute a broad range of molecules belonging to various biochemical classes which include carbohydrates,

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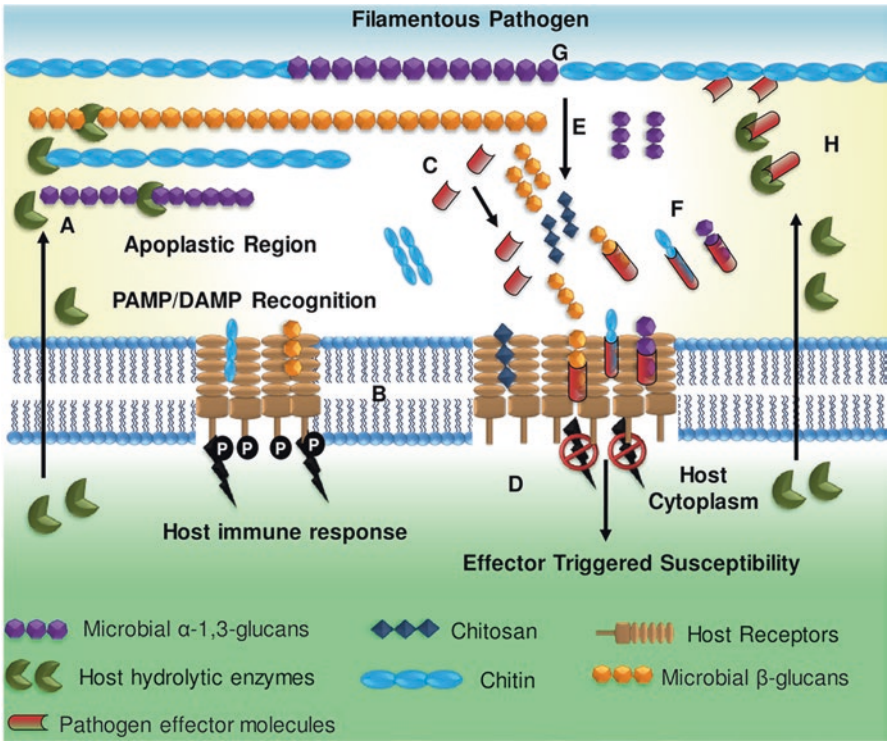

Fig. 4.1 Outline of glycan-triggered immune responses during pathogenic invasion. (**A**) Host-derived hydrolytic enzymes targets cell wall of invaded microbes which results in the release of glycan fragments (chitin, α- and β-glucans) thereby inducing innate defense response. (**B**) The pattern recognition receptors (PRRs) of host recognize these released glycans as PAMPs. (**C**) Pathogens secretes the effector molecules. (**D**, **E**,) Effector molecules combat against PTI defense responses producing less immunogenic chitosan from immunogenic chitin. (**F**, **G**,) Effector molecules mask the released PAMPs, to evade detection and there by remodel the cell wall components by the pathogen (e.g. accumulation of α-1,3-glucan)to mitigate the effect of enzymatic host hydrolysis on the microbial cell wall glycans and prevent hydrolysis. (**H**) Lastly, also certain effectors are secreted by the pathogen that directly inhibit host hydrolytic enzymes. (Adapted from Chaliha et al. [10] with permission under Creative Commons Attribution (CC BY) license)

lipopolysaccharides, proteins, lipids, peptides, glycoproteins and glycolipids [4, 7]. In recent times, researchers have garnered a special interest to study the role of carbohydrates during plant-pathogen interaction owing to the advances made in the area of functional glycomics [8]. Also, carbohydrate serves as essential energy required to fuel defense signalling cascade during plant-microbe interaction. This has led to the exploration of the role of carbohydrates as defense modulators in plants thereby giving rise to concepts like “sweet immunity” and “sugar enhanced defense” [4, 6].

In the context of glycan induced defense responses, several immunogenic microbial cell-surface glycans (e.g. chitin and β-glucans) and glycoconjugates (e.g.

glycolipids, glycoproteins, and lectins) have been identified, that promote plant defense signaling during infection [6, 9]. Within this chapter we present different classes of glycans and glycan conjugates and their strategic role during host-microbe interactions, whether establishing communication or circumventing recognition and their potential use as plant protection agents.

4.2 Simple Sugars

Sugars, including both mono and di-saccharides play a fundamental role during plant-microbe interactions, acting as signalling molecules that drive defense cascade in addition to serving as energy sources. Sugars, with ROS scavenging properties, are known to act indirectly as antioxidants and are considered an integral part of cellular redox networks [4, 11]. Thus, sugars are regarded as necessary components for metabolic coordination in connection with growth, stress responses, and response to environmental factors.

4.2.1 Glucose, Sucrose and Associated Metabolites

Hexokinase (HXK), a glucose sensor, responsible for the production of glucose 6-phosphate from glucose, has been studied for its role during microbial infection. The mitochondria-associated HXK1 is known to control programmed cell death (PCD) during infection and also mediate the regulation of pathogenesis related (PR) genes, during cell death due to the hypersensitive response (HR) [12]. Also, HXK acts as a catalyst for the activation of expression of several defense associated genes. For instance, in *Arabidopsis*, glucose facilitates the activation of *PR-1* and *PR-2* in the presence of *At*HXK1, in addition, overexpressing mitochondrial HXK in *Arabidopsis* lines is found to enhance resistance to the necrotrophic fungal pathogen *Alternaria brassicicola* due to higher expression of *PR* genes [13, 14].

Recent studies on sucrose show its involvement in regulating defense cascade during microbial attack, thereby showcasing its importance in plant sugar signalling pathways [15]. Glucose generated by hydrolyzing sucrose using cell-wall localized invertases is sensed by HXKs, which than act as signal fluxes activating defense signalling cascades [16]. Infection by *Fusarium oxysporum* in embryo axes of *in vitro* grown *Lupinus luteus* and *Lupinus angustifolius* germinating seeds, sucrose has been seen to activate defense response by the production of isoflavonoids and anthocyanins [17, 18]. In sugar signalling cascades, trehalose and trehalose-6-phosphate (T-6-P) have been discussed as important molecules known to modulate defense responses. Also, modulation of T-6-P pathway have been found to induce crop yield in maize (*Zea mays*), wheat (*Triticum aestivum*), and rice (*Oryza sativa*) significantly improving food security [19]. Activation of Phenylalanine Ammonia-Lyase (PAL) and Peroxidase (POX) by trehalose have also been observed upon

invasion by *Blumeria graminis*, the causal agent organism of powdery mildew in wheat [20, 21].

During biotic and abiotic stresses in plants, sucrose non-fermenting related kinase 1(SnRK1) has been found to regulate sugar metabolism wherein suppression of SnRK1 activity is mediated by plant cell derived T-6-P (Fig. 4.2) [22]. Interestingly, negative regulation of SnRK1 has been also found to be mediated by the external application of G-6-P and glucose [12]. Thus, the reduction in sucrose, G-6-P, glucose, and T-6-P level act as starving signals mediating the induction of SnRK1 [12]. PCD mediated protection in plants has been found to be driven by SnRK1 via inducing the activation of stress inducible genes aided by the heterodimerization of C-group and S-group bZIP transcription factors. However, variable responses to sugar level have been observed by different transcription factors in

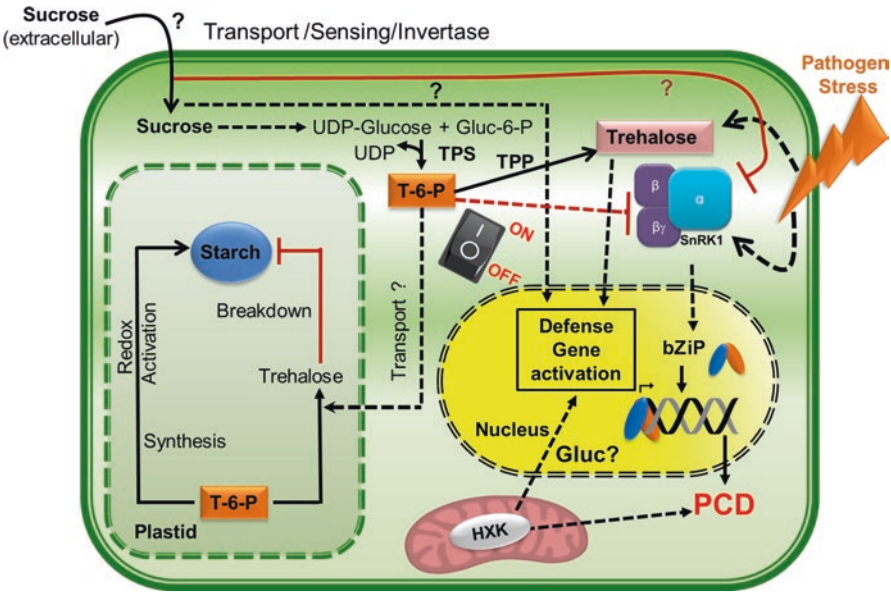

Fig. 4.2 Sugar fluxes regulating defense responses under pathogen attack. Metabolic switch between anabolic processes with energy utilization, such as-like starch synthesis and conditions of low sugar stress (during pathogenic invasion), is mediated by the stress regulator Trehalose-6-phosphate (T-6-P)which impacts on SnRK1. SnRK1 mediate defense signalling upon perceiving low sugar stress through transcriptional reprogramming by PCD and bZIP (basic leucine zipper) activated after post translational modification of key metabolic enzyme. However, Trehalose activates defense genes upon pathogen sensing and thereby blocks the starch synthesis pathway. Under low sugar stress conditions, sucrose mediates, immune responses positively by inducing secondary metabolite production and during normal conditions inhibit SnRK1. Also PCD and induction of defense gene during microbial attack are activated by mitochondrial HXKs. However, it is not yet known if the process is mediated by glucose and how sugars regulate the balance between homeostasis and stress signalling (as represented by '?'). (Adapted from Chaliha et al. [10] with permission under Creative Commons Attribution (CC BY) license)

Arabidopsis where in *At*bZIP11 is observed to be induced by sugar while and *At*bZIP1, *At*bZIP2 and *At*bZIP53 are on the other hand repressed.

In *Arabidopsis,* KIN 10 and KIN 11, the SnRK1 orthologues have been observed to mediate metabolic disruption under both biotic and abiotic stress via sugar metabolic pathway. Production of a very few metabolites (malate, alanine, glutamine, cinnamate and proline) has been reported in rice infected with *Magnaporthe grisea* in comparison to uninfected plants [23]. Thus, as described by Baena-Gonzalez [24] SnRK1 acts as a key inducer to activate catabolic pathways and repress anabolic pathways under stress conditions thereby restoring cellular homeostasis.

4.2.2 Raffinose Family Oligosaccharides

Raffinose family oligosaccharides derived from precursor galactinol by galactinol synthase (*GolS*), play a vital role in protecting plants from abiotic stresses, including temperature, drought, and injury by maintaining osmotic pressure via their accumulation. There are also key transporters of sugars via phloem and acts as storage carbohydrates [25]. The precursor galactinol has also been observed to be involved in plant defense against both biotic and abiotic stress, including drought, temperature and bacterial invasion via induced systemic resistance [26]. Induced systemic resistance by galactinol was first observed against *Pseudomonas chlororaphis* in tobacco and cucumber plants [27]. Also, induction of defense genes *PR1a*, *PR1b* and *NtACS1* (*Nicotiana tabacum* 1-aminocyclopropane-1-carboxylic acid synthase 1) by galactinol was observed to infer resistance in tobacco and cucumber against *Botrytis cinerea* and *Erwinia carotovora* infection [27]. In tobacco plants, H_2O_2 treatment induces galactinol level which act as ROS scavengers under drought conditions [28]. Similarly, a high level of galactinol synthase (*GolS*) was observed due to oxidative stress in *Arabidopsis* [29]. GolSprotein has been studied as osmoprotectant in various plants including *Coffea Arabica*, *Brassica napus*, grapevine, chestnut, *Medicago falcate*, *Salvia miltiorrhiza*, and *Cicer arietinum* L. In *Camellia sinensis* abiotic stress mediates regulation of *CsGolS1* and *CsGolS2,* while on the other hand *CsGolS3* was found to be regulated in response to both biotic and abiotic stresses [25].

4.2.3 Polyols

Polyols, the reduced form of aldose and ketose sugars and often referred to as sugar alcohols are widely studied for their role as osmoprotectant and ROS scavengers [30]. Polyols are often accumulate in plants under heavy metal stress, which severely affects sugar metabolic pathways. Mannitol, is reported to protect during salinity stress and has been found to possess ROS quenching ability species thereby

shielding against cellular damage [31]. Interestingly, mannitol is also widely present in algae, bacteria, and fungi as a common metabolite [32, 33]. In tomato, accumulation of mannitol in leaf apoplast was observed upon invasion with a virulent strain of *Cladosporium fulvum* [34]. Due to its ability to act as a ROS scavenger, mannitol is used by pathogens as a self-defense mechanism against plant antimicrobial strategies. Thus, secretion of mannitol by pathogenic microbes is often used by the pathogen to obstruct plant innate defense [35, 36]. In this context Patel and Williamson (2016) have comprehensively explained the mechanistic interplay between mannitol and mannitol dehydrogenase during mannitol secreting pathogenic interaction with plants and the counteraction of plant defense responses by the pathogen (Fig. 4.3) [32].

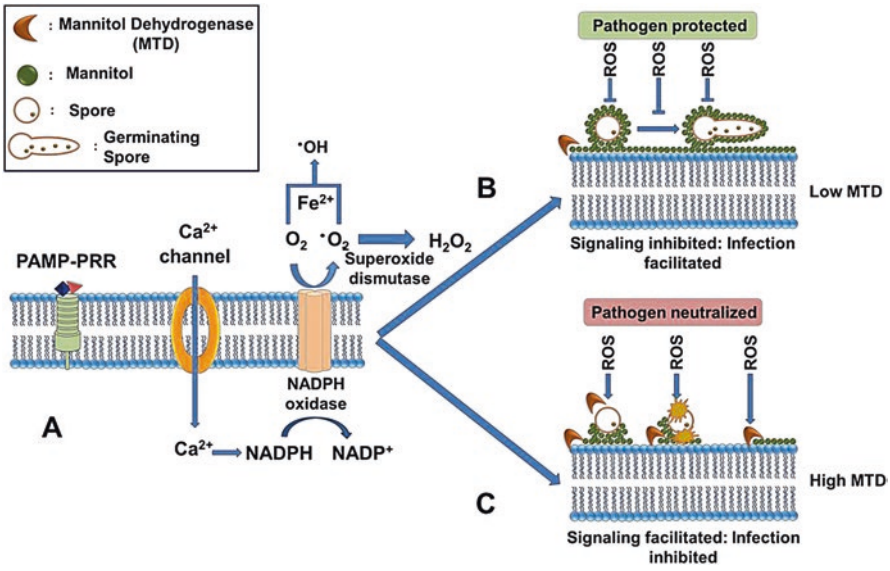

Fig. 4.3 Plant immune responses influenced by mannitol and mannitol dehydrogenase. (**A**) PTI induces an oxidative burst upon recognition of fungal PAMPs, which lead to the activation of membrane-bound NADPH oxidase and results in generation of reactive oxygen species (ROS) superoxide ($\bullet O_{2-}$), then converted into hydroxyl radical (•OH) and hydrogen peroxide (H_2O_2). (**B**) In response to the oxidative burst mediated by mannitol dehydrogenase, the pathogens forms a protective shield to mitigate against the plants oxidative burst by secreting mannitol (**C**) Mannitol dehydrogenase is secreted by plants to restore antimicrobial effect and ROS-mediated immune responses. (Adapted from Chaliha et al. [10] with permission under Creative Commons Attribution (CC BY) license)

4.3 Oligosaccharides

Oligosaccharides studied in plant-pathogen interaction are mostly the enzymatic breakdown products of polysaccharides found as pathogen effectors or those abundant in the pathogen cell wall [37]. The degree of polymerization is noted to play a key role in the elicitor activity of oligosaccharides [4].

4.3.1 β-Glucans

β-1,3−/ β-1,6-Glucans are extensively studied for their role as elicitors in plant-microbe interactions wherein the glucan oligomers are generated by the action of plant β-1,3-glucanases [38]. The first study reporting the isolation of β-glucans concerned from Phytophthora sojae wherein induced activity of PAL and chitinase were observed in plants [39]. Later, Schmidt and Ebel identified a binding site for glucan isolated from *P. megasperma* in tobacco that was responsible for phytoalexin accumulation. A variety of defense responses against microbial invasion are induced with β-1,3-glucans in different plants, including rice, bean, and alfalfa [40]. A β-1, 3-glucan with β-1,6-glycosidic side branches, laminarin isolated from marine brown algae *Laminaria digitata* has been found to elicit different defense responses including SA accumulation, H_2O_2 production, enhanced activation of PAL and lipoxygenase and induction of PR genes in tobacco against *Erwinia carotovora* [41]. In grapevine treated with laminarin, induction of innate defense responses against *Phomopsis viticola* and *B. cinerea* including oxidative burst, calcium influx, and MAPK activation was observed, which in later stages induced the expression of defense genes including the phenylpropanoid and octadecanoid pathway [42]. However, laminarin failed to elicit these defense responses in *Arabidopsis thaliana* (Col-0) [43]. Also, laminarin with five glucose units is reported to elicit defense responses in tobacco but not in rice. This suggests that degree of polymerization (DP) of oligosaccharides is a key factor in determining efficacy as an elicitor. In this context DPs in the range 10–16 for β-1,3-glucans and laminarin are considered to be optimal for activation of plant defense responses [44, 45]. For instance, as described by Fu et al. (2011), β-1,3-glucans with DPs 2–10 shows high induced resistance against tobacco mosaic virus infection of tobacco, as compared to β-1,3-glucans with DPs of 25–40 [46]. This suggests that the presence of different receptors for oligosaccharides of different DPs are used to recognize and elicit defense responses, as plants have been also found to respond in a distinct manner to specific structural form of β-glucans. For instance, branched β-glucan has been reported to recognized by rice and soybean while on the other hand tobacco is able to recognize linear β-glucan [47]. β-glucan with seven glucose units isolated from the culture medium of *P. megasperma* during germination have the ability to induce synthesis of phytoalexin and localized HR responses in alfalfa (*Medicago sativa*), soybean, lupin (*Lupinus albus*), bean (*Vicia faba*), pea, *Lotus japonicus* and *Medicago truncatula*

against *P. megasperma* [38]. In a different study related to chemical modification of β-glucan, addition of sulfate groups to laminarin with DP > 5 is shown to induce SA signalling in tobacco and *Arabidopsis* [43]. In this context acetylation of oligoglucuronans activates defense gene expression against *B. cinerea* (*viz.* PAL and chitinase and induced production of H_2O_2)in grapevine leaves [40].

In mammalian systems, fungal β-glucans are perceived byDectin-1 which induces homodimerization and phosphorylation of the ITAM (Immuno-receptor Tyrosine-Based Activation) motif of Dectin-1, activating a series of signalling cascade to produce of inflammatory chemokines/cytokines such as IL-1b, IL-6, IL-10, IL-23, TNFα, CCL2, CCL3, etc. [48, 49]. The C-type lectin domain of Dectin-1 harbors the Trp221 and His223 amino acids which mediate the recognition of β-glucan [50, 51]. This C-type lectin domain is known to share a 30% homology with that of soybean and *A. thaliana* [52, 53]. However, in case of plant C-type lectin domain of Dectin-1 orthologs, the structure of the binding groove is not known and it is assumed to possess a be a different set of amino acids [38]. Similarly, orthologues of for Dectin-2 and Dectin-3 hetero-dimers, known to produce inflammatory cytokinins in mice in response to α-mannans from *Candida albicans,* are yet to identified in plants (Fig. 4.4) [54].

To date, although various studies have reported β-glucans as important defense-priming agents, no studies have so far been able to identify the sequence/structural domain for its plant receptor. The possible reasons could be the restriction of studies to the Col-0 ecotype of *A. thaliana*, which is reported to show a weak immune response upon β-glucan treatment [38].

4.3.2 Cellodextrins

Cellodextrin consisting of a linear β-1,4- linked glucose backbone and derived from plant cell wall cellulose are widely studied for their ability as DAMPs to induce defense responses. Aziz et al. have discussed the induction of defense-related genes, oxidative burst, elevation of cytosolic Ca^{2+}, production of H_2O_2 and activation of chitinase and β −1,3-glucanase activity upon treatment with cellodextrin with DP > 7 in grape vine challenged with *B. cinerea* [55, 56]. Also, cellodextrin treatment has been shown to lead to increased innate immune responses with the enhanced production of lignin, phytoalexin and SA and activation of PAL genes. Similarly, chitinases (Chit3, Chit4c, and Chit1b) responsible for the degradation of chitin in fungal cell walls are induced by cellodextrin treatment [57, 58]. Cellobiose derived from cellulose has been found to activate MAPKs, enhance cytosolic calcium and expression of defense-related gene *WRKY30* against *P. syringae* infection in *Arabidopsis* [59]. However, in this context the role of signalling pathways involving cellodextrin induced defense responses are unclear and need further exploration.

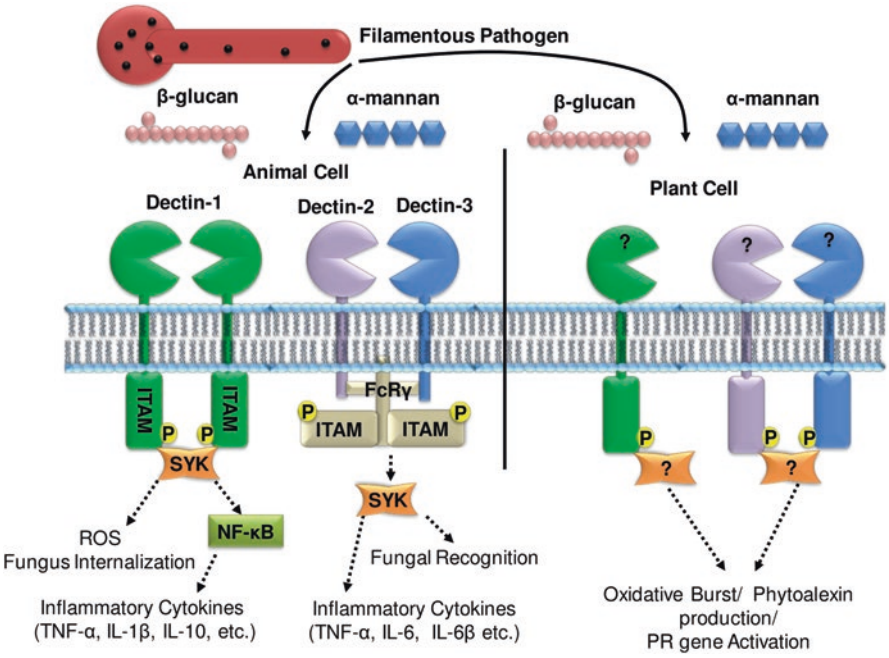

Fig. 4.4 Defense signalling in animal cells in response to β-glucan and α-mannan recognition alongside the assumed system in plants. In animals, upon perceiving β-glucan, homodimerization of Dectin-1 and phosphorylation of its cytoplasmic ITAM (Immuno-receptor Tyrosine-Based Activation) domain is induced. This activation leads to the induction of ROS pathways, activation of cytokines/chemokines/caspases and internalization of pathogen. On the other hand Dectin-2 and Dectin-3 heterodimerization perceive α mannans followed by the phosphorylation of the FcRγ-ITAM motifs leading to defense signalling by the production of inflammatory cytokinins (IL10, IL12, IL-6,and TNF-α, etc.). However, β-glucan perception in plants needs further understanding in order to appreciate what that leads to generation oh H_2O_2, activation of PR genes and accumulation of phytoalexin. The possibility of functional Dectin1/2/3 homolog in plants for recognition and defense signalling is not known (the assumed signalling pathways involved are represented in dotted lines). (Adapted from Chaliha et al. [10] with permission under Creative Commons Attribution (CC BY) license)

4.3.3 Chitin and Chitin Derivatives

Chitin is a water insoluble biopolymer of N-acetylglucosamine and is a major structural component of fungal cell walls. Studies have been reported on chitin as a PAMP to induce phytoalexin accumulation, HR response, lignin biosynthesis and membrane depolarization against fungal invasion [60]. These physiological changes along with induced systemic resistance by the activation of MAPK defense signalling pathway was reported in rice challenged with *Magnaporthe oryzae* [47, 61]. In citrus plants treatment with chitin has been found to induce production of secondary metabolites with antimicrobial properties, phenolic content and enhanced activity of PAL enzyme, along with SA dependent systemic defense responses [60]. In

Arabidopsis seedlings and rice cell suspension cultures use of chitin oligomers was observed to induced the activation of PR protein, PR-10 and enhanced ROS production [62]. These observation indicate the presence of chitin receptor in plants which activates defense signalling cascades against microbial invasion. Chitin Elicitor Binding Protein (*Os*CEBiP) was recognized in rice cells as the PRR for chitin which was responsible for the activation of defense signalling [63, 64]. Similarly, the OsCEBiPortholog, HvCEBiP was identified in barley as mediating innate defense resistance against *M. oryzae* [65]. In Arabidopsis, the chitin PRR AtCERK1 (also known as RLK1/LYK1) with an intracellular kinase domain and an extracellular carbohydrate binding lysine motifs (LysMs) mediated the defense signal transduction [66]. A recent report on *AtCERK1* has examined its efficacy to perceive β-1,3-D-$(Glc)_6$, hexasaccharide and to induce innate immune responses which include increased levels of cytosolic Ca^{2+} and PR protein and activation of MAPKs defense cascade [67]. In a recent study on M. oryzae interaction with rice MoChia1, a chitinase secreted by the pathogen was observed to trigger defense cascades in plants. However, MoChia1 also binds with chitin to suppress immune responses. In this scenario, OsTPR1, a rice tetratricopeptide repeat protein binds with MoChia1 in rice apoplast facilitating the availability of chitin to trigger plant defense responses. Induced expression of OsTPR1 in rice also results in enhanced levels of ROS on pathogen invasion [68]. Cotton apoplast chitinase 28 (Chi28) which was hydrolyzed by protease VdSSEP1 secreted by *Verticillium dahlia.* However, cotton apoplastCRR1 interacts with Chi28 to protect it from cleavage upon *V. dahlia* invasion, overexpression of CRR1 increased its resistance against *V. dahlia* and also related pathogenic fungi like *Botrytis cinerea* and *Phytophthora parasitica* [69]. Studies have been carried out on the LYS12 motif, an NFR5-type LysM receptor protein, from *P. palmivora* showing regulation of genes encoding chitinase, peroxidase and germin-like protein during interaction with *L. japonicus*. This receptor has been found to be responsible to distinguish between carbohydrates PAMPs originating from P. palmivora and DAMPs emerging from the plant host [70].

Chitosan is aβ-1,4-linked glucosamine containing oligosaccharide that is produced by the action of chitin deacetylases. Chitosan and its oligomers are considered as defense elicitors owing to their ability to induce synthesis of callose, H_2O_2 production, phytoalexin accumulation and to enhance the expression of PR genes in dicotyledonous plant [71, 72]. In wheat chitosan treatment induces lignin deposition and increased phenolic acids levels in leaves in response to microbial invasion while in melon plants chitosan oligomer activated chitinase activity [73, 74]. In *Arabidopsis* treatment with chitosan oligosaccharides was found to induce resistance against Tobacco Mossaic Virus via activating SA mediated systemic resistance [75]. In tomato plants, chitosan mediated defense signalling against Cucumber mosaic virus resulted in up regulating the expression of *PAL5* defense gene and accumulation of lignin and SA [76].

Chemical derivatives of chitin oligomers have been studied for their ability to elicit defense responses and they were also observed to initiate symbiosis signal transduction. For instance, lipochitooligosaccharides, chitin oligomers with lipid modifications play a role in initiating symbiosis between root-nodule symbiosis

(Nod factors) and arbuscular mycorrhizal symbiosis (Myc factors) [77]. In legumes, *L. japonicus, M. truncatula*, pea and rice chitin derivatives have been found to initiate accumulation of cytosolic Ca^{2+} during symbiotic association [78]. Hexaacetylchitohexaose (HC), a derivative of chitin oligomer from insect exoskeleton act as a herbivore-associated molecular pattern and initiate upregulation of various defense gene (*viz.* WRKY22, EDS1, PAL1, etc.) in citrus [60]. These studies thus show the apoplastic zone as the region that distinguishes between friendly microbes and pathogens through elicitation of symbiotic and defense signalling respectively.

4.3.4 Alginate and Fucans

Alginate oligomers are derived from sodium alginate extracted from brown seaweed and are made up of α-L-glucuronic and β-D-mannuronic residues linked via 1, 4- glycosidic bond and with DPs generally within the range of 2–10 [79]. These forms of oligomers have been characterized for their ability to act as elicitors for inducing PAL activity and enhancing phytoalexin accumulation in soybean [80]. Similar defense responses were observed against *M. oryzae* in rice with alginate treatment [37]. In a recent study in *A. thaliana* alginate oligosaccharides were found to mediate defense responses via induction of the oxidative burst, SA signalling pathway and through enhanced expression of PR1 genes against *Pseudomonas syringae* (*Pst* DC3000) [81].

Infucan oligomers made up of α-1, 4- and α-1,3- glycosidic linkages, chemical modification of fucose units with sulfate generates compounds that can induce defense responses in cell suspension cultures of tobacco, including enhancement of PAL activity [82]. Also, JA mediated defense signalling was observed in tobacco plants treated with fucan, inferring induced resistance against TMV [82].

4.4 Glycoconjugates

The fungal cell wall is composed of several complex carbohydrate structures modified, including N- and O-linked glycoproteins, and glycolipids in addition to β-glucans and chitin anchored loosely in cell surface layer.

4.4.1 Glycoproteins and Lectins

The first report on glycoconjugates mediating defense response in plants was in parsley infection with *P. sojae* wherein glycoprotein was observed to induce phytoalexin accumulation [83]. Later in tomato plants *BcGs*1, a glycoprotein isolated from the culture filtrate of *B. cinerea* was shown to act as an inducer of HR responses

and mediate SA, JA, and ET defense cascades. Treatment with *BcGs*1in tomato plants infer resistance against necrotrophic fungi via inducing the Prosystemin eliciting JA defense signalling, SA- transduced defense marker PR-1a, and the tomato protein kinase 1 (TPK1b), and an ET-mediated defense protein [84]. *Bc*IEB1, a glycoprotein abundant in the *B. cinerea* secretome acts as an elicitor on tobacco, onion, tomato and Arabidopsis plants where it induces the oxidative burst, cytoplasm shrinkage and electrolyte leakage [85, 86]. Structural studies of *Bc*IEB1 glycoprotein revealed glycosylation of two serine/threonine-rich residues with α-1,2−/α-1,3-linked mannose [87, 88]. Further studies on *Bc*IEB1 mediated defense responses have led to the discovery of osmotin, a stress protein of the PR5 family as its PRR in tobacco. Osmotin was also found to accumulate and promote multi-tiered defense response including PCD in fungi and ROS scavenging in response to fungal infection [89, 90]. Additionally, filamentous fungi are known to secrete small proteinaceous molecules that have elicitor activity that induces generation of ROS, necrosis, electrolyte leakage, cytoplasm shrinkage and HR responses thereby establishing necrotrophic/hemibiotrophic interactions [90]. Peptidogalactomannan (pGM) isolated from *C. herbarum* and composed of a main chain comprising (1 → 6)-linked α-D- mannopyranosyl units substituted with (1 → 2)-linked α-D-mannopyranose residues at O-2 was examined for elicitor activity in tobacco and was found to induced HR response and expression of defense related genes [91].

During defense signal transduction upon plant-microbe interactions, glycan-binding lectin proteins are reported to regulate many of the defense signalling pathways (Silipo et al. 2010). Lectins mediate the recognition of plant pathogens by using protein-protein interactions as well as protein-glycan interactions when perceiving distinctive epitopes or damage-associated patterns [92]. Lectin domains interact with carbohydrate signatures from microbes or saccharides obtained from plant cell wall injury leading to induced concentrations of ROS, elevation in cytosolic Ca^{2+} and MAPK-based signalling cascade activation [93]. Pi-d2 in rice and LecRK-I.9 in *Arabidopsis* are lectin with receptor-like kinase domains (LecRLKs) which are known to mediate resistance against *M. oryzae* and *Phytophthora brassicae* respectively [94]. *Arabidopsis* LEcRK-I.9 interacts with oligosaccharides through the Arg-Gly-Asp (RGD) tripeptide in the lectin domain to initiate defense cascade [95]. Similarly, glycans are perceived by the conserved hydrophobic domain present in the lectin domain of LecRK [96]. Also, a domain of LecRK-I.9 perceives extracellular ATP as ligand released by plants when invaded by microbes [97].

4.4.2 Glycolipids

Gram negative bacteria possess cell surface LipoPolySaccharide (LPS), a structural component constituting three functional domain *viz.* the di-glucosamine moiety lipid A (LA), an oligosaccharide core region and the O-antigen region with a varied number of oligomer repeat units [98]. LPSs act as PAMPs, with LA component perceived by different LPS receptors present as in extracellular and intracellular

form [99, 100]. RLK LORE (LipoOligosaccharide-specific Reduced Elicitation) belonging to bulb-type lectin S-domain-1 kinases (SD-RLKs) is the receptor for LPS in plants. It is reported to recognize *Pseudomonas* and *Xanthomonas* LPS as PAMPs in *Arabidopsis* thereby triggering PTI defense pathways [101]. However, this receptor does not have the ability to recognize LPS from *E. coli.* In mammalian systems TLR4/MD-2 (toll-like receptor 4/myeloid differentiation factor-2) is the LPS receptor that is well established for mediating defense signalling thereby triggering production of pro-inflammatory cytokines, antimicrobial peptides and activation of phagocytosis and interferons (Fig. 4.5) [102]. This cascade is activated by the mammalian glycoprotein CD14 upon recognition of bacterial LPS in a TLR independent fashion. On the other hand CD14 is responsible for the transfer of

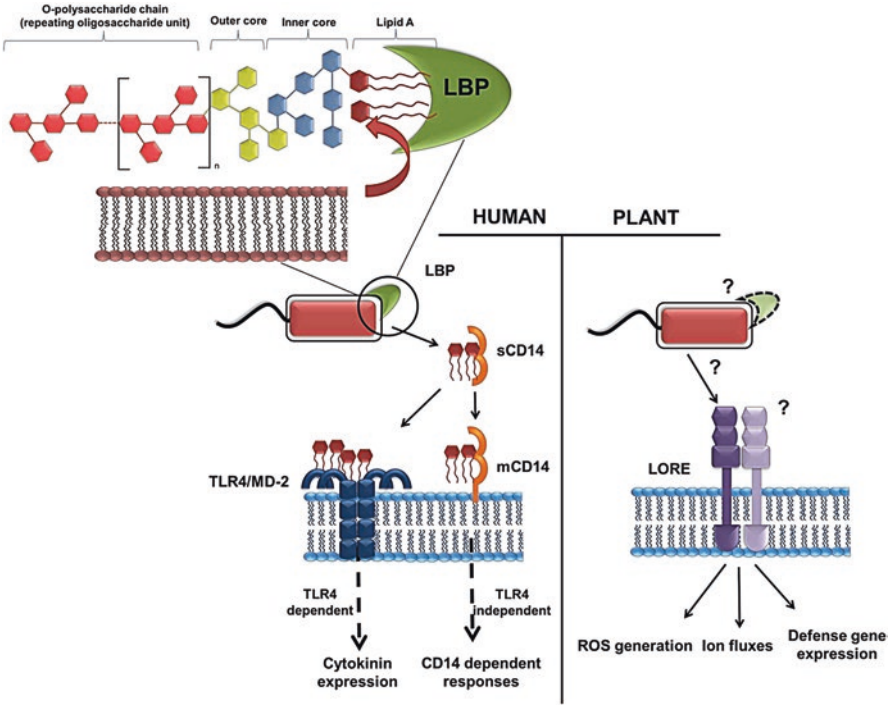

Fig. 4.5 Overview of the LPS receptor mechanism in humans and its plant analog. In humans disaggregation of lipid A (LA) domain of LPS from the bacterial membrane is mediated by LBP and then transferred to CD14 which mediates the transfer of LPS to TLR4/MD-2 receptor complex resident of either plasma membrane or in endosome. TLR4/MD-2/LPS complexes activate production of interferons or cytokinins through TIRAP/MyD88 or TRIF/TRAM signalling adaptors. However, CD14 also directly trigger defense response in a TLR4 independent fashion. In this scenario, the bulb-type lectin S-domain-1 RLK LORE (LipoOligosaccharide-specific Reduced Elicitation) in plants has been identified as the first LPS recognizes LA domain of Pseudomonas. But, the binding mechanism of LA to LORE following its downstream signalling is yet to be known. (Adapted from Chaliha et al. [10] with permission under Creative Commons Attribution (CC BY) license)

bacterial LA in a TLR dependent fashion by LPS-binding protein (LBP). In this context LORE is suspected to undergo dimerization during LPS sensing (Fig. 4.5).

Galactolipid is a plant glycerolipid with monogalactosyldiacylglycerol (MGDG) and digalactosyldiacylglycerol (DGDG) important for initiating systemic defense responses. For instance in the *Arabidopsis dgd1* mutant, deficient in DGDG synthesis and the *mgd1* mutant (deficient in MGDG) failed to initiate defense responses when infected with virulent pathogen *P. syringae pv. Maculicola* with prior priming with avirulent pathogen *P. syringae pv*. Tomato [103, 104]. However, the detailed mechanism initiating the SAR defense pathway is not known and needs further study.

Glycosylated lipids such as phosphoinositol sphingolipids, and glucosylceramides (GlcCer) are present in fungi with a C-9 methyl group on the long chain fatty acid base [105]. Rice plants treated with fungal GlcCer, display induction of defense responses, like PR protein synthesis and phytoalexin accumulation [106]. One of the predominant sphingolipids found in lipid extracts of plants is GlucosylInositolPhosphorylCeramides (GIPC) wherein the terminal hexose of GIPC is responsible for perceiving ethylene-inducing peptide 1-like (NLP) proteins and induction of necrosis in eudicots [107].

Natural rhamnolipids isolated from *Pseudomonas aeruginosa* have been found to possess elicitor properties to inducing defense responses in wheat, grapevins, *Arabidopsis* and tobacco cells via the innate immune system including ROS production oxidative burst and systemic acquired resistance [108, 109]. In this context, in tobacco cells, natural rhamnose based glycolipids enzymatically were synthesized in tobacco cells and used as elicitors to induce extracellular ROS production [110].

4.5 Conclusions

Carbohydrates have been widely investigated as PAMPs for priming defense responses in plants. In nature, plants are continually exposed to a wide variety of both beneficial and pathogenic microflora, highlighting the need for a detailed understanding of plant microbe interaction. In this context, the use of carbohydrate elicitors to combat pathogenic microbial invasion is reported as an effective means of providing resistance in plants. Also, in the context of sustainable agriculture, the defense signalling cascade is induced by the use of only micrograms or less of oligosaccharides for crop protection. Further, these carbohydrate elicitors also act as a safer and environmentally friendly alternative to the conventional chemicals currently used for crop protection in support of increasing needs for food security.

However, our understanding of the plant receptors of these carbohydrate elicitors is far behind the understanding of human-pathogen interaction. This roadblock may be addressed by improving techniques used for oligosaccharide extraction, purification and identification rather than using crude extracts for defense priming studies. Although a large number of carbohydrate receptors in plants *viz.* RLKs, RLPs, and lectins have been identified a better understanding of their biochemical and

structural properties would enable to the identification and optimization of the specific oligosaccharide candidates for field-based applications.

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Chapter 5
Biological Control and Need of a Strategic Shift in Plant Disease Management

Arun Kumar and A. K. Purohit

5.1 Introduction

It is hard to foresee and predict a total replacement of hazardous plant protectants with that of benign organic and Bio Control Agents (BCAs), yet a strategic make-over is being necessitated by the growing demand of quality food by a swelling number of health conscious populace. In view of a large number of growth and yield related constraints, plant diseases are major limitation in crop production in all agricultural systems. This review is particularly driven by the need of exploring this kind of strategic shift in plant disease management.

Plant diseases are caused by a variety of pathogens such as bacteria, fungi, and viruses. In general, losses due to diseases amount to 25% of world crop production per year [1]. In view of the fast changing agricultural scenario, a drift has resulted from sustenance to commercial farming. To maximize the yield, farmers are using high yielding varieties and hybrids with higher inputs of chemical fertilizers and pesticides to a large extent. The newly released hybrids, indiscriminate and excessive use of fertilizers and pesticides have resulted in susceptibility to various diseases and pests. Apart from this, a number of other problems, such as soil, water and air pollution, residual toxicity in fruit and vegetables, resistance to insects and pathogens, mortality of parasites, predators and pollinators, and resurgence with outbreaks of secondary pests have also cropped up. Besides resistant cultivars,

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existing disease management strategies are based on using pesticides. Injudicious reliance on chemicals for pest management has led to the development of resistance in pathogens, environmental degradation and contamination of food and ecosystem, necessitating search for viable alternatives [2]. Amid a spectrum of high throughput technologies, low external input sustainable agricultural (LEISA) technologies are in demand. A number of cultural practices such as growing genetically similar crop plants in continuous monoculture and plant cultivars susceptible to pathogens, and use of nitrogenous fertilizers at concentrations that promote disease susceptibility have actually enhanced the destructive potential of diseases. In the wake of prevailing situation the concept of biological control has been re-surfaced. Biological plant disease control is an alternative strategy for managing plant diseases.

The multitude of methods used in biological control can broadly be divided into two groups- (1) Antagonists- directly introduced into plant tissue and (2) Cropping conditions and other factors that can be modified in ways to promote the activities of naturally occurring antagonists. In other words the term 'Biological control' or "Biological suppression," i.e. reducing the population of the target pest to an acceptable level" includes plant disease resistance, biologically derived pesticides and cultural practices. Different definitions of biological control have been proposed in the past but according to recent definition by Chernin and Chet [3]: "the action of parasites, predators, or pathogens in maintaining another organism's population density at a lower average than would occur in their absence". However, in addition to antagonism BCAs have other activities like increasing plant vigour, competing out the pathogens from nutritional resources and occupation of ecological niche and inducing resistance in the host through elicitation of host defense mechanism. The beauty of elicitation of defense mechanism is that the biocontrol potential microbes play their role even without coming into direct confrontation with the pathogens [4]. Biological control agents interact with the components of the disease triangle to reduce the incidence of disease. The concept of biological control pyramid is formed by separating the biological control agents from the environmental component of the disease triangle. This biological control pyramid helps in conceptualizing the factors and their intricate interactions, which play a major role in disease control strategy [5]. The term 'Biological control' is commonly used and includes plant disease resistance, biologically derived pesticides, crop rotation etc. Here we are using the term as disease control-mediated by an additional organism(s), which changes the result of interaction between the environment, pathogen and host. Biotechnology for crop protection, is receiving considerable attention today. Many studies are now under way to improve the crop production through genetic engineering and expression of insect and virus resistant genes and microbial pesticides. An area of agricultural biotechnology in which fungi show considerable potential for the future is the biological control of pathogenic fungi, insect pests and weeds [6, 7]. Potential agents for biocontrol activity are rhizosphere-competent fungi and bacteria, which in addition to their antagonistic activity are capable of inducing growth responses either by controlling minor pathogens or by producing growth-stimulating factors. Biological control proves to be very successful economically, and even when the method has been less successful, it still produces a

benefit-to-cost ratio of 11:1. Those organisms which can be cultured with ease have maximum potential to be developed as commercial product. Unlike the past studies, more ecologically sound approaches involving a combination of organisms is currently being used by a number of workers [8]. Increasing use of fungi as mycoinsecticides and biocontrol agents for managing insect pests and plant diseases has opened a vast field of knowledge for studying this huge unexploited fungal resource [7, 8, 9, 10]. The objective of this paper is to review the new information on role of microorganisms used to control plant diseases, [1, 9] with emphasis on risk assessments, quality of food and cost effectiveness. The biocontrol methods, such as compost, seed bacterization, fungal biocontrol agents (*Trichoderma*), seed treatments, induced systemic resistance, genetic manipulation and induced resistance using pathogens and non-pathogens having practical relevance are discussed.

5.2 Biocontrol of Airborne Diseases

Many naturally occurring microbial biological control agents (MBCAs) have been used to control diseases on the aerial surfaces of plants. The most common bacterial species that have been used for the control of diseases in the phylloshpere include *Pseudomonas syringae; P. fluorescens, P. cepacia, Erwinia herbicola,* and *Bacillus subtilis*. Fungal genera that have been used for the control of air borne diseases include *Trichoderma, Ampelomyces*, and the yeasts *Tilletiopsis* and *Sporobolomyces*. Wan and Tian [10] studied the effect of ammonium molybdate (NH_4Mo) as an additive to improve biocontrol efficacy of antagonistic yeasts *Rhodotorula glutinis* and the use of NH_4Mo is a practical approach to improve the efficacy of *R. glutinis* for post harvest disease control.

Phytopathogenic bacteria possess several genes that encode phenotypes that allow them to parasitize plants and overcome defense responses elicited by the plant. In addition, phytopathogenic bacterial possess pathogenicity genes like *hrp*. Isogenic avirulent mutants can be produced by insertional inactivation of genes involved in pathogenicity. Antibiosis has been proposed as the mechanism of control of several bacterial and fungal diseases in the phyllosphere. Recently, the advances of plant and plant growth promoting bacterial (PGPB) interaction research focusing on the principles and mechanisms of action of PGPB (both free living and endophytic bacteria) and their potential use in biological control of plant diseases is reviewed by Compant et al. [11].

Molecular biological techniques could be used to enhance the efficacy of biocontrol agents that use antibiosis as a mode of action. Biocontrol agents must normally achieve a high population in the phyllosphere to control other strains, but colonization by the agent may be reduced by competition with the indigenous microflora. Integration of chemical pesticides and biocontrol agents has been reported with *Trichoderma* spp. and *P. syringae*. Biocontrol agents tolerant to specific pesticides could be constructed using molecular techniques.

5.3 Biocontrol of Soil Borne Disease

Chemical control of soil borne plant diseases is frequently ineffective because of the physical and chemical heterogeneity of the soil, which may prevent effective concentrations of the chemical from reaching the pathogen. Enrichment, conservation and management of microorganisms have been extensively used for the biological control of soil borne plant diseases as well as for promoting plant growth. Fluorescent pseudomonades are the most frequently used bacteria for biological control and plant growth promotion, but the species of *Bacillus* and *Streptomyces* have also been used commonly. Competition as a mechanism of biological control has been exploited with soil borne plant pathogens as with the pathogens on the phylloplane. Naturally occurring nonpathogenic strains of *Fusarium oxysporum* have been used to control wilt diseases caused by pathogenic *Fusarium* spp. Molecular techniques have been used to remove various deleterious traits of soil borne phytopathogenic bacteria to construct a competitive antagonist of the pathogen.

Chitin and β – (1, 3)-glucan are the two major structural components of many plant pathogenic fungi, except oomycetes, which contain cellulose in their cell wall and no appreciable levels of chitin. Biological control of some soil borne fungal diseases has been correlated with chitinase production. Bacteria producing chitinases or glucanases exhibit antagonism *in vitro* against fungi. A recombinant *Escherichia coli* expressing the *chi A* gene from *Sclerotium marcescens* was effective in reducing disease incidence caused by *Sclerotium rolfsii* and *Rhizoctonia solani*. In other studies, chitinase genes from *S. marcescens* have been expressed in *Pseudomonas* spp. and the plant symbiont *Rhizobium meliloti*. Shahnaz et al. [12] have reported biological control of soil borne, root-infecting fungi (*Fusarium* spp., *Macrophomina phaseolina* and *Rhizoctonia solani*) on mung bean and okra using strains of *Rhizobium* and *Bradyrhizobium* spp. All rhizobial treatments were effective in controlling the soil borne fungi on these plants. The rhizobial strains also increased nodulation as well as shoot and root growth of treated plants.

The effectiveness of certain on-farm weeds as soil amendments was ascertained against *Macrophomina phaseolina*, a soil-borne pathogen causing dry root rot of crops grown under rainfed conditions in arid regions. Mawar and Lodha [13] have reported significant reductions in the population of *M. phaseolina* with the weed residues. Accordingly, *Celosia* and *Euphorbia* residues completely eradicated viable propagules of *M. phaseolina*. Bio-agents and neem based seed treatment for management of root-rot complex in cluster bean has been studied by Jatav and Mathur [14]. They have observed maximum suppression of *Fusarium solani* by *Bacillus subtilis*. However, *Rhizoctonia solani* was successfully managed by *Trichoderma harzianum*. It was noted that for *R. solani* fungal biological control agents were more effective, whereas bacterial antagonists were effective against *F. solani.*

5.3.1 *Soil Solarization*

The soil biodiversity and microbial richness are involved in biological control of soil borne diseases. The interaction between the beneficial microorganisms of soil and plants facilitates plant growth and heightens defense against various pathogens. Soil solarization seems to be one of the field technologies directly related to the field of biological control of soil borne pathogens. For soil solarization, moist soil is covered with transparent, UV- resistant plastic and exposed to sunlight for a few weeks. Most of the fungi, bacteria and nematodes are sensitive to increased temperatures (45-55^{0}C). Accumulation of toxic volatile compounds such as alcohols, organic acids and aldehydes reduce the soil pH, which adversely affect the survival of soil borne pathogens. A number of studies have been carried out towards managing soil borne pathogens of arid legumes. The effects of soil solarization, residue incorporation, summer irrigation and biocontrol agents on survival of *Macrophomina phaseolina* have been worked out in the past. Results suggest that in hot arid regions use of Brassica residues can be a practical and feasible substitute for polyethylene mulching in managing soil-borne diseases [15].

5.3.2 Trichoderma – *An Environment-Friendly Biocontrol Agent*

Species of *Trichoderma* are one of the small groups of beneficial fungi, which have proven commercially viable as a biological control agent. This micro-organism is now registered as bio-fungicide in India, France, UK, Switzerland, Sweden, Belgium, Chile, New Zealand and the USA, and regulations are pending in several other countries. *Trichoderma* is completely safe for humans and livestock. Although, it is commonly considered as a contaminant that may cause infections in presence of certain predisposing factors. But, in 55 years of research there has been no account of recorded adverse reaction. The predatory qualities of *Trichoderma* are a big part of the appeal of this fungus along with other associated benefits for commercial applications. The thought of biological control of plant pathogens by mycoparasites (hyperparasites) dates back to Weindling [16]. He discovered that *Trichoderma lignorum* would parasitize a number of soil borne fungi in culture and suggested controlling certain pathogenic fungi by augmenting soil with an abundance of this mycoparasite. Comprehensive reviews on the subject have been published in the past [17–19]. Recently, the role of *Trichoderma* spp. has been extensively reviewed showing its mechanism of action along with its importance in developing plant resistance, increasing plant growth and in increasing crop production. The antagonistic activity involves mycoparasitism, antibiotics, competition for nutrients and also induces systemic resistance in plants. Commercial production of *Trichoderma* and its application in plant disease management are discussed by a number of workers [20–22].

5.3.3 Description and Natural Habitats

Trichoderma is a filamentous fungus that is widely distributed in the soil, plant material, decaying vegetation, and wood. *Hypocrea* spp. are the teleomorph of some of the *Trichoderma* species. *Trichoderma* thrives in the leaf litter or mulch, and it requires a minimum organic carbon level of 1% to ensure proliferation in cropping locations. This species is a myco-parasite or saprophyte, which feeds on pathogenic fungi. There are large number of photographic evidences highlighting this phenomenon where *Trichoderma* are seen actively parasitising several group of plant pathogens.

5.3.4 Species

The genus *Trichoderma* has five major species utilized in biocontrol of plant diseases viz. *T. harzianum*, *T. koningii*, *T. longibrachiatum*, *T. pseudokoningii*, and *T. viride*. Morphological features of the conidia and phialides help in differentiation of these species from each other.

5.3.5 Cultural Features

Colonies of *Trichoderma* grow rapidly and mature within 5 days at 25 °C. Its colonies develop as wooly and compact mycelium on potato dextrose agar (PDA) medium. At the time of sporulation scattered blue-green or yellow-green patches are formed. These patches may form concentric rings. They are more readily visible on PDA in comparison to Sabouraud dextrose agar. The fungal growth is pale, tan, or yellowish in colour on the reverse side of cultures.

5.3.6 Microscopic Features

Septate, hyaline hyphae, conidiophores, phialides, and conidia are observed. Some of the species like *Trichoderma longibrachiatum* and *T. viride* also produce chlamydospores. Conidiophores are hyaline, branched, and occasionally display a pyramidal arrangement. Phialides are hyaline, flask-shaped, and inflated at the base. They are attached to the conidiophores at right angles. The phialides may be solitary or arranged in clusters. Conidia (3 μm in diameter) are one-celled and round or ellipsoidal in shape. They are smooth- or rough-walled and grouped in sticky heads at the tips of the phialides. These clusters frequently get disrupted during routine

slide preparation procedure for microscopic examination. The color of the conidia is mostly green.

Screening of strains can be conducted in four ways: (1) selection of active strains in relation to plant pathogens (2) screening isolates which have high biotechnological indexes (3) analysis of pathogen properties for plant, useful insects, animals and peoples (4) search of low economic value substrates which are convenient for cultivation and saving of spores' activities. For developing effective biocontrol agent to combat damping-off in nurseries, we investigated fungal strains in the genus *Trichoderma* that was isolated from soil and fruiting bodies of *Ganoderma lucidum* [23, 24].

5.3.7 Pathogen Interaction

Mycoparasitism is a complex process, which include several successive steps. The interaction of *Trichoderma* with its host is specific. *Trichoderma* spp. have been intensely studied as biocontrol agents [25]. The first detectable interaction shows that the hyphae of the mycoparasite grow directly towards its host. This phenomenon appears a chemotropic growth of *Trichoderma* in response to some stimuli in the host's hyphae or toward a gradient of chemicals produces by the host. When the mycoparasite reaches the host, its hyphae often coil around it or are attached to it by forming hook like structures. In this respect, production of appressoria at the tips of short branches has been described for *T. hamatum* and *T. harzianum*. The possible role of agglutinins in the recognition process determining the fungal specificity has been recently examined. Indeed, recognition between *T. harzianum* and two of its major hosts, *R. solani* and *S. rolfsii*, was controlled by two different lectins present on the host hyphae. *R. solani* carries a lectin that binds to galactose and fructose residues on the *Trichoderma* cell walls. This lectin agglutinates conidia of a mycoparasitic strain of *T. harzianum*, but did not agglutinate the non-parasitic strains. This agglutinin may play a role in prey recognition by the predator Moreover, because it does not distinguish among biological variants of the pathogen, it enables the *Trichoderma* species to attack different *R. solani* isolates. D-glucose or d-mannose residues inhibited the activity of a second lectin isolated from *S. rolfsii*, apparently present on the cell walls of *T. harzianum*.

Following these interactions the mycoparasite sometime penetrates into the host mycelium, apparently by partially degrading its cell wall. Microscopic observations led to the suggestion that *Trichoderma* spp. produce and secrete mycolytic enzymes responsible for the partial degradation of the host's cell wall. The complexion and diversity of the chitinolitic system of *T. harzianum* involves the complementary modes of action of six enzymes, all of which might be required for maximum efficiency against a broad spectrum of chitin-containing plant pathogenic fungi. The level of hydrolytic enzymes produced differs from host-parasite interaction analyzed. This phenomenon correlates with the ability of each *Trichoderma* isolate to control a specific pathogen. It is considered that Mycoparasitism is one of the main mechanisms involved in the antagonism of *Trichoderma* as a biocontrol agent along

with chemotropic growth, secretion of extra cellular enzymes and lyses of host. Thus, the biocontrol ability of *Trichoderma* is most likely conferred by a number of mechanisms.

Efficacy of the culture filtrates of different species of *Trichoderma* against the powdery mildew (*Leveillula taurica*) of cluster bean has revealed that *T. viride* effectively managed powdery mildew, while *T. harzianum* recorded the highest yield with percent increase in yield over the control [26, 27].

5.4 Compost

Research on biological control and natural suppression of fungal plant pathogens has significantly increased worldwide during the last decade. The use of complex organic substrates has been shown to be effective in protecting plant health. Application of animal-derived residues that are high in nitrogen, such as manure or compost, can result in the production of ammonia gas, which is toxic to a wide range of pathogens and nematode pests.

It is known that there is a close connection between soil borne plant disease occurrences and the organic matter content in the soil. The importance of composted organic material in suppressing soil borne pathogens has often been documented. Stimulation of antagonistic microorganisms in the rhizosphere or induced defence reactions in the host plant tissue is considered responsible for the beneficial effects. In general, three approaches have been taken to use organic amendments for biological control: (1) compost amendments added to the soil to suppress powdery mildews; (2) seed treatment to suppress damping-off of seedlings; and (3) foliar application of liquid extracts from compost to suppress foliar diseases. Lodha and Burman [27] applied soil amendment of pearl millet and weed composts for higher seed yield of cluster bean and cowpea.

Trichoderma is more commonly found living in the soil rather than in plant tissues. More than two hundred strains of the organism have been identified to date, the majority of which are soil-dwellers. The species produce spores at a tremendous rate, rapidly colonizing the growing areas. Maintenance of adequate organic carbon levels is necessary as carbon is the home base for all beneficial microbes. Commercial products can simply be boom-sprayed or irrigated into the soil, but the best carrier is the compost. Good compost contains high humus and billions of microorganisms, some of which provide metabolites necessary for the proliferation of *Trichoderma*. The *Trichoderma*-inoculated compost provides huge numbers of thriving fungal protectors, set up in an organic carbon home-base, which help in ensuring their successful colonization.

5.5 Induced Resistance

Induced resistance is a new strategy for managing plant diseases. It is an alternative procedure to protect plants against disease by activating plants' own defense mechanisms using specific biotic or abiotic elicitors [28]. The basic tenet of IR lies in enhancing resistance in response to an extrinsic stimulus without altering the genome. The protection is based on the stimulation of defense mechanisms by metabolic changes that enable the plants to defend themselves more efficiently. A number of publications with different host-parasite systems have proven the efficacy of IR against fungi, bacteria and viruses through the manipulation of the host plant's physical and biochemical properties [2, 29, 30]. The elicitors secreted through bio-agents are non-specific and therefore, can be effective against a wide range of pathogens. These elicitors work by bringing about certain metabolic changes in plants to fight against infections. The landmark studies on the development of the classic Systemic Acquired Resistance (SAR) models were conducted during the 1980s in plants, such as common bean (*Phaseolus vulgaris* L.) and *Arabidopsis thaliana* (L.) Heynh, demonstrating that SAR was conserved across diverse plant families and was effective against a broad range of viral, bacterial, and fungal pathogens [31]. Additional interests in the biological control of soil borne diseases of plants led to the unexpected discovery of another form of induced resistance associated with the colonization of plant roots by certain plant growth promoting rhizobacteria (PGPR), referred to as induced systemic resistance (ISR) [32]. ISR is distinct from SAR in several types of physiological and biochemical phenotypes that are best defined in *A. thaliana*. Results of laboratory and field studies show that, like SAR, ISR is effective against a broad range of diseases caused by viruses, bacteria, and fungi [2, 5, 33–36]. It is likely that other forms of induced resistance exist that vary in their reliance on salicylic acid, ethylene, and Jasmonic acid and other as yet discovered plant regulators. However, it is the availability of chemical inducers of SAR, such as BTH, and the characterization of numerous PGPR strains, that makes the applied use of induced resistance in conventional agriculture a reality. Besides these agents, integration of these bio-agents with indigenous knowledge is also developing in modern times as a logical strategy to manage plant diseases [5, 33]. Milk has been demonstrated to effectively control powdery mildew, downy mildew and leaf curl virus [2, 5, 23, 36, 37]. Field experiments involving the effects of INA (2,6-dichloroisonicotinic acid) and BTH (benzo-1, 2,3 thiadiazole-7-carbothioic acid) on diseases of legumes have been reported [34, 35]. Reduced densities of uredinia of the rust fungus, *Uromyces appendiculatus*, on trifoliolates of common bean were obtained when INA was applied at least 7 day before inoculation, but not at 2 h before inoculation [35]. An additional application of INA during pod-set did not improve resistance of common bean plants to *U. appendiculatus*, as opposed to a single application to the first trifoliolate [35]. Repeated applications of INA to field-grown soybean (*Glycine max*) partially reduced symptoms of white mold caused by *Sclerotinia sclerotiorum* in field trials. INA was most efficacious in suppressing white mold on the susceptible cultivars.

Higher plants have a mechanism to protect themselves against biological stresses including pathogen attacks. Plant reactions to these factors are very complex involving array of genes, encoding different proteins. Such stresses induce physiological and biochemical changes in plants by producing pathogenesis-related (PR) proteins as a defence mechanism of plants [7]. These proteins accumulate locally in the infected and surrounding tissues. Among these PRPs chitinases and β-1,3-glucanases are important enzymes that are found in plants after infection by different types of pathogens. These proteins display high-degree of pathogen specificity. Numerous PRPs have been detected in rice, wheat, maize, sorghum, barley, tomato, pearl millet, bean, chickpea, soybean, pepper, sunflower, carrot, pepper, grape vine, alfalfa, celery, rubber and in many other plants [38].

5.6 Cross-Protection

The most commonly reported examples of cross-protection involving fungi are probably those used against vascular wilts. Inoculation with non-pathogenic *Fusarium* species, or with other fungi or bacteria, all have shown different levels of cross-protection. In general, the term biological control is not used in relation to viral diseases but cross protection with mild viral strains or control of insect vectors are imperative bio-control strategies [39].

5.7 Competition

Competition occurs between microorganisms when space or nutrients (i.e. carbon, nitrogen and iron) are limiting, and its role in the biocontrol of plant pathogens has been studied for many years, with special emphasis on bacterial biocontrol agents. An important attribute of a successful rhizosphere biocontrol agent would be the ability to remain at high population density on the root surface, providing protection to the whole root for a longer period of time. Mycorrhizal fungi can also be considered to act as a sophisticated form of competition or cross-protection, decreasing the incidence of root disease.

5.8 Antibiosis

The production of antibiotics by actinomycetes, bacteria and fungi has been adequately demonstrated *in vivo*. Numerous agar plate tests have been developed to detect volatile and non-volatile antibiotic production by putative biocontrol agents and to quantify their effects on pathogens. Species of *Gliocladium* and *Trichoderma* are well-known biological control agents that produce a range of antibiotics that are

active against pathogens *in vitro* [25]. Within bacterial biocontrol agents several species of *Pseudomonas* produce antibiotics to control plant pathogens.

5.9 Mycoparasitism

Mycoparasitism occurs when one fungus exists in intimate association with another from which it derives some or all its nutrients while conferring no benefit in return. Biotrophic mycoparasites have a persistent contact with living cells, whereas necrotrophic mycoparasites kill the host cells, often in advance of contact and penetration. Mycoparasitism is a commonly observed phenomenon *in vitro* and *in vivo*, and its mode of action and involvement in biological disease control has been reviewed. The most common example of Mycoparasitism is that of *Trichoderma* spp., which attack a great variety of phytopathogenic fungi responsible for the most important diseases, suffered by crops of major economic importance worldwide.

5.10 Control of Insect Pest

Over 400 species of fungi attack insects and mites, so there is great potential for the use of these organisms as biological insecticides. As insect biocontrol agents, fungi are markedly superior to other microorganisms because they are generally non-specific in their action and are useful against a wide range of insect pests. Most of these entomopathogenic fungi belong to the classes' Phycomycetes and Deuteromycetes of division Mycophyta (Table 5.1). Spores of these fungi attack the external or gut cuticle of their insect hosts. Death may result from the production of a toxin secreted by the fungus or following the direct utilization of the body fluids. Insecticidal toxins produced by fungi are non-enzymic in nature having low molecular weight, which can kill insects when present even at low concentrations. The best examples of the use of fungi to control insects are provided by species of *Beauveria* and *Metarhizium*. Almost all the acridid pests are highly susceptible to the fungus

Table 5.1 Principal deuteromycetes fungal species for biocontrol of insects

Species	Target pests
Aschersonia aleyrodis	Whiteflies
Beauveria bassiana	Colorado beetle
Beauveria brongniartii	Cockchafers
Hirsutella thompsonii	Rust mites
Metarhizium anisopliae	Beetles, bugs, grasshoppers,
Nomuraea rileyi	Caterpillars
Verticillium lecanii	Aphids, whiteflies

requiring about 1000 spores or even less to infect and kill 50% of a population in 10 days at 28° C. *Metarhizium* is promising as a mycoinsecticide for use against locusts and grasshoppers. Constant temperatures between 20 and 35 °C (optimum 28–30° C.) facilitate the fungal infestation of these insects. Fungus is effective under field conditions when sprayed at a rate of $1–5 \times 10^{12}$ conidia ha^{-1} using an oil-based ULV spray or an oil/water emulsion using a boom sprayer. There is great commercial interest in developing a product for the biocontrol of locust and grasshopper.

5.11 Fungal Metabolites

Biologically active secondary fungal metabolites produced are not only being evaluated as potential pesticides but also for controlling plant growth. These compounds have the advantage over conventional pesticides in being effective at very low concentrations while proving essentially non-persistent and harmless to the environment.

5.12 Mycorrhizal

Mycorrhizas are symbiotic associations between soil fungi and higher plants. There are around 150 species in Zygomycotina having obligate symbiotic association with agricultural crops. These associations are known to produce growth promoters and induce resistance to plants against different pathogens. It was soon recognized that mycorrhizal association could often greatly increase the rate of uptake of nutrients such as nitrogen and phosphorus from nutrient-deficient soils. This has led to the view that the inoculation of mycorrhizal fungi in soils should lead to an increase in the uptake of these essential plant nutrients.

Two types of mycorrhiza have been recognized, the endotropic or vesicular -arbuscular mycorrhiza (VAM), and the ectotrophic type. In VAM the fungal partner is restricted to the cells of the plant cortex where it grows within and without the cells, invading the host cells at intervals to form a dichotomously branched structure called the arbuscle, thought to be the site of nutrient exchange between plant and fungus. The fungal partner appears to have no independent existence is soil. Neither is the interaction specific, since a single species of fungus can infect a wide range of plant, including most crop species. In ectotrophic mycorrhizas, the fungal partner forms a tight sheath around the plant root and from this sheath hyphae grow into the outer cortex to form a network called the Hartig net. Ectotrophic mycorrhizas, unlike VAM, tend to be non-specific.

Vesicular-arbuscular mycorrhizas can directly enhance the uptake by plants of essential nutrients such as phosphorus, copper and iron on the other hand, zinc and manganese uptake may be reduced. Therefore, mycorrhizal associations protect some plants from the toxic effects of these elements. Ectotrophic mycorrhizas also show enhanced uptake of phosphorus, and by mineralizing organic nitrogen

facilitate availability of nitrogen to the plant. They may also protect their plant hosts from heavy metals and attack by pathogens, and they also help increase the uptake of water from soil to plants.

5.13 Conclusion

Disease management in crops is heavily dependent upon the application of synthetic fungicides for pathogen control. However, restrictions on fungicide use and widespread emergence of pathogen resistance has increased global demand for more sustainable production systems and driven research towards alternative disease control strategies. However, eco-friendly approaches such as use of beneficial fungi have significantly attracted the attention of workers worldwide due to their remarkable antagonistic properties against plant pathogens with successful applications. Trichoderma species, arbuscular mycorrhizas, endophytes, yeasts, and avirulent/hypovirulent strains of certain pathogens are among the main beneficial fungi with biocontrol capacity. Biological control, which includes elicitors of host defence, MBCAs and natural products, offers an attractive alternative to synthetic pesticides. Also mycoviruses and bacteriophages can be potential MBCAs against plant pathogens. In Australia, Brazil, Canada, Europe, Japan, New Zealand, and United States a total of 101 MBCAs has been registered in 2017 for disease control [40]. Biocontrol strategies exist in different forms viz. natural (organisms and environmental factors), classic (involve an active human role), augmentative (to increase population of biocontrol agents) and inundative.

A large number of plant diseases have been managed using biocontrol agents. What is important now is to discover and use the natural biological control mechanisms evolved so far against the diseases of crop plants. Present research trends include the increased use of bio-rational screening processes to identify microorganisms with potential for biocontrol, increased testing under semi-commercial and commercial production conditions, increased emphasis on combining biocontrol strains with other control methods and integrating biocontrol into an overall system. Meisner and Boer [41] have stressed the need of understanding pathogen suppression during and upon recovery to the drought and rainfall events to adapt agricultural ecosystems to changing climate scenarios. The challenge will be to find a strategy that allows managing both drought and waterlogged conditions as the microorganism that respond to drought will differ from the ones that survive waterlogged conditions.

Intensive activity is currently being geared toward the introduction of an increasing number of biocontrol agents into the market. Commercialized systems for the biological control of plant diseases are a few. However, they also pose possible environmental risks, so biological control interventions must be undertaken with great care [42]. In view of awareness toward nature-friendly management of plant diseases, use of biological control measures will be a most promising economic proposition for disease management.

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Part II
Use of Natural Compounds

Chapter 6
Potential Use of Polyphenolic Compounds Obtained from Olive Mill Waste Waters on Plant Pathogens and Plant Parasitic Nematodes

S. Leontopoulos, P. Skenderidis, and I. K. Vagelas

6.1 Introduction

Microorganisms such as fungi, bacteria, viruses etc., associate and interact in natural environment with plants promoting [1–3] or affecting their development stages, reproduction ability and ultimately yield production [3–5]. Most plants produce a broad range of secondary metabolites in very low quantities (less than 1% dry weight). Their production is mainly depending on development and physiological stage of the plants [6, 7]. Secondary metabolites are important in several physiological functions such as growth, reproduction, pigmentation, resistance to pathogens and many other essential for the plant acclimatization factors [3, 8–18] playing an important role in the adaptation of plants to their environment [19, 20], but have no vital role in the continuation of life processes in the plants [17]. So far, as a result of millions of years of plant-pathogens interactions more than 100,000 secondary metabolites are known to be involved in plant defense system [21]. Some of these secondary metabolites, apparently act as defence mechanism against microbes [22–25], or competing plants, and signal compounds to attract pollinating or seed dispersing animals, as well as protecting the plant from ultraviolet radiation and oxidants [26, 27].

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However, according to Jamwal [17] secondary metabolites not only play important role in plant-microbe interactions but also used by humans in modern and traditional industries as medicines, flavorings, pharmaceuticals, agrochemicals, fragrances, colours, biopesticides, food additives and drugs [28, 29]. Important types of plant secondary metabolites are Terpenes (monoterpenes, sesquiterpenes, diterpenes, sesterterpenes, triterpenes, sesquarterpenes, tetraterpenes, polyterpenes), Phenolics (cumarin, furano-cumarins, lignin, flavonoids, isoflavonoids, tannins), N containing compounds (alkaloids, cyanogenic glucosides, non-protein amino-acids) and S containing compounds (glutathione, glucosinolates, phytoalexins, thionins, defensins, allinin).

Among secondary metabolites, phenolic compounds such as flavonoids and hydroxycinnamic acid derivatives, are ubiquitous in plants [30] and are in great importance since most of them are used for defensive functions and have shown antimicrobial activities [31–33] controlling bacteria [34–38], fungi [39, 40] and viruses [26]. An appropriate response to attack by such organisms can lead to tolerance or resistance mechanisms as a natural response to the biotic stress. Phenolic compounds among the metabolites in nature with the above characteristics are polyphenols. According to Lattanzio, polyphenols arising biogenetically from either the shikimate/phenylpropanoid pathway, which directly provides phenylpropanoids, or the "polyketide" acetate/malonate pathway, which can produce simple phenols, or both, thus producing monomeric and polymeric phenols and polyphenols [41].

Interest in the isolation, examination and usage of plant tissues and agricultural wastes rich in natural polyphenols have been under consideration of many researchers [42–52] and companies such as Technidex fruit protection S.A. worldwide the last few decades even for production of carbon based nanomaterials [53]. This interest is due the uninterrupted and indiscriminate use of synthetic pesticides and fungicides which has not only led to the development of resistant strains, but results to the presence of toxic residues in soil [54] and crops [55, 56] used for human consumption and thus, substantially affect, human health and environmental quality [57–59].

Recovery of high added-value products from waste plant material such as OMWW constitute a cheap source in such components like phenolic compounds [60] which is therefore a significant issue in non chemical disease control [61] and can be used as alternative, eco-friendly treatments [27] contributing to farmers, applicant's and consumer's health protection [62–64].

The aim of this study is to exploit the utilization, the antioxidant and the antimicrobial effects of phenolic compounds extracted from OMWW as plant protective agents against important fungal and nematidical plant pathogenic species.

6.2 Polyphenolic Compounds Obtained from Olive Mill Waste Water

6.2.1 Olive Tree

Olive tree (*Olea europaea*) is a warm-temperate and subtropical xerophytic, evergreen tree of Oleaceae family which is comprised from 22 genus and about 500 species, most of them belonging to the subfamily Oleoideae. About 40 species of genus Olea with olive-growing heritage covered about 9.5 million hectares containing more than 900 million trees are cultivated worldwide [65]. Among its global distribution, olive trees are cultivated mostly in Mediterranean countries for its drupes which yield oil and consumed mainly as tabled olives [66–68].

According to archeological researches olive tree seems to be cultivated since prehistoric periods in the eastern Mediterranean region such as Syria and Crete. Like other cultivated plants in ancient and recent years, olive tree is closely related with human nutritional needs, medicinal, sociocultural and religious actions. Beside Mediterranean area, olive trees are also cultivated for commercial use in about 35 countries and regions like Australia, California, South Africa etc. and its luxurious product exported to more than 100 countries worldwide. Olive oil is produced by extraction techniques either traditionally by simple crushing-pressing equipments or mechanically by using modern widely varying designs equipments. According to the acid content and method of preparation olive oil is classified in 4 groups. Olive oil obtained by pressing olive fruit without further treatment is called virgin olive oil [68].

Mature olive fruit is composed by epicarp, mesocarp (or pulp) containing about 96–98% of the total amount of oil, and endocarp (or tone-pit) which contains the seed, and produces the remaining 2–4% of oil production. Olive tree parts such as fruits and leaves are rich in phenolic compounds [69] with pharmacological properties [70], antioxidant and antimicrobial activity [71–77], modifying the equilibrium of useful soil microorganisms [78] and inhibiting Gram positive microorganisms involved in olive fruit fermentation [79, 80].

Olive fruit pulp contains about 85–90% moisture and oil while the rest comprises organic matter, minerals such as organic acids (citric, malic, oxalic, malonic, fumaric, tartaric, lactic, acetic, and tricarballylic), potassium, monosaccharides (glucose, mannose, xylose, galactose, arabinose, mannitol, rhamnose), phenolic compounds such as oleuropein, lipid fractions and fatty acids, sterols, triterpene alcohols, dialcohols, and hydrocarbon fractions (Fig. 6.1).

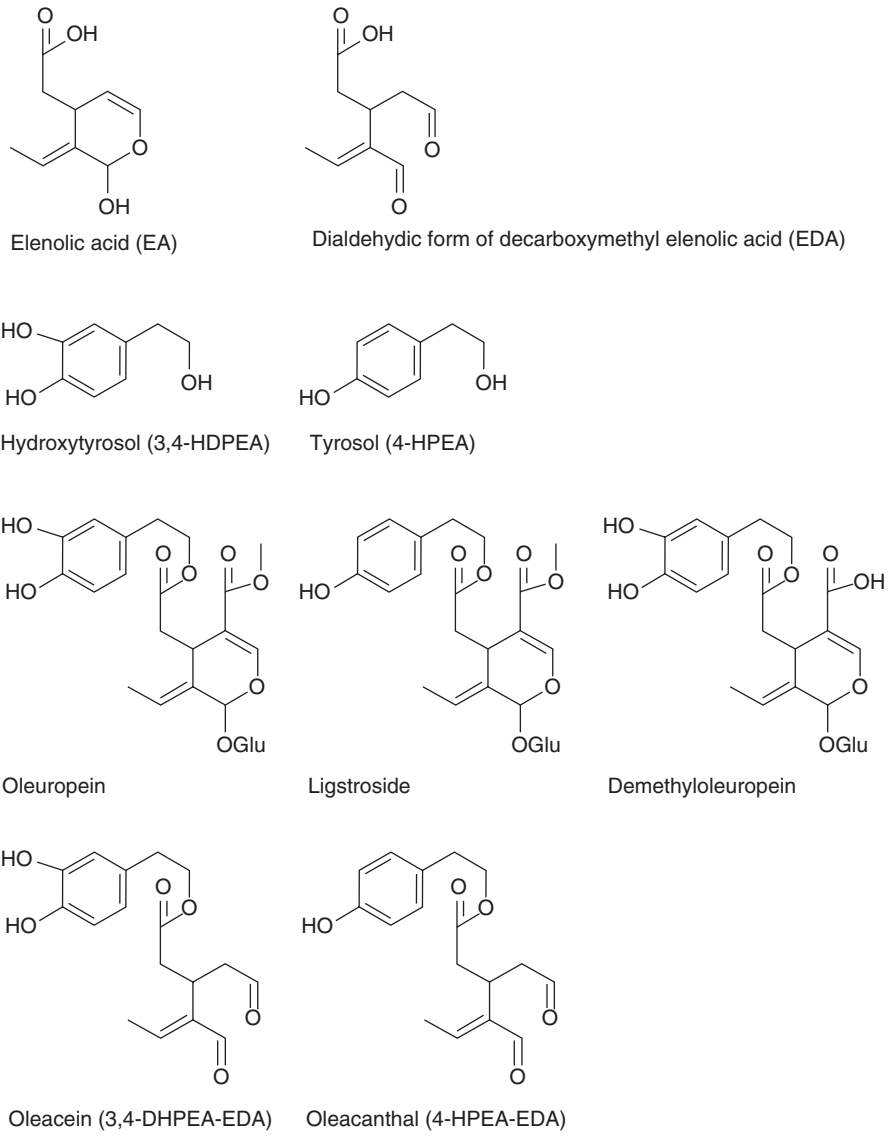

Fig. 6.1 Chemical structures of phenolic antimicrobial compounds in *Olea europaea* [76]

6.2.2 Olive Oil Phenols

Lipophylic components of olive fruits are transferred to the olive oil due to the extraction techniques used based on physical or mechanical pressure without the use of chemicals in contradiction with most vegetable oils which are extracted from seeds by solvents. The amount of phenolic compounds in olive oil depends on

several factors such as environmental conditions, cultivar varieties, cultivation techniques, fruit and tree maturation [81, 82], possible infestation by olive fruit fly *Bactrocera oleae,* [83], extraction (crushed and pressed temperatures) and elaboration techniques [84].

Oleuropein, hydroxytyrosol and tyrosol three compounds that are related structurally are the main phenolic compounds with the highest concentration found in olive oil [85]. Among them, oleuropein is the major phenolic compound in olive fruit (14% in dried fruit) and hydroxytyrosol is the major phenolic component in olive oil.

6.2.3 Bioactive Compounds Contained in Olive Mill Waste Water

After olive fruit elaboration, the extracted olive oil contains only 2% (50–1000 μg/g) of the total polyphenols contained in the olive drupe [86], while the rest 98% is transferred in the waste waters (approximately 53%) or in the pomace (approximately 45–90%) [87, 88]. According to Zouari [60, 89], OMWW is a blackish-red aqueous extract (83–94% water) after olive fruit elaboration for olive oil extraction which contains organic compounds (4–16%) and mineral salts (0.4–2.5%) such as polysaccharides, sugars, polyphenols, polyalcohols, proteins, organic acids, and oil. The organic fraction contains 2–15% of phenolic compounds [74]. Alternative, definition have been proposed by Tsagaraki who mention that OMWW is a mixture of olive oil and pulp, mucilage, pectin, soft tissues of the olive fruit, suspended in a relatively stable emulsion, and water used in the various stages of the oil extraction process, such as the water added during centrifugation, water derived from filtering disks, and from washing rooms and equipment [90].

The OOWW contains more than 30 different phenolic compounds Structure of OMWW phenolic compounds is very variable [91]. OMWW phenolic compounds are divided in two major categories, the low-molecular weight compounds such as caffeic acid, tyrosol, hydroxytyrosol, p-coumaric acid, ferulic acid, syringic acid, protocatechuic acid and the high-molecular phenolic compounds such as tannins and anthocyanins [89]. OMWW concentration of phenolics compounds is 0.5–1.8% and contains amounts of hydroxytyrosol, oleuropein, tyrosol, lactone, secoiridoid glucoside, verbascoside, catechol, 4-methylcatechol, p-hydroxybenzoic acid, vanillic acid, syringic acid, and gallic acid [92–95]. Among phenolic compounds occurring on OMWW hydroxytyrosol and tyrosol are the most abundant [96, 97]. The OMWW organic matter comprises an insoluble fraction consisting essentially of pulp of olives, suspended matter and colloidal, and a soluble fraction in the aqueous phase which contains sugars, lipids, organic acids, pectins, phenolic compounds, vitamins and traces of pesticides [98–100]. It has been calculated that for OMWW processing, about 20 million tones of water expanded per year and derived 30 million tones wastes water. According to Manios, [101] only in Greece are operating

about 2500–3000 olive mills producing a daily average of 15–20 tones of olive oil. Although OMWW is becoming a serious environmental problem due to its high concentration of organic matter (BOD ranging between $15 \times 10^3 - 50 \times 10$ mg/l and COD reaching about 220 g/l) Visioli, [92, 102], demonstrated that OMWW extracts can be used in preservative chemistry as cheap source of natural antioxidants since they contain large amounts of polyphenolic compounds (Fig. 6.2).

6.2.4 *Utilization of OMWW*

Olive oil production occurs mainly in Mediterranean countries where, Spain, Italy and Greece produces about 66,8% of global production of 2013. Among 19 million tons of harvested olive fruits globally, extracted virgin olive oil was about 2.6 million tons in 2013 [104] and 2.84 million tons in 2017 [105, 106]. It has been calculated that, annual production of solid or liquid olive mill residues in Mediterranean

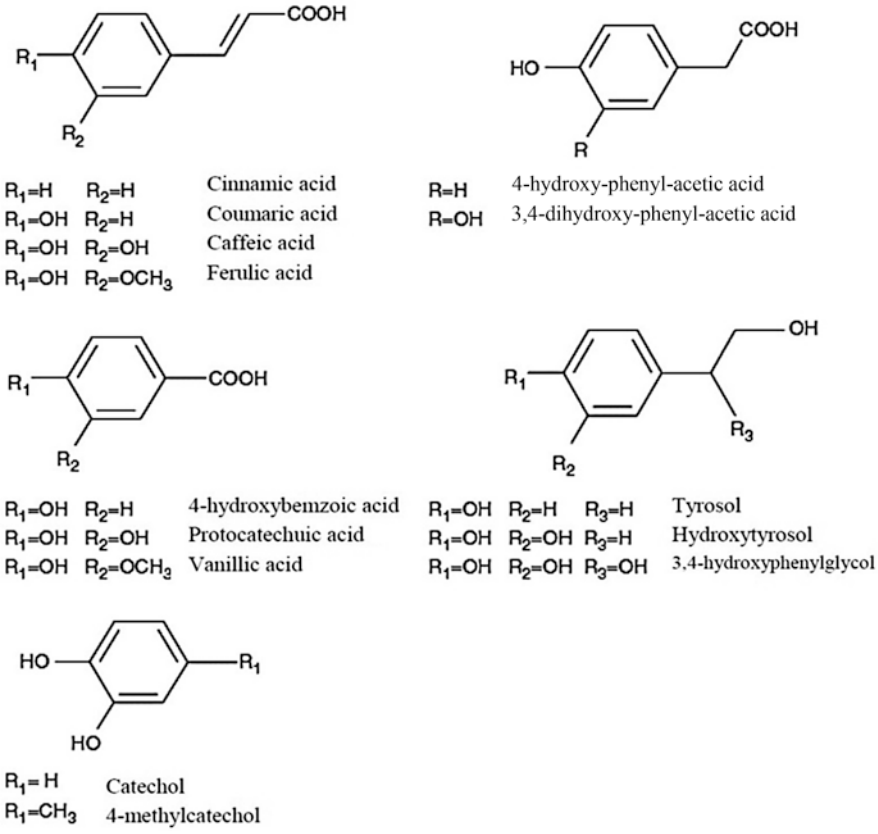

Fig. 6.2 Main phenolic compounds found in olive oil waste [93, 103]

countries varies widely from 10 to 30 million m^3 depending on different biotic and abiotic factors such as olive variety, weather conditions, soil cultivation technique, olive fruit harvesting time, use of pesticides and fertilizers, degree of ripening, duration of aging and technology (two phase or three-phase centrifugation process) applied for the extraction of olive fruit [107, 108]. Furthermore, the amount of OMWW generated is about 5 m^3/tn of produced olive oil [109]. It is mentioned that in three-phase centrifugation process, from 1000 Kg olives and 500 added water, produced 180 Kg olive oil, 720 Kg wastewater and 600 kg olive husk while in two-phase centrifugation process from 1000 Kg olives, produced 180 Kg olive oil and 820 kg olive husk [110, 111]. Ten years ago, among Mediterranean countries, only Spanish mills (about 97%) operated a two-phase decanter method due to its lower water consumption and waste streams, while in the remaining Mediterranean countries the three-phase extraction method was operated from about 95% of mills, due to the exorbitant cost of adopting the two-phase method [112]. However, the semi-solid residues (WOMW: wet olive mill waste), produced by this method, are more difficult to treat and dispose than wastewater derived from three phase centrifugation process [113]. The last few decades, this situation has been changed, since management of residues (liquid or solid) in both extraction methods is a major challenge issue for the olive mill operators from both economic and environmental perspectives [114]. However, in some cases like Greece high regional scattering of olive mills may conceal higher transportation costs that could make the re-use of the treated OMWW completely unaffordable [115].

Furthermore, environmental awareness, decrease of farmer's income and application of renewable energy techniques for energy purposes, has gained significant research interest and many studies have been conducted focused on the exploitation of olive mill solid and liquid wastes in order to create efficient, competitive, innovative and therefore more profitable and sustainable farm and olive industry businesses [114, 116–118]. The remained pressed solid or liquid residue byproducts such as olive cake, sludge and OMWW can be used for purposes such as energy production [107, 113, 119–125], animal feed [126–130], manure, N fertilization and soil improvement [115, 131], food additive [132–134] single-cell protein production [135], synthesis of highly luminescent carbon-based nano-materials [53], added as substrate supplements for the cultivation of *Pleurotus pulmonarius* impairing the growth of the mushroom pathogen, *Ps. tolaasii*, [136] etc.

The use of biomass is an emerging technology and seems to be an excellent and alternative energy source [119] after oil, coal, natural gas, sun and wind [107]. Olive oil mill waste (solid and/or liquid) contains large amounts of biomass residues, thus, many studies have been investigate the co-digestion of OMSW with OMWW [137–139]. Anaerobic digestion of wet olive mill wastes (WOMW) is a very difficult task due to their chemical characteristics such as acidic pH, high salinity, lack of nutrients, low biodegradability, and high concentration of polyphenols. For these reasons an adequate pretreatment is necessary [113, 140, 141]. Although, energy production is more efficient and easy for solid residues, where the energy potential of the yearly produced 79,000 t of olive solid wastes ascend in around 380 GJ [122], energy can also be produced from wet biomass using supercritical water gasification (SCWG)

instead of conventional gasification and anaerobic digestion suppressing also COD and TOC levels from OMW by 70–89% and 65–88% respectively [107]. Beside biogas production, Kıpçak and Akgün, studied the possibility of biofuel production from OMWW through its Ni/Al_2O_3 and Ru/Al_2O_3 catalyzed supercritical water gasification [124]. Their findings suggest that the employment of catalysts enhanced the gasification and biofuel production yield, which mainly involved methane and hydrogen.

Furthermore, many studies have been aimed in utilization of the produced OMWW, in agriculture as a nutrient fertilizer and/or organic amendment since it is rich in nitrogen [42, 108, 115, 142–145]. Mekki observed that OMWW is rich in organic matter, nitrogen (N), phosphorus (P), potassium (K) and magnesium (Mg) [146]. Thus, its use it could be potentially beneficial for soil improvement and crop fertilization, but mostly in areas suffering of water scarcity, where soil organic matter and nutrient contains is poor [142, 144]. Although, according to Brunetti before any crude OMWW amendment in soil, fresh OMWW should be subjected to appropriate treatments in order to eliminate environmental risks and to stabilize its organic matter through humification [147].

Piotrowska studied the use of OMWW for soil fertigation in a laboratory model system with and without removal its toxic phenolic compounds [142]. It has been found that within 0–14 days after the addition of crude or dephenolized OMWW several biological properties of the soil, changed suddenly. Studies done by Aharonov-Nadborny on the effect of OMWW on spreading and leaching of metal cations (Na, K, Mg, Mn, Fe, Cu, Zn) in several types and textures of agricultural soils have shown the contribution of OMWW on indigenous metal mobilization [148, 149]. Leaching of soil-originated metals from the sandy soil was substantially greater than from the loam and clay soils, while the clay loam was enriched with metals derived from the OMWW. The organic matter fraction of OMWW forms complexes with metal cations; these complexes may be mobile or precipitate, depending on the soil chemical and physical environment. Studies examined the impact of OMWW on contamination of groundwater due to leaching of nutrients and polyphenolic compounds, demonstrated the safe application in soil even in large rates. More specific, when OMW was directly applied, the acidity, electrical conductivity and polyphenols concentration of the leachate were always negligible with respect to the corresponding values of the incoming water. On the contrary, the amount of nitrate potentially reaching groundwater was dependent on the specific soil characteristics [144].

Furthermore, studies done by Meftah about the long term application effects of OMWW on the main properties of a Mediterranean soil under arid climate shown that irrigation of sandy soils by different doses of OMWW has influenced the soil physicochemical and microbiological characteristics even at 120 cm soil depth [108]. Likewise, results from Bargougui [145] showed an improvement of the organic matter, total nitrogen and minerals contents in soils treated with 25, 50, 75 and 100 $m^3\ ha^{-1}$ OMWW, while Mekki observed that addition of OMWW increase soil electrical conductivity but did not affect initial soil pH [150]. In contrary, Chartzoulakis reported that after 3 years of raw OMWW application, there were no

significant differences in pH, electrical conductivity, P, Na and organic rates between the control and OMWW treated soils [151].

The effects of OMWW on seed germination have also studied by many authors [152–154]. For example seed germination of *Triticum durum* [98, 146, 155], *Lycopersicon esculentum*, *Cicer arietinum*, *Vicia faba*, *Hordeum vulgare* [98, 146], *Zea mays* [98], *Sorghum bicolor* [145], *Lolium multiflorum* [156], *Amaranthus retroflexus, Solanum nigrum,* [157], *Lepidium sativum* [158] is affected by OMWW soil application while plant growth of many plants such as maize and sorghum irrigated with 25 m^3 ha^{-1} OMWW have showed a clear increase in stems length compared to those of control soil [115, 145, 159]. A higher OMWW dose application (50 or 100 m^3 ha^{-1}) can exert phytotoxic and nutritional disturbances for the soil plant interactions [156, 160–162].

Last but not least, according to De Marco phenols could be use as raw material for the isolation of valuable bioactive compounds by pharmaceutical, cosmetic and nourishment sectors [95, 163, 164]. Organic matter (nitrogen, phosphorus, potassium and magnesium), phenolic compounds, sugars and organic acids contained on OMWW could be possibly used as active ingredients for skin care [165].

6.3 Methods and Technologies Obtained Polyphenolic Compounds from OMWW

Although many studies have been completed about treating residues of OMWW, limitations in efficiency of the applied technology and no unified legislation or regulations from European Union has lead to major differences even between Prefectures of the same country [100, 164], in controlled disposal of OMWW. Thus, it remains one of the main environmental problems related to the olive oil industry [100, 166, 167]. So far, in many Mediterranean countries OMWW produced in excessive seasonal amounts from small scale olive mills and temporarily stored in evaporation ponds or lagoons [168] for further management [169] before their spreading in agricultural lands [170]. In order to identify olive oil waste disposal areas where huge quantities of wastes are produced different image analysis techniques could be applied to high resolution multispectral satellite data as sufficient and systematic tool [171–174]. Indeed, remote sensing data can provide a cost effective way to detect both legal as well as illegal OOMW open air disposal areas.

It is believed, that 1 m^3 waste mill have polluting power equivalent to 100–200 m^3 municipal waste [90]. For this reason OMWW is responsible for the severe environmental impact of wastewater mills. According to Borja [175] the waste material is characterized by strong unpleasant odor, high pollution parameters (COD up to 220 g/lt), pH between 3 and 5.9, high percentage of polyphenols (up to 80 g/lt) and high solids contains (up to 20 g/lt) [24]. The unmanaged disposal of such waste in natural receptors leads to serious problems in the ecosystem, rivers, lakes, seas and groundwater [176].

Innovative approach to the conversion of wet waste biomass into valuable materials is a major priority in modern agro-industry sector [177]. For this reason many efforts have been done for efficient recovery, valorization and use of agro-industrial wastes such as olive oil residues. According to Raza [178] so far, a wide range of stand-alone and integrated processes, technologies and management options such as direct application on soil, evaporation, solar distillation, centrifugation, filtration, flocculation, adsorption, electrocoagulation, fermentation, anaerobic digestion, aerobic treatments, oxidation, ozonation, membrane-integrated processes, bioremedation and biological degradation methods have been tested and proposed for the removal and/or elimination of phenolic compounds from waste water [63, 138, 141, 166, 179–196, 198]. Zagklis, studied sustainability of OMWW treatment methods and suggest that membrane filtration processes, electrolysis, supercritical water oxidation, and photo-Fenton, are considered the most effective processes in terms of the reduction of organics [197].

Among biological agents, studies focused on fungal species such as *Phanerochaete chrysosporium, Aspergillus niger*, *Aspergillus terreus,* and *Geotrichum candidum* have been used for to extract and remove polyphenols from OMWW [199, 200] with limited efficiency [183, 201, 202]. However, according to Nogueira biological treatment was more effective when no hydrogen peroxide was used in the pretreatment [195]. Furthermore, the treatment with *P. chrysosporium* promoted the highest reduction in toxicity, but *P. sajorcaju* was responsible for the best reduction in COD and TPC.

Among membrane technologies, membrane separation is considered one of the most efficient tools for abating phenolic compounds from waste water because of low capital cost, easy scalability, and ecofriendly production with the lowest emission of noxious compounds. Among membrane operations, microfiltration, nanofiltration, osmotic distillation, reverse osmosis, vacuum membrane distillation, diafiltration, membrane bioreactors are widely in use or are under test for their specific application [188, 203–214].

Dry thermochemical treatments for energy recovery [215], wet thermochemical processes [216, 217], valorization via hydrothermal carbonization in where liquid residues may contain a suitable amount of phenolic compounds and micronutrients [218–221], high performance membrane extraction technologies [222] for converting OMWW disposal into valuable compounds such as polyphenols used as food additives and organic fertilizers [223] are only some of the used techniques and methods for OMWW conversion reducing the organic load or recover polyphenols of OMWWs [224]. Ultra filtration fractions characterized by HPLC analysis were also used for the identification of phenolic compounds [95].

Forward osmosis is a membrane process that uses an osmotic pressure gradient as a driving force to transport water across an ideally semi-permeable membrane applied in many sectors [225–230]. According to Gebreyohannes forward osmosis is applied to de-hydrate OMWW within the logic of Zero Liquid Discharge and by-products valorization [231]. However, according to Ochando-Pulido, membrane separation technologies of OMWW still represent severe limitation due efficiency difficulties and operational and investment costs [232].

More recently, in study done by Annab powdered activated carbons beads (PAC-CA) was successfully synthetized and encapsulated in calcium alginate and was used for effective polyphenols absorption from OMWW effluents [233]. This application is a novel, efficient and low cost treatment process of olive mill byproducts, as both solid (OP) and liquid (OMWW) by-products can be recycled and reused for own benefit of the industry.

Furthermore, Dammak propose a novel method which provide a highly-concentrated polyphenols isolate (up to 75% (w/w)), with up to (510 mg/g) of the polyphenols content being hydroxytyrosol, using green technology, for obtaining a natural bioactive concentrate rich in polyphenols from OMWW [234]. In this method centrifugation, batch evaporation and drowning-out crystallization-based separation process are integrated for the separation of polyphenols from the different components present in OMWW, based on the solubility behavior changing after addition of ethanol. This product is also accepted as food grade.

Beside ethanol, a large number of solvents such as methanol [235], ethyl acetate [91], n-butanol, propanol, and tert-butyl methyl ether are used to recover polyphenols from OMWW. Several resins like XAD4 macroporous resin [236], amberlite XAD16 resin as the adsorbent and ethanol as the biocompatible desorbing phase are used in order to recover from 60% to 87% of hydoxytyrosol and 100% of tyrosol of the polyphenols [50, 237] while IRA96 polar resin recover about 76% [238].

For better results the absorbed polyphenols could be encapsulated in several excipients such as maltodextrin [51].

6.4 Antioxidant and Antimicrobial Activity of Polyphenolic Compounds of OMWW on Human Health as Food Additive

The treatment and disposal of OMWW is a critical problem where olive cultivation is widespread and a large volume of the OMWW is produced within a limited period of few months. As it was mentioned before, chemical substances contained in olive oil and in their wastes can be used as potential source of polyphenols in several applications. Numerous studies support the view that polyphenols found in olive oil and consequently in its wastes are capable of acting as phytochemical as presented in Fig. 6.3.

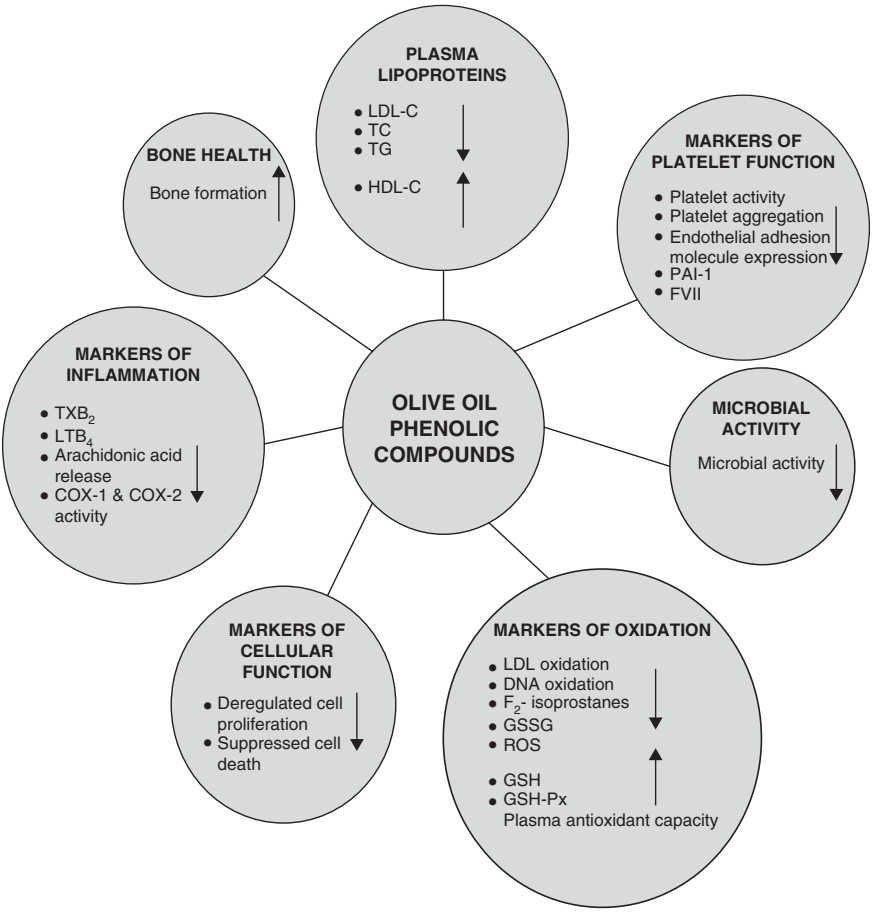

Fig 6.3 Positive effects of olive oil polyphenols as adopted from [239]

6.4.1 Antioxidant Activity of Polyphenolic Compounds of OMWW

Reactive Oxygen Species (ROS) are chemical reactive molecules produced by the normal metabolism of organisms. The level of the ROS increased under environmental stress and can damage lipids, DNA and proteins. The main ROS are superoxide radical ($O_2^{\bullet -}$), hydroxyl radical ($OH^{\bullet}$), peroxyl radical ($ROO^{\bullet}$), O_2 in normal condition, hydrogen peroxide (H_2O_2), hypochlorous acid (HOCl) and the nitric oxide (-NO).

In addition to oxidative damage, ROSs also involved in the activation of pre-anti-inflammatory cytokines resulting in inflammation. These two processes interact with and are responsible for a set of chronic conditions such as hypertension, insulin

resistance, CVD (cardiovascular disease) metabolic syndrome, cancer-related diseases and age [29].

Visioli has been shown that olive oil polyphenols scavenge ROS and has an antioxidant potential similar to vitamin C and E respectively and can also scavenging HOCl and –NO [240].

3-hydroxytyrosol (3,4-DHPEA-EDA), can play a remarkable protective role against ROS that cause oxidative damage to human cells, while small concentrations of this compound were able to protect red blood cells *in vitro* and prevent hemolysis [241], while Kohyama, observed that it can inhibit arachidonic lipoxygenase activity [242]. Furthermore, olive oil's phenolic components can reduce the oxidation of low density lipoprotein [243].

Many research studies have described the antiflammatory, atherosclerosis, cardiovascular and bone formation effects of oleuropein and hydroxytyrosol [243–257].

6.4.2 Antimicrobial and Antiviral Activity

Studies have shown that the phenolic compounds of olive oil (oleuropein, tyrosol and the dialdehyde form of decarboxymethyl oleic acid) have a strong bactericidal effect even greater than that of other phenolic compounds in food or synthetic biocides [258]. Yamada, presents in their study the antiviral activity of hydroxytyrosol, that can inactivated influenza A viruses including H_1N_1, H_3N_2, H_5N_1, H_9N_2 subtypes and can also inactivate Newcastle disease virus [259].

Furthermore, polyphenols obtained from OMWW showed antibacterial activity against phytopathogenic Gram-negative and Gram-positive bacteria like *Pseudomonas syringae, Pseudomonas savastanoi*, *Corynebacterium michiganense* [260]. Additionally, Roila, investigated the antimicrobial effects of OMWW polyphenol extract against 64 strains of *Pseudomonas fluorescens* which is the responsible microorganism for the discolouration of mozzarella cheese [261].

6.4.3 Use as Natural Antioxidant in Food

The addition of antioxidants is a well-established, relatively cheap and effective method to obtain oxidative stability improvements in food products. The use of OMWW polyphenols as natural antioxidants in food it has been reported by many authors. Veneziani, reported that extracted polyphenols recovered from OMWW increase significantly the oxidative stabilization of lard while extend also shelf life of a hamburger from white meat [262].

Moreover, Fasolato investigated the effects in the shelf life of fresh breast of chicken after the dipped in a crude OMWW extract [263]. Results of this study present an extension of at least 2 days of the shelf life and a significant reduction of the

TBAR values while the organoleptic characteristics like color and odor remained unalterable or even improved.

Furthermore, Lopez studied the effects of olive oil polyphenols against growth of moulds and yeasts on the surface of fermented sausages which they dipped before fermentation for 60 s in a 2.5% aqueous solution of a purified extract obtained from OMWW [264]. According to their results, an effective inhibition of spore germination against most assayed strains (excluding *A. parasiticus* and *A. flavus*), whereas a moderate impact on sensory traits was observed without any undesired effects. Same results also presented in the study of Balzan, who investigated the effect of olive phenols recovered from OMWW on the preservation of raw and cooked fresh pork sausages in two concentrations of 750 and 1500 ppm during aerobic storage at 2 ± 2 °C for 14 days [265]. Results from this study shown a decrease in pH, diacylglycerols, peroxide value and thiobarbituric acid reactive species (TBARS) and a reduction of the cholesterol oxidation products.

Recent work completed by Taticchi studied the possitive effects of OMWW extracts on carotenoids and other photochemical substances on tomato sauce by fortifying a refined olive oil that was used during cooking [266].

6.5 Biological Control of Plant Diseases

More than, 4000 parasitic organisms have been identified and they can be found in most major biomes. Thus, management of plant disease caused by parasitic organisms has become a challenging task to the researchers for sustainable agriculture [2].

Plant pathogens can enter to their host cells affecting cultivated plant health and thus global crop production. The control of plant pathogens is a major challenge in modern agriculture where many farmers in developing and developed countries are less likely to adopt biological approaches instead they prefer the use of chemical pesticides destroying the natural ecosystems [25]. Nowadays, it is required to explore eco-friendly alternatives, like plant based metabolites to control pathogens.

Moreover, according to Ganusova and Burch-Smith, plants have acquired a variety of adaptive mechanisms in order to eliminate biotic and abiotic stresses [4]. Plasmodesmata, these intercellular pores connecting adjacent plant cells, play an important role in intercellular trafficking, communication, signaling in plant development, infection by plant pathogens and plant defense responses [267]. Three major types of secondary metabolites viz. Phenolics, Terpenes and Nitrogen/Sulfur containing compounds are produced in plant's body [25].

Soil borne pathogens are in great importance since they interact with plants and other soil organisms such as free-living bacteria, fungi, foliar and root herbivorous insects and nematodes in the heterogeneous thin layer of soil directly surrounding the root system [268, 269].

6.6 Use of Polyphenolic Compounds from OMWW Against Fungal Plant Pathogens

Many fungal and bacterial species can affect plant growth causing severe yield losses in many crops worldwide affecting farmer's income. Resistant varieties, available chemical phytoprotective compounds, cultural techniques and market-consumers habits, may lead farmers to alternative crops [270]. Plant fungal pathogens can affect plant growth and cause symptoms in several parts and plant tissues such as roots, stem, branches, leaves, flowers, fruits and vascular tissues.

Soil borne pathogens are spread worldwide affecting plants in a wide range of seedlings in nurseries, glasshouses, gardens, crops and forests. Widely distributed soil borne species such as Pythium, Rhizoctonia, Phytopthora Gaumanomyces, Verticillium, Fusarium, Sclerotium, Macrophomina, Armillaria and many others, has a strong influence on their survival and capacity to cause a serious disease complex difficult to predict, detect and diagnose [271, 272]. Their ecological behaviour, extremely broad host range and the high survival rate of resistant forms such as chlamydospores and sclerotia under different environmental conditions make strategies to control soilborne diseases to be limited [273, 274].

Plant pathogens that affect foliar, fruit and flourish parts such as rust fungi, powdery mildews, *Ascochyta lentis* (that causes foliar necrosis), *Monillia laxa*, *Monillia fructigena*, *Eutypa lata* (that causes rots in several fruits), *Botrytis cinerea* (affecting flowers, leaves and especially fruits of several trees causes also post-harvest rots in plants such as strawberry, tomato, etc.,) *Cercospora beticola, Alternaria alternata* etc., are also important crop's pathogens and require methods for plant protection. Finally, many fungal species such as *Penicillium* sp., and *Aspergillus* sp., causes post harvest diseases in stored fruits and grains and release mycotoxins on them.

Recently, because of pesticide's environmental and deleterious effects on human health [275–278] there has been an increasing interest in the effects of polyphenolic compounds obtained from olive mill wastes in compost or liquid form as potential biodegradable pesticides against important plant pathogens for soil, foliar spray and hydroponic application [157, 279–281]. However, study done by Moreno about the response of soil microbial community to a high dose of fresh OMWW, reported that the addition of repeated and uncontrolled discharges of OMWW in field conditions was related to an increase in the fungi and Gram-positive bacterial biomass [62] (Table 6.1).

Although antimicrobial activity of compounds such as oleuropein contained on OMWW extract have been studied the last few decades, little research has been done on screening these extracts efficiency against economically important fungal and bacteria strains. Despite the environmental impact due accumulation and phytotoxic effects [48, 299, 300], phenolic compounds inhibit microbial growth [26, 33, 36, 38, 45, 281] and decrease pesticide mobility [301]. In low doses, OMWW soil application, provide a favorable environment for the development of soil microflora, which means recycling organic matter and enriching mineral elements that increase soil fertility [108, 302]. More specific, according to Bargougui, differences

Table 6.1 Studies on OMWW effectiveness against important phytopathogenic fungi

Studied organism Fungi species	Plant	References
Botrytis cinerea, Botrytis tulipae	Pepper, tulip	[47, 48, 157, 282–287]
Fusarium oxysporum f.sp., *melonis,*	Tomato, tulip, melon	[47, 48, 283, 285, 287–290]
Fusarium oxysporum f.sp., *lycopercici,*		
Fusarium culmorum		
Fusarium solani		[274]
Verticillium dahliae		[48, 280, 285, 286, 290–292]
Pythium sp.,		[48, 157, 285, 290]
Phytophthora parasitica var. *nicotianae, Phytopthora capsici*		[48, 157, 283, 290, 293]
Rhizoctonia solani		[46, 48, 157, 274, 294]
Phoma sp		[288]
Sclerotinia sclerotiorum, sclerotinia minor		[47, 48, 285, 290]
Armillaria mellea		[48]
Alternaria alternata, A., solani		[48, 283, 288, 289]
Cladosporium sp		(295, 296]
Cercospora beticola		[48]
Colletotrichum higginsianum	Brassica, Raphanus	[283]
Candida albicans		[297]
Penicillium italicum, P. digitatum, P. expansum, P. cyclopium,	Oranges, tulip	[48, 285, 287, 288, 295, 296, 298]
Aspergillus fluvus, A niger	Tulip, tomato	[48, 287, 288, 296, 297]
Rhizopus		[283]
Monillia laxa, M. fructigena		[48]
Eutypa lata		[48]
Paecilomyces sp.		[288]

in dose and incubation time of application of OMWW, affects soil cultivable aerobic microflora (CAM), fungi and sporulating bacteria [145]. Results from his study have shown that OMWW amendment has induced a clear increase and enhancement in the CAM number and fungal spore germination respectively. Nevertheless, this increase has been not proportional to the dose applied, above the dose 50 m^3/ha^{-1} (for CAM) and 75 m^3/ha^{-1} (for fungi) due to the antimicrobial inhibitory effects [160]. According to Aviani and Piotrowska large amounts of OMWW induce an anaerobic environment counteracted the beneficial effect of organic substrates provided, which promoted the growth and activity of indigenous microorganisms and inhibits aerobic germs [303, 304]. Thus, doses used for soil irrigation over 100 m^3/ha^{-1} and sometimes 200 m^3/ha^{-1} constitute an inhibitory dose for soil microflora. However, sporulating bacteria have shown a relative proliferation to OMWW applied doses in acid, anaerobic and saline conditions occurred when 100 m^3/ha^{-1}

applied [305]. Thus, a lower dosage of 50 m^3/ha^{-1} has been proposed to be the most suitable for the soil application.

Furthermore, according to Balk because of their low content in nitrogenous organic components and reachness in carbon sources, when OMWW applied in soil, they offer a highly favourable environment for the growth of free-living dinitrogen fixing microorganisms [42]. However, after the second year of implementation of polyphenols on the soil it appeared transient symptoms of phytotoxicity. Moreover, when oleuropein solution at concentration of 0,5% applied infiltrated directly to pepper wounded leaves, observed symptoms after 15 days, appear a strong toxicity effect [283] while spray application of pure and semipure oleuropein at concentrations of 0.01%, 0.05%, 0.1% and 0.5% did not show any toxicity symptoms.

Research completed by Papasotiriou studied the heat sterilization treatment of OMWW for plant disease control [280]. In this study compost amendment can lead in partial loss of its suppressiveness, pointing out the presence of microorganisms antagonistic to destructive vascular wilt soil fungus, such as *V. dahliae*. However, this type of treatment for disease control may be unaffordable for the re-use of the treated OMWW in case of large agricultural areas. Likewise, Vagelas, reported that filter sterilized OMWW significantly reduced the growth of important soil borne plant pathogens as *F. oxysporum* f.sp. *lycopersici, Pythium* spp., *S. sclerotiorum* and *V. dahliae* and tomato plants infested with *F. oxysporum* f.sp. *lycopersici* and OMWW, produced better developed plants compared with the plants infested only with *F. oxysporum* f.sp. *lycopersici* [285]. On contrary studies completed by Obied demonstrated that crude OMWW extract appeared to be unaffected on *C. albicans,* and *A. niger* fungi [306].

According to Mavrakis, oleuropein (derived from olive tissues) and its derivatives (derived from olive mills waste waters) have a variety of biochemical roles as natural antioxidants and as phytoprotective agents against economically important pathogens especially on organically grown crops [283]. These compounds have shown high antimicrobial activity against several phytopathogenic bacteria and inhibited/delayed radial growth and spore germination of some plant-pathogenic fungi such as *B. cinerea*, *A. alternata*, *F. oxysporum* f.sp., *melonis*, *Rhizopus* species, *C. higginsianum*, *P. parasitica* var. *nicotianae*, More specific, oleuropein was less efficient for *A. alternata*, *F. oxysporum* and *Rhizopus* sp. than *B. cinerea*, *C. higginsianum* and *P. parasitica*. *F. oxysporum* was the most tolerant to oleuropein with ED50 values after 4 days of incubation, varied from 8.87 and 7.45 mg/ml for semipure and pure oleuropein respectively. Furthermore, OMWW extract was less efficient for *A. alternata* and *C. higginsianum* compared to the control, but had less affect on *B. cinerea* and *Rhizopus*, where it only showed some delay in mycelium growth at high concentrations. However, it was effective for *F. oxysporum*, *C. higginsianum* and *P. parasitica* where a significant reduction in mycelium growth even at low concentrations was observed. Furthermore, oleuropein (semipure and pure) showed remarkable antibacterial activity and MICs values ranging from 0.2 mg/ml to 0.7 mg/ml [307, 308]. This observation confirmed the findings of others studies

reporting the antimicrobial properties of oleuropein [45]. Finally, application of OMWW on tobacco leaves infected with *P. parasitica* showed a negligible inhibition. These results are in agreement with those of radial growth in *in vitro* experiments.

In vitro and *in vivo* studies done by Leontopoulos about the effectiveness of different formulations of polyphenolic compounds obtained from OMWW on the growth of several fungal plant and food borne pathogens, suggest that liquid formed polyphenols obtained from OMWW could be used to control several plant fungal pathogens suppressing mucelium growth of species such as *B. cinerea* and *S. sclerotiorum* [48, 52]. However, according to Obied no antifungal activity was found for OMWW extracts against *C. albicans* and *A. niger* grown on olive mill solid waste [306]. According to Cayuela foliar application of OMWW compost extracts might have some positive application matter with certain crops as both a biocontrol for Botrytis and as a foliar growth promoter [157]. However, plant pathogenic fungi such as *R. solani*, *P. expansum* and *A. niger* affect plant growth in a very easy way, mainly due to their rapid mycelium growth, the large number of produced spores and the rapid dissemination into the tissues of the host.

Polyphenols also, act as phytoprotective compounds on fruits and vegetables during the growing season and after harvest, during storage, offering a promising solution for preventing losses of fruits and vegetables from post-harvest attacks such as those from the fungus *B. cinerea* [47]. Likewise, Vagelas investigated the antifungal activity of OMWW on *B. cinerea* Pers [284]. The effect of sterilized, filtered and non sterilized OMWW was tested *in vitro* on mycelium growth of *B. cinerea* and on strawberry and pepper fruits infected with the pathogen. The results show that the filter sterilized OMWW inhibits the growth of *B. cinerea* mycelium *in vitro* probably due to the activity of the phenolic compounds contained on OMWW. Furthermore, OMWW significant decreased fungus mold formation on the tested fruits.

Cayuela completed a series of laboratory bioassays to test the inhibitory effects of sterile water extracts of two-phase OMWW and OMWW composts with different degrees of stabilization on *P. capsici, P. ultimum, B. cinerea* and *R. solani* [157]. Results obtained from his study shown that the growth of *P. capsici* was consistently and strongly inhibited by all OMWW diluted extracts. In contrast, suppression of *P. ultimum* and *B. cinerea* was not as strong as expected. In addition mature compost of OMWW inhibited *P. capsici* and *B. cinerea* at dilutions as great as 1:50, w:v. while no inhibition effects was recorded for *R. solani*. However, Kotsou found that the addition of OMWW significantly suppressed growth of this root pathogen [46]. These different results could be explained on that soil application of OMWW is likely to have stimulated changes in the soil microbial populations favoring r-strategists that were hampering the growth and survival of *R. solani* [157]. Similar results were also observed for effectiveness of OMWW on *Phytophthora* sp., from Del Río, who suggested that phenolic compounds and especially tyrosol catechin and oleuropein are involved in the defense mechanisms of olive plants against *Phytophthora* sp., leading to greater suppression when they acted synergistically [293].

Likewise, in study completed by Alfano, olive waste compost water extracts appeared an inhibitory effect on the growth of *F. oxysporum* f.sp. *lycopersici*, *P. ultimum*, *P. infestans*, *S. sclerotiorum* and *V. dahliae* [290]. In pot experiments, the use of olive compost significantly reduced *P. ultimum* damping-off and *F. oxysporum* wilt diseases on tomato seedlings. Furthermore, studies completed by Papasotiriou demonstrated that application of OMWW compost was able to reduce the percentage of *V. dahliae* microsclerotia germination and the number of hyphae per germinated microsclerotium *in planta* [280].

Yangui also reported that the use of 20 µl of the substance extracted from OMWW inhibited the growth of mycelium of fungi that cause root rots such as *F. solani* and *R. solani* noting that there is probably a synergistic effect of the volatile polyphenols nucleotides and derivatives of proteins [274, 309]. Similar results against fungus *R. solani* were recorded from Kotsou in the *in vivo* test [46].

Studies completed by Lykas focused on the effect of OMWW on growth and bulb production of ornamental species such as tulips [287]. In this study filtered and sterilized OMWW was tested as mycelium growth inhibitor of important fungal pathogens such as *Botrytis tulipae*, *F. oxysporum*, *A. niger* and *Penicillium* spp. Results from this study demonstrated that only filtered OMWW application inhibited the mycelium growth of all tested fungi *in vitro*. Furthermore, when temperature increases because of sterilization procedure of OMWW at 121 °C for 20 min OMWW, antioxidant compounds seems to lose their properties [310]. However, OMWW on uninfected bulbs may appear limited, without further evolution scab-like lesions, caused by *A. niger* in a very early growth stage due to fungus ability to grow in the presence of OMWW [311–314]. Moreover, spraying uninfected bulbs with OMWW significantly increased shoot, leaf and flower malformations and reduce plant height compared to control bulbs due to possible phytotoxic effect. However, phytotoxic effects may be reduced applied saprobic fungi such as *F. oxysporum* 738 and *F. lateritum*. Results from Sampedro shown that these saprobic fungi were capable to transforming the dry olive mill residue after incubation of 20 weeks and were able to decrease the phenol content [315]. Thus, increase of growth and dry weight of tomato and soybean plants were observed.

Chaves-Lopez studied the antifungal activity of OMWW diluted in water and ethanol (4:1), found that 1.25% of OMWW had an inhibitory effect against several fungi such as *Penicillium expansum, Penicillium verrucosum, Aspergillus clavatus, Eurotium amstelodami and Cladosporium cladosporioides* while *Aspergillus parasiticus* was found to be resistant [296].

Finally, Xing mentioned that more effective and more specific targeted eco-friendly phytoprotective products are in great interest the last years for this reason an antifungal dispersion system was prepared by oleoyl-chitosan (O-chitosan) nanoparticles, and the antifungal activity against several plant pathogenic fungi was investigated [61]. Thus, one area of research interest in modern agriculture is the development and study of nanoparticles for the controlled release of active compounds. Likewise, Leontopoulos suggest that the encapsulation of a polyphenol in encapsulating agent may help to slow the degradation and protection from the frequent watering and runoff due to this action [48]. It has been found that

nanoparticles increase the efficiency of pesticides, further reducing their volatilization and decreasing toxicity and environmental contamination in crops

6.7 Use of Polyphenolic Compounds from OMWW Against Bacterial Plant Pathogens

OMW contain naturally several bacterial and yeast species in abundance. According to Vivas, Tsiamis, Kavroulakis and Ntougias the main bacterial representatives found on OMW are members of Alphaproteobacteria, Gammaproteobacteria, Betaproteobacteria as well as Firmicutes, Actinobacteria and fermentative bacteria [316–318]. Furthermore, high counts of infectious agents such as Acinetobacter, Enterobacter sp., and Pseudomonas have been also detected in OMW [319]. The main yeast biotas present in OMW are Pichia, Candida, and Saccharomyces-like species [320, 321]. Furthermore, according to Ntougias about 106 identified fungi belonging in Acremonium, Alternaria, Aspergillus, Bionectria, Byssochlamys, Chalara, Cerrena, Fusarium, Lasiodiplodia, Lecythophora, Paecilomyces, Penicillium, Phycomyces, Phoma, Rhinocladiella and Scopulariopsis genera, was revealed in a survey completed by the Greek Center for Biotechnology Information [322].

Mavrakis studied the effect of OMWW against important plant pathogenic bacteria such as *Clavibacter michiganensis* spp. *michiganensis*, *Ralstonia solanacearum*, *Pseudomonas syringae* and *Xanthomonas campestris* pv. *Vesicatoria* [283]. However, OMWW extract were not as effective as oleuropein against bacterial strains with MICs values ranged from 1 mg/ml to 2 mg/ml. More specific, results in Mavrakis, study, showed that het most significant inhibition of *C. michiganensis* (4040), *X. campestris* pv. *vesicatoria* (5075), *Erwinia atroseptica* (3217), and *Erwinia amylovora* (15) was observed on the samples with semipure and pure oleuropein. MIC after 2 days incubation and optical density (OD600) for *C. michiganensis* (4040), *X. campestris* pv. *vesicatoria* (5075), *E. atroseptica* (3217) and *E. amylovora* (15) was 0.07, 0.17mg/ml, 0.07 mg/ml and 0.07 mg/ml respectively [283]. However, OMWW extract was not as effective as the oleuropein against *C. michiganensis* and MIC for OMWW extract was 0.2 mg/ml. OMWW extract at the same concentration (0.07 mg/ml) showed 64.11% reduction in colony forming ability of *E. atroseptica* and 83.46% reduction in colony forming ability of *E. amylovora* (15) compared with the control. For *R. solanacearum* (819–6) and *P. syringae* pv *apii* (255a) MIC for pure oleuropein and OMWW extract was also 0,07 mg/ml and 0.15 mg/ml while optical density (OD_{600}) was 0,139 and 0.117 respectively. Furthermore, application of semipure and pure oleuropein on *Pseudomonas corrugate* (1157) and *Pseudomonas viridiflava* (Acant.2) showed greater colony forming ability compared to the control. After two days incubation time, OD600 was 0.148 and 0.125 respectively. OMWW extract, at the same concentration, showed 83.88% reduction in growth of *P. viridiflava* compared with the control while MICs for

Table 6.2 Studies on OMW effectiveness against important phytopathogenic bacteria

Studied organism Bacterial species	References
Clavibacter michiganensis	[283, 326]
Pseudomonas syringae	[283, 326]
Xanthomonas campestris.	[283, 326, 327]
Ralstonia solanacearum	[307, 308]
Agrobacterium tumefaciens	[274, 326]
Erwinia amylovora, Clavibacter sp., Pseudomonas sp., *Xanthomonas* sp	[326]
Bacillus megaterium	[323, 324]
Corynebacteriaceae	[323, 325]

OMWW extract was 0.15 mg/ml. Finally, results of OD_{600} on *P. syringae* pv. *tomato* (132) and *P. savastanoi* (1266) was 0.23 and 0.151 respectively, shown that semipure and pure oleuropein inhibited 100% growth of *P. syringae* pv. tomato at 0.05% (0.05 mg/ml MIC), while at the same concentration of OMWW extract colony inhibition was 84.04% compared with the control. In particular, samples with OMWW extract showed that delay but did not inhibit the growth of *P. savastanoi* even at high concentration (0.2%). Concluding, pure and semipure oleuropein showed remarkable antibacterial activity. MICs values ranged from 0.2 mg/ml to 0.7 mg/ml. OMWW extract were not as effective as oleuropein against the tested bacterial strains. MICs values OMWW extract ranged from 1 mg/ml to 2 mg/ml.

Furthermore, *in vivo* studies demonstrate that when 0.1% of OMWW extract was tested as precautionary treatment against bacterial speck disease caused by *P. syringae* pv. *tomato,* an impressive inhibitory effect appeared.

Earlier research by Paredes found that OMWW inhibit spore forming bacteria from the genus Bacillus [323]. Similar effects were reported by Rodriguez while β-glucosidase treated extracts from concentrated OMWW effectively inactivated *B. megaterium* [324]. However, further research was observed that there was no effect on species from the family of the Corynebacteriaceae [323, 325] (Table 6.2).

6.8 Use of Polyphenolic Compounds from OMWW Against Plant Parasitic Nematodes

Plant parasitic nematodes are a severe constraint on agricultural production that causes considerable annual losses among a broad range of crops grown all over the world [328] reducing agriculture productivity approximately up to 12.3% [329] especially because of difficulties reported due to their inhabitation and mode of parasitism [330]. More specific, according to Abad [331] and Singh [329] plant parasitic nematodes are responsible for an estimated yield loss of about $157 billion in world and $40.3 million in India.

The increasing rates in human population and longevity have lead to the increasing demand of agricultural production [332]. This can be attained by optimizing the productivity potential and by minimizing losses caused by plant-parasitic nematodes [2]. Although, chemical nematicides are widely distributed as a control strategy to combat this biotic stress, they believed that they are inappropriate and inadequate due to their adverse effect on bio-flora, fauna and natural enemies. Due to the environmental and regulatory pressure, use of potential biocontrol methods is the most welcomed way for nematode management by the farming community [2].

Mostly, nematodes attack underground part of the plants and cause serious reduction of agronomic performance, overall quality, and yield loss playing an important role in disease complex by penetrating and feeding on the roots of growing host-plants, absorbing nutrients, affecting growth and vigour and exposing the roots to other soil borne pathogens [333, 334]. Among nematode species *Meloidogyne* sp. (root-knot nematodes), *Heterodera*, *Globodera* sp. (cyst nematodes) and *Pratylenchus* sp. (lesion nematodes) considered as the most economically important due to their damage and infection level, wide host range and relationship with host plant attacking economically valuable crops like tomato and cucumber.

Nowadays, limitations in chemical nematicide availability, usage avoidance due to its hazardous nature to the non-targeted organisms, public health issues and environmental safety [335] and high costs in their development have created a need to discover alternative methods for controlling plant parasitic nematodes [336]. Hence, alternative nonchemical, eco-friendly strategy for nematode control is highly desirable. Various non-chemicals methods such as crop rotation, nematicide resistant cultivars, intercropping, deep ploughing and biological agents are available in order to limit damage, reduce crop losses and control plant-parasitic nematodes. Essential oils, from aromatic plants such as oregano (*Origanum vulgare*), citronella (*Cympopogon nardus*) and lavender (*Levandulla officinalis*), as well as other botanical compounds (plant extracts) compost extracts and biological agents have been reported to be efficient in controlling plant parasitic nematodes [337–346]. According to Timper, the use of biological agents and other biological control methods are the most effective and efficient way to suppress damage caused by plant parasitic nematodes and overcome nematodes stress [347]. However, due to the formation of protective cysts, gelatinous matrix and several survival adaptations, survival in the soil without host, making crop rotation, intercropping and deep ploughing unattractive [2].

Plant parasitic nematodes' biocontrol can be achieved mainly by nematode-trapping, endo-parasitic, and toxin producing fungi [348–350] and bacterial antagonists such as epiphytic, endophytic and endoparasitic bacteria through the mechanisms like parasitism, competition and antibiosis [342, 343, 351–356].

The use of agro-industrial wastes is another example for alternative nematode's control method but nematode control efficacy is not always satisfactory [357]. Phenolic compounds such as pyrocatechol, caffeic acid, and vanillic acid contained in these wastes could play an important role among phytochemical compounds in nematode's management [358]. Although 35 years ago it was believed that the efficacy of applied organic amendments rich in phenolic compounds was not clear

[359] it is now believed that olive mills waste (soilid or liquid) rich could be used against plant parasitic nematodes affecting J_2 mobility and number of egg hatching increasing subsequently plant resistance [157, 358].

OMW contains compounds with nematicidal action and has been suggested for use as a biopesticide by Cayuela [157]. High concentration of sterile water extracts of two-phase OMW have shown strong inhibitory effect on egg hatching and mobility on second stage juvenile (J_2) of *M. incognita* 7 days after extract's application, disrupting the nematode life cycle. These results suggest that OMW extracts have significant hatch suppression on eggs due to possibly existence of bioactive compounds that are able to spread through nematode eggs shell. J_2 mobility was inhibited more than 95% at all dilutions.

However, mature dry olive mark compost application found to limit nematotoxic activity, suggesting that the decomposition process in compost eliminates the inhibitors [360, 361]. More specific, Nico reported that amendment of the potting mixture with the mixture of dry-olive marc + dry-rice husk (1:1 v/v) composts did not influence root galling or the final nematode population, irrespective *Meloidogyne* species or rate of amendment, thus these composts while suitable for plants, have limited nematicidal activity and therefore are not suitable for the management of root-knot nematodes [361].

6.9 Conclusions

The recovery of high added-value products from waste plant material such as OMWW is a significant issue in non chemical disease control as non-hazardous organic phytochemical and plant protective substances. Phenolic compounds in *Olea europaea* tissues and OMWW are considered as natural antioxidants with antimicrobial activity against several fungal, bacterial and nematode's species. Also, OMWW in high doses inhibit seed germination and affect vegetative growth of plants appearing phytotoxic effects. Thus, OMW could be used as pre-plant biopesticide or in crops on which they have no phytotoxic effect. So far it is agreed that suppression of soil-borne pathogens is not only due to the antimicrobial effect of phenolics present in OMW but it as complex situation combining several biotic and abiotic factors such as spore germination, germ growth inhibition, competition for nutrients and ecological niches, secretion of antimicrobial agents, and lysis via hydrolytic enzymes. Many compounds present in OMW and OMWW could represent a promising option for the control of pests and diseases in the areas where olive oil production occurs. So far, despite its potential agronomic value, the phytopathogen suppression capacity of OMWW has been barely investigated and extended and more specific field research is needed. Therefore, it is necessary to show that the phenolic content of OMWW retain their biocide activity after large-scale application allowing sustainable development of the agro-economy.

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Chapter 7
Alternatives to Synthetic Fungicides Using Small Molecules of Natural Origin

Christian Chervin

7.1 Introduction

There are many plant diseases, among which downy and powdery mildew and gray mold, causing important pre- and post-harvest losses, including yield drops, storage losses and food contamination with hazardous biogenic compounds [1]. Most commercial crops are susceptible to such fungi. To counteract this disease development a wide panel of synthetic fungicides is in use nowadays. However, solutions to limit pre-harvest treatments with synthetic fungicides are of particular interest as chemical residues are limiting access to many markets, and there is a diminishing number of antifungal compounds that are still registered [2]. Moreover, some pathogen strains may develop resistance to some pesticide, thus alternative strategies are required. One of the most sustainable alternative strategies would be to develop cultivars that are naturally resistant to fungi or other pathogens, and there are available genotypes for this trait [3]. But it is a long term development and most food industries are relying on specific cultivars, around which all marketing efforts have been made for decades. So finding alternative treatments with existing cultivars is still of interest for most agro-industries. There are strategies to develop plant induced defences, and these have been reviewed [4].

The purpose of this review is to list small compounds of natural origin that have been tested, with or without success, as alternatives to synthetic fungicides. Some of them may induce plant defences. These compounds are ranged by alphabetical order.

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7.2 Acetaldehyde

As this compound has such a low boiling point (20 °C), it is very volatile at ambient temperature, so most trials were performed for postharvest applications using it in the vapour phase [5]. As most aldehydes, it has a strong bactericidal and fungicidal potential, as aldehydes are very oxidative compounds. One must attract attention to users that they are very toxic compounds to manipulators and quite oxidative to many parts of the equipment (Fig. 7.1).

Kim et al. [6] classified acetaldehyde as a biogenic volatile organic compounds (BVOC) and showed it can be emitted from wounded plant parts.

Utama et al. [7] showed that aldehydes were more effective that alcohols in blocking fungus growth *in vitro*. They stated that acetaldehyde was quite effective against various fungi: *Rhizopus stolonifer, Penicillium digitatum, Colletotrichum musae, Erwinia carotovora*, and *Pseudomonas aeruginosa*, showing germicidal effects at concentrations below 1 mmole/per dish, as the assays were run in Petri dishes; but they did not report trials *in vivo*. These germicidal concentrations corresponded approximately to 60 µmoles acetaldehyde/litre of air, in the vapour phase, after 1 h of application, and they went down to 20 µmoles/litre of air in 5 days at 25 °C. When applied on 'Sultanina' and 'Perlette' grape berries grapes with low sugar content and high acidity, the acetaldehyde was found to increase total soluble solids, to decrease acidity, and to enhance sensory preference [8], but these authors did not report effects on fungus. These observations were reported later by Avissar and Pesis in 1991 [9], who showed that acetaldehyde was controlling the decay of table grapes in a postharvest trial.

Acetaldehyde is probably present in most plant and fruit, but at very low concentration around a few nmoles.g_{FW}^{-1} [10], so whether it is effective or not at this natural concentration is not known. However, there was an interesting report by Miyake and Shibamoto [11] showing that acetaldehyde can be produced in aerobic and relatively mild conditions by oxidation of L-ascorbic acid. Thus during the oxidative stress following a fungus infection, there may be some acetaldehyde produced, and it may be part of the natural defence; this has been shown in the case of resistance of potato plants to *Phytophthora infestans* [12].

7.3 Acetic Acid

The use of acetic acid fumigation for postharvest control of fungi has been reviewed by Tripathi and Dubey [13]. These authors referenced the fact that acetic acid is a natural metabolite occurring in several fruit, and that fumigation with acetic acid onto grapes has been proven an effective treatment to control gray mold (Fig. 7.2).

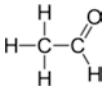
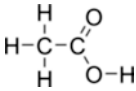


Fig. 7.1 Acetaldehyde (MW: 44 g; BP: 20.2 °C, 68 °F)

Fig. 7.2 Acetic acid (MW: 60 g; BP:118.1 °C, 245 °F)



Venditti et al. [14] showed that postharvest repeated treatments with acetic acid vapors preserved the quality of table grapes, mainly by reducing gray mold incidence. Camili et al. [15] showed that acetic acid might stimulate natural defences in Italia grapes, as the best control of Botrytis was obtained when the fruit was treated with acetic acid vapours 48 h prior inoculation with Botrytis spores. To my knowledge, there no or very scarce data about a potential control of grape disease by spraying acetic acid in vineyards, but the trials may be worth it.

Interestingly, sprays of indole-acetic acid on tomato seedlings were shown to reduce the symptoms caused by a phytopathogen, *Pythium ultimum* [16]. The indole-acetic acid, one of the auxins, is obviously quite different to acetic acid, however no application has been tested onto grapevines to check if such an effect would be observed with grape specific pathogens.

In a more comprehensive approach, Martin and Maris [17] tested the antifungal and antibacterial efficacy of 17 organic and mineral acids against several strains of bacteria and fungi, known as food contaminants. They found interesting inhibitory effects by formic, mandelic and lactic acids.

7.4 Aldehydes (Other than Acetaldehyde)

The remarks regarding their toxicity, outlined in the acetaldehyde paragraph, are valid for the following compounds too.

The hexanal and hexenal are two natural compounds, oxidation products of lipids. They have been shown to harbour anti-fungal properties against *Botrytis* sp., *Alternaria* sp., and *Penicillium* sp. among others ([13] and references therein). This has been confirmed by Song et al. [18], as hexanal vapours gave a excellent control of *Monilinia fructicola* on peaches and a very good control of *Botrytis cinerea* on raspberries at doses around 900 $\mu l.l^{-1}$. And hexenal isomers have been found to limit mould development on strawberries [19] (Fig. 7.3).

In planta experiments have shown that Arabidopsis over-producing C6 aldehydes (e.g. hexanal and hexenal) were more resistant to *Botrytis* infection, mainly through a direct effect of the aldehydes on the fungus growth rather than through an elicitor role that the aldehydes might have had [20]. This study also leads to think that the spraying of such aldehydes on grapevines may reduce the development of various fungi, however the cost and the hazard of manipulating such molecules have to be considered.

Utama et al. [7] tested in vitro the bactericidal and fungicidal properties of several aldehydes listed above and also benzaldehyde, which was shown to be slightly more efficient than acetaldehyde. He also listed effects by cinnamaldehyde. This

Fig. 7.3 Hexanal (MW: 100 g; BP: 120 °C, 248 °F)

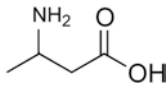


Fig. 7.4 β-aminobutyric acid (MW: 103 g, solid at ambient temperature)

compound is part of the cinnamon aroma. It can be used as a food additive, and has anti-microbial properties at quite low concentration around 10 mM [21]. A report shows the potential of such a treatment or with other compounds listed in this chapter: Viazis et al. [22] showed that cinnamaldehyde limits the viability of an enterohemorrhagic Escherichia coli strain on leafy vegetables, when sprayed at 0.5% (v/v).

7.5 Aminobutyric Acids

These compounds are involved in plant induced resistance [23, 24]. Plant perception of β-aminobutyrate has been described by Luna et al. [25], and this non protein amino acid is produced by plants [23]. Its chemical structure is close to the γ-aminobutyrate which is a well-known neurotransmitter. Thus, for in-field applications to various crops, the toxicity to humans has to be considered (Fig. 7.4).

The β-aminobutyrate was shown to induce resistance to a wide range of plant stresses, biotic or abiotic, and there are links to other plant metabolisms such as abscisic acid [24]. Other aminobutyrates are also active in plant defences, such as the γ-aminobutyrate metabolism which is involved in tomato resistance to bacterial wilt [26], thus more is to be discovered regarding the anti-fungal properties of aminobutyrates.

7.6 Ascorbic Acid

It is a natural compound which accumulates in many fruit, particularly in citrus [27], but also in grapes [28] (Fig. 7.5).

Whether it shows some antifungal or antibacterial direct activity at natural levels is still unknown. Authors have observed antimicrobial effects of ascorbic acid at higher concentration such as 2.5% [29]. However, Khalil et al. [30] showed that ascorbic acid can enhance antifungal activity of curcumin.

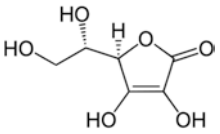


Fig. 7.5 Ascorbic acid (MW: 176 g, solid at ambient temperature)

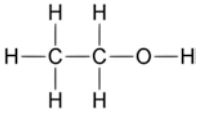


Fig. 7.6 Ethanol (MW: 46 g; BP: 78 °C, 172 °F)

7.7 Ethanol

This compound is also naturally present in plant and fruit tissues, and can be found under normal aerobic conditions when the inside of the cells become too acidic or under hypoxic conditions ([31] and references therein) (Fig. 7.6).

A study by Hann et al. [32] suggests that it can be used by plants to sense and modulate responses to stress or microbes. Our work regarding applications of ethanol has been initiated by reading an article by Beaulieu and Saltveit [33]. They observed that exogenous ethanol was stimulating ethylene production and tomato fruit ripening, so we tested it on grapes in order to modulate ripening and anthocyanin accumulation [34] and wine colour [35] using hand-held sprayers directed towards the bunches. Later we found that ethanol sprays with commercial sprayers increases mostly the berry diameter [36].

And then we tested ethanol for its efficacy to limit fungus growth. The first application was post-harvest, we will present pre-harvest applications later. The idea came from a paper by Lichter et al. [37] showing that dipping grapes at harvest in ethanol solutions was decreasing the *Botrytis cinerea* growth. The ethanol dip has two drawbacks which are the need to promptly dry the grapes after treatment to prevent berry cracking [38], and the possible cross-contamination with fungus spores from a previously infected grape crate, when working with low ethanol concentrations. We adapted these ethanol treatments to commercial practices using ethanol in the vapour phase [39]. Indeed, the industry is already using fumigation with SO_2 in adapted chambers, or in crates with SO_2 pads releasing the SO_2 in contact with air humidity, so if ethanol was going to be efficient and accepted by the industry, a simple change of the active ingredient was possible. The application of ethanol vapours was optimised over two seasons for 'Chasselas' table grapes and at a dose rate of 2 $ml.kg^{-1}$ of grapes, the ethanol vapour was as effective as sulphur dioxide pads to prevent rot development, caused by *Botrytis cinerea*, and stem browning. Further tests with consumer panels showed no significant difference in sensory perception between controls and treated grapes. The application of evenly distributed ethanol vapours is critical, as higher concentrations of ethanol may enhance stem browning. Materials releasing ethanol are already on the market, such as the "ethanol powder" [40]. Postharvest applications of ethanol may also present potentials to reduce berry shatter [41] but these need further development.

Then, we tested pre-harvest applications of exogenous ethanol in the vineyard, to prevent fungus development ahead; the results that are detailed below have been reported [42]. The idea came from the reading of an article by Karabulut et al. [43]. These authors found that spraying 1 litre per 5 vines of a solution at 50% ethanol, 24 h prior harvest, was effective in reducing the rots over the postharvest period. We then adapted this to commercial practices, reducing the amount of solution to 150 litres per hectare (using a mist blower) and no treatment in the last 2 weeks prior harvest. The treatments were performed every 2 weeks from veraison, we tested a late harvest, scheduled 2 months and a half after veraison, was chosen for these trials, so the conditions were optimised for high *Botrytis* development. The fungus development was assessed at harvest and after 4–6 week cold storage. We always found a higher impact of the treatments after cold storage when Botrytis development had occurred at higher rates.

In preliminary trials, we found that even a very low concentration of 2% ethanol was reducing the Botrytis growth. It is not likely that ethanol would have had a direct effect on fungus growth at this low percentage, as Lichter et al. [44] showed that at least 30% EtOH is necessary to prevent *Botrytis cinerea* spore germination. Thus the 2% EtOH dose is more likely to induce plant defence.

The optimal dose of ethanol to reduce *Botrytis* growth by pre-harvest spraying was found to be around 16%. However, to match the industry demand, we had to combine it with calcium chloride in order to further reduce the gray mold growth. This was done after reading an article by Nigro et al. [2] in which the authors reported the efficacy of various salts to reduce the *Botrytis* development. This will be detailed in a paragraph below in this chapter. Thus we reported that pre-harvest applications of a 16% ethanol solution, containing 1% $CaCl_2$, reduced gray mold development. At harvest the losses due to rotten clusters dropped from 15% in controls to 5% in grapes treated with ethanol & $CaCl_2$. Over 6 weeks of cold storage, the losses due to gray mold were reduced by 50% in bunches treated with ethanol & $CaCl_2$, compared to untreated controls. These treatments did not induce significant changes in fruit quality assessed by sensory analysis of healthy berries.

Part of the plant response to ethanol may be due to acetaldehyde, into which it partially oxidises, by enzymatic and non-enzymatic ways.

The ethanol has a much higher boiling point than acetaldehyde and is not oxidative, which renders it safer to use; it is already used by industry as a wetting agent or for its solvent properties. As it is flammable, precautions are necessary. Ethanol is rather cheap to produce, and its worldwide production is increasing, mainly due to its use as ethanol fuel, so its cost will decrease.

7.8 Ethylene

It is a gas at ambient temperature and it plays important roles as phytohormone in most plants [45]. These authors review all aspects of ethylene metabolisms in plant biology, including production and perception, while a more recent review details new findings in perception [46] (Fig. 7.7).

Ethylene is involved in many plant defence mechanisms [47], in a complex signalling network that includes jasmonic acid, salicylic acid and abscisic acid. Some of these will be detailed in paragraphs below.

Regarding the ethylene role in grape defence, there has been a series of works by J.M. Merillon's team using 2-chloroethylphosphonic acid, an ethylene precursor, also called ethephon, which led to a decrease of fungus growth when sprayed onto grapevines, through elicitation of natural defences [48]. And a series of microarray analyses, using mRNAs extracted from berry tissues after exogenous application of ethylene on grape clusters, has revealed some genes involved in plant defence [49].

7.9 Jasmonic Acid and Methyl Jasmonate

Jasmonates are signaling in plants for resistance to many biotic and abiotic stresses [50]. Contents vary according to plants or organs, for example, jasmonic acid is present naturally in grape berries, particularly in seeds [51] up to 50 pmoles/seed, and methyl jasmonate is also present to a lesser extent (about 10 times less) (Fig. 7.8).

Treatment of avocado fruit with exogenous methyl jasmonate has been shown to help controlling the anthracnose disease [52]. Methyl jasmonate has also been shown to simulate grape defences against *Erysiphe necator* [53].

7.10 Salicylic Acid and Methyl Salicylate

The salicylic acid is well-known to be part of the SAR: Systemic Acquired Resistance [54]. Park et al. [55] showed that both conversions of salicylic acid to methyl salicylate and methyl salicylate to salicylic acid were essential for SAR in tobacco, and they concluded that methyl salicylate is a SAR signal in this plant (Fig. 7.9).

In strawberries, Babalar et al. [56] showed that pre-harvest sprays of salicylic acid had potential to limit fungal decay. The concentration that gave a good control

Fig. 7.7 Ethylene (MW: 28 g; gaz at ambient temperature)

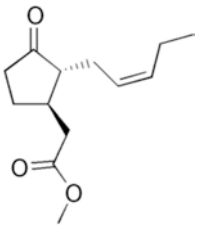


Fig. 7.8 Methyl jasmonate (MW: 224 g; BP: 89 °C, 192 °F)

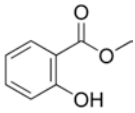


Fig. 7.9 Methyl salicylate (MW: 152 g; BP: 222 °C, 432 °F)

was 2 mM, and several sprays were necessary: at the vegetative growth stage, then the fruit development stage and postharvest. Salicylic acid may also interest companies and growers as it seems to be available at low cost. Methyl salicylate was also shown to delay ripening of cherries [57], thus this effect leads to a positive spin-off on the fruit quality.

A study reports that infection of grapes with Botrytis leads to fast responses of salicylate and other hormonal metabolisms, which induce grape resistance/ tolerance to infection [58].

In addition to controlling the fungal diseases, salicylic acid and derivatives might have other potentials for fruit growers. Indeed, methyl salicylate was also used to "recruit" beneficial insects in vineyards [59]. These authors showed that use of controlled-release methyl salicylate in a crop could increase recruitment and residency of populations of certain beneficial insects. This strategy may have the potential to enhance the efficacy and reliability of conservation biological control in crop pest management.

7.11 Salts (e.g. Sodium Bicarbonate, Calcium Chloride, Copper Sulfate)

Salts are cheap, accepted by consumers, with minor environmental impact at the effective concentrations, which are non-toxic, and they are already used by the food industry. One review was published about efficiency of Generally Recognised As Safe (GRAS) salts [60]. The GRAS salts antifungal properties have been known for a long time, but formal studies were initiated in the 1990's. One of the most comprehensive studies about their use as antifungal agents in vineyards has been published by Nigro et al. [2]. These authors tested 19 different salts, from sodium phosphate dibasic, the most efficient against Botrytis, to potassium carbonate the less efficient in a small trail using artificially infested berries. Then they ran larger field trials in which the most efficient salts against bunch rots were calcium

chloride, sodium bicarbonate and sodium carbonate at concentrations around 1% (w/v). the spray timing was variable, some treatments preformed 90 and 30 days prior harvest in the last trial, but also 20 and 5 days prior harvest in earlier trials. The salts were as efficient as the classical chemical treatments in some field trials. Calcium signal is well described for its impact in plant defence mechanisms [61].

Another well-known salt used in viticulture is the copper sulfate pentahydrate, which has a blue colour, and its natural form is called chalcanthite. It is one of most common antifungal when it is mixed with lime, particularly against downy mildew, and it is named Bordeaux mixture. It is one of the rare antifungal treatments, with sulphur, allowed in organic vineyards. However, its intensive use is known to 'pollute' soils and to render some cultures difficult after several years of vine growing, as other crops are not so tolerant to high Cu concentrations in soils (Fig. 7.10).

7.12 Sorbic Acid

It is a well-known food preservative, and it can be used as sodium or potassium salts, among others. Karabulut et al. [62] showed that potassium sorbate when applied on 'Thompson Seedless' grapes after harvest, at concentrations around 1%, reduced the incidence of gray mold over storage. Feliziani et al. [63] found that preharvest sprays of potassium sorbate were reducing gray mold incidence in table grapes after 6 weeks of cold storage.

7.13 Sulphur

Sulphur remains a very efficient and simple alternative to synthetic fungicides against powdery mildew and other pathogenic organisms (e.g. mites). Reports by Crisp et al. [64] confirmed this fact. They compared the efficacy of several compounds, such as milk, whey, canola oils and potassium bicarbonate to sulphur, and this latter was most often the best blocker of powdery mildew. Whey showed some potential, however in field trials the acceptable yield (i.e. bunches with less than 5% powdery mildew infections) was lower than when treated with sulphur. The whey compounds are mainly lactose and lactoglobulin. Why these compounds or the whey pH might have an inhibitory effect on the fungus is not discussed in the article. The antifungal effects of sulphur salts were also described for storage of root crops, such as carrots and potatoes [65].

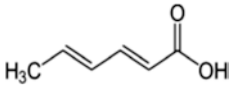


Fig. 7.10 Sorbic acid (MW: 112 g; solid at ambient temperature)

7.14 Combinations

Combinations are always interesting, as they can generate additive or synergistic effects. One example has been detailed above when combining ethanol with calcium chloride during field sprays gave a better control of gray mold at harvest, and after storage [42]. Another paper is reporting such effects with ethanol and potassium sorbate [62]. Belhadj et al. [66] have tested with success the combination of methyl jasmonate and sucrose to induce accumulation of polyphenolics in grape cell cultures.

Many combinations have been tested on different fruit, giving ideas for grape future treatments. For example, Spadaro et al. [67] have tested with some success combinations of hot water, baking soda and ethanol against *Penicillium expansum* and *Botrytis cinerea* over apple storage, but the potential may be extended to grapes and field trials. Wang et al. [68] showed that the combined treatment with methyl jasmonate and ethanol resulted in a greater control of green mold due to *Penicillium citrinum* and also improved antioxidant capacities of bayberries. These authors reported a clear synergistic effect of the combination of methyl jasmonate and ethanol leading to approximately 10% decay in bayberries when each treatment led to 40% decay and the controls showing 80% decay. Interestingly the combined treatments led to a better sensory appreciation of the fruit by a sensory panel than for all other treatment.

There is an infinite number of combinations, (i) as the number of potential individual treatments is big, (ii) as the combination order may vary (e.g. treatment A before treatment B, or B before A), and (iii) as the number of repeated applications over the pre- and post-harvest periods (e.g. one treatment every week over the ripening period or one treatment every fortnight). Combinatorial optimisation has an obvious interest in such approaches.

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Chapter 8
By-Products from Pine: A Prospective Tool for Pest Biocontrol

Julien Gabaston, Josep Valls, Caroline Rouger, Tristan Richard, Pierre Waffo-Teguo, Stéphanie Cluzet, and Jean-Michel Mérillon

Abbreviations

IC_{50}	Concentration of a product inhibiting 50% of the observed effect
IC_{90}	Concentration of a product inhibiting 90% of the observed effect
LC_{50}	Concentration of a product killing 50% of the organism studied
LC_{90}	Concentration of a product killing 90% of the organism studied
PB	Pine bark
PC	Pine cone
PK	Pine knot
PN	Pine needle

8.1 Introduction

Nowadays, the agricultural field is mainly dominated by the use of chemical inputs to fight various pests and related diseases. These products are generally based on synthetic chemistry as well as some metals such as copper used in viticulture. However, the use of these products gives rise to ecotoxicological problems with risks towards the soil ecosystem, the development of resistant strains, the appearance of metabolic deregulation of crops and also potential risks in human health [1–4]. In this context, farmers currently face several legislations on the reduction of chemical inputs. Indeed, national and/or European plans have emerged in recent years, such as the Ecophyto plan in France, which aims to reduce the use of plant protection products by 25% in 2020 and up to 50% in 2025 [5]. Similarly,

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some inputs such as copper, which is the main anti-mildew treatment in viticulture, have recently undergone a reduction in their applied quantities (6 kg/ha/year to 4 kg/ha/year) at the European level [6]. In this sense, the short or medium-term decline in the use of synthetic pesticides or metals implies the need to develop other products to replace or supplement the historical treatments.

For a decade now, research has focused on the use of plant extracts as a natural pesticides. These potential sources of antimicrobial compounds have the advantage of being biodegradable, environmentally friendly and safe to human health. Different strategies have been initiated to find plant extracts that are both active and economically sustainable. A first approach concerns the study of medicinal plants since their biological activities in human health, often antimicrobial, can find an echo in plant health. For example, a study on 20 medicinal plants has revealed an oomycide activity of seeds extracts from *Azadirachta indica* (neem, Meliaceae), *Ocimum sanctum* (sacred basil, Lamiaceae) and *Catharanthus roseus* (Madagascar periwinkle, Apocynaceae) [7]. However, these plants used for their therapeutic properties, such as *C. roseus* (anticancer alkaloids), could not be a viable source for recovery in plant health. Another approach consists in performing screening from libraries containing thousands of plant extracts. This *a priori* strategy allows to highlight extracts without apparent links with plant health but with real pesticide potential [8]. Unfortunately, these extracts often remain difficult to obtain in sufficient amount due to the marginal cultures of their producing plants that have few known applications. Thus, the question of abundant and sustainable sourcing must be raised. In this sense, a strategy based on the valorization of vegetal by-products from agriculture or forestry is an interesting approach. Indeed, these residual plant materials are an abundant biomass, generally low-cost, containing active compounds and being nature-friendly. The plant by-products may thus be an alternative to synthetic products.

Among the components of the forest-wood industry, pine is one of the most cultivated trees in the world. *Pinus*, with more than 100 recognized species, is the most extensive genus of conifers [9]. Geographically, pine is distributed mainly in the Northern hemisphere especially in Eurasia (latitudinal boundary of 70°-N) and North America (latitudinal boundary of 65°-N) with an exception at the level of the equator where *Pinus merkusii* is produced in Sumatra (latitudinal boundary of 2°-S). Almost all species are distributed only in America, Europe or Asia [10]. There are nearly 66 species in America, including *P. banksiana*, *P. contorta, P. elliottii, P. palustris*, *P. radiata*, *P. strobus* and *P. taeda*. Twelve species grow in Europe, particularly *P. brutia, P. cembra, P. halepensis, P. mugo, P. nigra, P. pinaster*, *P. pinea and P. sylvestris*, while about 27 species are found in Asia including *P. densiflora, P. koraiensis, P. merkusii* and *P. thunbergii.* It can be noted that only two species occur in both Europe and Asia, namely *P. sibirica* and *P. sylvestris* [10–12]. Nowadays, the pine is economically used for the manufacture of pulp as well as in the fields of carpentry, joinery (paneling) and crates (packing box) owing to the quality of its wood, that is heavy and dense [13, 14]. However, the exploitation and process of obtaining pine wood is accompanied by the formation of many by-products such as knots (PK), bark (PB), needles (PN) and cones (PC) (Fig. 8.1). These biomasses are rich in high value-added compounds and constitute an abundant and renewable deposit.

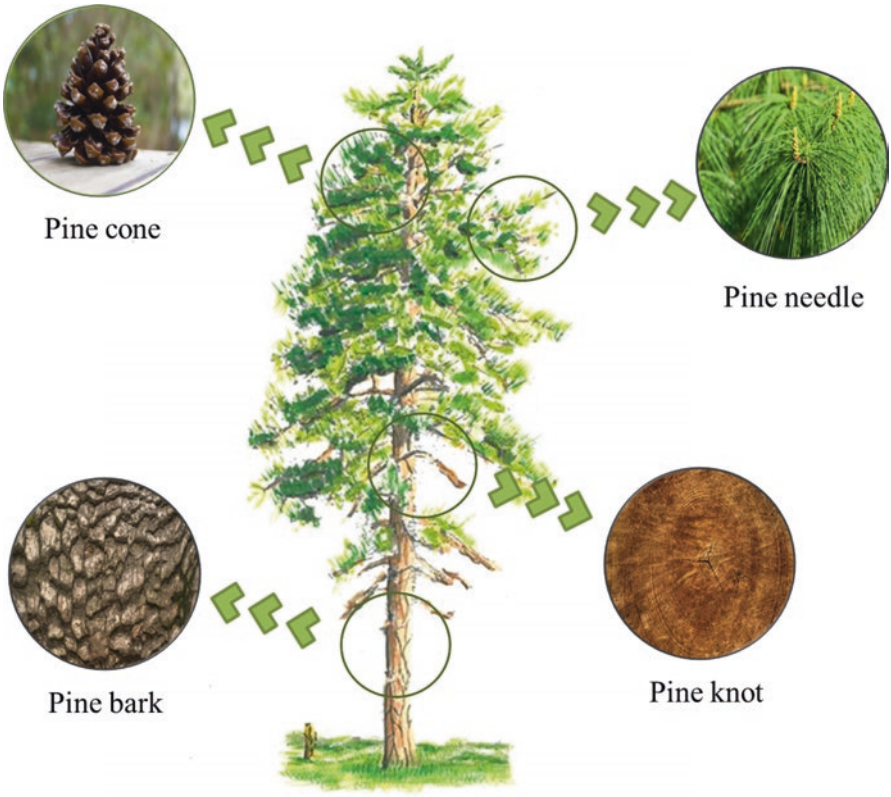

Fig. 8.1 By-products from pine: knot, bark, needle and cone

In this chapter, we describe the several pine by-products in terms of botany, biomass volume, chemical characterization as well as bioactivities in plant health. We investigate the recent advances in the use of pine by-products as biocontrol tool against fungi, oomycetes and insects with the aim of considering potential recovery of these forest wastes.

8.2 By-Products from Pine

8.2.1 Pine by-Products from Forest Industry

The various industrial applications of *Pinus* species generate several by-products including PK. These parts of the plant correspond to the insertion of the branches on the trunk. Their shape, size and number in the tree depend on tree species, age of tree, site conditions and stand composition. Usually, knots are considered as

defect since they cause losses in value of wood. Indeed, knots are the most common flaw in timber and in other wood based products, due to more dense, harder and high resin content than that of the surrounding wood tissue [15]. In this sense, the PK reduce the tensile strength of the wood, therefore are undesirable in lumber manufacturing while the high content in resin makes knots an unwanted material in the papermaking process. In pulp processing, the PKs can be separated from the wood before or after firing. Most often, the knots are subjected to firing and are collected after sieving of the dough, damaging the compounds present in knot. However, a separation of knots after grinding the wood by density difference but before firing can allow maintenance of PK and as a consequence a preservation of bioactive compounds [16].

Usually, the PKs correspond to 1–2% of the total weight of the tree [17]. Focusing on the maritime pine (*P. pinaster*), which is one of the most produced *Pinus* in France, a volume of wood around 3.6 million m^3 was marketed in 2013 [16]. In the maritime pine, the cubic meter corresponds to a mass between 450 and 575 kg, making it possible to estimate the annual production of *P. pinaster* at 1.8 million tons [18]. Thus, it can be calculated a production between 18,000 and 36,000 tons of PKs per year in France. These results are consistent with current production data from a single French production site, which uses between 1000 and 2000 tons of wood per day, thus generating between 10 and 40 tons of knots per day, i.e. between 3500 and 15,000 tons of knots per year on one site [19].

Used as a source of energy in the absence of a real economic valuation, PKs are nevertheless a deposit of important raw material in France as well as in the pine producing countries. The qualitative and quantitative diversity of the compounds present in these co-products thus aroused an interest for their exploitation in various fields.

In the papermaking process, the entire first outer layer of the tree called bark is also removed prior to the shredding process due to the high lignin/polyphenol content, which complicates the processing of the wood [20]. The bark is the trunk, branches and roots covering allowing to protect the tree against various biotic and abiotic stresses. Anatomically, the bark is a protective envelope including periderm, itself consisting of phellem, phellogen and phelloderm, and liber. The bark is formed from a cell multiplication zone called phellogen. On the periphery of the phellogen, a tissue called phellem or suber (or cork) made up of flattened cells secreting suberin, an impermeable wax, allows a protection from moisture and gases [21]. This process of wall suberification causes the death of cells and the inclusion of air, resin compounds, tannins or other compounds in the cellular interior space thus acting as a barrier against parasites [19]. In contrast to periphery dead cells, the phellogen produces towards the inside of the trunk the phelloderm which contains two types of cells; thick-walled lignified cells with many inclusions and a layer of thin-walled cells that are radially expanded [22]. Finally, the bark is formed of the secondary liber or phloem which contains screened cells and other parenchymal cells ensuring a conduction function of the sap or storage of steroid crystals, starch granules or tannins [23].

As a rule, the bark usually corresponds to 10–15% of the total weight of the tree [24]. Within the pine species, the bark represents 7% of the total weight in *P. sylvestris*, 11% in *P. pinaster*, 9% in *P. nigra* and 9% in *P. halepensis* [18]. In terms of amount of bark generated, a French pulp mill uses nowadays between 1000 and 2000 tons of dry wood a day. Thus, it can be considered that the PB can reach a deposit between 100 and 300 tons/day and between 36,000 and 110,000 tons/year for a single French production site [19]. As France has a total of five pulp mills in his territory, a maximum PB volume of 550,000 tons/year could be generated in France [18]. At the European level, Spain and more specifically in the Galicia region, a production of 350,000 tons/year of *P. pinaster* bark and 87,500 tons/year of *P. radiata* bark is recorded for a total of nearly 450,000 tons/year [25], while in Portugal a production of 600,000 tons/year of pine (*P. pinaster*) bark is estimated [26].

The bark extracted from the logs is generally used as fuel in the boilers, but a considerable surplus is still rejected as waste residue. The presence of high added-value compounds makes pine bark a valuable source to value.

8.2.2 Pine Waste from Natural Biological Process

The industrial exploitation of pine also generates other by-products such as PN or PC. Indeed, the pine branches are covered in vegetation period with leaves, also called needles due to their shapes, and flowers and fruit, also known as cones, during the breeding season [19]. If these by-products are generated during the preparation of wood and the cutting of logs, they can also be obtained naturally when they fall to the ground each year.

In the *Pinus* species, the foliage is made up of "true leaves" (or euphyllous) as well as "false-leaves" (or pseudophylls). In fact, the true leaves appear in very early stage development but have a brown color owing to a chlorophyll deficiency, therefore rendering them inefficient for tree except for a horizontal extension. Conversely, dwarf shoots present in tree lateral branches are differentiated at the apex in 1–8 green needles which are photosynthetically very active. In this sense, these portions of chlorophyllian branches are called "false-leaves" or pseudophylls [27]. Generally, pseudophylls or needles are grouped by 2, 3 or 5 depending on the *Pinus* species. For instance, *P. pinaster* and *P. sylvestris* are composed of 2 pseudophylls while *P. strobus* has 5 needles [27]. In terms of life expectancy, the needles are usually in place between 3 and 14 years of age with a turnover every year. Thus, the PNs fall throughout the year, with a small fall during the winter/spring period (December-May), a first peak of fall during the summer (May-August) while the most strong fall occurs in autumn with a peak in November [28]. Regarding the fallen needle mass, a fall of 2.0 tons/ha/year for *P. halepensis*, 3.3 tons/ha/year for *P. sylvestris*, 3.4 tons/ha/year for *P. palustris*, 4.5 tons/ha/year for *P. elliottii*, 4.8 tons/ha/year for *P. roxburgii* and 5.0 tons/ha/year for *P. taeda* were reported [28–32]. Thus, pine species generate an average between 2 and 5 tons of needles per hectare over a year.

Knowing that *P. pinaster* covers more than four million hectares in Europe, with France, Portugal and Spain averaging 1.3 million ha each, an estimation of 8–20 millions of tons of PNs in Europe and 2.6–6.5 millions of tons of PNs in the main countries can be illustrated [33]. Moreover, *P. sylvestris*, mainly present in Scandinavia and Western Europe (Russia, Balkans), exceeds now 28 million hectares in Europe expecting more than 90 millions of tons of PNs [34].

Focusing on PCs, a difference between male and female cones occurs. The male cones are considered as a single flower formed of small leaves spirally inserted around a small solid axis and forming as a whole a small cone. Each of these leaves carries two pollen sacs (or microsporangia) on its underside and is, therefore, called microsporophyll. At maturity, they escape into the air in the form of what are commonly called pollen grains. Unlike male cone which is a single flower with many stamens, the female cone is made up of a large number of female flowers spiraling on a common axis and therefore constituting an inflorescence. Each of its flowers consists of a lignified carpel supporting a small number of ovules and a naked bract axillating this carpel. At maturity, the female cones can reach 30 or 40 centimeters depending on *Pinus* species [27]. For its reproduction, pine is an anemogamous plant with male cones that produce pollen scattered by the wind to fertilize female cones in order to maintain a genetic diversity. Each year, 1–6 tons of cones of *P. pinea* per hectare in adult stands are formed suggesting a fall of the cones in the same order of magnitude owing to a natural turnover [35]. A study on litter fall in *P. halepensis* forest on Mallorca (Spain) exhibited a fall of PCs at 0.78 tons/ha while another study estimated the total cone fall of *P. sylvestris* between 0.6 and 0.7 tons/ha/year [28, 30]. Thus, as *P. pinaster* and *P. sylvestris* exceed more than 30 million hectares in Europe, a PCs amount can be estimated at 21 million tons with a ratio of 0.7 tons/ha/year. In France, mainly composed by *P. pinaster* (1.3 million ha), a deposit of PK can reach more than 910,000 tons per year.

Currently, PNs and PCs are poorly valued in view of the mass generated each year. The chemical content with high added-value compounds makes the pine by-products an interesting biomass to value.

8.3 Chemical Compositions of Pine by-Products

8.3.1 Pine Knotwood

Focusing on PKs, these by-products are particularly rich in lipophilic compounds, usually concentrated in resins. Extracted using apolar solvents such as hexane or dichloromethane, the major nonpolar compounds are non-volatile terpene acids named resin acids that can reach 26 g/kg in *P. pinaster* knot and between 85 and 300 g/kg in *P. sylvestris* knot (Table 8.1) [36, 37]. The most abundant resin acids are the abietane-type with abietic acid, dehydroabietic acid, palustric acid and neoabietic acid as well as the pimarane-type with pimaric acids [38]. The fatty acids are

Table 8.1 Summary of contents according to the different classes of compounds in pine by-products

	Polyphenols	Lipophilic compounds	Mono-, polysaccharides	Carotenoids
Pine knot	+++(+)	++++	+++	–
Pine bark	+++(+)	+(+)	++	–
Pine needle	++(+)	+++	++	+
Pine cone	+++	+++	++	–

Notation: ++++, >100 g/kg; +++, 10–100 g/kg; ++, 1–10 g/kg; +, <1 g/kg; -, absence. Crosses in brackets indicated that some *Pinus* spp. did not reach the upper threshold

also dominant compounds in the PK with the notable presence of oleic acid (18:1 n-9), palmitic acid (16:0), linoleic acid (18:2 n-9,12), pinolenic acid (18:3 n-5,9,12) and stearic acid (18:0) [36]. To a lesser extent, the knotwood also includes juvabiones which are sesquiterpenes from the methyl ester of todomatuic acid, as well as the presence of esters (β-sitosterol) and triglycerides [39].

PKs are notably known to possess a rich spectrum of extractable phenolic compounds, including stilbenes, lignans and flavonoids (Fig. 8.2) [40]. Stilbenes (1,2-diphenylethylene) are polyphenolic compounds formed from two phenyl rings connected to each other by an ethylene bridge generating a C_6-C_2-C_6 structure. The double bond makes it possible to generate *trans*- (*E*) and *cis*- (*Z*) forms while the aromatic nuclei are generally substituted by different functions such as hydroxyl, methyl, methoxy, prenyl or geranyl groups [41]. Monomeric units can also be coupled leading to the construction of dimers, trimers, tetramers and up to octamers [42]. Lignans are polyphenols initially formed by the coupling of two units of coniferyl alcohol and resulting in a bond at the C-8 central carbon forming a characteristic $(C_6$-$C_3)_2$ structure [43]. The neoformed structure is the cornerstone of this metabolic pathway from which will emerge several distinct subgroups depending on the presence or absence of oxygen in the skeleton and the various cyclization patterns [44]. Flavonoids are compounds with a common structure formed by two benzene rings connected to each other by an oxygenated heterocycle generating a C_6-C_3-C_6 structure. From this central unit flows a multitude of subclasses according to the presence of alcohol and/or ketone functions and unsaturation on the oxygenated heterocycle as well as substitutions on the benzene rings (hydroxylation, methylation, glycosylation) [45]. The PKs, through extracts obtained in an acetone-water mixture, showed phenolic compound contents up to 20 g/kg in *P. pinaster* knot, 30 g/kg in *P. contorta* knot, and between 14.5 and 105 g/kg in *P. sylvestris* (Table 8.1) [36, 37, 40]. Among the phenolic compounds, lignans are generally the major compounds with mainly nortrachelogenin, followed by pinoresinol, isolariciresinol and secoisolariciresinol (Fig. 8.2). The lignans content reaches 10 g/kg in *P. pinaster* knotwood and 29 g/kg of *P. sylvestris* knotwood for instance [36, 37]. Flavonoids are also predominant in PK (2 g/kg in *P. sylvestris* knot and 8 g/kg in *P. pinaster* knot) with pinocembrin, pinobanksin, dihydrokaempferol, taxifolin and pinobanksin-3-acetate in descending order [46]. Stilbenes including pinosylvin and pinosylvin monomethyl ether and to a lesser extent pterostilbene and pinostilbene are also mainly present in PK with total stilbenes content close to 2 g/kg in *P. pinaster* knot,

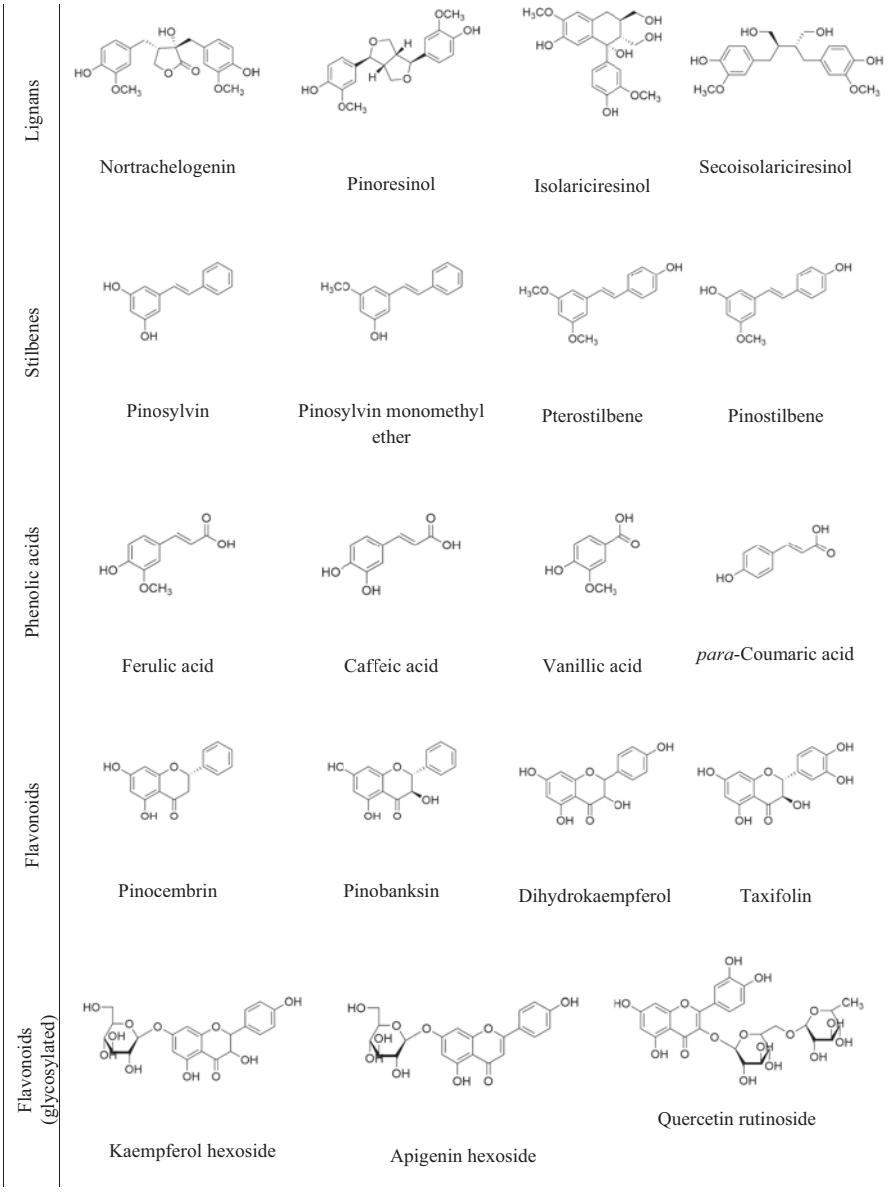

Fig. 8.2 Structure of the main polyphenols present in pine by-products

Table 8.2 Summary of contents according to the different classes of polyphenols in pine by-products

	Polyphenols				
	Lignans	Stilbenes	Flavonoids	Phenolic acids	Condensed tannins
Pine knot	+++	++(+)	++	+	–
Pine bark	+(+)	+(+)	+(+)	+	+++(+)
Pine needle	–	–	++	+	+
Pine cone	+	+	+++	+	++

Notation: ++++, >100 g/kg; +++, 10–100 g/kg; ++, 1–10 g/kg; +, <1 g/kg; -, absence. Crosses in brackets indicated that some *Pinus* spp. did not reach the upper threshold

2.5 g/kg in *P. radiata,* and between 10 and 80 g/kg in *P. sylvestris* knot (Table 8.2) [36, 37, 47, 48]. Phenolic acids such as caffeic acid or ferulic acid are also present but in lesser quantities [39].

In addition, PKs are composed of parietal compounds that constitute the cell wall of wood such as cellulose, hemicellulose and lignin [49]. It can be noted that aqueous solvent extraction of PKs leads to the degradation of hemicellulose and to the production of numerous derivatives. Hemicellulose, mainly galactoglucomanan, leads to considerable concentrations of oligosaccharides such as mannan-oligosaccharides (2–10 units of oses) and polysaccharides such as mannan (> 10 units of oses) with values reaching 81.5 g/kg of knotwood. Monosaccharides (mannose, xylose, galactose or arabinose) are also found with a content of 1.7 g/kg of knotwood (Table 8.1) [46, 49, 50].

8.3.2 Pine Bark

The bark of *Pinus* species is known to be rich in polyphenolic compounds with content oscillating between 20.5 g/kg in *P. banksiana*, 30 g/kg in *P. merkusii*, 70 g/kg in *P. pinaster* bark, 75 g/kg in *P. pinea* and up to 100 g/kg in *P. oocarpa* and *P. radiata* (Table 8.1) [20, 51, 52]. The main compounds are monomers and condensed flavonoids also known as condensed tannins (Fig. 8.2). The usual monomers found in pine bark are flavan-3-ols with mainly catechin and epicatechin, as well as their gallic acid esters, epicatechin gallate, epigallocatechin, and epigallocatechin gallate [53]. In *P. pinaster* bark extract, the monomers catechin and epicatechin represent 18.9% and 0.2% of total compounds respectively, for a total monomers content of 19.1% while in *P. sylvestris* the monomers content reaches 37% [54, 55]. In bark of *P. wallichiana*, *P. gerardiana* and *P. roxburgii*, Willför et al. (2009) reported catechin content at 5.8 g/kg, 6.2 g/kg and 13.3 g/kg of PB, respectively [56]. The condensed tannins (or procyanidins) are oligomeric ($n < 6$) or polymeric ($n > 6$) forms of flavan-3-ols. They are present in nature with a great diversity of structures due to the structural characteristics of the monomeric flavan-3-ols as well as the type of inter-flavan bond or the degree of polymerization. The most common interflavonoid linkages are the C_4–C_6 or C_4–C_8 linkage and may contain gallic acid

esters (Fig. 8.2). In such cases, an additional linkage could be present between the C_2-C_5 or C_2-C_7 [57]. The PBs are mainly composed by dimers of flavan-3-ols and to a lesser extent by trimeric and tetrameric forms [54]. Dimers represent 40.9% of total phenolic compounds and the more complex forms 19.0% of total phenolic compounds in *P. pinaster* bark extract for total procyanidins content at 59.9% [54]. However, the total procyanidin content in bark is *Pinus* species dependant since values swing from 5% in *P. banksiana* to 93% in *P. densiflora* [20]. For instance, the proanthocyanidins (or condensed tannins) content can reach 22 g/kg in *P. geradiana* bark, 25 g/kg in *P. roxburgii* bark, 72 g/kg in *P. pinea* bark and up to 160 g/kg in *P. wallichiana* bark (Table 8.2) [23, 56]. Some authors reported that pine bark could contain procyanidins from monomers through decamers and higher polymers [58]. Other flavonoids are also present in PB especially taxifolin (0.5 g/kg in *P. geradiana* bark, 2.7 g/kg in *P. roxburgii* bark) as well as some minor flavonoids such as dihydroquercetin, dihydroquercetin-3′-β-D-glucopyranoside, dihydroquercetin-7-β-D-glucopyranoside and kaempferol 3-β-L-rhamnopyranoside [56, 59]. Additional polyphenolic compounds were also investigated in PB such as stilbenes (resveratrol glycoside, monomethyl pinosylvin, dihydro-monomethyl pinosylvin and 3,4′,5-trihydroxy-*trans*- stilbene 4′-*O*-β-D-glucopyranoside) reaching 9.6 g/kg of pine (*P. wallichiana*) bark, phenolic acids (caffeic acid, ferulic acid, gallic acid and coumaric acid) which represent 0.5 g/kg in pine (*P. roxburgii)* bark and several lignans rising up to 7.2 g/kg in *P. gerardiana* bark (Table 8.2) [56, 59].

In addition to polyphenolic compounds, PBs contains a large amount of polysaccharides (from 4.5 g/kg in *P. pinaster* bark to 70 g/kg in *P. wallichiana*) [56, 60]. Several polysaccharides were found using different molar ratios of monosaccharides with mainly D-glucose, followed by D-galactose, D-mannose, L-arabinose, D-xylose, L-rhamnose, and D-ribose [61].

Lipophilic compounds are also present with mainly terpenic compounds ranging from 0.97 g/kg in *P. pinea* bark to 2.9 g/kg in *P. pinaster* bark (Table 8.1) [62]. Fatty acids (1.1 g/kg in *P. pinea* bark and 0.8 g/kg in *P. pinaster* bark), long-chain aliphatic alcohols (0.2 g/kg in *P. pinea* bark and 0.1 g/kg in *P. pinaster* bark), and sterols (0.2 g/kg in *P. pinea* bark and 0.6 g/kg in *P. pinaster* bark) are also prevalent in PB. Regarding the main lipophilic metabolites, the diterpene dehydroabietic acid is the major constituent of both *P. pinea* and *P. pinaster* lipophilic fractions, accounting for 0.45 g/kg and 0.95 g/kg of total compounds, respectively [62].

8.3.3 Pine Needle

Regarding the chemical composition of pine needle, it appears that polyphenolic compounds are mainly present with concentrations at 2 g/kg in *Pinus eldarica* needles, 10 g/kg in *P. sylvestris* needles, 12.2 g/kg in *P. mugo* needles, 13.3 g/kg in *P. nigra* needles and 14.1 g/kg in *Pinus peuce* needles (Table 8.1) [63, 64]. The main compounds are flavonoids and more particularly glycosylated flavonoids with the prevalent kaempferol-, apigenin- and laricitrin- hexoside, isorhamnetin pentoside,

myricetin-, quercetin-, laricitrin-, isorhamnetin-, syringetin-, kaempferol coumaroyl-, kaempferol feruloyl- rutinoside for a total content of flavonoids reaching 7.2 g/kg in PNs (Fig. 8.2) [63]. The flavan-3-ols catechin and epicatechin as well as procyanidin dimers were also present in pine (*P. elderica*) needles at 0.9 g/kg [64]. Some phenolic acids were also identified such as ferulic acid, vanillic acid, *para*-coumaric acid, *ortho*-coumaric acid, gallic acid and quinic acid for a total content estimated at 0.8 g/kg in *P. elderica* needles (Table 8.2) [63, 64].

In addition, PNs contain polysaccharides which are composed by various monosaccharides such as pinitol (5 g/kg of PNs), glucose (4.7 g/kg of PNs), and fructose (3.9 g/kg of PNs) for a total content at 14.2 g/kg in pine (*P. nigra*) needles [65]. These by-products are also composed by lipophilic metabolites mentioned in *P. koraiensis* and *P. sibirica* (21-95 g/kg of PNs) with mainly bornyl *para*-coumarate, heterocyclic 15-*O*-functionalized labdane type acids (lambertianic acid), 10-nonacosanol, sterols and their esters [66]. The PNs are also known to contain carotenoids, especially β-carotene, α-carotene, lutein, neoxanthin as well as zeaxanthin, antheraxanthin and violaxanthin [67]. The total content of carotenoids can reach 0.15–0.30 g/kg in pine (*P. nigra*) needles (Table 8.1) [68].

Furthermore, PNs are known for the essential oils generated by their distillation owing to several volatile terpenes present in these by-products. Essential oil yields of 4.6 mL/kg from *P. cembra* needles, 3.0 mL/kg from *P. mugo* needles, 2.7 mL/kg from *P. nigra* needles, 2.6 mL/kg from *P. sylvestris* needles and 2.2 mL/kg from *P. pinaster* needles were reported [69, 70]. Among the various essential oils, the main class of terpenes was the monoterpenes which represented between 37.4 and 67.3% of total compounds followed by the sesquiterpenes with content comprised between 19.8 and 49.0% [69, 70]. On the contrary, the diterpenes as well as oxygenated mono-, sesqui- and diterpenes were in smaller quantity. The usual main compounds are the volatile monoterpenes α-pinene, limonene, β-phellandrene, δ-3-carene, β-thujene, and/or myrcene as well as the sesquiterpene germacrene D and caryophyllene [69–71].

8.3.4 Pine Cone

Concerning the chemical composition of PC, polyphenols are predominant with total phenolic compounds that can reach 53.3 g/kg in *P. halepensis* cones and 80.7 g/kg in *P. densiflora* cones (Table 8.1) [72, 73]. Among them, flavonoids are mainly present (7.1 g/kg in *P. densiflora* cones and 24.1 g/kg in *P. halepensis* cones) especially glycosylated flavonoids (apigenin hexoside, phloretin-*C*-hexoside, quercetin-3-*O*-pentoside, myricetin-glucoside, and quercetin-acetyl-rutinoside hexoside) [72–74]. The flavanonol taxifolin (0.1 g/kg in *P. halepensis*), the flavan-3-ols catechin and gallocatechin (1.3 g/kg), the stilbene pinosylvin (0.09 g/kg), the lignan isolariciresinol (0.08 g/kg), the phenolic acids (ferulic acid, caffeic acid, chlorogenic acid and protocatechuic acid with content less than 0.1 g/kg) as well as ellagic tannins are reported (Fig. 8.2, Table 8.2) [74, 75].

Similarly to PBs, PKs and PNs, PCs contain polysaccharides mainly formed with arabinitol, pinitol, mannitol and glucose for a total content of carbohydrates at 1.7 g/kg of pine (*P. nigra*) cones (Table 8.1) [65]. Fatty acids were also identified (2% of total weight in *P. halepensis*) with richness in satured fatty acids (41.5%) such as undecanoic acid (11:0), pentadecyclic acid (15:0), palmitic acid (16:0) and lignoceric acid (24:0). Monounsatured fatty acids such as oleic acid (18:1 n-9) and undecenoic acid (11:1), polyunsatured fatty acids such as *cis*,*cis*-linoleic acid (18:2 n-6) and *cis*,*cis*,*cis*-α-linolenic acid (18:3 n-3) as well as *trans* fatty acids such as *trans*,*trans*-linoleic acid (18:2 n-6) were prevalent in PCs [76].

Like PNs, PCs can generate essential oil through a hydrodistillation procedure. For instance, *P. koraiensis* cones yielded 10.7 mL/kg of essential oil which was almost three times the amount of essential oil extracted from PNs. Analyses revealed more than 87 compounds in the essential oil. The most abundant oil components in PCs were similar to PNs with the monoterpenes limonene (27.90%), α-pinene (23.89%), β-pinene (12.02%), 3-carene (4.95%) and β-myrcene (4.53%) [77].

In a context of sustainable development, investigation of plant extracts to fight diseases and pests raised a renewed interest. Recent studies have focused on the pine industry by-products recycling such as knot, bark, needle and cone owing to a wide spectrum of compounds. Promising biocontrol products were investigated against fungi, oomycetes and insects.

8.4 Biocontrol by Pine by-Products

8.4.1 Antifungal Activities

Some works have studied the potential application of PK extracts and derivatives against several fungi affecting agricultural crops or forest stands. Several extracts (dichloromethane, acetone, toluene/ethanol and water) of *P. merkusii* knots were assessed against the development of the tree fungi *Trametes versicolor*, responsible for the white-rot (degradation of both cellulose and lignin) and *Poria placenta*, responsible for the brown-rot (degradation of cellulose). Experiments were performed in Petri dishes with the several extracts solubilized in the medium contacting a small portion of the studied fungi, allowing an evaluation of mycelium growth by measuring the diameter of the colony. The acetone extract of PKs possessed the highest antifungal activities whereas the water extracts showed no influence on mycelium growth [38]. The high content in stilbenes in PKs may be the explanation of these antifungal activities. In fact, a study has focused on the application of *P. sylvestris* heartwood extract containing a high content in stilbene pinosylvin and pinosylvin monomethyl ether against the brown-rot *Coniophora puteana*, *Gloeophyllum trabeum* and *Rhodonia placenta*. Methodologically, wood blocks (*P. sylvestris*) are impregnated with a stilbene-enriched fraction obtained from an ethanolic extraction of *P. sylvestris* pinewood. The tests showed that the impregnation with a high

concentration of stilbenes (60 mg/g dry wood) significantly suppressed the growth of fungi and slowed down the decay process of wood blocks. These results allowed to underline the paternity of stilbenes in the antifungal activity of pine wood [78]. In addition, another study investigated the effect of pure pinosylvin, pinosylvin monomethyl ether and pinosylvin dimethyl ether as well as a stilbene mixture against the growth of white-rot fungi (*T. versicolor* and *Phanerochaete chrysosporium*) and brown-rot fungi (*Neolentinus lepideus*, *G. trabeum* and *Postia placenta*). The results showed that pure stilbenes inhibited the growth of white-rot and brown-rot fungi according to the nature of stilbenes, their concentration, the fungi and the bioassay test used highlighting the major role of stilbenes in antifungal activity of PK extract [79]. Regarding the potential mechanisms of action, brown-rot fungi are highly destructive wood decaying organisms that use free radicals in the initial stages of decay. A study reported the ability of *P. sylvestris* knot extractives to inhibit radical-based degradation, with a focus on the Fenton reaction. The antioxidant assays exhibited good radical scavenging activity for extracts and pure pinosylvins, suggesting that the antioxidant activity of extractives may play a role in inhibiting brown-rot decay [80]. Furthermore, some studies have shown that stilbenes may be associated with the generation of ROS that might induce apoptosis in fungi and they may also be involved in the inhibition of ergosterol biosynthesis present in membrane cell [81].

Antifungal assays were also performed with *Pinus* spp. bark extracts. For instance, pine (*Pinus brutia*) bark extracts were tested against two types of white-rot fungi (*T. versicolor* and *Pleurotus ostreatus*) and two types of brown-rot fungi (*Fomitopsis palustris* and *G. trabeum*). The results showed that PB extracts did not exhibit any antifungal activities against the studied fungi even at high concentration (until 12%). Moreover, the PB extract seemed to stimulate the development of fungi as the decay was higher than the control. These findings suggested that tree fungi could use the PB extracts as food source [82]. Another work focused on the antifungal effects of pine (*P. sylvestris*) bark ethanolic extract against the phytopathogenic fungi *Botrytis cinerea*, *Colletotrichum acutatum*, *Phytophthora cactorum* and *Mycosphaerella fragariae*. In contrary to the findings above, the PB extract showed a strong inhibition of mycelial growth (70–100%) as well as of the sporulation with a high concentration at 10 or 20 g/L. For a lower concentration (0.1 and 1 g/L), the inhibition was more moderated (20–30%) [83]. Thus, the powerful antifungal activity of PB extracts seemed to be link to the targeted fungi and obviously to the concentration applied.

Likewise, PN extracts were tested for their fungicidal activity. Several extracts from *P. elliottii* needles such as hexane, ethyl acetate, ethanol and water extracts were assessed against the white-rot fungus *Coriolus versicolor* and the brown-rot fungi *G. trabeum* and *Polyporus vaporaria* in bamboo. It appeared that the hexane extract at 32 g/L exhibited a strong antifungal activity against both white- and brown-rot fungi (antifungal index higher than 75% and 80%, respectively). The ethyl acetate and ethanol extracts showed a strong antifungal capacity against the brown-rot fungi while the activity was mitigated against the white-rot fungus. As for the water extract, it showed a weak antifungal activity (< 40%) [84]. Antifungal

assays with essentials oils obtained from hydro-distillation of pine (*P. strobus*, *P. wallichiana* and *P. monticola*) needles were also conducted against *Fusarium verticillioides* (causal agent of kernel and ear rot of maize). Mainly composed by monoterpenes (α-pinene, β-pinene, limonene and myrcene), all the essential oils evaluated showed inhibitory activities on the growth of *F. verticillioides*. *P. wallichiana* and *P. monticola* essential oils were the most active inhibitors with a minimum inhibitory concentration at 40 µL/L [85]. Another work studied the antifungal activity of essential oil from pine (*P. pinaster*) needles against ten agricultural fungal species (*Gibberella avenacea*, *Fusarium culmorum*, *F. oxysporum*, *F. subglutinans*, *F. verticillioides*, *F. nygamai*, *Rhizoctonia solani*, *Microdochium nivale*, *Alternaria alternata* and *Bipolaris sorokiniana*). The results showed that the essential oils reduced the growth of fungal species by 50% for a concentration of 4 mL/L [86]. The antifungal activity appeared to be related to the high content of monoterpenes as well as some sesquiterpenes in PNs. In fact, some authors highlighted that the monoterpenes α-pinene, β-pinene or limonene possessed strong antifungal effects (4.0–13.0 mL/L) against strains of mushroom pathogenic fungi *Verticillium fungicola* and *Trichoderma harzianum* [87]. Therefore, PNs through organic extracts or essential oils could be a useful source of biocontrol products.

The PCs as potential antifungal products were also tested against several fungi. Indeed, extracts of pine (*P. sylvestris*) cones were assessed against eight brown-rot fungi, three white-rot fungi and four soft-rot fungi. The extraction was carried out with water and then with acetone/water (75/25, v/v) allowing to recover polyphenols, especially flavan-3-ols. The results showed that the development of several brown-rot fungi was inhibited at 0.25–0.5 g/L by the PC extract while the extract was not effective against the white-rot and soft-rot fungi [88]. Thus, the success of the antifungal effect of PC appeared to be dependent on the fungi species. Furthermore, an essential oil obtained from pine (*P. koraiensis*) cones was also assessed for its antifungal effect against human fungal pathogens such as several *Candida* strains, *Aspergillus fumigatus* and *Cryptococcus neoformans*. The findings indicated a minimal inhibitory concentration of essential oil at 0.14 g/L against *C. neoformans* and 0.55 g/L against *C. glabatra* [77].

Overall, the pine by-product extracts exhibited a significant antifungal activity which strongly depends on the fungi species as well as the nature of by-product and its derivatives.

8.4.2 Anti-oomycete Activities

For a long time, the oomycetes were parts of the Fungi kingdom before being reclassified among Chromists kingdom due to their close relationship with photosynthetic organisms such as brown algae and diatoms [89]. The reclassification is thus due to structural divergences between oomycetes and fungi such as the cell wall of oomycetes which is composed of cellulose while that of mushrooms is composed of chitin [90]. Oomycetes are the causal agent of mildew in several agricultural crops.

Therefore some studies have focused on the potential application of pine by-products extracts and main pine compounds against oomycetes, hoping a success as biocontrol products.

For instance, a pine (*P. pinaster*) knot extract was tested against *Plasmopara viticola*, an oomycete causing grapevine downy mildew. The bioassay was performed on grapevine leaf disks and PK extract provided a total inhibition of downy mildew at 0.5 g/L and had an IC_{50} value of 0.08 g/L. This antifungal effect was explained by the high content of stilbenes (pinosylvin and pinosylvin monomethyl ether) and flavonoids (pinocembrin) in PK which were able to strongly block zoospore mobility and to inhibit mycelium development with IC_{50} values comprised between 18 and 34 μM [17].

An European project has also focused on the valuation of the bark of eight forest species including *P. sylvestris* as a natural oomycide against grapevine downy mildew (*P. viticola*). The ethyl acetate and dichloromethane extracts of *P. sylvestris* bark showed intermediate efficacies between 50% and 80% against downy mildew at 1 g/L. In addition, *P. sylvestris* extracts were also significantly active (40–60% efficacy) at the lower tested concentration, i.e. 0.25 g/L [91]. The oomycide effect of *P. sylvestris* bark extracts is due in part to the presence of the diterpene 15-hydroxydehydroabietic acid which produced 99% inhibition of mildew at a concentration of 1.4 g/L. Two other compounds, 7-oxo-15-hydroxydehydroabietic acid and 7α,15-dihydroxydehydroabietic acid, also showed inhibitory effects of 72–73% at 1 g/L and 94–96% at 2 g/L [91].

These findings suggested that pine by-products, especially knot and bark, could be used as a sustainable tool for controlling oomycetes. More research on oomycide effects of PN and PC could bring useful information in pine by-products recycling.

8.4.3 Insecticide Activities

Insects are other major phytopathogens of agricultural crops and forest stands that generated since several decades the use of chemical insecticides to curb their impact without long-term vision of their potential harm [92]. Nowadays, there is a new impetus to develop natural products that are safe for human and ecofriendly. In this sense, some works have been interested in assessing pine by-products extracts as botanicals insecticides.

Recently, a pine (*P. brutia*) bark extract was tested against the larvae of the wood-damaging insect *Spondylis buprestoides* (Coleoptera: Cerambycidae). The PB extract rich in tannins was dissolved in water until its impregnation in wood blocks used in bioassays. At the end of the 6-month experiment, the numbers of dead and live larvae were accounted. The results showed that larvae in the PB extract at a concentration of 6% caused damage until the end of the fifth month while the PB extract at 12% mitigated the impact of larvae until the end of the third month [93]. Another work was interested in the application of pine (*P. merkusii*) bark extract against the mosquito *Aedes aegypti* which is the vector responsible for the

transmission of dengue fever, chikungunya as well as yellow fever. Several concentrations of PB ethanolic extract were added to a set of third instar larvae in order to evaluate after 24 h of inoculation the larval mortality. The results revealed a high larval mortality against the larvae of *A. aegypti* in the presence of the extract, with LC_{50} at 96.3 mg/kg and LC_{90} at 298.4 mg/kg after 12 h, and LC_{50} at 58.4 mg/kg and LC_{90} at 125.7 mg/kg after 24 h. The total larval mortality after 24 h was obtained with a concentration of extract at 160 mg/kg. These data strongly suggest that extract from PBs could be exploited as new bioinsecticides [94].

PN extracts were also tested against several insects. A hexane extract from *P. banskiana* needle was assessed against various *Neodipirion* (Hymenoptera: Diprionidae) sawflies known as pine pests. The results showed that PN extract (1 g/L) exhibited antifeedant activity up to 52 h after treatment and three times more larval mortality than control [95]. Another study was conducted on insecticide effects of pine (*P. taiwanensis*) needle methanolic extract against *Musca domestica* (house fly) and *Aedes albopictus* (mosquito). An applied dose of 1 g/L allowed a fourth instar larvae (*A. albopictus*) mortality of 56.3% and a pupation rate disrupted of 21% (control at 98.3%) 72 h after treatment. Focusing on *M. domestica* adults, a concentration of 10 g/L of PN extract exhibited 53.3% mortality after 24 h and 96.7% after 48 h. These findings showed that PN were highly potential to be botanical insecticides [96].

Besides, a recent work studied the insecticide activity of pine (*P. banksiana*) cone ethanolic extract against the Solanaceae pest *Leptinotarsa decemlineata* (Coleoptera: Chrysomelidae) also known as Colorado potato beetle. More particularly, the mechanism of action was investigated through the inhibition of glutathione S-transferase (GST) activity which is a detoxifying enzyme involved in defense mechanism of insects. The results showed a GST median IC_{50} of 10 mg/L and an inhibition of 90% for a PC extract concentration at 53 mg/L. Several steps of purification allowed to identify the polyphenol taxifolin (flavanonol) as major compound in PC which exhibited a strong GST inhibition activity with IC_{50} value of 4.2 mg/L. Thus, these findings suggested that the PC can be a source of natural insecticide by unsettling the detoxification process in insect [97].

Furthermore, the study of PK extract as botanical insecticide remains unexplored to our knowledge. The PKs contain mainly stilbenes, flavonoids and lignans known for their insecticide effects and could be an interesting way to value.

8.5 Conclusions

Nowadays, the agricultural field is prone to the development of new strategies to cope with historical changes in pest treatment. Various studies aim at proposing new solutions and more particularly the valuation of plant extracts containing a wide range of antimicrobial compounds. This chapter has focused particularly on forest-based co-products such as knots, bark, needles and pine cones with a focus on their antifungal, anti-mildew and insecticide activities.

The pine by-products represent several million tons of biomass each year in forest industries or forest stand, commonly used as a source of energy or simply discarded as residual waste. In France, one of the major pine producers in Europe, the PK amount can reach 18,000–36,000 tons per year, the PB can be estimated at 550,000 tons per year, the PN can arrive between 2.6 and 6.5 million of tons per year and the PC can reach up to 910,000 tons per year. These raw materials are thus available in huge amount, renewable and cheap.

The interest of these by-products lies in their diversified chemical composition. In fact, the pine by-products are generally rich in polyphenols belonging to different classes depending on each material. As such, PKs are composed of lignans, stilbenes and flavonoids, PBs contain mostly condensed tannins while PNs and PCs have glycosylated flavonoids and flavan-3-ols. Pine by-products also contain lipophilic metabolites such as terpene, fatty acids and sterols as well as polar compounds like polysaccharides. The qualitative and quantitative variabilities in the chemical space of the pine by-products components allowed to expect a broad spectrum of bioactivities owing to various molecular targets.

Indeed, several studies highlighted the antifungal, oomycide and insecticide activities of the pine by-products. For instance, pine by-products mitigate the impact of white-rot and brown-rot fungi, downy mildew as well as coleoptera and mosquitoes.

The pine by-products appeared to be a prospective tool to manage agricultural crops and forest stand against various pathogens. As a consequence, these findings pave the way for major breakthroughs in sustainable agriculture.

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Chapter 9
Stilbenoid-Enriched Grape Cane Extracts for the Biocontrol of Grapevine Diseases

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9.1 Introduction

Grapevine production and wine quality largely depend on the use of various agrochemicals to control the development of grape pathogens [1]. Indeed, Treatment Frequency Index (TFI) in viticulture is among the highest ones within crop production with an average of 20 treatments per year. Almost the three quarters of these treatments are used to control fungal diseases especially downy mildew, powdery mildew and gray mold respectively caused by *Plasmopara viticola*, *Erysiphe necator* and *Botrytis cinerea* [2]. Risks of pesticide exposure on human health and environment have been reported by several reviews [3–5]. To meet the societal demand of pesticide reduction, public authorities set up programs to develop alternative pest control strategies as proposed by the French Ecophyto plan. Since the past decade, EU required to minimize pesticide use through the Sustainable Use Directive (2009/128/EC [6]). Unfortunately, there is still a gap between the promotion of pesticide-free agriculture and the number of alternative solutions available for farmers. Whereas pesticide restrictions are increasing, only few alternatives are being proposed and there is an urgent need for efficient crop protection suitable for sustainable agriculture. Among several diversification methods towards more sustainable agriculture, the use of biocontrol is a promising option to be considered and

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plant extracts are gaining more importance in pest control programs as ecofriendly biopesticides [7, 8].

Active substances of plant extracts are specialized metabolites that derive from plant primary metabolism and act as constitutive or induce plant defenses against biotic aggressors. *Vitis vinifera* L. and more broadly, plants of the Vitaceae family, accumulate a peculiar class of polyphenol called stilbenoids harboring the 1,2-diphenylethylene unit. From the common precursor *E*-resveratrol, a vast array of derivative compounds is formed consecutive to hydroxylation, glycosylation, methoxylation or oligomerization [9, 10]. Stilbenoids are grape phytoalexins *i.e.* locally-induced molecules upon pathogen infection, involved in disease resistance. *In vitro* antifungal assays have demonstrated different activity levels of grape stilbenoids against *Plasmopara viticola*, *Botrytis cinerea* and causal agents of wood diseases. Additionally, recent studies reported insecticide potential of GCE for the biocontrol of *Spodoptera littoralis* [11].

In this chapter, we present the state of the art in the knowledge on GCE composition, the different extractive technologies and the biocontrol activities of pure stilbenoids as well as stilbenoids-enriched extracts against plant pathogens and finally the sources of variation of GCE.

9.2 Grape Canes Represent a Valuable Biomass for Stilbenoid Sourcing

Grapevines are widely cultivated worldwide with a total vineyard surface of 7.6 million hectares [12]. Grapevines are cultivated for the production of wine, juice, table grapes and dried fruits, but in parallel, this production generates tons of byproducts. Grape pomace is the first byproduct in abundance [13] and represents a promising source of dietary fibers [14, 15] and antioxidants [16, 17] that could be used for animal, pharmaceutical, cosmetic and food industry. Grape canes are the second byproducts in term of volume, they are produced during winter pruning and represent a volume of 1–5 tons per hectare and year depending on planting density, climate and grapevine vigor [18]. Although grape canes are mainly recycled for soil amendment, the remaining biomass still constitutes a huge volume that is poorly valorized. In France, these unvalorized portion of grape canes has been estimated at 20%, representing an available biomass of 276′200 tons of dry weight per year [19]. This biomass has been identified as a source of valuable polyphenols, particularly stilbenoids that could not be retrieved from grape pomace. Considering a yield extraction of 5%, that is common for polyphenol, it represents a total polyphenol mass of 13′810 tons per year only from French vineyards. Given that stilbenoid extraction process from grape canes requires dedicated industrial equipment, we proposed for the future the deployment of phytoextraction units allocated in accordance with wine-growing areas. Investment towards industrial scale-up should be determined in accordance with economic potential. Rayne et al. [20] estimated

between 2000 and 3000 USD per hectare and per year the potential of valorization of grape canes extracts.

9.3 Metabolomic Profiling of Grape Cane Extracts Highlights an Original Set of Biomolecules

Metabolomics is the holistic study of the small-molecule metabolites contained into a cell, organ, organism or biological system [21, 22]. The ideal metabolomics study (1) provides a snapshot of the whole metabolites present in a biological system [23, 24]. Analytical methods using Ultra High Performance Liquid Chromatography in tandem with Diode Array Detection and Mass Spectrometry (UPLC-DAD-MS) have been developed offering rapid and comprehensive metabolomic analyses of GCE [25]. Targeted metabolomics analyses using both electrospray ionization in positive (ESI^+) and negative mode (ESI^-) were employed to characterize grape cane compounds based on retention times, UV and mass spectra. Polyphenolic compounds presented a more reliable fragmentation pattern through ESI^- and peak annotation was performed by comparison with pure standards available for purchase or isolated in our laboratory [10]. The method allowed the annotation and relative quantification of 42 polyphenols [25]. Figure 9.1 presents the elution profile of the ten major biomolecules presents in GCE following an extraction step by maceration in ethanol/water solution (60:40; v/v) and shaken at 83 °C for 30 min. By elution order, the following molecules were identified; peak 1(RT = 4.35 min) catechin (λmax = 204.0, 229.0, 279.2 nm; $[M-H]^-$ m/z 289.1, $[2\ M-H]^-$m/z 579.0), peak 2 (RT = 5.02 min) epicatechin (λmax = 204.0, 227.3, 279.2 nm; $[M-H]^-$ m/z 289.1, $[2\ M-H]^-$ m/z 579.0), peak 3(RT = 7.66 min) ampelopsin A (λmax = 227.9, 281.6 nm; $[M-H]^-$ m/z 469.0, $[M-H + HCOOH]^-$ m/z 515.1, $[2\ M-H]^-$ m/z 939.4), peak 4 (RT = 7.85 min) *E*-piceatannol (λmax = 227.9, 281.6 nm; $[M-H]^-$ m/z 243.0, $[2\ M-H]^-$ m/z 487.2), peak 5 (RT = 9.42 min) *E*-resveratrol (λmax = 216.3, 305.3 nm; $[M-H]^-$ m/z 227.0 $[M-H + H_2O]^-$ m/z 242.9), peak 6 (RT = 10.46 min) hopeaphenol (λmax = 227.7, 281.7 nm; $[M-H]^-$ m/z 905.1), peak 7 (RT = 10.77 min) isohopeaphenol (λmax = 228.8, 283.9 nm; $[M-H]^-$ m/z 905.2), peak 8 (RT = 11.89 min) *E*-ε-viniferin (λmax = 228.8 and 283.9 nm; $[M-H]^-$ m/z 453.0, $[M-H + HCOOH]^-$ m/z 499.1, $[2\ M-H]^-$ m/z 907.2), peak 9 (RT = 12.19 min) *E*-miyabenol C (λmax = 227.7, 294.6, 324.2 nm; $[M-H]^-$ m/z 679.1, $[M-H + HCOOH]^-$ m/z 725.0), peak 10 (RT = 14.09 min) *Z*/*E*-visitin B (λmax = 227.7, 324.2 nm; [M-H]- m/z 905.3).

The biosynthetic pathway of stilbenoids in grape canes is only partially described. From L-phenylalanine, the consecutive activity of three enzymes: phenylalanine-ammonia-lyase (PAL), cinnamate 4-hydroxylase (C4H) and 4-coumarate ligase (4CL) leads to the formation of *p*-coumaroyl-CoA (Fig. 9.2). From this metabolite derives avast array of polyphenol structures including groups that are ubiquitous in plant kingdom (*i.e.* flavonoids) and others that are restricted to a limited range of

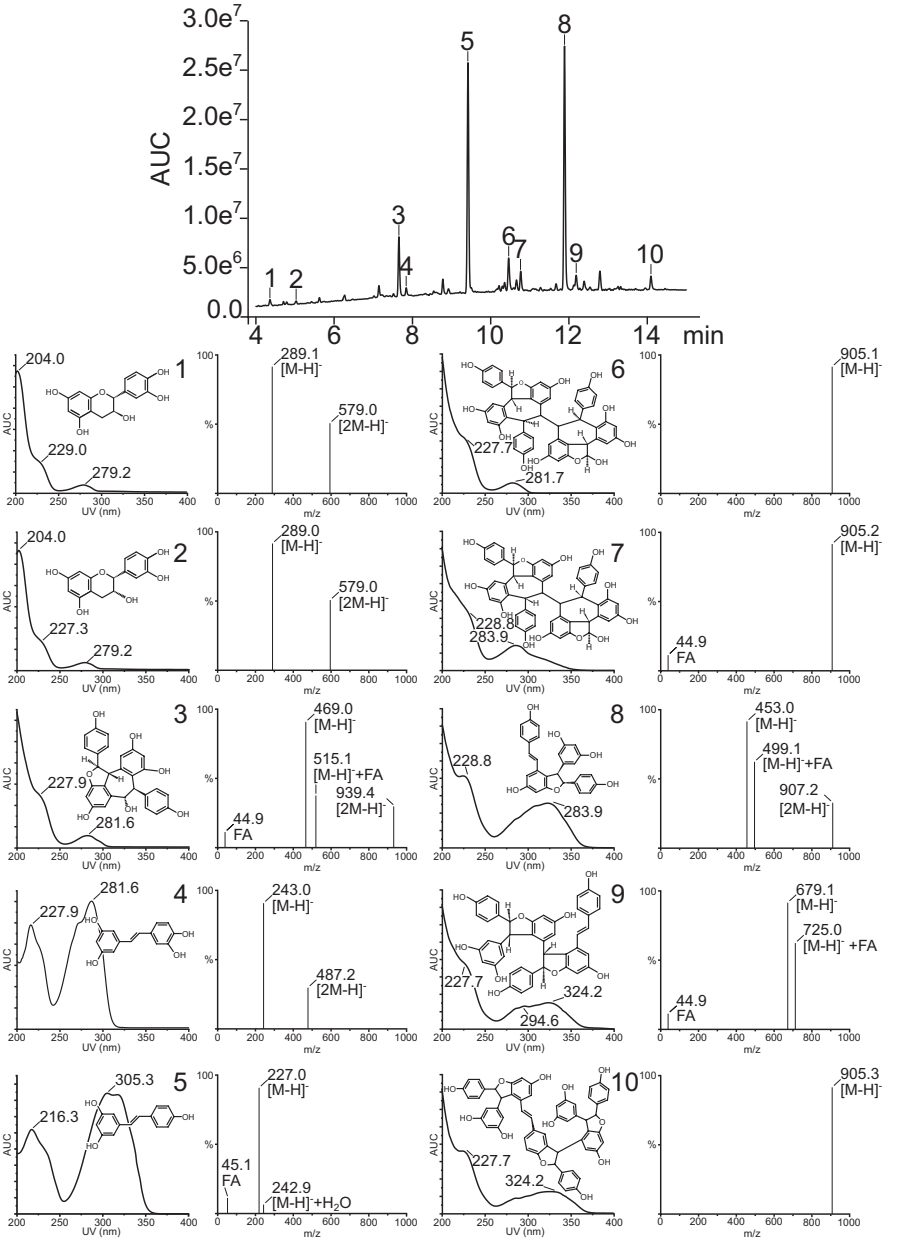

Fig. 9.1 UPLC-DAD-MS analyses of grape cane extracts with UV and mass spectra of the ten major compounds. (1) catechin, (2) epicatechin, (3) ampelopsin A, (4) *E*-piceatannol, (5) *E*-resveratrol, (6) hopeaphenol, (7) isohopeaphenol, (8) *E*-ε-viniferin, (9) *E*-miyabenol C, (10) *Z*/*E*-visitin B

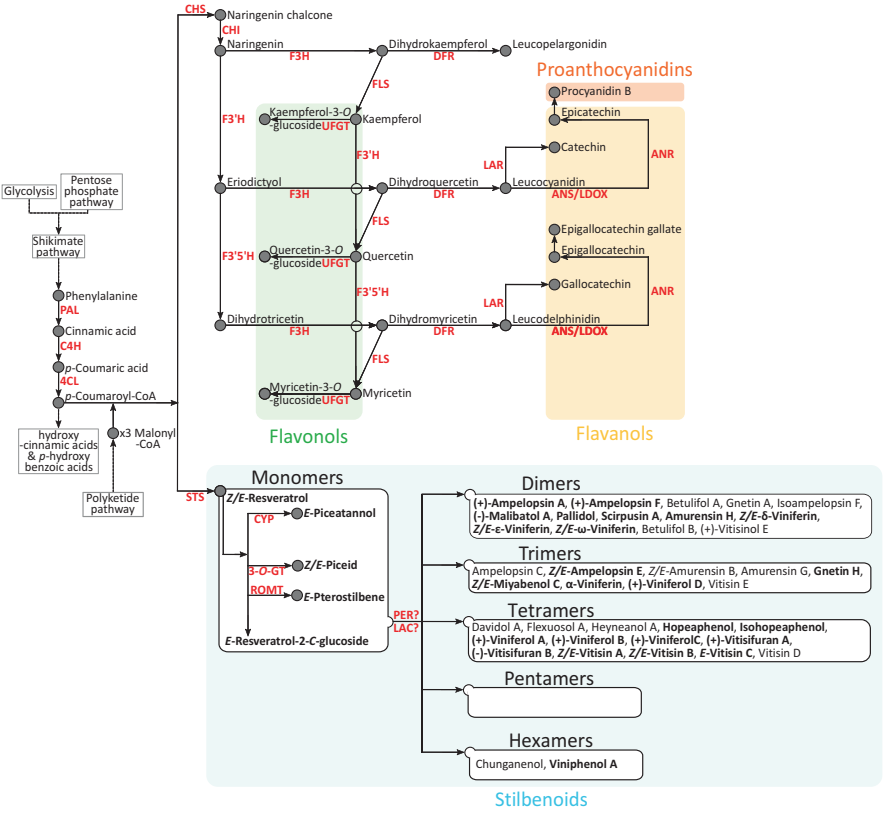

Fig. 9.2 Polyphenol biosynthetic pathway in grape canes. Biosynthetic enzymes are presented in red: phenylalanine ammonia-lyase (PAL); cinnamate 4-hydroxylase (C4H); 4-coumarate:CoA (4CL); chalcone synthase (CHS); chalcone isomerase (CHI); flavanone 3-hydroxylase (F3H); flavanone 3′-hydroxylase (F3'H); flavanone 3′,5′-hydroxylase (F3'5'H); flavonol synthase (FLS); UDP glucose:flavonols-3-*O*-glucosyltransferase (UFGT); dihydroflavonol (DFR); leucoanthocyanidin reductase (LAR); anthocyanidin synthase (ANS); leucocyanidin oxygenase (LDOX); anthocyanidin reductase (ANR); stilbene synthase (STS); 3-*O*-glucosyl-transferase (3-*O*-GT); (*E*-resveratrol)-*O*-methyltransferase ((R)-OMT); cytochrome P450 (CYP); peroxidase (PER); laccase (LAC). Stilbenoids in bold are present in *V. vinifera* L. while others refer to non-*vinifera* grape varieties

taxa like isoflavonoids and stilbenoids [26]. Grape canes represent polyphenolic-rich botanical extracts containing numerous polyphenols that belong to flavonols, flavanols, proanthocyanidins and stilbenoids resulting from activities of several biosynthetic enzymes described in Fig. 9.2. From the same substrates *p*-coumaroyl-CoA and three units of malonyl-CoA, two polyketide synthase; chalcone synthase (CHS) and stilbene synthase (STS) produce the same linear tetraketide intermediate. However, CHS performs a Claisen condensation giving the naringenin chalcone core, whereas STS performs an aldol condensation coupled to a decarboxylation giving the stilbenoid skeleton of *E*-resveratrol [27]. It is assumed that competitive

activity between these two enzymes regulates metabolic fluxes towards flavonoids or stilbenoids biosynthesis. Whereas in berries anthocyanins are mainly accumulated consecutive to CHS activity, in grape canes stilbenoid accumulation is preponderant, indicating higher STS activity in lignified tissues. In grape canes, only two CHS downstream products are accumulated; catechin and epicatechin [28]. However, a vast array of STS downstream products are present as presented in Fig. 9.2. About 60 resveratrol derivatives have been characterized so far in *Vitis vinifera* grape canes (bold compounds in Fig. 9.2) and beyond, a higher chemical diversity has been described in non-*vinifera* grape varieties [29]. Whereas flavonoid biosynthesis is well known, stilbenoid pathway is not so far explained and only few enzymes have been characterized (Fig. 9.2). Identification of these enzymatic steps, particularly enzymes responsible of stilbenoid oligomerizations, will be a great challenge towards the comprehension of stilbenoid accumulation in *Vitis vinifera* particularly in a context of plant defense and for the selection of resistant varieties.

9.4 Preparation of Raw Biomass and Polyphenol Extraction

9.4.1 Storage Period

Interestingly, polyphenol accumulation in grape canes can be induced after pruning and the accumulation rate is influenced by storage conditions. A *E*-resveratrol increase after a 6-month storage of grape canes has been observed in numerous varieties [30, 31]. The storage conditions also induced vanillin accumulation in Airén and Cencibel varieties [32]. *E*-resveratrol amount in grape canes was induced by 40 fold in only 6 weeks when stored as 10-cm sections at 15–20 °C in the obscurity [18]. Whereas PAL, 4CL and C4H genes were constitutively expressed throughout this storage, STS was induced during the first 4 weeks of storage, suggesting that grape canes are transcriptionally active even after pruning [18]. *E*-resveratrol accumulation relied on *de novo* biosynthesis and not on oligomer degradation [18]. In a recent study, our group took advantage of the stress response of freshly-pruned grape canes to induce stilbenoid biosynthesis [33]. As a result, cutting the grape canes in sections of 0.5–1 cm immediately triggered a transient expression of PAL and STS genes, followed by a rapid *E*-resveratrol and *E*-piceatannol accumulation in only 2 weeks. The simultaneous induction of jasmonate signaling, PR proteins and stilbenoid metabolism suggested a global defense response in freshly-pruned canes [33]. Finally, this induction process enables shortening the storage step from 6 months to only 2 weeks. At industrial scale, it represents a great advantage for the preparation of raw biomass before stilbenoid extraction.

9.4.2 Grinding Process

Grinding of raw biomass could produce different sizes of particles affecting extraction yield. Smaller particles allow better extraction yields. Adequate biomass fragmentation provides better solvent access to plant tissues. Therefore, the grinding process is a key factor to reach high extraction yield. When grinding grape canes, Riquelme et al. [34] reported that the reduction of sample size from 2 to 1 cm, increased the extraction yield by 37%, while the reduction from 1 to 0.5 cm increased extraction yield again by 7%. The particle size reduction produced by grinding positively affects polyphenol recovery from grape matrix and promotes surface increase between solid and liquid phase [35]. Soural et al. [36] showed that grinding process is crucial in obtaining higher yields of extraction. They compared extraction of cut versus powdered grape canes by various extraction methods. Higher extraction yields were always obtained with powdered materials. Bucić-Kojić et al. (2007, [37]) studied the influence of grape seed particle size on polyphenol extraction. Four particle sizes were tested; >0.63 mm, 0.63–0.4 mm, 0.4–0.16 mm and 0.16–0.125 mm. In this study, highest extraction yields were obtained for the smallest particle class. Following the grinding of grape canes, an average particle size of less than 1 mm is usually obtained [18, 38]. We currently perform a grinding in two steps; before and after lyophilization. First, using a cooled analytical grinder for 2 min (Ika-Werke, Staufen, Germany) and then by using a cutting mill (Polymix PX-MFC 90 D, Kinematica AG, Switzerland) [33].

9.4.3 Extraction Using Maceration

Extracting stilbenoids from grape canes by maceration was optimized through several parameters including solvent type, temperature and solid-liquid ratio. At first, Karacabey and Mazza (2008, [39]) obtained a maximal extraction for *E*-resveratrol and *E*-ε-viniferin at 83.6 °C with 58% and 68% of ethanol in water, respectively. Then, they found an optimal solid-liquid ratio of 103.6 mg.L^{-1} with a solvent mixture of ethanol:water (55:45; v/v) at 83.6 °C [39]. Using response surface methodology, Karacabey and Mazza (2010, [40]) optimized the antioxidant activity of grape cane extracts. Ethanol concentration and temperature impacted the antioxidant capacity of grape cane extracts to a greater extent than solid-liquid ratio [40]. Riquelme et al. [34] also studied the optimal conditions for stilbenoid extraction from stored grape canes. Optimal extraction for *E*-resveratrol, *E*-ε-viniferin and *E*-piceatannol was confirmed by using ethanol:water mixture (80:20; v/v), cane sections from 1.0 to 0.5 cm, solid-liquid ratio 1:10 (m/v), temperature 108 °C and extraction time 50 min. High temperatures resulted in better solvent penetration into the grape cane matrix, by reducing the solvent viscosity and surface tension. On the other hand, extraction yield decreased to 53% after next 40 min, probably due to

thermal degradation of the compounds. As previously described, 80 °C was recognized as the optimal temperature [34].

All these experiments only focused on *E*-resveratrol and *E*-ε-viniferin which have similar polarities and close solubility. Stilbenoid oligomers like trimers (*i.e.* *E*-miyabenol C) and tetramers (*i.e.* hopeaphenol, isohopeaphenol and vitisin B) are less polar and more lipophilic than monomers and dimers and may need higher ethanol concentration or the use of another solvent to reach their optimal extraction conditions. Modification in extraction solvent by mixing solvents with various polarities can enhance the solubility of the desired solute. The change in solvent mixture composition will affect the physical properties of the mixture such as density, dynamic viscosity, and dielectric constant. The solubility of compounds, thus, can be modified by a change in ethanol concentration or applying another solvent component [39]. Lambert et al. [28] isolated polymeric stilbenoids (hopeaphenol and *E*-miyabenol C) with a solvent mixture of methanol and water (1:1; v/v) at room temperature. The resulting mixture of stilbenoids was rich in the desired compounds including more polar like *E*-resveratrol and also less polar like vitisin B. The application of low temperature can be beneficial not only for temperature-sensitive polyphenols and higher extraction efficiency but also for economic reasons. Each extraction process should represent a good compromise between cost (*e.g.* energy consumed, solvents used and time required) and efficiency in the scope of industrial scale-up.

9.4.4 Ultrasound Assisted Extraction

Ultrasounds are known to favor solvent penetration into cells and lead to fast release of cell content through cell wall disruption [41]. In addition, it implements solvent substitution by non-toxic ones, reduces energy consumption compared to conventional methods and minimizes the process times [42]. Ultrasound frequency is a key factor of efficiency and a low frequency range (20-40 kHz) ensure high extraction yields [43]. It is also well adapted to heat-sensitive compounds allowing high extraction yield at low temperature [43]. *E*-resveratrol has been extracted from grape leaves by ultrasound-assisted extraction (UAE) with aqueous ethanol. Various parameters were investigated including ethanol:water ratio, temperature, sample:solvent ratio and extraction time. The maximum *E*-resveratrol concentration was obtained when the extraction was performed with 40% aqueous ethanol solution at 50 °C for a minimum of 30 min. Extraction yield decreased when the temperature exceeded 50 °C due to the degradation of *E*-resveratrol at higher temperatures [44]. According to further research, resveratrol oligomers are more resistant to high temperatures. Piñeiro et al. [45] analyzed the influence of temperature, ultrasound amplitude, ultrasonic cycle duration, ultrasonic probe type, time, sample:solvent ratio and solvent type (mixtures of ethanol and water) on stilbenoid extraction. Best results were achieved at 75 °C in 80% ethanol using single extraction cycle. Main stilbenoids isolated from grape stems (*E*-piceatannol, *E*-resveratrol,

isorhapontigenin, *E*-ε-viniferin and vitisin-B) were extracted for 15 min. The optimal sample:solvent ratio applied in the experiment was 1:30 (m/v), diameter probe tip 7 mm, ultrasound amplitude 70% and the cycle time 0.7 s. Less polar resveratrol oligomers, like vitisin B was only extracted with higher content of ethanol (80%). Later, Piñeiro et al. [46] reduced extraction time to 10 min using ultrasound assisted extraction at 75 °C for a sample:solvent ratio of 1:40 (m/v) in a solvent mixture of ethanol:water (60:40; v/v).

9.4.5 Microwave Assisted Extraction

Microwave assisted extraction (MAE) employed electromagnetic radiations with frequency ranging from 0.3 to 300 GHz and offered a rapid energy delivery through the heating of both solvent and sample matrix [41]. Microwave radiations lead to high localized temperature and pressure and triggered cell disruption, thus facilitating the migration of targeted compounds in surrounding solvent [47]. Employed on wine lees, MAE provided better polyphenol extraction efficiency than conventional extraction methods and reduced the extraction time from 24 h to 17 min [48]. On grape canes extract, extraction time was reduced to 5 min using ethanol:water (80:20; v/v) mixture at 125 °C [49]. Despite high extraction yields in a short time with low solvent volume, MAE is limited to polar compounds and useless for volatile metabolites [41].

Selvamuthukumaran and Shi (2017, [50]) showed also that MAE offers high extraction yield in short time, saving energy and costs compared to super critical CO_2 extraction.

Several studies reported that modifications of MAE can enhance extraction yield of stilbenoids from grape canes. The applied electromagnetic field frequencies of 915 MHz and 28 GHz with the combination of pressure between 5,000 and 95,000 Pa allowed to increase MAE yield. Four stilbenoids (*E*-piceatanol, *E*-resveratrol, *E*-ε-viniferin and vitisin B) were isolated using ethanol/water mixture comprising from 10 to 70% of ethanol (v/v) at room temperature during 30–125 min. The dry extract contained 2 times more *E*-ε-viniferin, and 3–6 times more vitisin B than conventional ethanolic extracts [51].

9.4.6 Pulsed Electric Field

Pulsed electric field (PEF) assisted extraction increased cell membrane permeabilization *via* the creation of high electrical potential differences [52]. It facilitates cell compound releasing through cell semi-permeability loss [53]. PEF was employed to increase extraction yields in food industry for the purification of chlorophyll, carotenoids, sucrose and oils as well as anthocyanidins and wine polyphenols [54]. The

PEF assisted extraction is a non-thermal treatment and might be useful to recover heat-sensitive compounds [55]. This extraction technic was used on grape seeds and fresh grape canes and allowed an electrical disintegration index Z of 0.16 for an energy input of 100 $kJ.kg^{-1}$ [56, 57]. High voltage electrical discharges (HVED) assisted extraction required little changes to PEF apparatus, but enhanced the Z value to 0.546 with the same applied energy [56]. It generates electron avalanche that occurs from the positive to the negative electrode and triggers secondary effects that lead to particle fragmentation and cell structure damage and thus enhance the molecule extraction from cells [58]. This method of extraction was used to obtain polyphenols from grape pomace and peel. Two methodologies were compared: PEF and enzymatic hydrolysis of polyphenolic compounds at room temperature. The application of the PEF showed better results than the pectinase treatment. Therefore, for better extraction efficiency, the use of PEF is recommended [59].

9.4.7 Supercritical Fluid Extraction

Supercritical fluids exhibit both gas and liquid properties and supercritical state is reached when solvent temperature and pressure raise over their critical values [41]. Supercritical fluid extraction (SFE), which uses primarily CO_2 as extraction medium, has been widely used for the extraction of nonpolar substances from natural plants. Recent studies showed that it can also be used for extraction of polar compounds, such as flavonoids and polyphenols, by adding a proportion of polar solvent, such as methanol and ethanol, as modifier [60]. Supercritical CO_2 is characterized by low supercritical point (31 °C and 74 bar, [61]) and can replace organic solvent in non-explosive, non-toxic and inexpensive ways [62]. SFE enables clean (without solvent remaining), rapid and selective extraction and is well adapted to the analysis of thermolabile and volatile plant products [63, 64]. SFE was successfully employed on grape skins and pomace [65–67]. On grape waste containing stalks, supercritical fluid extraction allowed the recovery of bioactive antioxidant molecules [68]. To our knowledge, the SFE was not assayed on grape canes solely but might provide good results on the extraction of highly bioactive compounds. Wenli et al. [60] used this method with ethanol as co-solvent for the extraction of resveratrol and its glycoside piceid from *Polygonum cuspidatum*. The yield obtained by SFE was comparable with those obtained by traditional organic solvent methods, indicating that SFE is an alternative method for extraction of the two stilbenoids. *E*-resveratrol extraction in supercritical fluid conditions was also described by Pascual-Martí et al. [69]. Extraction process conditions like pressure, the modifier concentration (ethanol), and extraction time were optimized. The optimal extraction conditions developed in this research were 40 °C, 150 bar, 7.5% ethanol and extraction time in 15 min. Interestingly, SFE of *E*-resveratrol from grape-skin samples provided a clean-up suitable for HPLC analysis. There was no need for solvent evaporation or sample purification. The SFE method is suitable for routine analysis in quality control of industrial processes. It should be soon tested for stilbenoids extraction from grape canes.

9.5 Biocontrol Activities of Grapecane Extracts Towards Several Major Grapevine Diseases

Grapevine culture is susceptible to numerous fungal diseases depending on cultivar resistance and climatic conditions. Grapevine production is highly dependent on the use of various pesticides. Treatment frequency index (TFI) in viticulture is the highest of all crops after apple and potato production. Within a single growing season, the control of grape diseases required between 8 and 25 treatments per year in French vineyards [2]. Most of the fungicides are used to control *Plasmopara viticola*, *Erysiphe necator* and *Botrytis cinerea*, the respective causal agents of downy mildew, powdery mildew and gray mold [2]. Botanical extracts containing natural antifungal compounds like polyphenols of GCE represent an eco-friendly alternative to agrochemicals. Here, we present the recent developments that evaluate the potential of GCE to control several important diseases of grapevine.

9.5.1 Downy Mildew

Plasmopara viticola (Berk.& Curt.) Berl.& De Toni is a biotrophic obligate endoparasite oomycete causing downy mildew. This parasite affects both quantity and quality of wine production. During vegetative period, *P. viticola* develops only in living tissues where it competes for host assimilates, afterwards it overwinters as oospores in leaf litter and soil where it can maintain in dormant state for 3–5 years. Under favorable climatic conditions (relatively warm and humid), oospores germinate and release zoospores [70]. First contaminations spread with rain drops and reach grapevine leaves. Following early infection, disease propagation in vineyards depends on the rate of secondary infection cycles that could be limited to 5 days in optimal conditions. Early symptoms appear as yellowish, oily lesions on upper leaf surface. Following disease development, sporangia and sporangiophores will form a white downy mycelia on leaves and bunches. Ultimate stages result in premature defoliation and reddish brown berries. Initial bioassays using purified stilbenoids [71] highlighted properties of *E*-resveratrol and viniferins to inhibit germination, zoospores mobility and sporulation of *P. viticola*. Table 9.1 gives the whole picture of *in vitro* studies conducted on *P. viticola* with IC_{50} (concentration that inhibited 50% of the disease development) for many grape stilbenoids. Tetramers of resveratrol were the most effective compounds with by descending order: *E*-vitisin B, hopeaphenol, *E*-pterostilbene, *E*-ε-viniferin, α-viniferin and δ-viniferin (Table 9.1). Additionally, stilbenoid-enriched extracts of grape canes also cause damages on *P. viticola* cells. Sporangia cell shrinkage as well as cell membrane and organelle disorganization were observed after 6-h treatments leading to the complete coagulation of cytoplasm after 24 h [72]. In greenhouse experiments, the use of a GCE against *P. viticola* reduced disease incidence and severity respectively by 59–69% and 83–88% [73]. As observed for pure stilbenoids, GCE inhibits germination and

Table 9.1 Stilbenoids expressed as concentration (μM) causing 50% inhibition (IC50) against *Plasmopara viticola*

	IC_{50} (μM)			
	Plasmopara viticola			
Molecules	Spores germination	Zoospores mobility	Sporulation	References
E-resveratrol	>877	>877		[95]
	>877	>877		[96]
	145	192		[71]
	121	122		[72]
			484	[97]
E-ε-viniferin	41,6	27,5		[95]
	41,6	27,5		[96]
	71,2	73		[71]
	63	66		[72]
			155	[97]
δ-viniferin	>220	>220		[95]
	14,7	14,6		[71]
	63	66		[72]
α-viniferin	51,6	16,2		[95]
	51,6	16,2		[96]
E-pterostilbene	17,5	8,9		[96]
	12,7	28,3		[71]
Ampelospin A	282	124		[72]
			934	[97]
E-miyabenol C			103	[97]
Hopeaphenol	23	17		[72]
			18	[97]
Isohopeaphenol			45	[97]
Ampelopsin H	282	92		[72]
Vitisin A			20	[97]
E-vitisin B	12	13		[72]
			12	[97]

zoospore sporulation [73]. In field trials, GCE reduced average disease incidence by −35% and −38% and disease severity by −35% and −43% on leaves and clusters, respectively [74]. Under artificial infection, the ability of GCE to control downy mildew development was compared to different dilution of copper sulfate (Bouillie bordelaise). As a result, the activity of GCE corresponded to 1 $g.L^{-1}$ copper sulfate treatment [74].

9.5.2 Powdery Mildew

Ersiphe necator, the causal agent of powdery mildew on grape, is a biotrophic obligate fungus that triggers ash-grey to white powdery structures mainly visible on the upper side of leaves. During winter, the fungus survives in infected buds and also on bark as chasmothecia, tiny spherical fruiting structures colored from yellow to dark. Primary infections occur under wet and temperate conditions to produce conidiospores that spread by wind dispersal. Conidiospores germinate and penetrate epidermal tissues through haustoria that absorb nutrients from grapevine. Several infection cycles can occur during the growing season within 5–12 days depending on temperatures. Along with the powdery mildew growth, the disease progressive extension is accompanied by islets of necrotic cells. Following infection by *E. necator*, Schnee et al. [75] highlighted that stilbenoids, particularly δ-viniferin and ε-vinferin, are key components of grapevine defense mechanism. Later the same authors reported *in vitro* efficacy of a methanolic fraction of GCE to inhibit the conidial germination of *E. necator* [72]. Although these results seem encouraging, no further data are available concerning *in vitro* toxicity of pure stilbenoids and their possible mode of action.

9.5.3 Gray Mold

Botrytis cinerea, the causal agent of gray mold also called Botrytis bunch rot, is an ubiquitous polyphagous necrotrophic fungus that causes severe yield losses by infecting ripe berries. The pathogen overwinters in mummified berries, dead grape tissues and other plant hosts [76]. During spring, when temperatures reach 15–20 °C with 95% of relative humidity, conidia are produced and spread by wind and water causing primary infections. *B. cinerea* spores germinate easily on wet leaves and produce a germ tube that penetrate the cuticle by destructing cell wall. Berries are covered by a grey mycelium and become shriveled. *In vitro* assays showed the toxicity of pure stilbenoids on *B. cinerea*. The most active compounds by descending order were *E*-pterostilbene, α-viniferin and *E*-ε-viniferin (Table 9.2). Note that these stilbenoids are less active on grey mold than on downy mildew. The toxicity of *E*-resveratrol tetramers was not reported but might contribute to the total activity of GCE. *E*-resveratrol led to cytological abnormalities of *B. cinerea* conidies [77]. *E*-pterostilbene triggered ribosome, endoplasmic reticulum, mitochondrial and nuclear membrane destruction as well as conidial respiration inhibition and germination [78–80]. Ethanolic and methanolic extracts of grape canes exhibited growth inhibition of *B. cinerea* mycelium [72]. The mode of action of GCE on *B. cinerea*

Table 9.2 Stilbenoids IC_{50} against *Botrytis cinerea*

Molecules	IC_{50} (μM) *Botrytis cinerea* Growth	Spores germination	References
E-resveratrol	438	>877	[98]
		>877	[95]
		>877	[96]
		395	[99]
	500		[100]
E-ε-viniferin		220	[95]
		220	[96]
δ-viniferin		>440	[95]
α-viniferin		143	[95]
		72	[96]
E-pterostilbene		70	[96]
		70	[99]

Table 9.3 Stilbenoids IC_{50} against causal agent of wood diseases

Molecules	IC_{50} (μM) *Eutypia lata* Fungal growth	*Diplodia seriata*	*Neofusicoccum parvum*	References
E-resveratrol	>500			[100]
E-piceatannol	>500	299	>500	[90]
E-ε-viniferin	>500	260	>500	[90]
E-pterostilbene	251	163	250	[90]

has been investigated. GCE as preventive treatment was able to reduce pathogen development on infected leaves. GCE was able to inhibit mycelium growth and also to activate a general plant defence mechanism including H_2O_2 production, activation of mitogen-activated protein kinase (MAPK) and phytoalexins accumulation [81]. Despite *B. cinerea* produces a laccase-like stilbene oxidase able to detoxify *E*-pterosilbene and *E*-resveratrol [82, 83], this activity can be inhibited by some flavan-3-ol-type polyphenols [84] thus limiting the *B. cinerea* defence against GCE (Table 9.3).

9.5.4 Grapevine Trunk Diseases

Grapevine trunk diseases (GTD) is probably the most relevant challenge for viticulture nowadays [85]. The loss of grapevine increased from 1.8% in 2003 to 10.5% in 2007 [86]. During the 1990s, the grapevine planting boom participated to the spread of potentially contaminated materials. Moreover in 2003, the prohibition of the most efficient chemical products in several countries due to human health concerns,

increases the impact of GTD. Esca, Eutypa and Botryosphaeria dieback represent the main wood diseases [87]. These pathogens cause the apoplexy of the vine aerial part [88] and lead to foliar wilting, necrosis and white mold on the trunk [89]. The potential of pure grape stilbenoids against GTD has been assessed. Esca associated strains as *Neofusicoccum parvum* and *Diplodia seriata* and *Eutypia lata*, the causal agent of Eutypia, were submitted to *in vitro* assays (Table 9.4). Best results were obtained for *E*-pterostilbene with IC_{50} evaluated at 163, 251 and 250 μM for *D. seriata*, *E. lata*, and *N. parvum*, respectively [90]. In any case, stilbenoids showed a fungistatic activity and not a fungitoxic activity demonstrating the tolerance of GTD pathogens towards *E*-resveratrol and its derivatives *E*-pterostilbene, *E*-ε-viniferin and *E*-piceatannol.

9.6 Understanding the Variation in Composition of Grape Canes

9.6.1 Towards Less Quantification Bias

Grape canes extracts are composed of ten major polyphenols including *E*-ε-viniferin, hopeaphenol, *E*-resveratrol, ampelopsin A, isohopeaphenol, *Z*/*E*-vitisin B, *E*-miyabenol C, catechin, *E*-piceatannol and epicatechin [74]. As depicted in the previous paragraph, these molecules are active against several important grapevine diseases and consequently, their concentration in GCE is often monitored in studies dealing with the biocontrol of grapevine diseases by GCE. Because very few stilbenoids from GCE are commercially available, absolute quantifications have been sometimes estimated as resveratrol equivalent [31, 91, 92]. In this case, the quantification of resveratrol oligomers are underestimated. Indeed, it is known that the molar extinction coefficient of resveratrol oligomers decreases progressively with polymerization degree. As an example, when *E*-ε-viniferin content is expressed as resveratrol equivalent, the quantity is then underestimate by a factor two [28] and this quantification bias is even higher when trimers and tetramers are estimated as resveratrol equivalent. Accordingly, in-lab purification of stilbenoids using CPC and preparative HPLC methods must be encouraged to generate analytical standards of high purity. The use of calibration curve for each stilbenoid is a prerequisite for the reliable quantification of active compounds in GCE. Additionally, another bias specific to *E*-resveratrol and *E*-piceatannol quantification has been identified. Before pruning of grape canes in vineyards, these two molecules are present at very low concentrations. They largely accumulate in freshly harvested grape canes as a stress response following cuttings. Consequently, their presence in large amount in GCE is the consequence of a post-harvest accumulation with variable rates according to storage conditions [18, 33]. Before evaluating the total stilbenoid amount in GCE, it is therefore fundamental to ensure that *E*-resveratrol and *E*-piceatannol induction in freshly-pruned grape canes reached a plateau.

Table 9.4 Stilbenoids concentrations in *Vitis vinifera* L. grape canes expressed as $mg.kg^{-1}$ of dry weight

Variety	*E*-resveratrol	*E*-piceatannol	*E*-ε-viniferin	*E*-ω-viniferin	*E*-ampelopsin A	Miyabenol C	Hopeaphenol	isohopeaphénol	*E*-vitisin B	Total	References
Cabernet sauvignon	871 ± 202	735 ± 142	2379 ± 1123			30 ± 12	1346 ± 294		420 ± 109	5781	[28]
	1948,5 ± 132,5	212 ± 15	512,5 ± 42							2673	[92]
	1621 ± 14	573 ± 31	2585 ± 68	NQ	ND				2159 ± 81	6938	[94]
	2407 ± 110	283 ± 8	333 ± 10				79 ± 14		NQ	3102	[31]
	1639 ± 15		2203 ± 29							3842	[30]
Carmenère	2811 ± 98	212 ± 23	354 ± 7				NQ		NQ	3346	[31]
	2493 ± 155	186 ± 4	414 ± 20							3093	[92]
Chardonnay	190 ± 87	190 ± 67	2089 ± 334			NQ	766 ± 149		NQ	3235	[28]
	3175 ± 159	312 ± 16	3759 ± 188	229 ± 11	1896 ± 95		1527 ± 76	260 ± 13	178 ± 9	13,452 ± 673	[18]
	2859 ± 270	353 ± 65	3033 ± 318		2533 ± 299	72 ± 13	2606 ± 516	560 ± 123	160 ± 45	12,176 ± 1649	[25]
Chenin	794 ± 161	1227 ± 267	2218 ± 274			35 ± 23	623 ± 175		NQ	4897	[28]
	4908 ± 245	480 ± 24	6059 ± 303	354 ± 18	148 ± 7		1361 ± 68	182 ± 9	411 ± 21	16,732 ± 837	[18]
	1625 ± 587	277 ± 133	2667 ± 1190		308 ± 164	67 ± 40	2216 ± 975	244 ± 155	205 ± 93	7609 ± 3337	[25]
Cinsault	486 ± 226	298 ± 268	1629 ± 100			106 ± 12	339 ± 96		NQ	2858	[28]
	446 ± 7	90 ± 3	266 ± 4							803	[92]
	1506 ± 48	143 ± 1	324 ± 10				NQ		NQ	1974	[31]
Gamay	980 ± 201	843 ± 138	1828 ± 157			NQ	1085 ± 182		102 ± 53	4838	[28]
	5803 ± 290	551 ± 28	4857 ± 243	288 ± 14	4215 ± 211		2950 ± 147	415 ± 21	1420 ± 71	22,367 ± 1118	[18]
	2923 ± 906	382 ± 111	2808 ± 371		3679 ± 453	87 ± 13	3466 ± 583	845 ± 265	773 ± 133	14,963 ± 2835	[25]
Gewürztraminer	649 ± 290	490 ± 150	2199 ± 379			NQ	1118 ± 357		1116 ± 380	5572	[28]
	4628 ± 568	457 ± 38	744 ± 105							5829	[92]
	3599 ± 117	233 ± 19	542 ± 29				72 ± 7		50 ± 1	4446	[31]
Malbec	3589 ± 485	235 ± 3	603 ± 91							4428	[92]

Variety	*E*-resveratrol	*E*-piceatannol	*E*-ε-viniferin	*E*-ω-viniferin	*E*-ampelopsin A	Miyabenol C	Hopeaphenol	isohopeaphénol	*E*-vitisin B	Total	References
	3316 ± 166	363 ± 18	5920 ± 296	349 ± 17	290 ± 14		999 ± 50	223 ± 11	185 ± 9	14,613 ± 731	[18]
	1869 ± 625	381 ± 84	3352 ± 488		395 ± 68	52 ± 17	1277 ± 276	303 ± 87	41 ± 13	7670 ± 1658	[25]
Merlot	1181 ± 189	947 ± 353	2263 ± 220			22 ± 14	642 ± 163		146 ± 48	5201	[28]
	1936 ± 65	170 ± 8	294 ± 12				32 ± 3		NQ	2432	[31]
	2409 ± 103		1656 ± 355							4065	[30]
Moscatel de Alejandria	1038 ± 79	97 ± 11	316 ± 41							1451	[92]
	4941 ± 128	288 ± 22	343 ± 23				NQ		NQ	5571	[31]
Pinot Noir	1526 ± 293	1710 ± 224	3737 ± 421			73 ± 22	1126 ± 294		313 ± 156	8485	[28]
	3676 ± 353	284 ± 52	700 ± 60							4660	[92]
	2827 ± 61	202 ± 16	598 ± 23				76 ± 11		130 ± 13	3833	[31]
	1908 ± 124		2790 ± 123							4698	[30]
	4725 ± 236	771 ± 39	6563 ± 328	322 ± 16	1116 ± 56		1270 ± 64	301 ± 15	307 ± 15	19,113 ± 956	[18]
	2615 ± 391	315 ± 92	3537 ± 611		1449 ± 354	110 ± 23	2411 ± 696	645 ± 176	491 ± 131	11,573 ± 2474	[25]
Riesling	605 ± 258	270 ± 101	1716 ± 441			174 ± 12	1468 ± 601		88 ± 54	4321	[28]
	1994 ± 34		1928 ± 96							3922	[30]
Sauvignon Blanc	730 ± 34	607 ± 294	2697 ± 167			36 ± 17	841 ± 263		369 ± 212	5280	[28]
	1360 ± 20	182 ± 14	508 ± 44				74 ± 6		NQ	2125	[31]
	2010 ± 124		3329 ± 296							5339	[30]
	2908 ± 145	563 ± 28	6644 ± 332	550 ± 28	1359 ± 68		1883 ± 94	238 ± 12	2896 ± 145	19,082 ± 954	[18]
	804 ± 366	162 ± 81	2673 ± 1319		840 ± 433	90 ± 45	1812 ± 955	276 ± 136	646 ± 335	7303 ± 3670	[25]
Sémillon	872 ± 263	471 ± 205	2448 ± 186			NQ	287 ± 124		252 ± 106	4330	[28]
	2112 ± 18	216 ± 5	621 ± 13				NQ		NQ	2949	[31]
Syrah	481 ± 373	460 ± 253	2507 ± 462			38 ± 14	586 ± 456		182 ± 244	4254	[28]
	3591 ± 188	261 ± 14	368 ± 14				NQ		NQ	4221	[31]
Tintorera	2331 ± 58	179 ± 8,5	358,5 ± 5,5							2868	[92]
	4074 ± 125	308 ± 12	512 ± 2				59 ± 10		NQ	5003	[31]

9.6.2 Screening of Grapevine Varietal Diversity

Common grapevine (*V. vinifera* L. subsp. *vinifera*) presents a huge varietal diversity estimated at 10,000 varieties. This diversity originated from several domestication centers from wild grapevine populations (*V. vinifera* L. subsp. *silvestris*), spontaneous crosses between domesticated and undomesticated vines and from varietal selection [93]. *V. vinifera* germplasm is linked to important agronomical traits including phenology, fertility and organoleptic characters as well as adaptation to environmental constraints like pathogenic pressure and water stress. It is assumed that the polyphenol content in GCE might change according to the variety, giving rise to different degrees of efficacy to control pathogen development. In this way, several studies have compared the polyphenol content in diverse varieties. The Table 9.4 presents only the reports that used calibration curves based on purified stilbenoids. Lambert et al. [28] analyzed the grape cane content of 16 different varieties and reported an amplitude of total stilbenoid concentration from 3469 (Grenache) to 8485 mg.kg^{-1}DW (Pinot Noir). Similarly, in a study performed on 8 varieties of the French Loire Valley, a two-fold difference was found between Chardonnay (13,452 mg.kg^{-1}DW) and Gamay (22,367 mg.kg^{-1}DW [18]). Therefore, it is obvious that varietal choice strongly influences the stilbenoid composition of GCE, leading to different degree of toxicity on grape diseases. Further analyses on large germplasm collection will help to screen for polyphenol-rich grape varieties.

Table 9.4 also enables the comparison of stilbenoids levels from the same grape variety over different studies implying different geographical origins. The case study of *E*-ε-viniferin content is relevant. In Cabernet Sauvignon, *E*-ε-viniferin content varied from 333 to 2585 mg.kg^{-1}DW over five different studies where grape canes originated from France [28, 94], Chili [31, 92] and Germany [30]. In Pinot Noir, *E*-ε-viniferin content varied from 598 to 6563 mg.kg^{-1}DW over six different studies where grape canes were harvested in France [18, 28], Chili [31, 92] and Germany [30]. It is therefore obvious that not only the varietal origin should be taken into account when assessing stilbenoids variation in GCE but also the geographical origin. Besides location, the year of collection should also be considered. Indeed, stilbenoids are phytoalexins and their concentration might vary according to environmental conditions including soil, climate and agronomical practices. As an example it has been reported that downy mildew infection impacts stilbenoid composition in grape canes [18]. Downy mildew strongly induced *E*-ε-viniferin and depleted *E*-resveratrol contents. Besides varietal impact, it is then assumed that terroir key-elements like vintage, climate and soil factors will determine the concentration of active compounds in GCE.

9.7 Conclusions

Grapevine is widely planted to produce wines and table grapes. This production generates by-products in large amounts, whereas pomace is well recycled for distillation, grape canes remain largely under-valorized despite the presence of stilbenoids useful for the biocontrol of grape diseases. These compounds showed good inhibition capacity against downy mildew and grey mold, however activity against powdery mildew needs additional results. Stilbenoids showed also fungistatic activity against grapevine trunk diseases, a real challenge for viticulture. During the prospection of efficient biocontrol solutions suitable for sustainable agriculture, a decisive step is the demonstration of real activity in field conditions. A first large scale study in the vineyards showed the ability of GCE to control downy mildew development on three different susceptible cultivars in three growing zones of France. Therefore, stilbenoid-enriched GCE can be seriously envisaged as an eco-friendly alternative to classical fungicides. Future steps will cover the registration of GCE as biopesticides. European Union supports the use of less harmful substances through the current Regulation (EC) No. 1107/2009, although no formal definition of biopesticide exists at the European level. Within the Regulation GCE might be approved in the categories “low risk” or “basic substances”.

The scale up of industrial stilbenoid extraction from grape canes will require the selection of appropriate extraction method to ensure proper composition and titration of GCE. The application of alternative methods including extraction assisted by microwaves, ultrasounds, pulsed electric fields or supercritical fluids have proven efficient extraction yield for stilbenoids. These processes are not commonly used by phytoextraction industries and require dedicated equipment with new investment costs. While the concentration of active compounds has a decisive impact on the quality of the extract, it is also necessary to consider economic aspects in the choice of a given method. At this point, the best proven and most reliable extraction method is classical maceration. As water-ethanolic extracts were proven to be effective against grapevine diseases, the classical extraction remains the optimal solution for reliable and economically sustainable valorization of natural substances from grape canes. Apart from the extraction method used, the choice a stilbenoid-rich variety might be a lever to increase GCE efficacy against grapevine diseases.

There is a growing interest of science, industry and society for the exploitation of agronomical by-products as potential sources of natural active ingredients particularly for the development of new biopesticides. In viticulture, the valorization of grape cane extracts for biocontrol purposes participates in the development of a circular bio economy where by-products of grape pruning from a grapevine growing zone will be routed toward a local extraction unit for the production of stilbenoid-enriched extracts. In turn, the remaining wood wastes will serve as biomass for bioenergy plants cogenerating heat and power. European Union has already identified industrial symbiosis as one of the key solutions to obtain mutual benefits and simultaneously moving toward through a circular economy. The development stilbenoid-enriched extracts from grape canes for biocontrol fits perfectly this trend.

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Part III
Use of Biological Agents

Chapter 10
Biological Control of Postharvest Diseases by Microbial Antagonists

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10.1 Introduction

The postharvest phase has been considered a very suitable environment for successful application of biological control agents (BCAs), since the first work on the biological control of brown rot disease of stone fruit was reported by Pusey and Wilson [1]. Sure enough, the conditions of constant temperature and high humidity seem to offer more chances to BCAs, increasing their antifungal activity [2]. BCAs are living organisms and act following different antagonistic strategies depending on pathogens, host and environment. Knowledge of their modes of action is therefore essential to enhance their viability and increase their potentiality in disease control.

In general, antagonists used for biocontrol of postharvest diseases are yeasts and bacteria, and to a lesser extent fungi, and they have been widely reviewed [3–7].

Antagonists can display a wide range of modes of action, at different stages of their activity, relating to different hosts, pathogens; sometimes-different modes act simultaneously, and it is therefore difficult to establish which individual mechanism has contributed to a specific antifungal action. Considerable information is available with respect to their efficacy, their application under storage conditions, and their mixture with safe substances or according to the formulation. However, the mechanisms by which BCAs exert their activity against pathogens have not yet been fully elucidated [5] and sometimes, in order to achieve maximum effectiveness in postharvest phase, were combined with physical and chemical methods including heat treatments, gamma or UV-C irradiation, and controlled atmosphere (CA).

The bottleneck of the biocontrol matter remains the BCAs formulation often done in association with private companies, due to the high costs of production and the regulatory barriers to BCAs registration in different countries that often do not

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encourage their dissemination. Also, a formulation often could reduce the activity of antagonists with respect to the fresh cells [2].

10.2 Mechanisms of Action of Biocontrol Agents

Several modes of action exerted by BCAs against the main postharvest pathogens have been described in these last years, notably antibiosis through the production of volatile and non-volatile compounds, competition for space and nutrients, induction of resistance in the host, parasitism and biofilm formation.

10.2.1 Antibiosis

The antibiosis is considered a biological process by which antagonists produce substances that inhibit or kill potential pathogens (bacteria or fungi) occurring in close proximity [7]. Antibiosis is found more in bacteria than yeasts and fungi [8]. Among the most common antibiotic compounds are included iturin produced by *Bacillus* spp. [9, 10]; pyrrolnitrin produced by *Pseudomonas* spp. [11] and syringomycin produced by *Pseudomonas syringae* [8]. Other metabolites such as bacillomycin, surfactin, and fengycin, all included in the cyclic lipopeptide (LPs) families are synthetized by *Bacillus* spp. and showed antibacterial, antifungal activity [12] and sometimes stimulated the resistance response in fruit [13].

Arrebola et al. [12] showed how iturin A produced by *Bacillus amyloliquefaciens*PPCB004 was found to be active against three postharvest fungal pathogens (*Alternaria citri*, *Colletotrichum gloeosporioides* and *Penicillium crustosum*), while an iturin deficient mutant of *B. amyloliquefaciens* (called PPCB004itu-) displayed a higher disease incidence in comparison with the wild type PPCB004. Nevertheless, the production of antimicrobial compounds by BCAs is often influenced by several abiotic factors such as oxygen, temperature, carbon and nitrogen sources, and microelements. For example, the production of syringomycin *in vivo* is dependent on the physiological state of the *P. syringae* and on the nutrient availability, but also on the presence of plant signal molecules like arbutin, phenyl-beta-D-glucopyranoside and salicin [8] for the activation of genes responsible for syringomycin biosynthesis [14]. Therefore, the antibiosis observed *in vitro* trials is not necessarily correlated with inhibition of the pathogens *in vivo* [15]. The production of antimicrobial compounds was not generally detected in fruit and Bull et al. [8] found that the treatment of wounds with pure syringomicyn to control *Penicillium digitatum* of lemons required 1000 times the concentration needed for pathogen inhibition in *in vitro* trials, suggesting the participation of other mechanisms of action not dependent on syringomycin production. Although the antibiotic producing bacteria represent an important opportunity for the biological control of postharvest diseases, the public opinion is always inclined on the possible use of non-antibiotic producing

antagonists also avoiding the possibility of an appearance of pathogen resistance towards these antimicrobial substances [16].

Furthermore, microorganisms are known to produce a wide range of VOCs [17], mainly studied for their biotechnological potential in the food or agricultural industries [18] but also involved in the biological control of several postharvest fungal diseases of fruit.

The volatile organic compounds (VOCs) mainly produced by BCAs belong to the alcohol group (ethanol, 3-methyl-1-butanol, 2-methyl-1-butanol, 2-phenylethanol) [19], to the esters group (ethyl acetate, ethyl octanoate) [20], aldehydes group (2-methyl-2-hexenal and 2-isopropyl-5-methyl-2-hexenal) [21] and alkane group (3-hydroxy-2-butanone, thiophene) [22]. VOCs produced by *Aureobasidium pullulans* strains L1 and L8 were found to be active against *Botrytis cinerea*, *Colletotrichum acutatum* and *Penicillium* spp. in *in vitro* and *in vivo* trials [19]. The VOCs emitted by these BCAs provide only a limited contribution to the biological control of pathogens, since they frequently show a fungistatic activity. Conversely, *Muscodor albus* [23] one of the most promising BCAs active against some fungal postharvest diseases, probably produce high toxic volatile substances that could compromise their commercial use, since a recent study showed DNA damage and cytotoxicity on bacteria cells from VOCs produced by *M. albus* [24]. Similarly, other antagonists such as *Sporidiobolus pararoseus* [25] and *B. amyloliquefaciens* [22] inhibited the *B. cinerea* growth of strawberries and cherries respectively, by the emission of volatile compounds. The biological control through the production of VOCs could represent a very interesting method to control postharvest pathogens avoiding the direct contact between the antagonist, the pathogen and the host.

10.2.2 Competition for Nutrients and Space

Competition is intended as the request for the same macro and micronutrients or space by two or more microorganisms. The competition for nutrients and space is generally considered one of the main modes of action of BCAs because it involves the nutritional requirements of both antagonist and pathogen [26, 27], and is important mainly against wound pathogens that are typically dependent on exogenous nutrients for their development.

This mode of action is considered a key factor by which BCAs suppress postharvest pathogens [4] and among the BCAs, mainly the yeasts, compete for space and some nutrients with the pathogens [28], being able to grow rapidly during the first 24 h after treatment, deplete quickly available nutrients and physically occupy the wounds [29, 30]. As the main postharvest fungal pathogens are wound parasites, the ability of antagonists to colonize these niches and rapidly increase their population appears strategic for the success of control. In addition, nutrient disposal is correlated with host species since different fruits can support different nutrients,

explaining for example how the activity of some antagonists on citrus was less effective than on strawberry [31].

The competition for nutrients was widely investigated; however, the non-destructive method adopted by Janisiewicz et al. [32] that separates the pathogen from the antagonist cells but keeping both in the same medium, was used in several antagonist/pathogen interactionssuch as *A. pullulans*/*Monilinia laxa* and *B. cinerea* [29, 33], *A. pullulans/P.expansum* [34] and *Kloeckera apiculata/Penicillium italicum* [35]. During the interaction between *A. pullulans*/*M. laxa* or *B.cinerea,* when the tests were performed using high concentrations of fruit juice, BCAs proved less active against pathogens probably because there is a greater availability of nutrients for it. Analogous results were obtained with 'Navel' oranges, using *P. italicum* and *K. apiculata* as pathogen and antagonist respectively [35]. Increased concentrations of orange juice stimulated the conidial germination of the pathogen and reduced the inhibitory effect of BCA. As the concentration of juice increased, the efficacy of the antagonist decreased respectively. However, competition for nutrients is only one of the numerous modes of action of a BCA; in fact, antagonists can display a wide range of mechanisms of biocontrol sometime acting simultaneously [2]. Also, the addition of exogenous nutrients resulted in a reduced efficacy of the BCA that was able to compete with the pathogen mainly when nutrients were scarce. Bautista-Rosales et al. [27] showed how *Colletotrichum gloesporioides* in mangoes increased its pathogenicity after the addition of sucrose and fructose.

Furthermore, the competition for nutrient can be associated to the deamination of different amino acids by some BCAs as nitrogen source [29, 32, 33] explaining their behavior differences always related to different hosts. *In vivo* trials showed that a strain of *A. pullulans* (Ach1-1) active against *P. expansum* assimilated apple amino acids better than the pathogen, most particularly serine, glycine and glutamic acid; among these amino acids, serine in particular appeared to be the most limited nutrient [34]. In addition, it is note that not all organisms are able to synthesize amino acids [36] so as to better explain their antagonistic activity through other mechanisms of action.

It is known that the majority of postharvest diseases derive from wounds, a niche where an abundant availability of nutrients occurs, so the rapid colonization of this site is dependent on the antagonist competition for space ability and also on host species that could be more favourable for certain BCAs than others. Besides competition for nutrients, competition for iron may also play a role in the biocontrol interactions [37] by the production of siderophores, molecule able to chelate low weight ferric compounds enhancing the effectiveness of BCAs by subtracting iron to pathogen development and inhibiting its growth and metabolic activity [38]. Competition for iron may thus play an important role in the antagonist-pathogen interactions [37] although it is still not well understood. Among nutrients, iron is one of the most critical microelements for fungal development, to the point that it can be a limiting factor for fungi growth; for example, conidia require a large intake of iron to germinate [39], so it is understandable that the presence of siderophores, the low molecular weight ferric chelating agents, may delay or reduce pathogens

conidial germination. High siderophore concentrations could help to control disease without increasing the population of the applied antagonist [40].

10.2.3 Induced Resistance

The induction of host plant resistance was demonstrated to be one of the modes of actions in controlling postharvest pathogens [41–43].

Induced resistance by microorganisms in fruit or plant concern the activation of PR proteins (β-1,3-glucanase, chitinase) [43, 44] or defence-related enzymes such as phenylalanine ammonia-liase-PAL, peroxidase, polyphenoloxidase [7, 41, 45, 46].

PAL is responsible for the biosynthesis of p-cumaric acid derivates, phytoalexins, and lignin, and an increase in PAL activity in fruit tissue wounds treated with antagonists can favour the cicatrisation processes, reducing the possibility of the pathogen becoming established in wounds [47]. For these reasons could be much more effective applied the antagonists before the pathogen inoculation to induce a resistance process in fruit skin.

One of the first barriers against invading pathogens is the accumulation of reactive oxygen species (ROS) that also work as signals for plant defence reactions. When yeasts are applied to fruit wounds, the ROS-generated oxidative stress can affect their viability and performance, and their tolerance to oxidative stress is consequently important for biocontrol [48].

On the other hand, antagonist yeasts can influence ROS production and defence signalling in fruit tissues, stimulating both antioxidant gene expression and antioxidant enzyme activity in peach fruit tissues [49]. Now, with the help of new technologies is possible provide insight on the origin of these mechanisms of defence by the different changes and regulations of genes expression [48].

10.2.4 Parasitism

Direct parasitism is represented by a close contact and recognition between an antagonist and a pathogen. Often both microorganisms interact by the secretion of cell wall lytic enzymes or by the direct development of the antagonist in the fungal pathogen [50].

The direct parasitism is demonstrated also through pathogen hyphae deformation or consumption [51, 52] by the use of scanning electron microscopy (SEM) or transmission electron microscope (TEM). In fact, *Pichia guilliermondii* cells had the ability to attach to the hyphae of *B.cinerea* and *P. expansum* [51], leaving the hyphal surface concave, as if the fungi cell walls were attached also through the production of lytic enzymes like β-1,3-glucanase, and chitinases (endo- and exo-) [53].

Yu et al. [54] showed how *Bacillus* spp. and *Pseudomonas* spp. are considered effective chitinolytic microorganisms for their chitinase direct action. Moreover, the presence of fungal cell wall significantly increased the β-1,3-glucanase activity compared to that observed in media containing only laminarin. Different levels of activity of this enzyme were observed with respect to the incubation time; for example, the level of the exo-β-1,3-glucanase reached a maximum after 24 h of incubation at 25 °C. Di Francesco et al. [43] showed how the activity of cell wall hydrolytic enzymes such as chitinase and glucanase in *A. pullulans* cell filtrates was constitutive and encoded by gene expression from 24 h onward of cell growth. Also, the gene expression demonstrate different expression levels varying according to the two *A. pullulans* tested strains (L1 and L8).

Biofilm formation has been proposed as a possible mechanism of biocontrol in some antagonist yeasts [55] and in particular intend as a form of parasitism. The biofilm is a barrier constitute by micro colonies enclosed in a hydrated matrix of proteins, nucleic acids, and polysaccharides [56] and interposed between the host surface and the pathogen creating an exclusion condition.

One of the main issues of biofilm is the initial attachment of microorganisms, since it is the necessary first step of biofilm formation. The ability to form biofilms has been also proposed as an effective mechanism of action in some biocontrol yeasts [57].

Metschnikovi apulcherrima and *Wickerhamomyces anomalous* were found to be effective against gray mould decay by wounds colonization and *A. pullulans* against sour rot of citrus fruit [30]; conversely *Saccharomycescerevisiae* showed lower wound colonization, no ability to form a biofilm and to reduce *B. cinerea* disease incidence [58]. The yeasts cell attachment is often mediated by specific cell wall adhesive glycosylphosphatidylinositol (GPI)-anchored proteins [59] regulated by two genes families HWP1and ALS thatplaya critical role in host cell recognition, adhesion, invasion, and biofilm formation [60]. Different yeast species expressed different adhesin genes that reflect their species lifestyle.

In light of this, the detection of BCAs biofilm production could represent a starting point to discriminate the more effective antagonists able to create a mechanical barrier interposed between the wound surface and the pathogen.

10.3 Use of Biocontrol Agents Combined with Physical, Chemical and Natural Treatments to Control Postharvest Diseases

Against the main postharvest fruit diseases, different combinations of treatments could represent a solution to optimize the BCAs activity. The exclusive use of BCAs to control postharvest fruit diseases is still constrained by the lack of high levels of disease control required in the postharvest phase, almost more than 95% [61]. One of the major difficulties of the application of formulated BCAs is obtain a shelf

stable product with a biocontrol activity similar to that of the fresh cells [7, 62]. For these reasons, integrated strategies were explored and continue to be one of the fields most investigated in postharvest fruit disease control, in order to achieve maximum effectiveness.

Among the principal physicals methods were included heat treatments by hot water or air, gamma or UV-C irradiation, and controlled atmosphere (CA).

In addition, the combination of low doses of fungicides and BCAs was also explored, appearing to be one of theirliable options for large-scale utilization of microbial antagonists in the control of postharvest fungal rots of fruit and vegetables [63], even if contradictory.

Infact, non-chemical technique have gained enormous interest in worldwide to control various postharvest diseases due to the total absence of residues in the final product and minimal environmental impact [64]. In the case of natural treatments, BCAs were combined with compounds such as chitosan, plant extracts, representing a valid sustainable and environmental friendly solution to contain the main postharvest fruit diseases.

10.3.1 Biocontrol Agents and the Main Postharvest Physical Treatments

Thermal treatment is the application of heat as a short treatment before cold storage [65]. Among the most used efficient physical methods, heat treatment has been shown to be effective in reducing postharvest fruit fungal diseases. The combination of heat treatment with a biological control agent may have an additive effect on fruit such as berries since heat may partially disinfect the fruit surface, allowing the biological control agent to become established more quickly and completely [66].

The thermal treatments has been used to control fungal pathogens to render them harmless and at the same time to induce fruit resistance [67].

The combined use of BCAs with hot water (HW) has been shown to be effective in reducing postharvest diseases of fruit. For example, strawberries dipped in water at 55 °C for 30 sand treated with a suspension of *Cryptococcus laurentii* (10^8CFU/mL) showed an incidence of *Rhizopus*rot lower than fruit treated with HW or BCA alone [68]. *Rhodotorula glutinis* combined with hot water (46 °C) for 10–20 min was able to significantly reduce blue mold decay of pear fruit by 86.7% for combined treatment and 33.3% for control after 15 days of storage at 20 °C. The same treatments led to a reduction by 93.3% for combined treatment and 53.3% for control after 60 days at 4 C followed by 15 days at 20 °C [69].

Also for an isolate of *A. pullulans* (PL5) when used alone, the *M. laxa* infections on peaches was of 25.8%, compared with 74.2% for the control; when applied together with HW (55 °C per 50 s) the brown rot incidence decreased to 17.5%, while when HW was used alone the disease incidence was 30%. These results suggest that HW greatly increased the effectiveness of the antagonist [70].

The mechanism by which HW enhanced the biocontrol efficacy of the antagonistic yeasts could be related to the elicitation of biochemical defense responses in fruit.

The postharvest phase is characterized by some conditions such as the controlled atmosphere (CA) and low temperatures in order to delay fruits senescence and to avoid the development of various decay-causing fungi. In fact, effective BCAs must be able to survive at refrigerated temperatures (0–1 °C) and low oxygen levels.

In fact, Conway et al. [71] reported that CA conditions had no adverse effect on the growth of two antagonists *M. pulcherrima* or *C. laurentii* when compared to growth in air storage. Both antagonists rapidly colonized apple wounds, growing well at low temperatures and oxygen levels, proving useful for commercial application on apples designated for CA storage.

Conversely, two *A. pullulans* isolates after 4 months of kiwifruit storage significantly reduced gray mould disease in normal refrigeration (RN) and CA, but in NR, their efficacy resulted higher (80% both) with respect to CA (30% and 60% respectively) [33].

In addition, the combination of hot-air treatment (45 °C for 4 h) and *P. guilliermondii* had notable inhibitory effects on *Rhizopus stolonifer* and *P. expansum* infections in peach fruit wounds. In addition, the individual hot-air treatment or *P. guilliermondii* could improve quality indexes to varying degrees, but the combination of the above two treatments could achieve the highest efficacy [72]. Furthermore, the combined treatment induced the highest activities of superoxide dismutase and catalase, improving the content of total phenolic compounds.

Included on the physical methods to combine with BCAs is included the UV-C irradiation, often used to control postharvest diseases management [73].

Guo et al. [74] showed how *M. pulcherrima* combined with UV-C treatment was able to control both natural decay and artificial wound infection caused by *Alternaria alternata* on winter jujube by 77% and 84%, respectively.

The yeast *Candida guilliermondii* with UV-C irradiation exhibited a synergistic effect in reducing blue mold and grey mold caused by artificial inoculation with *P. expansum* and *B. cinerea* on pear fruits [75].

Pichia cecembensis was evaluated for its ability to control *Fusarium oxysporum* and *A. alternate* of melon fruits alone or with UV-C. The combination with UV-C provided a superior level of decay control on artificially inoculated and naturally infected fruit, compared to either treatment alone, not impairing fruit quality [76].

A possible synergistic effect between *D. hansenii* and UV-C irradiation in controlling *Monilinia* spp. incidence on peaches was observed by Stevens et al. [77], where the capability to control deep-seated infections such as latent infections was attributed to the physical treatment combined with the fruit superficial control exerted by the BCA.

The application of UV-C irradiation must be carefully carried out in order to prevent the DNA damage due to the dimerization of thymine [78], the damage of the cell membrane and degradation of essential proteins (enzymes) in the fruits [79].

10.3.2 Biocontrol Agents Combined with Defense Elicitors and Natural Products

Elicitors are compounds that trigger chemical defense in plants and various biosynthetic pathways [80]. Between the principal defense elicitors are included chitosan, salicylic acid, methyl salicylate, benzothiadiazole, benzoic acid, and pectin, which affect production of phenolic compounds and activation of various defense-related enzymes in plants.

Chitosan has been proven to control numerous pre and postharvest diseases [81] mainly affecting the morphology of the pathogen hyphae, inducing defense reactions in plants correlated with enzymatic responses [7]. It possesses antimicrobial properties and acts as an elicitor of plant defenses against pathogens [82]. This polymer is obtained from chitin deacetylation and plays a substantial role in the control of postharvest diseases in fresh products also extending their shelf life by inducing the synthesis of lignin-like material, a semi-permeable coating formed on the fruit surface that minimize respiration and water loss [81]. The chitosan combination with the yeast *C. laurentii* was evaluated against the *P. expansum* of pear [83] showing that the most effective concentration able toenhance blue mold control was 0.5% when combined with the BCA. Furthermore, the combination of pre-harvest treatment with *C. laurentii* and chitosan enhanced the control of fruit decay [84]. Many studies showed that chitosan coating increased antioxidants, peroxidase and catalase in treated fruit, which led to delayed ripening of fruits during cold storage [54, 85, 86]. Recent experiments confirmed that the combination of the treatments have superior level of control on fruit [87, 88].

Food additives have been used to extend the shelf life of commodities, and are extensively used in postharvest storage practices, not only to improve the quality and safety of fruits and vegetables, but also to stimulate the efficiency of antagonistic microorganisms [7, 89]. Among the most common food additives, sodium bicarbonate (SBC) at a concentration of 5% showed a reduction of 62.3% of green mold of citrus fruit [90].

The combination with *Rhodosporium paludigenum* was effective as a fungicide reducing the infected fruit by 95.8%.

The yeasts *K. apiculata* and *Metschnikowia fructicola* combined with SBC at 1% w/v led to a reduction of decay incidence caused by *B. cinerea* and *P. expansum* without negatively affecting the quality of sweet cherry fruit during cold storage [91].

In addition, many other studies showed a greater reduction of fruit postharvest fungal diseases by using BCAs combined with SBC [71, 92–94] respect to the treatment applied alone.

To control papaya anthracnose, SBC was incorporated in wax coating to enhance yeast activity against the pathogen [95].

Among the main food additives, calcium chloride (CCH) showed significant effects when integrated with *A. pullulans* against brown rot of sweet cherries [92].

Significant effects were also shown when calcium chloride (CCH*)* was integrated with *Vishniacozyma victoriae* and *Pichia membranifacien*s against *P. expansum* and *B. cinerea*, the causal agents of blue and grey mold of pear fruits [96].

While CCH had no direct antifungal activity against postharvest pathogens like *P. expansum* and *B. cinerea* when applied alone to fruit wounds, its combination with the antagonist was more effective to control moulds of pear fruit than *C. laurentii* alone [83]. The BCA could act as a primary defense line, inhibiting the initial attack by the pathogen, while CCH could induce resistance defense responses in fruit tissue [7].

By salt ammonium molybdate, despite the modest concentrations required, were achieved great reductions of gray mold in grape fruits or blue mold in peach fruit when combined, respectively, with *Hansenia sporauvarum* [97] and *P. membranifaciens* [98].

Phytic acid is a natural substance, extracted from cereals bran, characterized by antioxidant properties and some studies on postharvest treatments indicated that 4 μmol ml^{-1} of this compound combined with *R. mucilaginosa* control the decay of strawberries caused by *B. cinerea* [99]. Among antioxidants, ascorbic acid (AA, 250 μg ml^{-1}) was reported to improve the biocontrol efficacy of antagonistic yeast *P. caribbica* against blue mold on apples [100].

Commonly, the bioactive compounds can be described as natural non-nutritive components of food plants, which are non-toxic, that provide beneficial health effects when consumed. The possibility to increase the activity of BCAs with natural substances was investigated for different combinations of substances and antagonists. Burdock fructo-oligosaccharide (BFO) is a natural elicitor from *Arcitum lappa* and if integrated with *R. mucilaginosa* greatly improved its biocontrol efficacy against *Rhizopu*s decay and blue moldof peaches [99].

Essential oils (Eos) such as those derived from garlic, cinnamon, thyme, oregano, clove, basil, coriander, citrus peel, eucalyptus, ginger, rosemary, peppermint, thymus, and savory have been reported to have antimicrobial, antifungal and anticancer properties [101–103].

Nevertheless, the mechanism of action of these potential fungicidal natural compounds are yet to be completely elucidated [104]. Sukorini et al. [105] showed how a mixture of crude extract of *Eugenia caryophyllata* (15,000 mg/L) and *C. utilis* (10^8 CFU/mL) was found to be the best combination to attain a reduction of 90.3% of *P. digitatum* on citrus fruit. The combination of thyme and lemongrass oils with *B. amyloliquefaciens* PPCB004 was tested against *B. cinerea* and *Rhizopus stolonifer* of peach [12]. In addition, the study showed an increase of BCA biofilm formation, as a mechanism of action against the pathogens, more in presence of lemongrass oil than time oil. In fact, the monoterpene components of Eos increased the biofilm formation in gram-positive bacteria [106].

10.4 Formulation of Biocontrol Agents

The formulation represents the bottleneck of BCAs application in large–scale. One factor limiting commercial interest in biocontrol could be the high cost of production, mainly due to the high cost of substrate, low biomass productivity, or limited economies of scale [107].

In the last years, the number of registered strains and microbial products has been steadily increasing, mainly due many principal reasons such as (a) the reduction of pesticide use; (b) consumers' demand of zero-residue food; (c) development of resistance to agrochemicals by weeds, pests, and disease organisms [108].

The aim of a formulation process is to obtain a product with a significant shelf-life of at least 6 months or more, at environmental conditions [109] and for the mass production of BCAs, both solid and liquid formulations have been used. Depending on the type of disease and the mode of action, BCAs are typically applied as seed treatment, root dip, soil amendment, foliar spray or biofumigation. However, for a better product maintenance dry environment is preferable to liquid formulations since BCAs can be handled using the normal distribution and storage channels. Unfortunately, not all microorganisms are able to survive drying conditions; they can lose viability during the drying process and storage through denaturation of proteins [110–112].

Nevertheless, different cryoprotecting media were assessed during the freeze-drying treatments for conserving microorganisms such as a mixture of skimmed non fat milk [113] (or e.g. cells or polymers, sugars, albumin, salts, honey, polyols or amino acids) that enabled the 98% viability of the cells after 4 years of storage showing only a slight decrease with respect to fresh. In many cases, additives were found to be effective toward protection of dried microorganisms because they replace structural water in cell membrane after rehydration and prevent unfolding and aggregation of proteins [114].

Components of the formulation media have two main functions in preserving the viability of formulated cells: to provide a dry residue and thus acting as a receptor in the rehydration process and to protect biochemically the cells against damage during the drying process [115–117].

A biocontrol product to be commercialized should be a shelf-stable formulation [113], economical to produce, easy to distribute to the intended environment, should contain enough colony forming units (CFU) and finally maintain the efficacy against plant pathogens similarly to fresh cells [117].

To make development and commercialization more successful, it would be beneficial to broaden the spectrum of action of these products to different hosts and pathogens [118, 119], since the major postharvest BCAs were limited in their potential market size.

The introduction of BCAs in wax or oil-based coating was also widely investigated, above all for citrus fruit, papaya or mango that are treated by application of the coating after harvest [109]. Carnauba wax or paraffin oil coating containing *B. amyloliquefaciens*JBC36 (10^8 CFU/mL) efficiently decreased the incidence of

P. digitatum on Satsuma mandarin with control efficacy of 91 and 80.9%, respectively [83]. The efficacy of the formulated product of *P. guilliermondii* strain Z1 in combination with rosin maleic wax increased significantly in the control of postharvest pathogen *P. italicum* on wounded orange fruit [120].

Papaya fruit naturally infected by *C. gloeosporioides* and dipped in wax coating containing sodium bicarbonate and *C. oleophila* suspension resulted in significantly lower anthracnose incidence and severity than fruits dipped in wax coating containing only sodium bicarbonate. Wax coating probably creates a modified atmosphere around the fruit, sustaining the antagonist growth [95].

However, one of most challenging barrier to overcome for the bio-formulations commercial success is improve the microbial viability during the storage by ensuring the effectiveness under commercial conditions against the target pathogen.

The achievement of a formulated microorganism must take into consideration a number of field or greenhouse trials, executed in different locations, in meaningful tests, using specific methods for applying the antagonist in large-scale. This makes the trials costly and they are therefore often done in association with a private company wishing to commercialize the biocontrol product [108].

10.5 Conclusions

The use of BCAs as an alternative to synthetic products has been a research focus in the last 30 years by many researchers and several commercial enterprises worldwide [121]. Nevertheless, postharvest disease management needs the development of alternative strategies, like bio fungicides since fungicides remain the primary method of controlling diseases.

The growing concern about the residue of synthetic products on fruit has led to a demand for the development of alternatives to control postharvest diseases for the well-being of human health and environment. The most studies are focused on the BCAs modes of action and surely need to be more developed to better understand the interaction between antagonist and environment in particular postharvest conditions [109]. However, each mode of action can interact with each other varying to yeast-pathogen-host system, always determining innovative solutions for sustainable fruit production. However, the common objective is the development of BCAs products for the reduction of chemical contaminants in food [122].

However, the process before to realize a commercial product with BCAs is very long and complex. BCAs should be isolated, selected based on their efficiency and before to released into the market, it should pass different studies, and the production process should be scaled up to an experimental level to obtain sufficient quantities of the product to carry out its assessment in greenhouse, field, warehouse, or packing plant [121].

Available at commercial level, there are different bioformulations, which are considered first-generation biocontrol products for postharvest and whose active ingredients are antagonist yeast and bacteria such as: *Candida oleophila* (Aspire,

Ecogen, Langhorne, PA, USA) and (Nexy, Leasafre, Lille, France) [123], *Candida sake* (Candi fruit, Sipcam Ibérica, Valencia, Spain) [124], *B. amyloliquefaciens* (Amylo-X, made by Biogard CBC in Grassobbio, Italy) [125]. Recent studies on antagonistic microorganisms have made evident their potential use in other sectors such as medical and the food industry [126–128] but also in these cases, their effective application is still under a study phase.

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Chapter 11
Sorghum Allelopathy for Sustainable Weed Management

Józef Sowiński, Franck E. Dayan, Lilianna Głąb, and Katarzyna Adamczewska-Sowińska

11.1 Introduction

Weeds constitute a crucial problem in agricultural fields. Their negative impact is a result of competition with crops for nutrients (they are at the same trophic level as the crops), vie for light, water and surface area. According to Oerke and Dehne [1], crop losses resulting from weed infestation amount to 32%, while insect pests and crop diseases contribute to 18% and 15% reduction in crop yield, respectively.

The introduction in the 1940s of synthetic herbicides heavily increased the efficacy of crop protection as well as labor productivity. This method has developed rapidly, becoming a standard method contributing to decrease the significance of other weed control methods, such as agronomic, mechanical or biological.

Today, the use of weed control chemicals is being reevaluated because of their potential negative impact on food safety, human health and the environment [2, 3]. Moreover, herbicides used in simplified crop rotation contribute to the selection of weed resistance and reduces their efficacy (Fig. 11.1). In many crops and regions of the world, herbicide-resistant weeds are becoming increasingly common and consist a major challenge to science and modern agriculture.

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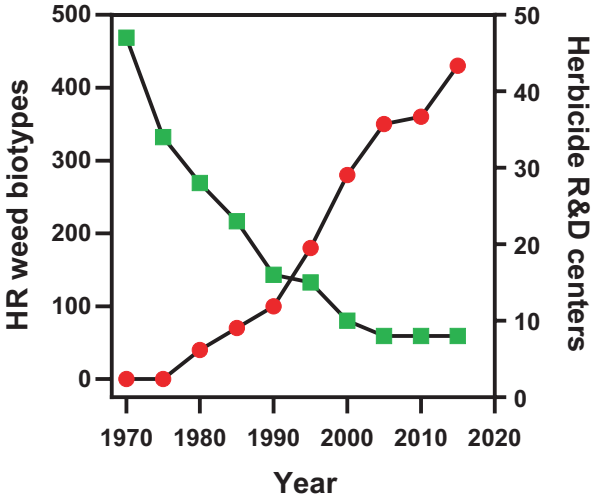

Fig. 11.1 Trends of herbicide research and development (R&D) centers (■) and herbicide-resistant (HR) weed biotypes (●) [4, 5]

Duke [6] reports that in the 1950–1970s, during the initial period of application of herbicides, new active substances (*Mechanism of action* – MOA) were commercialized every 2.5–3 years and currently 18 MOAs are used in the production of herbicides (Table 11.1). A flurry of activity has recently emerged with several new MOA being reported [7].

In the mid-1990s, the first genetically-modified crops resistant to glyphosate were introduced to agriculture production. The mechanism of glyphosate resistance has been transferred to cultivated species and transgenic crops now occupy 189.8 million hectares worldwide [8]. Many farmers use only glyphosate to manage weeds and are not actively using any other herbicides. The popularity of transgenic species is mainly due to the reduction of weed control costs and the effectiveness of weed control. The widespread use of this herbicide has contributed to the selection of glyphosate-resistant weed species and currently 45 weed species have evolved resistance to this active ingredient: *Amaranthus hybridus* L. (syn: *quitensis*), *Amaranthus palmeri* S. Watson, *Amaranthus spinosus* L., *Amaranthus tuberculatus* (Moq.) J.D. Sauer *(=A. rudis), Ambrosia artemisiifolia* L., *Ambrosia trifida* L., *Bidenspilosa* L., *Bidens subalternans* D.C., *Brachiaria eruciformis* (Sm.) Griseb, *Brassica rapa* L. *(=B. campestris), Bromus catharticus* Vahl., *Bromus diandrus* Roth., *Bromus rubens* L., *Chloris elata* Desv., *Chloris radiata* L., *Chloris truncate* R.Br., *Chloris virgate* Sw., *Conyza bonariensis* L., *Conyza canadensis* L., *Conyza sumatrensis* (Retz.) E. Walker, *Cynodon hirsutus* (L.) Pers., *Digitaria insularis* (L.) Fedde, *Echinochloa colona* (L.) Link, *Eleusine indica* (L.) Gaertn., *Hedyotis verticillate* (L.) Lam., *Helianthus annuus* L., *Hordeum murinum* L. ssp. *glaucum* (Steud.) Tzvelev., *Kochia scoparia* (L.) Schrad., *Lactuca saligna* L., *Lactuca serriola* L., *Leptochloa virgata* (L.) P. Beauv., *Lolium perenne* L., *Lolium perenne* ssp.

Table 11.1 Mechanism of action of currently used herbicides [7]

Group of herbicides	Mechanism of action
Amino acid metabolism	Glutamine synthetase
	Acetolactate synthase
	EPSPS
Synthetic auxins receptors	Auxin receptor F-box proteins
	ABCB auxin transport proteins
Carotenoid synthesis	Deoxyxylulose-5-phosphate synthase
	Phytoene desaturase
	p-hydroxyphenylpyruvate dioxygenase
	Solanyl diphosphate synthase
Cellulose synthesis	Cellulose synthase
Folate synthesis	7,8-dihydropteroate synthase
Lipid synthesis	Acetyl-CoA carboxylase
	Fatty acid thioesterases
	Very long-chain fatty acid elongases
Mitosis	Tubulin
Photosynthesis	Electron diverters from PSI
	Blocking electron at D-1 of PSI
Porphyrin synthesis	Protoporphyrinogen oxidase
Protein phosphatase	Serine/threonine protein phosphatases
Uncoupler	Membrane disruptors

multiflorum (Lam.) Parn., *Lolium rigidum* Gaud., *Parthenium hysterophorus* L., *Paspalum paniculatum* L., *Plantago lanceolate* L., *Poaannua* L., *Raphanus raphanistrum* L., *Salsola tragus* L., *Sonchus oleraceus* L., *Sorghum halepense* (L.) Pers., *Tridax procumbens* L., *Urochloa panicoides* P. Beauv., at 30 countries and 311 locations [9].

This forced manufacturers of plant protection products to increase their spending on the search for new active substances. Gerwick [10] reported that between 1980 and 2009, 137 biologically active herbicides were launched in the market. In perspective, protection against weed infestation cannot involve new herbicides based on previously introduced mechanisms of action or on new transgenic plants resistant to marketed herbicides. The search for new MOAs is also very costly, quit often only for short-term and sometimes doomed to failure [5].

Many authors [11–13] revealed a better understanding of weed ecology in order to make greater use of integrated weed control methods. This should be based on a strong link between biology basic research and weed biology. Understanding the biology and ecology of weeds and the interaction between plants should be an integral part of sustainable methods to reduce weed infestation.

A promising phenomenon is the development of weed control based on natural products that are produced as by-products of microorganisms or plants. Only a small part of the microbiological and plant diversity has been tested for weed control. In the 1980s and 1990s, many innovative biotechnology companies discovered

and investigated active compounds that were potentially of great importance as bioherbicides, bioinsecticides or biofungicides. Obtaining glyphosate-resistant crop species in rapid development of biotechnological processes resulted in the abandonment of work on the search for biopesticides. Currently, the development of molecular techniques, genomics and metabolomics allows for more targeted and conscious research to commercialize the discovered mechanisms of activity of compounds of biological origin.

The basis for future plant protection is the understanding of physical, microbiological, hormonal and chemical inter-species and intra-species interactions. Understanding and defining the plant-plant, microorganism-plant interaction will be the foundation for the development of plant protection and its scientific basis in the future. The development of a weed control strategy will be incomplete without taking into account all available methods, in particular biological control of weeds especially implementing bioherbicides.

It is particularly difficult to develop effective and economic methods to reduce weed infestation on organic farms and weed control on such farms must be complementary used preventing, agricultural (both biological and technical) and biological methods [14]. Many different components are competitive or allelopathic in character, but also targeted in terms of activities resulting from technological development and understanding of phenomena occurring in the agricultural environment (Fig. 11.2) [15].

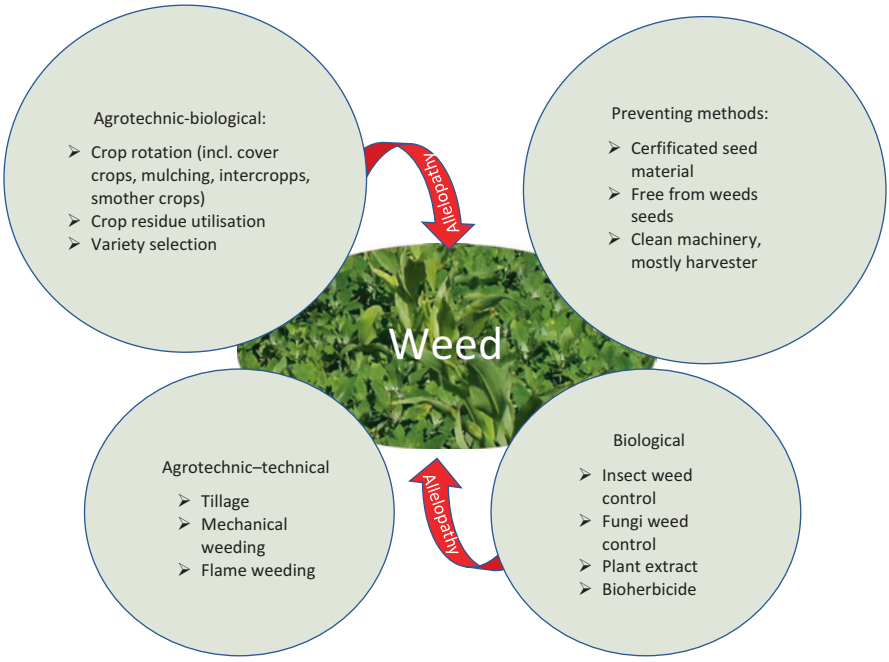

Fig. 11.2 Weed management methods. (Adapted from Kalinova) [15]

11.2 Application of Allelopathy as a One of the Methods for Biological Weed Control: Perspective and Challenges

Crop rotation and management were used for 1000 years for reduce weed abundance and biodiversity [16]. Until the 1940s, weed infestation has been managed using crop rotation systems and interventional mechanical weed control [17]. In the last several decades, chemical weed management practices have had some impact on the environment. Using knowledge of organisms for natural weed control methods are recommended [18]. Theoretically, only competitive interaction between plant species will provide the plant community with a proper structure and diversity [19]. Unfortunately, high-productivity communities – such as agricultural crops biocenosis – are characterized by less diversity due to the targeted competitiveness and reduced growth of species with less capacity to use available environment resources [19].

The plant-plant interactions are very sophisticated and difficult to distinguish character and occur at various levels. These complex interactions are based on two general relationships: competition and allelopathy (Fig. 11.3). On the basis of many studies, the interactions between plants can be successfully used in agricultural systems where the use of industrial inputs (fertilizers and pesticides) is sought. These days, there is a great need to search eco-friendly methods of weed control in modern low-input sustainable crop production systems [20]. Various studies have reported

Fig. 11.3 Competition and allelopathy differences on the mechanism as well as nature of that processes. Graph based on Qasem and Foy publication [26]

that allelopathic potential of some plants could be considered as promising alternative technique of weed management to herbicide application [17, 20, 21].

Many crops, such as alfalfa, buckwheat, corn, rice, rye, sunflower, wheat, but also sorghum have a strong impact through root exudate and realizing allelochemicals during the decomposition of biomass on weed and crop germination. Therefore, it is necessary to know the biochemical and physiological processes, but also to understand the morphological features of plants that affect the external or internal species interaction, allowing their use in limiting the growth and development of weeds.

Irrespective of the many studies confirming the stimulatory or inhibitory effect of allelopathy the advisability of its practical application in field conditions is still being questioned [22]. Detailed information has been included in review article of Głąb et al. [23]. Sometimes scientists and authors of review articles claim that "full proof of allelopathy may never be attained" [24]. Allelopathy directly and indirectly affects not only, the nutrient circulation and plant growth, but also the growth of mycorrhizal microorganism, intra-species competition and diversity as well as attractiveness for insects and other herbivorous species consist complementary natural mechanism of weed reduction [25].

González and Reigosa [22], based on studies carried out on a slope (slope direction: up – left, down – right part of graph), showed different ways of plant interaction with another plant when active compounds are exudate into soil (Fig. 11.4).

Reinhardt et al. [27], however, distinguished the following strategies for reducing weeds using the phenomenon of allelopathy:

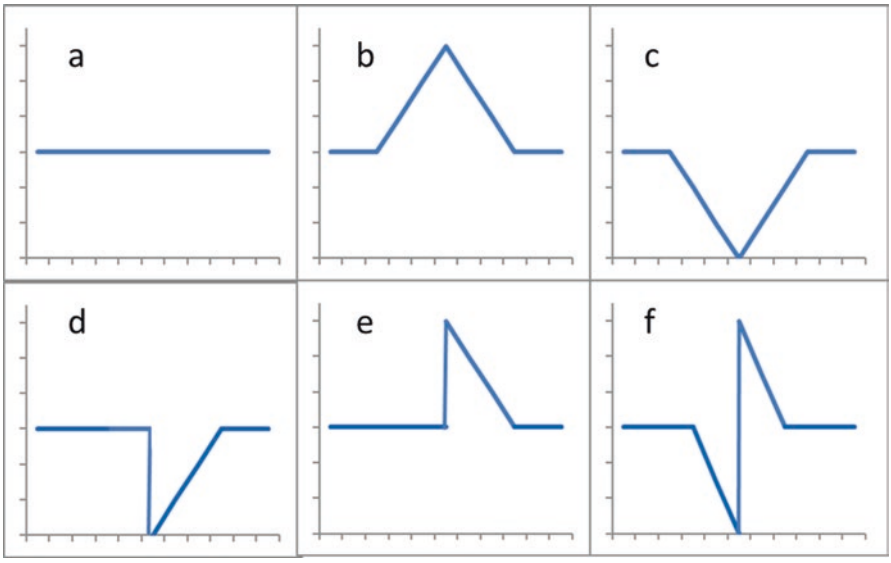

Fig. 11.4 Differences of allelochemical interaction on slope: (**a**) neutral, (**b**) stimulation, (**c**) competition, (**d**) inhibitory allelopathy, (**e**) stimulatory allelopathy, (**f**) inhibitory and stimulatory allelopathy. Based on article of González and Reigosa [22]

- use of weed smother species and breeding of these species in order to preserve such traits,
- the introduction of species with allelopathic properties for crop rotation and/or the use of post-harvest residue for mulching the field,
- isolation of allelochemicals from higher plants or microorganisms and their use as bioherbicides.

11.3 Allelopathic Effect of Living Sorghum and It's Residues on Weeds Cultivation and Succeeding Crops

11.3.1 Sorghum in Crop Rotation

The evaluate of the allelopathic effect of sorghum on cultivated species in crop rotation, under controlled laboratory conditions and in field experiments. This effect results from the accumulation of allelochemicals in the sorghum and their slow release during biomass degradation in the soil. The subsequent effects of compounds found in various parts of the sorghum plant and the sorghum hybrid with Sudangrass have been well documented and have been the subject of much research in the last 40 years (Fig. 11.5 – adapted from Weston et al. [28]). The phytotoxicity of sorghum and sorghum hybrid with Sudangrass ranged from several to over 90% and depended on the species that was tested and also part of the plant whose

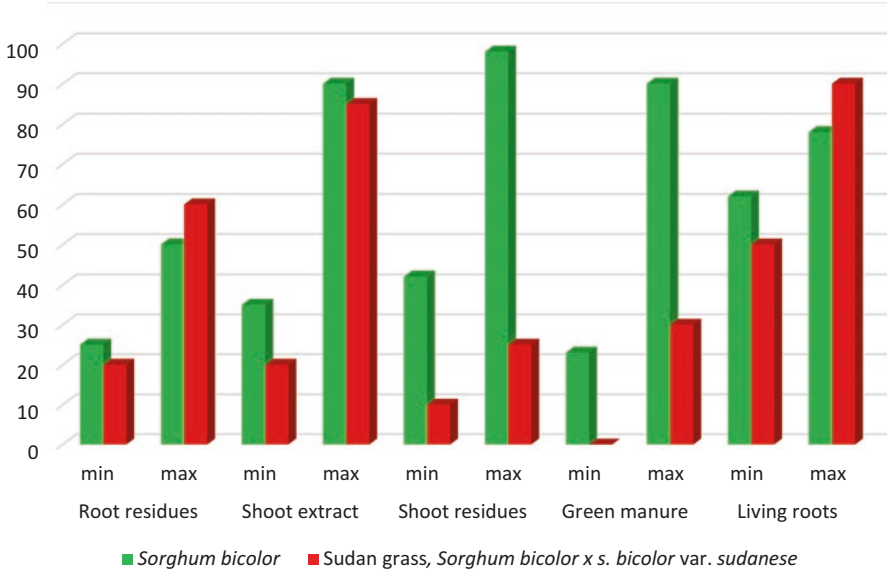

Fig. 11.5 Sorghum plants part phytotoxicity in % (base on many articles published from 1983 to 2012 years). (Adapted from Weston et al. [28])

allelopathic effect was assessed. The effect of compounds found in sorghum and sorghum hybrid with Sudangrass also depended on the weed species to which the toxic effect was directed, the development phase of the crop and weed as well as environmental factors.

Extensive global research has evaluated the after-effects of sorghum and other crops from this genera in the following areas [28]:

- use of sorghum in crop rotation and impact on other crop species,
- using an extract from various sorghum plant parts,
- use of post-harvest sorghum residues and as a cover and mulch species,
- use of sorghum as a smother species,
- use of sorghum as a component of intercropping and crop mixtures,
- utilization of allelopathic properties of sorghum with the combined use of herbicides in a reduced dose.

During the decomposition of sorghum biomass, large amounts of organic compounds are released into the environment, which may have a negative effect on the following plants, e.g. cotton germination [29, 30]. Under controlled conditions, a significant reduction in the growth of Canadian Judas (*Cercis canadensis* L.) has been demonstrated, regardless of whether fresh or dry sorghum mass is mixed with the soil [28]. The inhibition of successive plant growth was proportional to the amount of biomass introduced. This negative effect was, however, the greater where greater was the share of roots residues than stems. The effect of dried residues was also lower than fresh sorghum biomass. In the studies conducted under controlled conditions by Weston and Czarnota [31], there was an adverse follow-up effect on lettuce seedlings when the seeds were sown in rows in which sorghum had previously been grown. The authors observed that the allelopathic effect was stronger when cultivating species with small seeds. It manifested itself as dwarfism, chlorosis and, as a consequence, death of seedlings. Petersen et al. [32] reported that small-seeded species are more susceptible to phytotoxic action of residues containing allelochemicals.

In other studies, sorghum cultivation and its subsequent effects had a beneficial impact on the growth, development and yield of *Fabaceae* and *Liliaceae* family plants [33]. The root system of both sorghum and Sudangrass, secreted biologically active compounds that subsequently, positively influenced the growth of Alexandria clover, field beans, onions and contributed to a higher yield of these species. The same studies did not show a beneficial effect of sorghum and Sudangrass on plants belonging to the *Poaceae* and *Chenopodiaceae* families [33]. The assessment of allelopathic action of sorghum biomass on weeds is presented in the study conducted by Chauhan et al. [34]. Increasing the amount of post-harvest sorghum residues limited germination of *Chloris truncate* R.Br. and with 8 tons of sorghum biomass per ha, the seeds of this weed did not germinate at all (Fig. 11.6).

Post-harvest sorghum residues and associated compounds released from the residues limit the growth of many weed species in various regions of the world, e.g. *Phalaris minor* Retz., *Chenopodium album* L., *Rumex dentatus* L., *Lolium rigidum* Gaud., *Lolium temulentum* L., *Malva parviflora* L., *Carthumus oxycantha* M. Bieb.,

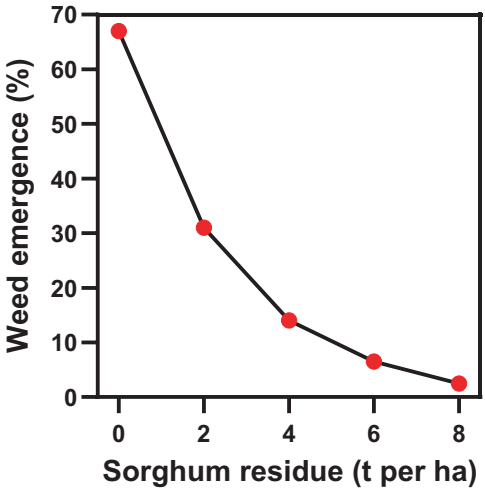

Fig. 11.6 Effect of sorghum residue biomass on *Chloris truncate* R.Br. emergence [34]

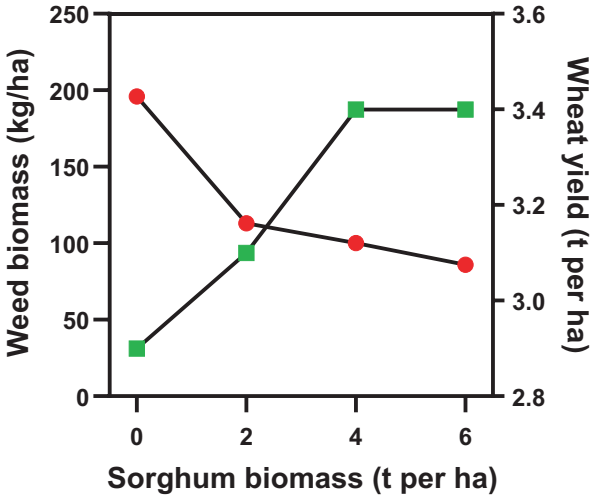

Fig. 11.7 Effect of sorghum biomass on wheat yield ■ and weed biomass ●. (Adapted from Cheema and Khaliq [39])

Silybum marianum (L.) Gaertner., *Melilotus indica* L., *Beta vulgaris* L., *Polypogon monspeliensis* L. (Desf.), *Trifolium repens* L. and *Plantago ovata* Forssk. and *Convolvulus arvensis* L. [35–37]. Sorghum biomass caused a reduction of weed mass in wheat cultivation and had a positive effect on the yield of this species (Fig. 11.7) [35]. The toxic effect of plowed sorghum biomass was observed already 1 week after the beginning of sorghum biomass degradation and it continued up to 8–10 weeks (depending on the amount of biomass absorbed) [38]. The effect on the length of *Chenopodium album* L. seedlings depended on the amount of biomass to

be broken down in the first 6 weeks after plowing, and also resulted from varietal differences and phytotoxicity of the plowed biomass.

Sorghum allelopathic potential results from different content in grains, husks, leaves, stems and roots of phenols and in particular: ferulic, *p*-coumaric, *p*-hydroxybenzoic, vanillic and syringic acids and their slow release during the decomposition of post-harvest sorghum residues. The allelopathic potential depends more on the quality of phenolic compounds than their amounts. Mallik et al. [40] reported that among gallic, syringic, chlorogenic, vanillic, caffeic, ferulic, and coumaric acids, only chlorogenic acid manifested allelopathic action on *Chenopodium album* L. In addition, the extraction of individual compounds is expensive and cumbersome from the technological point of view; and what is more frequently used instead of a mixture of compounds or water extract.

11.3.2 Crop Mixtures and Intercropping

Intercropping and crop mixtures are used in some parts of the world, mainly on small farms in tropical and subtropical zones. Environmental, production and economic effects are the main determinants of this method of plant cultivation by farmers. The scientific justification for the advisability of intercropping also emphasizes protection against erosion, limiting the rate of reduction of soil organic matter, the content and availability of nutrients, increasing soil microbiological activities and limiting weed infestation with troublesome weed species, e.g. *Striga hermonthica* (Del.) Benth [41].

In crop mixtures and intercropping, the productive effect, apart from the fundamental constituents of the environment, is also influenced by the interaction between species and access to the limiting factor of the habitat. The decision on crop mixing or intercropping depends on the degree and possibility of reducing weed infestation and infection by diseases and pests. The production technology used on a farm is another condition that should be taken into account. Therefore, the selection of plant species in intercropping should be complementary so that the cultivated species use basic environmental factors in different ways. It is necessary to analyze their suitability for such cultivation and choose agricultural technology adapted to the requirements of plants.

In the available literature, for the most part, the research results confirm that intercropping and crop mixing are more effective in reducing weed infestation than homogeneous crops [42]. Schoofs and Entz [43] and Cheema et al. [17] recommended the inclusion of intercropping as one of the basic methods of integrated weed control. Limiting the growth and development of weeds in such a system occurs through two ways, i.e. interspecies competition and the secretion of allelochemicals into the rhizosphere through the root system and their allelopathic (inhibiting or stimulating) development of the cultivated species [44]. Allelopathic interaction provides a larger balance area than just competition for an element of the environment (Fig. 11.8).

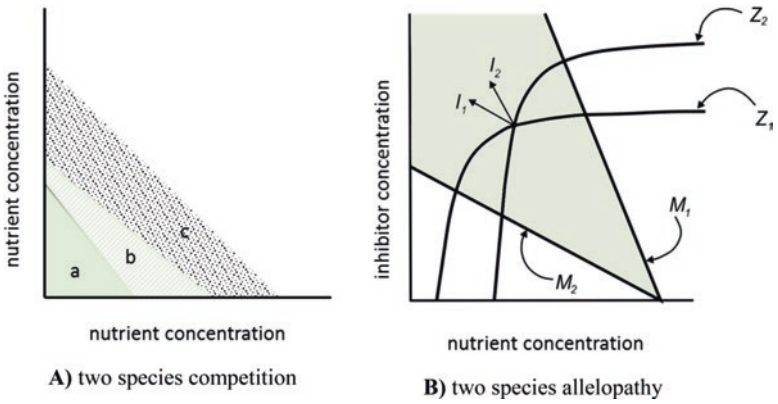

Fig. 11.8 Competition and allelopathy. Differences on Lotka-Volter model [47]. (**A**) Competition between two species: a – area for development of both species, b – area for development only for one species, c – area not favorable for both species. (**B**) Effect of allelopathy between two species compete for nutrient as an element of environment. The marked area for populations 1 and 2 (indicated by Mi) subjected stable coexistence (indicated by vectors Ii). (Adapted from Grover [48])

Growing sorghum with other crop species (mainly *Fabaceae*) is common in India, Pakistan, many African countries, as well as North and South America [28]. Many publications have confirmed that the use of sorghum as a component of such cultivation has contributed to the effective method for weeds control. Cultivation of sorghum with cotton reduced the number of *Cyperus rotundus* (L.) plants by 70–96% and the dry weight of this weed by 71–97% [44]. Similarly, intercropping of sorghum and maize significantly reduced the number of the weed species *Cyperus rotundus* (L.) by 52%, *Convolvulus arvensis* (L.) by 73% and *Trianthema portulacastrum* (L.) by 69% [45, 46]. Cultivation of sorghum with peanut and soybean proved to be very effective in limiting the number of *Striga hermonthica* (Delile) Benth., with 12% to 70% and 3% to 54% reduction in parasitic plants when sorghum was grown with peanut and soybean, respectively, compared to sorghum monoculture.

11.3.3 Sorghum as a Cover, Smother and Catch Crop

Limiting weed infestation in crop rotation without the use of herbicides is possible by sowing cover crops, smother (shading) plants, living mulch, catch crops, intercrops and protective crops [49–52]. Both smother, ground cover and catch crops are sown as a crop rotation element or after harvesting the main crop, when the remaining vegetation period allows their cultivation. The goal is not to obtain a crop that will be used for different exploitation purposes. In such cultivation methods, the produced biomass performs mainly protective functions and limits: erosion, nutrient losses and weed growth. Species sown as smother or cover plants cover the soil

and limit the access of light to weeds and inhibit their growth and competition. Limiting the growth of weeds through the cultivation of ground cover plants is supported by the secretion by the root system of chemicals that inhibit weed seed germination.

Cultivation of ground cover plants not only reduces the occurrence of annual weed species. Species used as ground cover can be used as covers to restore the naturally occurring perennial sward (plants species composition) to restore the original character of plant communities [25]. The most important species with such properties include: buckwheat (*Fagopyrum esculentum* Moench.), foxtail millet (*Setaria italica* (L.) P. Beauv.), rye (*Secale cereale* L.), sorghum spp., alfalfa (*Medicago sativa* L.), sunflower (*Helianthus annus* L.) and some cruciferous plants [53]. Sorghum and sorghum hybrids with Sudangrass can be sown after early crops or in regions where the cultivation of other species is risky due to limited water resources [54]. The size of the aboveground mass and the ability to cover the surface make sorghum attractive as a smother and cover species (Figs. 11.9 and 11.10). During the 50–60 day vegetation period, sorghum in plastic tunnels obtained from 11.6 to 14.5 t of dry matter from ha, similar to that obtained in field conditions at 120–140 days of vegetation, and the amount of water used was up to 5 times lower than in field cultivation [54].

High value of sorghum as a smother species was reported in studies conducted by Milchunas et al. [25]. The goal of research conducted in Colorado was to restore prairie vegetation on arable land. Sorghum and wheat were sown as smother plants, and after their harvesting a mixture of prairie meadow species was sown in the following proportions:

Fig. 11.9 Sorghum as cover crops. (**A**) stand of 30 days after sowing (DAS), on high densities – 60 plants per square meter (3 times higher than standard). (**B**) Sorgo x Sudangrass hybrid at 60 DAS at first harvest cut. (Photos: J. Sowiński)

Fig. 11.10 Sorgo x Sudangrass hybrids ratoon after first cutas cover crops 3 days frost (**A**), 3 weeks after frost (**B**). Tomato cultivated on sorghum straw cover (**C**) (Photos: K. Adamczewska-Sowińska, J. Sowiński)

Pascopyrum smithii Rydb. – western wheatgrass	30%,
Bouteloua gracilis Willd. ex Kunth – blue grama	20%,
Bouteloua curtipendula Michx. Torr. – sideoats grama	20%,
Nassella/Stipa viridula Trin. – green needlegrass	10%,
Panicum virgatum L. – switchgrass	10%,
Dalea purpurea Vent – purple prairie clover	10%.

Sowing the mixture after wheat cultivation caused an increase in the share of annual species by 50% and exotic species by 67% compared to the botanical composition obtained when sowing was carried out after sorghum cultivation. In contrast, sowing after sorghum cultivation (as a smother species) caused an increase in coverage by native species by 245%, permanent grass species by 270% and western wheatgrass by as much as 811% compared to the coverage of surface after wheat sown as a smother plant. The high usefulness of sorghum resulted from the limited availability of nitrogen, which contributed to the increase in the share of annual species, in particular kochia (*Bassias coparia* (L.) A.J. Scott.) and Russian thistle (*Salsola tragus* L.) after using wheat as a smother species. In addition, the allelopathic effect of sorghum sown as a smother plant limited the growth of alien, invasive species and contributed to the good development of western wheatgrass [25].

Difficult conditions during the occurrence of drought as well as the type of soil can potentially affect the effectiveness of allelopathy and allelopathic activity of compounds found in individual plant species.

The phenomenon of allelopathy and the presence of rhizosphere fungi and other microorganisms mean that crops using sorghum as a ground cover contribute to improving the physical and chemical properties of the soil, and also allow the renewal of land and restore natural communities.

11.4 Effect of Sorghum Allelochemicals on Weeds

In an ecosystem, many important interactions are based on chemical regulations and a wide group of chemical compounds that directly or indirectly affect plants. These relationships occur between populations or between processes occurring within a population, taking various forms: commensalisms, competition, mutualism, and pathogenesis. These compounds interact in different ways, and relationships between species are from neutral through favorable to unfavorable (Table 11.2). The most important of them belong to the following groups: enzymes, vitamins, hormones, chelates and allelochemicals. Groups of chemical compounds that are secreted into the environment by leaching, decomposing, volatilizing or root secretions and at the same time have an impact on the biological processes that occur between plants are called allelochemicals [15]. In many species they occur in all parts of plants such as: leaves, stems, flowers, pollen, seeds and fruits, and roots.

Sorghum is a crop with high allelopathic ability and its active compounds are distributed in different parts of the plant. The range of action of allelochemicals is wide – from changes in physiological and biochemical processes, through the activation of cell division and anatomical changes in the cell. Some of them inhibit the process of photosynthesis and respiration and increase oxidative stress, contributing to the accelerated process of cell death and, consequently, the entire weed plants [55]. The assessment of the suitability of plants as an allelopathic species is often possible by determining their total content of phenolic compounds.

During the growing season as well as during the decomposition of biomass, compounds released to the environment are usually an organic mixture that can interact through synergism modified by other environmental factors. In the conditions of rainfall deficiency, high temperature, severe disease and pest infestation or nutrient deficiency, the allelopathic effect is stronger [56]. In conditions of high soil moisture, cloudy weather and intensive rainfall, the content of allelochemicals and their activity is lower [57]. High soil moisture stimulates biological activity and sorption

Table 11.2 Different interaction between plants

Interaction type	Species first	Species second
Mutualism[a]	↑	↑
Commensalism[a]	↑	=
Competition	↓	↓
Allelopathy	↑	↓
Herbivory[a]	↑	↓
Predation	↑	↓
Parasitism[a]	↑	↓
Amensalism	↓	=

[a] some specific interaction is called symbiosis – Mutualism, Commensalism, Herbivory, Parasitism
Interaction unfavourable (↓), favourable (↑), neutral (=)

of allelochemicals by soil particles and as a consequence, allelochemicals become biodegraded by microorganisms.

Sorghum contains many substances that have allelic character and allelopathic effect. The basic one is sorgoleone, produced by root hair. It has a strong limiting effect on the growth of other species, including crops [58].

There are many compounds in sorghum biomass and their usefulness has been evaluated in various conditions (laboratory, controlled and field): chlorogenic, *m*-coumaric, *p*-coumaric, caffeic, *p*-hydroxybenzoic, ferulic, vanillic, syringic, gallic acids, and *p*-hydroxybenzaldehyde [36, 38, 59, 60].

In the aboveground parts of sorghum and sorghum hybrid with Sudangrass, there are hydroxybenzoic acid and *p*-hydroxybenzaldehyde, which inhibit the growth of seedlings of annual weed species [28]. However, better toxic effects were obtained under controlled conditions than in the field ones. The authors account for the differences with the rapid rate of degradation in non-sterile field conditions. Similarly, the activity of phenolic compounds was short-lived and unstable in field conditions [60].

11.4.1 Allelochemicals in Aboveground Sorghum Parts as a Source of Sorgaab

Water extracts of organic acids from sorghum plants prepared according to the procedure described by Cheema and Khaliq [39] are called sorgaab. They can be made from fresh and dried parts of sorghum plants. Preparation of sorgaab is easy, cheap and does not require a specialized laboratory. The sorghum material (leaves, stems or whole plants) cut into 2 cm sections are soaked at room temperature in distilled water in 1: 20 ratio for 24 h. For easier use after preparation, the extract should be filtered and sterilization at 100 °C for 20 min is recommended. Sorgaab can be used fresh, immediately after preparation or stored frozen (−15 °C) and applied at any time depending on the needs.

Sorgaab contains various water-soluble compounds. Mahmood [61] distinguishes 14 chemicals that are water-soluble and easily go into solution. Iqbal and Cheema [62] determined the occurrence of the following phenolic compounds: gallic, protocatechuic, syringic, vanillic, *p*-hydroxybenzoic, *p*-coumaric, and benzoic acids. Parveen [63] and Nielsen et al. [64] showed the presence in sorgaab of the following: caffeic, ferulic, chlorogenic, syringic and vanillic acids, as well as dhurrin and *p*-hydroxybenzaldehyde.

The limiting effect of sorghum plant extracts on weeds and their beneficial impact on cultivated species has been confirmed in many publications. In the studies by Cheema et al. [35] the concentration of sorgaab used and the number of treatments carried out had an impact on the number and weight of weeds and increased wheat yield (Fig. 11.11).

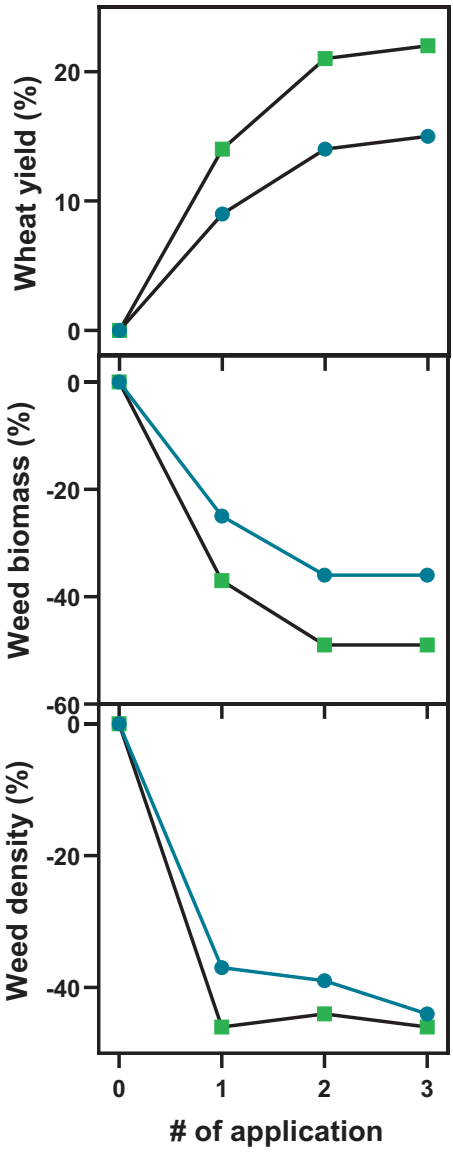

Fig. 11.11 Effect of number of sorgaab application applied as either 5% (●) or 10% (■) concentration (w/v) on wheat grain yield, weed biomass and weed density. Sorgaab was applied either at 1 – 30 DAS (days after sowing), at 2 – 30 and 60 DAS or at 3 – 30, 60 and 90 DAS compare to control (0) without sorgaab application

In the conducted tests, the most sensitive weed species to the applied sorgaab were: *Chenopodium album* L., *Phalaris minor* Retz., *Avena fatua* L., *Convolvulus arvensis* L. *Coronopus didymus* L. (Sm.), *Fumaria parviflora* Lam. and *Rumex dentatus* L. On the other hand, however, the sorghum water extract stimulated the growth of *Melilotus parviflora* Desf. [35].

11.4.2 Sorgoleone – The Main Sorghum Allelochemical as a Bioherbicide

Sorghum is an allelopathic crop that represses the growth of weeds by exuding a number of lipophilic benzoquinones (referred to as sorgoleone) from its root hairs. The most abundant form is 2-hydroxy-5-methoxy-3-[(Z,Z)-8′,11′,14′-pentadecatriene]-*p*-benzoquinone [65] (Fig. 11.12) and its resorcinol derivative, which accounts for 90% of compounds that are present in the root exudates [66, 67]. The remaining 10% of root exudate components include sorgoleone analogues with vary in the degree of saturation of the aliphatic side chains and their respective resorcinols derivatives [68].

11.4.2.1 Herbicidal Activity

Sorgoleone extracts are not very potent when applied postemergence. This is due to the extreme lipophilic nature of this molecule. It does not readily absorbed nor translocated in mature leaves, although sorgoleone does penetrate into hypocotyls and cotyledons [37]. Herbicidal activity was improved via formulation of sorgoleone as a wettable powder [4.6WP]. Broadleaf species were more susceptible than grass weed species. Preemergence application of sorgoleone completely suppressed germination and growth of broadleaf weed species at 0.2 g a.i. L^{-1} active. *Rumex japonicus* Houttuyn. and *Plantago asiatica* L. were most sensitive to sorgoleone, with 100% control following postemergence application of 0.4 kg a.i. ha^{-1}sorgoleone. Most other broadleaf weeds were 90% controlled at that rate. On the other hand, crop species were less sensitive to sorgoleone, with no more than 30% inhibition at the highest rate of 0.4 kg a.i. ha^{-1} [69].

Another approach has been to mix sorgoleone extracts with extracts from other plant species.

A mixture of sorgoleone and root extract of tartary buckwheat (*Fagopyrum tataricum* Gaertn.) was much more active than either extracts alone. Consistent with other studies, broadleaf weed species (e.g., *Galium spurium* L., *Rumex japonicus* Houttuyn., *Aeschynomene indica* L., and *Amaranthus retroflexus* L.) were more susceptible than grass weed species. This example of enhanced suppression of weed growth by sorgoleone and with tartary buckwheat root extract suggests interesting possibilities for effective weed management under organic farming situations [70].

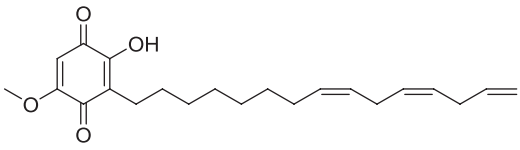

Fig. 11.12 Structure of the main sorgoleone analogue

11.4.2.2 Mechanisms of Action

Detailed studies on the phytotoxic activity of sorgoleone demonstrated that its mechanism of action targets the electron transport chains. With regard to photosynthetic electron transport [71, 72], sorgoleone is structurally similar to plastoquinone (a lipid benzoquinone) (Fig. 11.13a), resulting in competition with the natural electron acceptor at the plastoquinone binding site on the D1 PSII protein (Figs. 11.13b, c and Fig. 11.14) [37, 73].

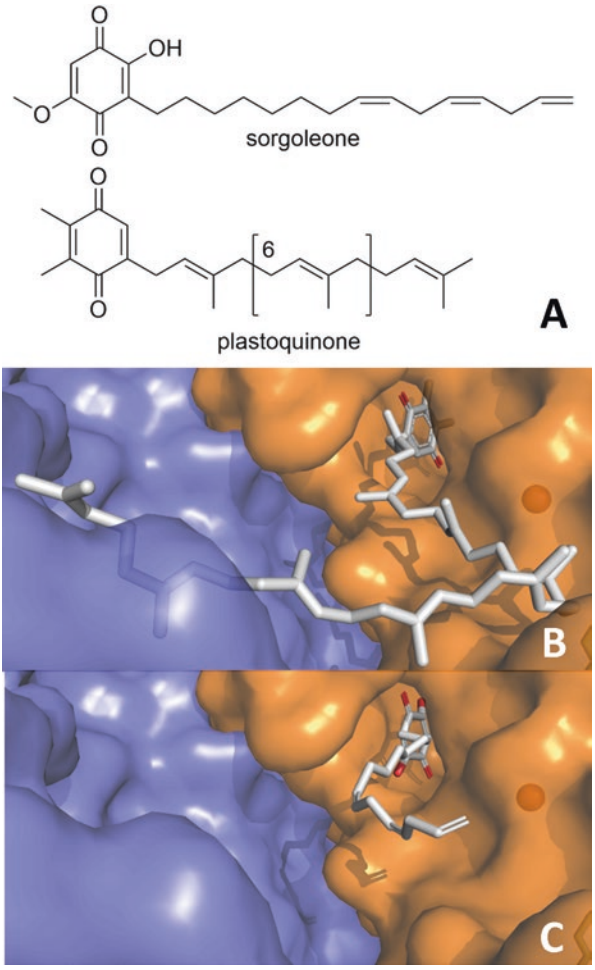

Fig. 11.13 (**A**) Structure of sorgoleone and plastoquinone. (**B**) Plastoquinone binding domain (Q_B) on the D1 protein (gold color) of photosystem II obtained from the crystal structure analysis of photosystem II complex (3wu2) [74], with a close view of plastoquinone binding on QB. (**c**) Modeling of sorgoleone binding in the plastoquinone binding site. Structure of minimized sorgoleone was obtained from Lebecque et al. [75]

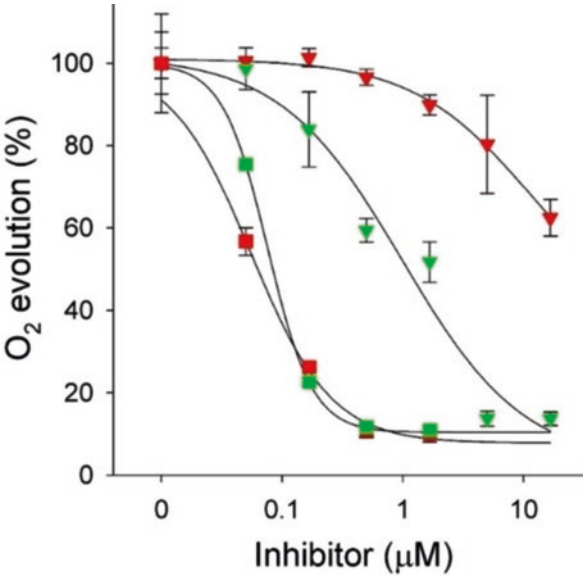

Fig. 11.14 Effect of sorgoleone (square) and atrazine (triangle) on oxygen evolution from thylakoid membranes isolated from wild-type and triazine-resistant redroot pigweed (*Amaranthus retroflexus* L.). ▼= wild type with atrazine; ▼= resistant with atrazine; ■ = wild type with sorgoleone; ■ = resistant with sorgoleone adapted from Dayan et al. [37]

An additional mechanism of sorghum phytotoxic activity [76] is the reduction of carotenoid production through inhibition of *p*-hydroxyphenylpyruvate dioxygenase (HPPD), a key enzyme in carotenoid synthesis and the target site for triketone herbicides. Carotenoid reduction leads to a decreased amount of chlorophyll and subsequent reduced photosynthetic capability. Sorgoleone was tested along with 33 other natural products of various structural classes on HPPD. Recombinant HPPD from arabidopsis is sensitive to several classes of natural compounds including sorgoleone. While the triketone natural products were competitive tight-binding inhibitors (showing parallel lines in the protein titration assays) (Fig. 11.15a), sorgoleone did not bind tightly to HPPD (showing conversion lines in the titration assay) (Fig. 11.15b).

Additionally, sorgoleone lowers the membrane activity of H^+ ATPase, which, in turn, leads to disturbances in water uptake [77]. While the participation of this activity on weed control is not well understood, it is interesting that this natural product interacts with more than one target site, suggesting that evolution of resistance to sorgoleone may not be very likely.

11.5 The Area of Future Research

Based on the experience gained during the last 50 years of intensive use of herbicides, we should understand that by introducing new MOAs we will not be solve the problem of weed infestation. Herbicides, as well as mineral fertilizers and other plant protection products, have contributed to the increase in the productivity of

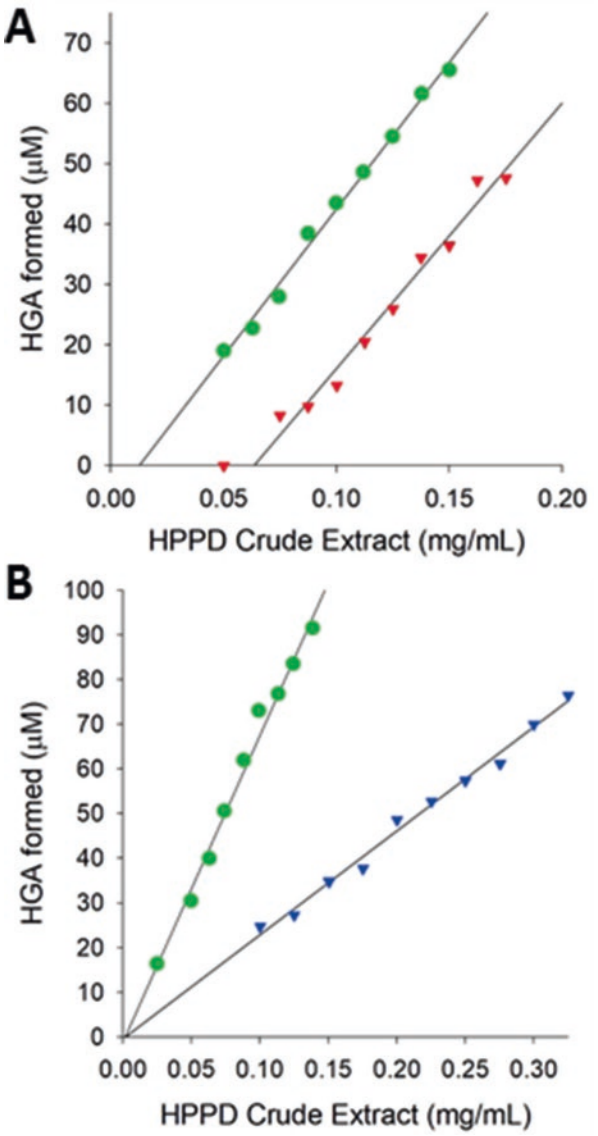

Fig. 11.15 HPPD inhibition kinetics of (**A**) the β-triketone usnic acid, (**B**) the *p*-benzoquinone sorgoleone. ●=no inhibitor; ▼ = 0.03 μM(−)-usnic acid and ▼ (= 1 μM sorgoleone). (Adapted from Meazza et al. [76])

arable crops, while heavily burdening the environment [78]. We should learn that herbicides are only a small part of the solutions that can be used in weed control [79]. Sustainable weed control is a key action for both organic and conventional agriculture. Reducing the occurrence of weeds requires the introduction of new comprehensive methods in addition to the already existing ones.

The use of the phenomenon of allelopathy and organic compounds produced by plants should be a future-oriented area of intensive research and implementation. Sorghum and its forms contain many significant substances that affect other plants and animals (dhurrin). Sorghum with its allelopathic properties should be used as an element of crop rotation, sown as a ground cover, mulch plant or in intercropping. The importance of sorghum in crop rotation and the use of its post-harvest residues should result not only from the increase in soil organic matter content, but also its effect on reducing weed infestation.

In the future, the main area of research and implementation should be focused on the use of compounds present in sorghum, in particular sorgoleone. This is due to the following properties of this compound:

- it is toxic to dicotyledonous and monocotyledonous weeds in very low i.e. 10 μM concentrations [80, 81].
- its postemergence application at a dose comparable to atrazine (0.6 kg a.i. ha^{-1}) inhibits the growth of most 14-day-old weed seedlings [82].
- its pre-emergence application is toxic to small-seeded weed species [31].

This is confirmed by the advanced work on sorghum gene mapping and the recognition of the SOR1 gene, which codes fatty acid desaturase (FAD), the enzyme responsible for the synthesis of sorgoleone in sorghum roots [83].

The expression of this gene is strongly differentiated in sorghum plant parts and the relative values according to Yang et al. [83] were as follows (assuming the initial content in the stems):

Stem	1.0
Immature leaf	1.3
Panicle	1.6
Root with hair removed	4.1
Mature leaf	4.4
Root hair	4369.7

More recent work characterized the function of the fatty acid desaturases responsible for the biosynthesis of sorgoleone [84]. Research attempting to transfer the genes encoding key enzymes involved in the production of this natural herbicides to other plants is on-going.

Research is currently underway to determine the importance of plants in influencing on and modification of the nitrification process. Many studies in this area confirm the ability to reduce nitrification by the secretion of secondary metabolites into the environment by root hairs of many plant species [85, 86]. This process is called biological nitrification inhibition (BNI) and it has been well described in

species such as *Brachiaria* [87, 88]. The results of the first research conducted on cultivated plants showed that sorghum (specifically sorgoleone) manifests strong ability to modify the nitrification process [89].

Interesting results were obtained by Maqbool and Sadiq [90] after applying sorgaab in the form of spraying on maize seedlings. Phenolic compounds from sorghum increased maize resistance to drought and net photosynthesis, the efficiency of water utilization was highest when 1.0–1.5 mL of phenolic compounds per 1 litre of solution was applied.

11.6 Conclusions

One of the many common, transdisciplinary goals for scientists working in the field of agriculture should be to decrease weed infestation with limited or no negative impact on the environment. From a social and demographic points of view, it is also important to ensure food security for the world's growing population up to 9 billion in 2050 [91]. To sum up, we should be optimistic that this must be the case and that future herbicides along with their new modes of action will be discovered through the integrated use of biological methods, i.e. modern "-omics" techniques of genomics, proteomics or metabolomics in combination with traditional biology [92].

Biotechnology-based transgenic plant breeding has been developing actively since the mid-1990s. In addition to the unquestionable benefits for the global economy and food security, new threats are emerging, such as weed resistance through the transfer of the gene responsible for modification of the gene from the crop to weeds. Corrective actions should be taken now and solutions for the future should be sought. In contrast, compounds found in plants also in sorghum provide biological protection through the production and secretion of compounds that can be used to limit the growth and development of weeds.

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Chapter 12
The Fungal Genus *Chaetomium* and Its Agricultural Applications

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12.1 Introduction

12.1.1 Chaetomium *Biology and Taxonomy*

Chaetomium is a fungal genus belonging to the phylum Ascomycota and the family Chaetomiaceae. Several authors reviewed the taxonomy of *Chaetomium* genus [1–12]. Members of this genus are known for their ability to degrade cellulose and grow on various substrates like paper, wood, seeds, straw, dung birds, feathers, textiles and construction materials. They are important agents of decomposition of plant matter and some species are known to be important inhalant allergens and producers of a variety of bioactive metabolites, such as mycotoxins and microbial volatile organic compounds. Also, they have been reported as endophytes, that is, fungi live inside plant tissues without inducing symptoms in their hosts. Besides, they can behave as pathogens as a soft-rot fungus for softwood and hardwood timber and fruit rot in apple [12–15].

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More than 400 species have been described in *Chaetomium* genus. *Chaetomium globosum,* the type species of the genus, is cosmopolitan and it can be found in a diversity of environments such us soil, dung, remains plant materials, seeds and other cellulolytic substrates, as well as indoor and marine environments [15–17]. In addition to its cellulolytic ability, its potential use as plant growth promoter and biological control agent to plant diseases has been reported [18, 19]. By the other hand, there are researches where it is cited as producer of secondary metabolites with biological activities (antitumor, cytotoxic, antibiotic, phytotoxic) and other industrial applications [20, 21].

Chaetomium globosum was described for the first time on 1817 by Kunze and Schmidt [22]. Before the review by von Arx et al. and Wang et al. [12, 23], assigned the taxonomic concept of *C. globosum* to species that produce globose to ovate or obovate ascomata with texture intricata of the wall covered by ascomatal hairs ranging from erect, flexuous to regularly coiled. Clavate, evanescent asci, and ascospores limoniform and bilaterally-flattened shaped with an apical germ pore (Fig. 12.1). Then, Asgari and Zare [24] and later Wang et al. [12, 25] re-defined the taxonomic concept of *C. globosum.* Wang et al. [12] in their last research, confirmed the affiliation of *Chaetomium globosum* with *C. globosum* species complex through of phylogenetic analyses of DNA-directed RNA polymerase II second largest subunit (rpb2), β-tubulin (tub2), ITS and 28S large subunit (LSU) nrDNA sequences, together with morphological comparisons with related genera and species. *C. globosum* species complex was confirmed as a monophyletic clade which is called *Chaetomium sensu stricto.*

This complex contains a variability of morphological characters within the *Chaetomiaceae* family. Wang et al. [12] define this complex by the following features: ascomata globose, ellipsoid to ovate or obovate, ostiolate or non-ostiolate; ascomata with texture intricata or epidermoidea of the wall, asci clavate or fusiform with eight biseriate ascospores and evanescent; limoniform, globose to irregular, bilaterally flattened and length of more than 7 μm. The asexual morphs (*Acremonium*) is only known to species *C. angustispirale*, *C. elatum*, *C. rectangular* and *C. subaffine*, and this acremonium-like.

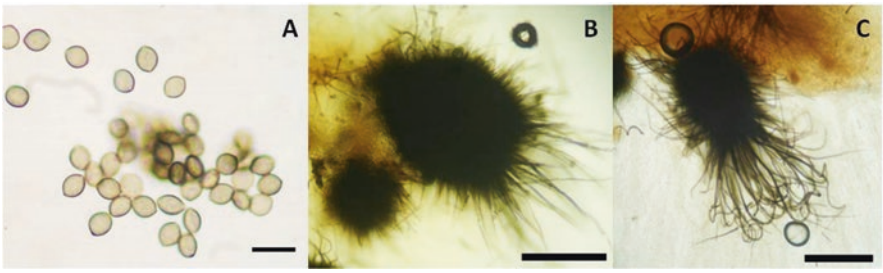

Fig. 12.1 Microscopic structures of *Chaetomium globosum*. (**a**) Unicellular, lemon-shaped ascospores OM 40X scar = 10 μm. (**b**) Ascomata globose or oval ostiolar with irregular hairs without undulations OM 40X scar = 200 μm. (**c**) Ascomata with straight or undulate hairs OM 40X scar = 200 μm

Table 12.1 *Chaetomium* species within the *Chaetomium globosum* species complex in accordance to Whang et al. [12]

Group I	*C. globosporum;, C. grande, C. megalocarpum; C. nozdrenkoae; C. contagiosum; C. cervicicola; C. madrasense; C. ascotrichoides; C. fimeti; C. subfimeti and C. interruptum*
Group II	*C. globosum; C. unguicola; C. tenue; C. afropilosum; C. pseudoglobosum;, C. umbonatum; C. novozelandicum.*
Group III	*C. olivaceum; C. cucumericola; C. undulatum; C. subglobosum; C. spiculipilium; C. pseudocochliodes; C. cochliodes; C. subaffine; C. telluricola; C. capillare; C. angustispirale; C. graminiforme; C. elatum; C. rectangulare; C. spirochaete* and *C. pilosum*
Species in basal lineage	*C. coarctatum* and *C. citrinum*

According to this research, thirty-six species were recognised in this complex. They were gathered in three groups clustered in two main clades (Table 12.1).

12.1.2 Metabolites

A secondary metabolite is any organic molecule produced by a microorganism, which is not essential for its growth, development and reproduction, but under certain conditions plays an important role in interaction with other microorganisms [26]. Many of these compounds have reported significant biological activities, such as antitumor, antimalarial, cytotoxic, enzyme inhibitory, antibiotic and phytotoxic [20].

The *Chaetomium* genus produces around 200 secondary metabolites with a great diversity. The main chemical structures of the metabolites that have been elucidated, belong to the following groups: chaetoglobosins, epipolythiodioxopiperazines, azaphilones, depsidones, xanthones, anthraquinones, chromones, terpenoids and steroids. Within the metabolites that *Chaetomium globosum* produces it can be mentioned chaetoglobosins A, B, C, D, E, F, G, J, Q, R, T, U, V, W; prochaetoglobosins I and II; gliotoxin; methylthiogliotoxin; fumitremorgins; echinuline; chetomin; cochliodinol; cerebrocides, chaetoviridins A and B, allantoin, chaetomugilins A to H; epichaetomugilin A; chaetoglocins A and B; penochalasin A and epichaetoviridin A [20, 27].

To determine the structure of secondary metabolites, the extraction and isolation of the compounds must be done. The fungus is cultivated in liquid broth, which is extracted with organic solvents and it is subjected to column chromatography on silica gel and Sephadex. The fractions obtained were further subjected to preparative HPLC (High Performance Liquid Chromatography) to afford pure compounds. The structures of secondary metabolites are determined through spectroscopic analysis, including UV (UltraViolet), IR (InfraRed), MS (Mass Spectrometry), and NMR (Nuclear Magnetic Resonance). The absolute configurations of several

compounds can be established by X-ray analysis, CD spectra together with computational chemistry [20].

12.2 *Chaetomium* in Agriculture

12.2.1 Biological Control Agent

Biological control, in plant pathology, refers to the use of microbial antagonist to suppress diseases. The biocontrol could be due for competition for nutrients, space or substrate, antibiosis, mycoparasitism or the combination of them. Antagonism between organisms results on a negative effect for one or for both. Antibiosis occurs when microbes have the ability to produce or to secrete one or more compounds with antibiotic activity. The antibiotics have the particularity of suppressing growth of the target pathogen *in vitro* and *in vivo* conditions. Competition within and between species results in decreased growth of the interacting organisms because non-pathogens compete with pathogens for nutrients, space or substrate around the host plant. Parasitism is a symbiosis in which two organisms coexist in a period of time. In this type of association, one organism (the parasite) can benefit and the other (the host) is the damage one [28].

Some species of *Chaetomium,* principally *Chaetomium globosum,* have the ability to control seedborne and soilborne phytopathogens, as well as its properties have been reported for the control of nematodes, aphids and beet armyworms [29, 30]. Istifadah and McGee [31] evaluated the effect of *C. globosum* on *Pyrenophora tritici-repentis* in wheat. Leaves were inoculated with a spore suspension and with filtrates of the antagonist. Both treatments decreased the disease but the degree of disease reduction was variable, this was due to time of application and wheat cultivar. Mandal et al. [32] inoculated *C. globosum* in soil and in wheat leaves to control *Bipolaris sorokiniana*. The soil application of antagonist decreased the browning on collar region of the seedlings and the foliar application reduced the disease severity. On the other hand, Gurucar et al. [33] pelleted the soybean seeds with *C. globosum* to evaluate the effect on *Macrophomina phaseolina*. They observed that the treatment in seeds caused a greater germination and a lower incidence of the disease under greenhouse conditions. At the same time, plants growth and the average of grain yield increased. In field conditions, the treatment was also effective, because incremented the field emergence and decreased the incidence of the disease. Dhingra et al. [34] observed that the inoculation with ascospore suspension of *C. globosum* on soil-surface of soybean stubble reduced the primary inoculum of *Diaporthe phaseolorum* f. sp. *meridionalis* in field conditions. In the results obtained by Vannacci & Harman [35], *C. globosum* was one of the more effective strains to control *Alternaria brassicicola* and *Alternaria raphani* on radish seeds.

In relation to soilborne phytopathogens, Shantiyaa et al. [18] studied the biocontrol of *C. globosum* on *Phytophthora infestans* in potato. Tubers and leaves were treated with a spore suspension. Also, the soil was inoculated. All the treatments

lower the disease incidence in comparison with control, and the combination of all of them was more effective that each treatment alone. Di Petro et al. [36] evaluated the effect of *C. globosum* on *Pythium ultimum* on sugar beet. They treated the seeds with a spore suspension of the antagonist and inoculated the soil with wheat bran colonized by the antagonist. Also, they combined the treatments with chemical fungicide and observed that the combination was more effective that each treatment alone, and the soil inoculation was at least the double more effective that seed treatment.

Yan et al. [37] studied the nematicidal effect of *C. globosum* isolated from cucumber seedlings. They found that *C. globosum* was one of the more effective antagonists against *Meloidogyne incognita* because reduced significantly the numbers of galls formed. This could be due to the production of nematicidal compounds and compounds that affected root-knot motility. The researchers concluded that the strain of *C. globosum* had the best potential to be used as seed treatment to control the agent for root-knot nematode. Also, Zhou et al. [30] reported that *C. globosum* strain TAMU520 inhibited root-knot nematode (*Meloidogyne incognita*) infection in cotton crop and affected negatively the fecundity of both cotton aphids (*Aphis gossypii*) and beet armyworms (*Spodoptera exigua*). These results showed that the use of a single fungus can control a range of invertebrate herbivorous in a major crop.

Soytong et al. [38] patented and registered the only bioformulation formed with *Chaetomium* strains (Ketomium®). This was formulated as bio-pellet and biopowder, with twenty-two strains of *C. globosum* and *C. cupreum*, which demonstrated antagonistic capacity against various plant pathogens. Ketomium® was registered as mycofungicide, for the control of plant diseases. It has also registered as a biological fertilizer, for degrading organic matter, for induction of plant immunity and as growth promoter. These researchers evaluated the effect of Ketomium® in field for more than 4 years, with the application of Ketomium® as a broad-spectrum mycofungicide. It has been shown that this biological product can be applied to the field-soils infested with the test pathogens. This report has shown that Ketomium® is effective and demonstrates its usefulness in disease control. However, it is necessary to carry out other field studies and observations to establish a better approach on the use of Ketomium®. They consider for increase the effectiveness of Ketomium® in plant disease control, especially soilborne pathogens, it must be integrated with other control measures such as cultural practices: sanitation, improving water drainage, pruning, removal of disease plant parts, adding organic compost and liming. Tomilova and Shternshis [39] evaluated the fungicidal activity of this bioformulation from *Chaetomium globosum* and *Chaetomium cupreum* (Ketomium®) against *Rhizoctonia solani* and *Fusarium oxysporum*. In both phytopathogens, the bioformulate had inhibitory effect. A high inhibition, on the third day, against *Rhizoctonia solani* was observed and then decreased, on the contrary with *Fusarium oxysporum* the inhibition was increased progressively. They found that the inhibition activity depended on its concentration, duration of storage and growth characteristics of cultures of the pathogens. To assess changes in the activity of the preparation, pure cultures of the bioformulation were examined after 1 and 2 years of storage. The activity of the biological preparation decreased after 1 year, but after 2 years showed

antifungal activity. They did field experiments on potato plants against *R. solani*, introducing Ketomium® on soil. The disease incidence decreased after treatment.

Other species of *Chaetomium*, with biological control properties, had been studied. Tann and Soytong [40] evaluated the effect of *Chaetomium cupreum* on *Curvularia lunata*, causal agent of brown leaf spot in rice. The rice seedling treated with spore suspension and nanoparticles of *C. cupreum* demonstrated that incidence of disease was lower in relation to the control. So, in greenhouse conditions, all the treatments were effective against the pathogen. Soytong [41] studied the effect of *C. cupreum* on *Fusarium oxysporum* f. sp. *lycopersici* in tomato and observed that the treatment with the antagonist lower the disease incidence and was more effective than the chemical treatment. On the other hand, Vilichet al. [42] treated the seeds of barley with *C. globosum* and *Chaetomium funicola*, against *Erysiphe graminis* f. sp. *hordei*. They inoculated the seeds with spore suspension, and observed that treated plants were more resistant to the pathogen in relation to the control.

12.2.2 Metabolites

Many researchers have evaluated the antagonistic capacity of *C. globosum* through culture filtrates in which the presence of antifungal substances is suggested. Rajakumar et al. [43] tested the efficacy of the filtrates of *C. globosum* against to *Ascochyta rabiei* isolated from chickpea. They observed that the filtrate decreased the conidia germination and growth of the colony. *In vivo*, the severity of the disease in the pre-inoculation and the post-inoculation was reduced with respect to the control. In concordance, Mandal et al. [32], Aggarwal et al. [44] and Istifadah et al. [45] evaluated the efficacy of the culture filtrates of the antagonist against the wheat pathogens. Mandal et al. [32] proved that the filtrate of *C. globosum* reduced the conidial germination of *Bipolaris sorokiniana* and in bioassays, decreased the severity of the disease. This agree with Aggarwal et al. [44], which not only tested the culture filtrates effect but also analyzed the crude extract of the filtrates, through TLC (Thin Layer Chromatography). They concluded that the production of antifungal compounds was related with the antagonism against *B. sorokiniana*, being the most effective the isolate with the higher production of metabolites *in vitro* and *in vivo*. Istifadah et al. [45] showed that the culture filtrates of *C. globosum* were the most effective in the reduction of the mycelial growth of *Pyrenophora tritici-repentis*, in comparison to the filtrates of other species of *Chaetomium.*

Kumar et al. [46] evaluated extracts of *C. globosum* EF18, isolated from *Withania somnifera*, against *Sclerotinia sclerotiorum*. Ethyl acetate and methanol extracts were more effective than hexane extract. They isolated the bioactive compound from ethyl acetate extract following bioassays, using Vacuum liquid chromatography (VLC), Column chromatography and preparative HPLC. In concordance, Awad et al. [47] studied the petroleum ether and ethyl acetate extracts of *C. globosum* against *Rhizoctonia solani*, *Pythium ultimum*, *Fusarium solani*, *Fusarium oxysporum* and *Candida albicans*. They observed, in concentration of 100 mg of the

petroleum ether extract, the maximum antifungal effect against *C. albicans, R. solani* and *P. ultimum* and moderate antifungal effect against *F. solani* and *F. oxysporum*. In the ethyl acetate extract at concentration of 100 mg, they recorded higher antifungal effect against *C. albicans*, *F. solani* and *P. ultimum* and moderate effect against *F. oxysporum* and *R. solani*. The researchers registered a positive correlation between the increase of the pathogen inhibition and concentration of both extracts. They isolated the compounds and found in the petroleum ether extract, undecyl benzene, ergosterol, methyl tetradecanoate and methyl 9-tetradecanoate. Also, prenisatin, chrysophanol, chrysazin, chaetoviridins A and B were isolated from the ethyl acetate extract.

Charoenporn et al. [48] evaluated crude hexane, crude ethyl acetate and crude methanol extracts of *C. globosum* N0802 and *C. lucknowense* CLT for inhibition of *F. oxysporum* f. sp. *lycopersici.* They demonstrated that these substances could break the conidial cells of the pathogen. Also, assayed formulations with spores of the antagonist fungi and observed a disease reduction of tomato wilt of 44.68% and 36.28% respectively.

Zhang et al. [49] proved the efficacy of the *C. globosum* filtrates against *Setosphaeria turcica* and observed a remarkable suppression capacity of the pathogen growth in presence of the filtrate. Also, these researchers characterized the metabolites in the culture filtrate through Mass spectrometry (MS) and Nuclear Magnetic Resonance (NMR), and determined the presence of chaetoglobosins A and C. They evaluated the efficacy of chaetoglobosins A *in vitro* and observed a high inhibition capacity of this metabolite against the mycelial growth of the pathogen. In the same way, Biswas et al. [29] identified five metabolites in the culture filtrates of *C. globosum*: BHT, cochliodinol, mollicelin G, chetomin and chaetoglobosins. Of these, chaetoglobosins and chetomin were the most effective in the growth suppression *in vitro* against the pathogens *Bipolaris sorokiniana, Fusarium graminearum, Pythium ultimum, Macrophomina phaseolina* and *Rhizoctonia solani*. Di Pietro et al. [50] evaluated the effect of the filtrates of different isolates of *C. globosum* against the pathogen *P. ultimum* isolated from sugarbeet. They found that the filtrate effect in the growth suppression of the pathogen, depends not only on the antagonist isolate but also on the culture growth temperature. Only one of the antagonist isolates produced mycelial growth inhibition and the presence of chetomin was identified. The researchers concluded that the isolate that produced chetomin in broth was the more effective against *P. ultimum*. Coinciding with these researchers, Zhao et al. [51] evaluated the effect of the metabolites of *C. globosum* against a wide range of phytopathogenic fungi: *Rhizoctonia solani*, *Fusarium moniliforme*, *Magnaporthe grisea*, *F. graminearum*, *Rhizoctonia cerealis*, *Sclerotinia sclerotiorum*, *Phytophthora capsici*, *Alternaria solani* and *Botrytis cinerea.* These authors observed that the mycelial growth of all phytopathogens was inhibited in different levels. They focused the studies on *Sclerotinia sclerotiorum* and registered that a certain concentration the filtrates inhibited the 90% of sclerotia germination. The metabolites identified were chaetoglobosins A-E and Vb, which had a strong antifungal effect against *S. sclerotiorum*. In concordance, Jiang et al. [52] determined the presence of chaetoglobosins A in filtrates of *C. globosum* and evaluated the effect on the mycelial growth of *Fusarium sporotrichioides*. The

metabolite inhibited the growth mycelial and the conidial germination. Zhang et al. [53] also determined the presence of the chaetoglobosins principally A, C, D, E, G and R among other metabolites. The authors evaluated the effect of the chaetoglobosins A on *Rhizopus stolonifer* and *Coniothyrium diplodiella* and observed that it had inhibitory effect.

Hu et al. [54] registered that the chaetoglobosins, principally chaetoglobosins A, were present in the culture filtrate of *C. globosum* and were the most predominant group of metabolites. These authors evaluated the nematicide capacity of the filtrate and found that it had significant inhibitory effect on the second stage juvenile of *Meloidogyne incognita.*

On the other hand, Yue et al. [27] identified through the analysis of the crude extract of the *C. globosum* the presence of chaetoglobosins C and T, epichaetoviridin A, prochaetoglobosins I, chaetoviridins E and A. They determined that the latter was the most efficient in the control on *B. sorokiniana* isolated from wheat. In coincidence, Park et al. [55] proved the effect of the chaetoviridins and concluded that the chaetoviridins A and B were the most effective in the growth inhibition of *Magnaporthe grisea*, *Phytophthora infestans* and *Puccinia triticina*. Between both metabolites, chaetoviridin A was more efficient because in lower concentrations it had a greater effect.

Other species of *Chaetomium* have been registered as producers of secondary metabolites. In Thailand, Soytong [41] and Soytong et al. [38] determined that a specific isolate of *Chaetomium cupreum* produced secondary metabolites that significantly suppressed tomato wilt caused by *Fusarium oxysporum* f. sp. *lycopersici* in tomato fields. Kanokmedhakul et al. [56] confirmed that this isolate of *C. cupreum* produced rotiorinols A, B and C and rotiorin, which also exhibited antifungal activity against *Candida albicans*. Phonkerd et al. [57] identified from two strains of *Chaetomium cochlioides*: cochliodones A, B, C and D, chaetoviridines E and F and a new epi-chaetoviridin A. It has been reported that *Chaetomium elatum* produces, prochaetoglobosin III, chaetoglobosins V, B, C, D, F and G, and isochaetoglobosin D [58]. Also, Vilavong & Soytong [59] assayed the hexane, ethyl acetate and methanol crude extracts from *C. cupreum* CC3003 for the control to *Colletotrichum gloeosporioides.* These extracts showed a significative inhibition. Moreover, the authors worked with coffee plants and proved a spore suspension, a bio-formulation in powder and nano-rotiorinol (nano-particles). Anthracnose disease was reduced in 54.77% by the powder bio-formulation while the nano-rotiorinol and spore suspension reduced its incidence in 46.23 and 18.59%, respectively.

12.2.3 Plant Growth Promotion

The microbial community, including fungi, is an important component of the soil-plant system that affects the development of plants and could promote their growth. Representatives of the genus *Chaetomium* can promote plant growth acting as: biofertilizers, phytostimulators, biopesticides and bioremediators. In the first case,

cause the mobilization of nutrients in soils, which increases the availability of nutrients such as nitrogen or phosphorus. By the other hand, as phytostimulators through the production of numerous regulators of plant growth, such as gibberellins and auxins (IAA). Also, they can protect plants from phytopathogens, through their control or inhibition or they can improve soil structure and bioremediate contaminated soils [60, 61].

Several researchers have studied the effect of production of phytohormones by *Chaetomium* spp. on plants. Mahmound et al. [19] evaluated the effect of one strain of *Chaetomium* spp. on the seeds vigor of durum wheat. At the same time, they assessed the production of indol acetic acid (IAA) and hydrocyanic acid (HCN) by this strain. These researchers observed that the treated seeds showed a higher germination, a longer length of seedlings and a higher dry and fresh weight for aerial part and roots. In this sense, Khan et al. [62] studied the effect of *C. globosum* isolated from chilli pepper on pepper plants. They observed that inoculated plants had a longer root and more shoot biomass, more chlorophyll content and bigger leaf area. They also determined that this isolate produced IAA and active and no active gibberellins, but the proportion of active gibberellins was higher.

Several studies have been conducted to evaluate the effect of species of *Chaetomium* spp. as biofertilizers. Kowapradit et al. [63] determined that the application of *C. lucknowense* improved soil fertility and caused higher yield in rice because increased the number of tillers, plant height, number of grains per panicle, number of panicles and grains weight per panicle. Moreover, Yadav et al. [64] observed that *C. globosum* mixed with organic matter improved the yield of clusterbean because the content of organic nutrients and the availability of nitrogen, potassium and phosphorus were higher. It must be taken into account that most agricultural soils are characterized by phosphorus deficiency. This is because they generally present high amounts of insoluble phosphorus that is not found in their available form for plants. In this sense, the solubilization of phosphorus by microorganisms is very important, since it releases or solubilizes them [26]. Mahmound et al. [19] reported that the strain of *Chaetomium* spp. which they worked with, increased the solubilization of phosphorus. Also, Tarafdar and Gharu [65] observed the positive influence of the inoculation by *C. globosum* in the pearl millet and wheat, apparently as a result of the release of phosphatases and phytases which mobilized phosphorus and made it available for plants.

The effect of *C. globosum* as soil remediator, has been reported. Abou Alhamed & Shebany [66] studied the effect of inoculation by *C. globosum* in soil contaminated by copper on seedling of maize. They observed that the seedling with the treated soil had higher weight and the toxic effect was reduced, possibly due to the presence of solutes that allowed maintaining a favorable osmotic potential in the presence of copper. On the other hand, Ortiz et al. [67] reported that *C. cupreum* had high tolerance to metalloids in soil, principally copper. They observed that the inoculation with *C. cupreum* in *Eucalyptus globulus* increased plant growth (principally shoot and root dry weight) and mitigated the toxic effect by copper. This could be due to *C. cupreum* increased the siderophores and IAA production, under Cu presence. Siderophores form complexes with other metalloids which would

imply the possible absorption and mobilization of copper, decreasing its availability in soil.

As well, it has reported the effect of *Chaetomium* spp. as biopesticide focusing its effect as plant growth promoter. Soytong et al. [68] cited that the application of *Chaetomium* spp. in tomato improved the plant stand and could decrease the wilt of tomato caused by *Fusarium oxysporum* f. sp. *lycopersici*. On the other hand, Soytong & Ratancherdchai [69] observed that the treatment of potato tubers with *Chaetomium* produced longer tubers and they did not observe differences among this treatment with chemical treatment. Soytong et al. [70] determined that *Chaetomium* spp. produced microbial elicitors that raised the yield of tomatoes and induced immunity against phytopathogens.

12.2.4 Production of Chaetomium *Enzymes*

The filamentous fungi have been reported as producers of a variety of enzymes as β1,3-glucanase, chitinases, proteases, cellulases, xylanases, esterases, alkaline phosphatases, lipases and MAPK kinases [71–73]. These enzymes are used by species of the genus *Chaetomium* spp. for survival, adaptation to environmental stress conditions, biological control processes and mycoparasitism [74–77].

Many of the enzymes produced by *Chaetomium* spp. are used for biotechnology purposes in food, paper and fuel industries [78, 79]. Furthermore, some species of *Chaetomium* release phytases and phosphatases that mobilize and make available nutrients for plants, allowing its use as a biofertilizer and plant growth promoter [80]. The broad spectrum of enzyme production makes this fungus widely studied for potential application for industry and agriculture.

12.2.4.1 Xylanases

Xylanases are responsible for the degradation of xylan to xylose and xylo-oligosaccharides. A variety of xylans can be found in nature depending upon the side chains attached to the xylan backbone. It is the major component of hemicelluloses in plants. These enzymes have received great attention on account of applications such as the pulp and paper, alimentary and fuels industries. The xylose as a source of xylitol is used as a sweetener in foods and beverages developed for diabetic people and also, is used for the production, by fermentation, of alternative fuels such as ethanol [78, 79, 81].

Xylanases are composed by three groups of enzymes: endo-1,4-b-D xylanases, exoxylanases and b-D-xylosidase. Ganju et al. [81] isolated two xylanases (I and II) out of *Chaetomium thermophile* var. *coprophile,* a thermophillic fungus.

These enzymes have also been reported as responsible of plant pathogen control. Ahammed et al. [74] purified partially xylanase of *C. globosum* and compared antifungal activity of the enzyme and culture filtrate against *Bipolaris sorokiniana.*

Purified xylanase at 100 μg ml^{-1} concentration caused 100% inhibition of conidia germination of *B. sorokiniana*, whereas the culture filtrate was 33% less effective.

12.2.4.2 Glucanases, Cellulases and Chitinases Enzymes

The glucanases and chitinases enzymes are very important during the mycoparasitism process since fungal cell walls contain different kinds of β-glucans and chitin as structural components. The cellulolytic enzymes play a special role with plant pathogenic oomycetes, which contain glucan and cellulose in its cell walls.

Ahammed et al. [75] purified, characterized and studied the β-1,3-glucanase activity enzyme produced by *C. globosum* (Cg 2). Also, they evaluated the biocontrol capacity of partially purified glucanase fraction at 100 μg/ml and showed it inhibited 93.5% conidial germination of *B. sorokiniana.*

Sandhu and Puri [82] examined the production of endoglucanase and β-glucosidase enzymes in extracellular and intracellular fractions during development of *Chaetomium erraticum*. They found that the relative distribution of these enzymes varied with the age of the culture and that these multiple molecular forms were greatly differentiated by their response to temperature, pH, ethylenediaminetetraacetic acid, and metal ions multiple. Shanthiyaa et al. [18] evaluated the ability of biocontrol of eight *C. globosum* isolates against the oomycete pathogen *Phytophthora infestans* and assayed its exo- and endo-glucanase activity. Isolate Cg-6 showed greater exo- and endo-glucanase enzyme activity compared to other isolates. Also, they reported that the same isolate produced cellulolytic enzymes on carboxy methyl cellulose (CMC) and cellulose as source of carbon. Similarly, Ahammed et al. [83] registered the production of cellulolytic enzymes of *Chaetomium* isolates on CMC and cellulose. On the other hand, Umikalsom et al. [84] reported higher cellulolytic activity in the culture filtrate of fast growing *Chaetomium* isolates.

Studies of genes of *Chaetomium* species also can provide useful information about its involvement in biocontrol mechanisms. Yang et al. [85] evaluated biocontrol related genes from *Chaetomium cupreum, C. globosum* and *Trichoderma harzianum*, through analysis of their expressed sequence tags (ESTs) and showed that more than 100 genes from the fungi are plant disease biocontrol related genes. Also, Liu et al. [86] studied the expression of the chitinase gene (*chi46*) from *C. globosum* and demonstrated that it can be highly induced by exposure to the cell walls of plant pathogens as *Rhizoctonia solani, Fusarium oxysporum, Sclerotinia sclerotiorum, Valsa sordida, Septoria tritici,* and *Phytophthora sojae*, showing its involvement in biocontrol process.

Endopolygalactouronase gene is another important gene that helps in biocontrol mechanism. A study about this gene of *C. globosum* showed high values of homology of the blast with *Rhizoctonia solani* (HQ197932.1 and HQ197933.1) and *Thanatephorus cucumis* (FJ544455.1). The identification of this gene in *C. globosum* was reported [87].

12.2.4.3 Proteases and Heat Shock Proteins

Proteases participate in the fungal cell wall degradation and as inactivators of pathogens enzymes. They include aspartic proteinase, subtilisin and trypsin-like proteases [88, 89].

Studies have been performed on proteins that give resistance to organisms against factors of environment stress like high temperatures and salinity.

Li et al. [90] purified and characterized two thermostable proteases from the thermophillic fungus *C. thermophillum* that gives resistance to the fungus to high temperatures. Besides, the presence and function of the heat shock proteins (*Hsps*) in *C. globosum* through the clonation and *Hsps* 22.4 gene expression was reported [91].

Aggarwal et al. [77] cloned a small heat shock protein gene and determined that the expression of this gene in *Escherichia coli* transformants gave resistance to NaCl and Na_2CO_3.

12.2.4.4 MAP Kinases

Mitogen activated protein kinases (MAPK) are involved in osmoregulatory pathways, oxidative stress and maintaining fungal cell wall integrity [73, 92]. Also, the genes involved in biocontrol and mycoparasitism encode transduction pathways that include the kinase (MAPK) cascade, protein G signaling and cAMP pathway [72]. In accordance with a research by Reithner et al. [93], the signal transduction pathways of MAP-kinases from *Trichoderma* are involved in the production of hydrolytic enzymes and secretion of antibiotics.

Zhang et al. [76] analyzed the expressed sequence tags (ESTs) of *C. cupreum* to identify genes with biocontrol function expressed during the mycoparasitism. These genes included, among others, endochitinase, b-1,3-exoglucanase, a and b-glucosidase, serine protease, endo-1,4-xylanase and MAP kinase.

12.2.4.5 Phosphatases and Phytases

As mentioned in the Sect. 12.2.3, *Chaetomium* affects the development of plants and could promote their growth through the production of phosphatases and phytases [65]. In this sense, Vaghasia et al. [80] evaluated the response of bio-phos (*C. globosum*) on yields of castor seeds and compared its efficiency with applied inorganic phosphorus through fertilizer under field conditions. They observed an increase in seed yield of castor by seed inoculation with bio-phos with respect to the treatments with fertilizer.

12.3 Current Status in Argentina

There is scarce literature respecting *Chaetomium* spp. as endopyhyte from agricultural crops in Argentina. *Chaetomium globosum* was registered on wheat grains and leaves of tomato, soybean, wheat and beet [94–97]. Also, it was cited as endophyte of *Celtis occidentalis* leaves, a naturalised tree [98].

As antagonist and biocontrol agent, it is cited on wheat plants [99]. *In vitro* and *in vivo* assays were performed against *Alternaria tritici-maculans, Bipolaris sorokiniana, Pyrenophora tritici-repentis* and *Septoria tritici.* The results registered that *C. globosum* caused from 41% to 60% of necrotic leaf area reduction against *P. tritici-repentis*. At a microscopical level this fungus showed significant effect on all the pathogens, as plasmolysis of the mycelium and spore inhibition. Larran et al. [15] carried out several combined treatments of *C. globosum* with other fungi and *Bacillus* spp., confirming a reduction in the spore germination rate and alterations in the morphology of *P. tritici-repentis.* These authors have also studied the biocontrol of *C. globosum* on *P. tritici-repentis* in wheat leaves, and observed that the treatment with the antagonist reduced significantly the disease severity in comparison with the control.

There are references on barley against *Bipolaris sorokiniana*, the causal agent of spot blotch and on *Pyrenophora teres*, responsible for the net blotch disease [100–102]. In assays of dual culture, inhibition of *B. sorokiniana* and *P. teres* accounted for 30,5% and 38%, respectively compared with the control [101]. The mechanisms of action against *B. sorokiniana* was antibiosis while competition and mycoparasitism were for *P. teres*. Microscopic observation revealed deformed conidia in *B. sorokiniana* and in *P. teres* plasmolysis, coiling and orange pigmentation were observed (Fig. 12.2).

Cipollone & Sisterna [102] confronted in dual cultures nineteen *C. globosum* isolates against *B. sorokiniana* and selected three of them with significative differences. They registered between 18% and 42% of micellar inhibition. Also, conidia

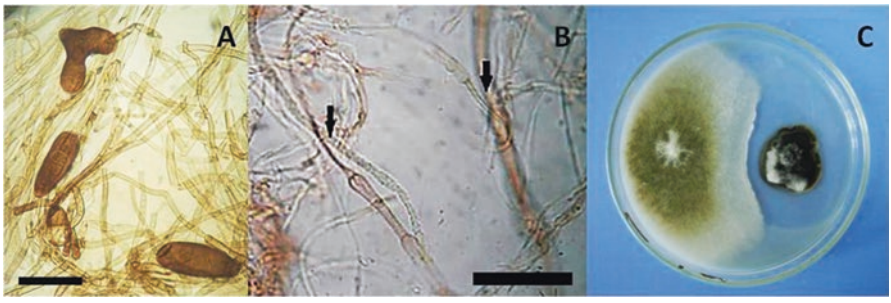

Fig. 12.2 *In vitro* antagonistic interactions of *C. globosum - B. sorokiniana*: (**a**) *B. sorokiniana* abnormal conidia. OM 40X scar = 50 µm. (**c**) Antibiosis. Microscopic interaction area of *C. globosum – P. teres* (**b**) plasmolysis OM 40X scar = 50 µm

and mycelium alterations were observed. Respecting *in vivo* assays, barley seeds naturally infected by *B. sorokiniana* were inoculated with a spore suspension of *C. globosum.* Infection was reduced between 32% and 55% compared with control. In artificial inoculations on leaves, percentage of necrotic lesions (between 23.5% and 29%) was lower in treatments with the antagonist than in controls (between 25% and 48%) [100].

12.4 Conclusions

Nowadays, practices in the agricultural systems lead to looking for new management alternatives. Although in the modern agriculture better results and more stable yields were obtained, there has been an unbalance between diseases and their control. The indiscriminate use of chemical products has brought negative consequences for human health and the environment. Thus, the option of sustainable alternatives for crops is necessary and urgent.

Among them, biocontrol may have lower economic costs and be more efficient compared to traditional control practices, since the action of microbial antagonists may be more stable and lasting over time than chemical control. In this latter case, the effect is often temporary and it is necessary several applications to achieve adequate crop protection. Nevertheless, biocontrol has restrictions, limitations and requirements that must be known. The biocontrol organisms are much more sensitive to external conditions than chemical products. They have ecological limitations as inactivation by the presence of other organisms and by the effect of chemical substances. Therefore, for efficient use, knowledge of its biology, ecology and mechanisms of action on the pathogens to be controlled is required.

Microorganisms as *Chaetomium*, used for disease management, do not generate the negative effects produced by chemical synthesis products such as fungal resistance to fungicides. In the last years, studies for agriculture as biocontrol agent, plant growth promoter and secondary metabolites producer were developed. Besides, it was studied as enzymes source for agricultural and industrial application. At present, there are commercial formulations for the mentioned uses, oriented principally to diseases caused by soil fungi. For this reason, it would be necessary to develop new research to expand the spectrum towards other pathologies.

In the coming years, we envisage a broader appreciation of the benefits of alternative methods and expect to see synergistic combinations of biopesticides with other technologies that will enhance the effectiveness and sustainability in integrated control. *Chaetomium* and its metabolites combined with new technologies as nanotechnology, are giving good results and have a potential field of research. Nanotechnology is transforming economy, industry and agriculture worldwide. Its various applications have a direct impact on the environment, health, food production and agriculture, as precision agriculture to reduce costs and maximize production.

The results of the present review let know the actual state of *Chaetomium* knowledge for crop management. They are the start point for new researches and future biotechnology developments in a sustainable approach.

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Chapter 13
Fusaria Strains as Biocontrol Agents: The Case of Strain Fo47 and *Verticillium dahliae*

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13.1 Introduction: *Verticillium dahliae*, the Pathogen, the Disease and Its Management

13.1.1 Verticillium dahliae *Kleb*

Verticillium is a genus of filamentous fungi that was established in 1817 by Nees von Esenbeck for *Verticillium tenerum*, now *Verticillium luteo-album* [1], and that was isolated from a stem of hollyhock (*Alcea rosea* L.) in Germany [2]. The genus *Verticillium* belongs to the family *Plectosphaerellaceae* in the subclass Hypocreomycetidae of the class Sordariomycetes, which belongs to the phylum Ascomycota and order Hypocrealses [2, 3].

Within this genus, 10 *Verticillium* species are distinguished [3]: *V. dahliae*, *V. albo-atrum*, *V. longisporum*, *V. tricorpus*, *V. theobromae*, *V. nubilum*, *V. nigrescens*, *V. alfalfa*, *V. nonalfalfae*, *V. zaregamsianum*, *V. isaacii* and *V. klebahnii*. *V. dahliae* was used as the type species when this genus was redefined, and *Verticillium* species which belong to the same clade as *V. dahliae* are referred to as *Verticillium sensu stricto* (s.s.), therefore excluding *V. theobromae* [2]. The reproduction of *Verticillium* spp. appears to be strictly asexual; it has not been discovered any sexual state in the genus, being all species potentially heterothallic [2, 3]. *Verticillium* spp. produce survival structures called microsclerotia (black melanised clumps that are derived from budding of mycelial cells); microsclerotia usually germinate and form hyphae. *Verticillium* hyphae grow toward plant roots and penetrate root epidermis by means of the hyphopodium [4]. Then, hyphae grow between

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epidermal cells and eventually colonize xylem vessels due to fungal secretion of effector proteins [5, 6]. Once the fungus colonizes plant roots, hyphae grow upstream and conidia and microsclerotia are produced into the vascular system, thus impeding water and nutrient transport across the plant [5]. Among *Verticillium* spp., *V. dahliae*, *V. albo-atrum* and *V. longisporum* cause the most important agronomic diseases, and are the three causal agents of the so-called "Verticillium wilt" [7]. The other *Verticillium* species attack a fewer number of hosts, are not so globally distributed as the three species mentioned above, and cause less crop losses [2, 3].

Verticillium dahliae Kleb. is an asexual, vascular-colonizing and soil-borne Deuteromycete fungus [3]. This plant pathogen has a broad host range, being the species with more hosts among *Verticillium* spp. Approximately, 300–400 plant species are susceptible to this pathogen (some examples are shown in Table 13.1), causing billions of dollars in annual crop losses. Herbaceous annuals and woody perennial hosts including crops, flowers, and vegetables are infected by *V. dahliae* in greenhouses and in open field and at any growth stage [2, 3, 8–10].

V. dahliae is also able to infect and colonize plants without triggering symptoms. In this way, *Verticillium* inoculum remains dormant and can initiate epidemics of Verticillium wilt disease [10]. Thus, *V. dahliae* is considered by some authors as a hemibiotroph with an initial biotrophic phase within root xylem without causing visible symptoms and a second necrotrophic phase with the development of symptoms in the aerial parts of the plant [11]. Other authors classify *V. dahliae*as anecrotroph due to its appressoria, which are used to infect plants [12]. There are controversies about *V. dahliae* life style, and authors have not reached a consensus yet.

Table 13.1 Some horticultural crops attacked by *Verticillium dahliae*

Family	Scientific name	Common name
Solanaceae	*Capsicum annuum*	Pepper
Solanaceae	*Solanum lycopersicum*	Tomato
Solanaceae	*Solanum melongena*	Eggplant
Solanaceae	*Solanum tuberosum*	Potato
Brassicaceae	*Brassica oleracea*	Cabbage
Brassicaceae	*Brassica oleracea* var. *italic*	Broccoli
Brassicaceae	*Brassica oleracea* var. *botrytis*	Cauliflower
Rosaceae	*Prunus persica*	Peach
Rosaceae	*Fragaria* x *ananassa*	Strawberry
Asteraceae	*Lactuca sativa*	Lettuce
Asteraceae	*Cynara scolymus* var. *scolymus*	Artichoke
Vitaceae	*Vitis vinifera*	Grapevine
Oleaceae	*Olea europaea*	Olive tree
Amaranthaceae	*Spinacia oleracea*	Spinach
Cucurbitaceae	*Cucurbita pepo*	Pumpkin
Cucurbitaceae	*Citrullus lanatus*	Watermelon

Fig. 13.1 *Verticillium dahliae* symptoms in pepper (*Capsicum annuum*). (**a**) Dwarf plant and (**b**) Wilted plant. Blank arrow in (**a**) shows the dwarf plant. Blank arrow in (**b**) shows the asymmetric wilting caused by *V. dahliae* while the white arrow shows the part of the plant which does not show wilting

When symptoms caused by *V. dahliae* occur (commonly known as "wilt disease symptoms"), these consist of dwarfism (Fig. 13.1a), leaf loss of turgor (Fig. 13.1b), abscission and epinasty, foliar chlorosis and necrosis (Fig. 13.1b), vascular discoloration and, in extreme situations, plant death [5, 8–10]. The pathogen colonizes water-conducting tissues within a plant resulting in wilting and tissue necrosis at the end of the disease cycle [5, 9, 10]. Symptoms can vary among hosts and true wilt does not always occur after *Verticillium* infection [9]. *V. dahliae* is a systemic fungal pathogen, therefore symptoms can affect all the parts of the plant. Generally, symptoms in the aerial part start in the lower leaves, then spread acropetally and eventually can affect the whole plant or be confined to one side instead (sectoring, Fig. 13.1b) [5, 9, 10].

V. dahliae causes a monocyclic disease, which means that only one cycle of disease and inoculum production takes place during a growing season [9]. *V. dahliae*-cycle consists of two phases (Fig. 13.2). In the first phase (Phase I; asexual development), *V. dahliae* microsclerotia (spreading structures) germinate as a response to root exudates and the fungus enters the plant root through epidermis or wounds to reach immature xylem elements [5, 10]. After crossing the endodermis,

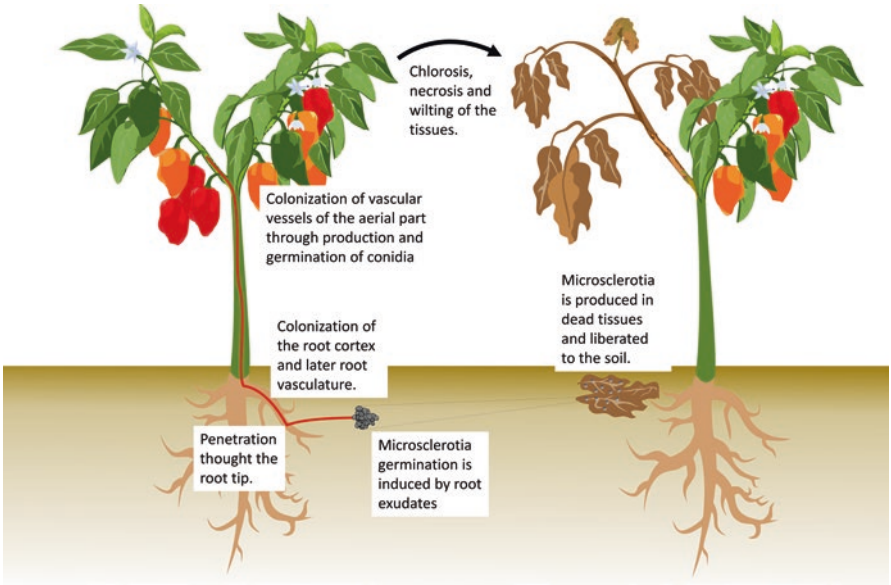

Fig. 13.2 The cycle of Verticillium wilt disease caused by *Verticillium dahliae*

the second phase (Phase II; disease cycle) begins, in which hyphal proliferation and conidia production (budding) occur; conidia germinate and colonize the vascular elements and the upstream vessels through the sap stream and they are trapped in pit cavities or at vessel end walls (trapping sites) [5, 10, 13]. When foliar senescence takes place, *V. dahliae* enters a saprophytic stage and colonizes the surrounding nonvascular tissues in shoots and roots, so that new microsclerotia are produced in the dying stems and leaves and will constitute a new source of inoculum in the soil [5, 10]. While the host plant is alive, *V. dahliae* usually colonizes only host xylem vessels, and the hyphae compete with plant tissues for nutrients. When the host dies, the fungus colonizes senescent tissues [5, 10]. Microsclerotia are the main inoculum in the field, where they can survive for 10–15 years even in the absence of a host [14]. Generally, the density of microsclerotia in the soil is host-dependent and is proportional to the incidence of the disease [15]. Thus, *V. dahliae* is able to remain in a dormant phase in the soil as microsclerotia [10].

Different strategies have been used to reduce the disease caused by *V. dahliae*, and some of them are more effective than others. Currently it is claimed the necessity to find an effective control strategy of this pathogen without harming the environment and human health, a strategy economically viable, and easy to apply for farmers.

13.1.2 Current Control Strategies of Verticillium Wilt

Control of the soil-borne disease caused by *V. dahliae* is difficult for different reasons. The disease cannot be treated once crops are affected due to the inaccessibility of *V. dahliae* once it reaches the vascular plant tissue, and the long persistence of its microsclerotia in the soil [9, 13, 15, 16]. Moreover, this fungus has a wide host range, so there is no single effective treatment for all affected crops by this pathogen. In addition, *V. dahliae* can be introduced in the soil via infested seeds and/or spread locally from field to field by harvesting crews or equipment [15, 16]. Therefore, it is necessary to raise awareness among farmers about the importance of cleaning equipment and tractors before entering a new field to prevent the spread of soil-borne pathogens such as *V. dahliae*.

Plant diseases can be controlled through chemical, mechanical and biological ways, but the use of pesticides is the most widespread practice. Thus, soil fumigation with fungicides is used to reduce the inoculum of *V. dahliae* in the soil, but its application is restricted due to the environmental and ecological risks, as well as the appearance of resistances to the fungicides used [15, 16]. Methyl bromide was the main soil fumigant to treat Verticillium wilt, but its use was banned under the Montreal Protocol on Substances that Deplete the Ozone Layer in 2005 due to environmental concerns [15].

Currently, control of Verticillium wilt is focused on different IPM (Integrated Pest Management) strategies, including crop rotation [15]. This practice is generally of limited use for Verticillium wilt management due to the broad host range and the long-term persistence of *V. dahliae* microsclerotia. Biofumigation (use of biologically active plant substances), soil solarization (mulching the soil and covering it with tarp to trap solar energy), soil steam sterilization, flooding, anaerobic soil disinfestation (anaerobic decomposition of organic soil amendments), use of fertilizers consisting of growing plants that are ploughed back into the soil (green manures), organic amendments or crops grown for the protection and enrichment of the soil (cover crops) are also common IPM strategies to control soil-borne pathogens [16]. For example, flooding [17] and anaerobic soil disinfestation [18] proved to be effective in suppressing *V. dahliae* in pepper. However, all these environmentally friendly approaches have their specific drawbacks and limitations, both economic and practical (expensive equipment, high temperature and water requirements, site-specific variability) [15, 16].

Grafting on resistant rootstocks is commonly used to protect plants against soil-borne pathogens. However, this technique is not always effective to control Verticillium wilt [19, 20]. It has been shown that grafting can trigger the emergence of new virulent races of the pathogen, therefore changing its population [21]. Genetically resistant varieties and development of transgenic varieties also play an important role in solving the Verticillium wilt problem [16, 22]. In resistant tomato varieties, resistance against Verticillium wilt caused by race 1 is mediated by the *Ve* locus, which comprises *Ve1* and *Ve2* genes; however, no resistant varieties against race 2 have been found [22]. In *C. annuum* there is an important number of genes

conferring resistance against various pathogens [23]; nevertheless, to our knowledge, no resistance genes to *V. dahliae* have been found in *C. annuum*. However, Barchenger et al. [24] recently found an allele-specific cleaved amplified polymorphic sequence (CAPS) in a *Ve1* homolog of *C. annuum*; this probably will help in the future to improve Verticillium wilt resistance in pepper.

The exogenous application of natural compounds that activate plant defenses and, therefore, induce resistance, such as DL-3-aminobutyric acid, methyl jasmonate or ASM [25, 26] has been used successfully to control Verticillium wilt. On the other hand, biocontrol makes use of biological control agents (BCAs), which are living organisms that directly or indirectly control pathogens or pests. In next section of this chapter we are going to address this kind of disease management.

13.2 Biological Control of Verticillium Wilt: An Overview

BCAs are usually bacteria (i.e., actinomycetes and Plant Growth Promoting Rhizobacteria (PGPR)) and fungi (i.e., entomopathogenic fungi, root endophytic fungi, Plant Growth Promoting Fungi (PGPF) and mycorrhiza), that reduce the severity and incidence of several diseases in many fruits and vegetables of economic importance [27–33]. The BCAs were regulated at the European level by the European Regulation 1107/2009 and the European Directive 2009/128/EC where the use of pesticides was reduced to reach a sustainable level by combining it with biocontrol and other new control techniques [21]. Currently, there is a global trend to increase the use of BCAs in crop protection because of their easy accessibility and large-scale propagation, without negative repercussions on the environment or human health.

An ideal BCA should be able to survive in the environment and colonize different organic substrates with high cell viability during a long period of time, produce large amounts of inoculum in the fastest way possible, and germinate and grow faster than the pathogen [34, 35]. The BCAs should be ecologically competitive, act against a wide spectrum of diseases, persist in soil after its application with a similar population size to that of the native population, and do not imply a risk for the other non-target microorganisms present in the ecosystem, in addition to do not negatively influence human and animal health or the environment [34, 35]. Moreover, the BCA has to be easily prepared, transported and applied, stable at room temperature, and economically profitable for farmers [34–36]. In the case of BCAs that will be intended to control Verticillium wilt, their proposed desirable traits are: affect the viability of microsclerotia, colonize the tissues of the plant and compete for nutrients and/or space, trigger induced resistance and cause the promotion of plant growth [37].

Plant and soil microbiota present where the pathogen is located can be a source of potential BCAs, since they are adapted to the same environmental conditions and can compete for nutrients and/or occupy secondary lesions and displace the pathogen. BCAs are first selected and isolated according to their antibiotic production

and root colonization abilities [32, 38, 39]. The study of BCA persistence in the roots helps to know the optimal number of BCA applications and also the proper time of application.

Several microorganisms have been reported as BCAs against *V. dahliae* (for a review see: [37], and they offer an eco-friendly alternative to chemical fungicides. In Table 13.2 it is shown a list of those potential BCAs that were assayed successfully in pepper against *V. dahliae*.

The process of commercialization of biological products can be a disadvantage for biological control development, due to the high patent and registration costs of new products, especially in EU. In USA, the US Environmental Protection Agency (EPA) created the "Biopesticide Pollution and Prevention Division" (BPPD) to accelerate this process and reduce the costs involved. Most of the biological products currently registered are fungi-and bacteria-based [45]. In EU, the existence of potential BCAs against Verticillium wilt according to the literature has not lead to the registration of many biopesticides for that specific use [37] (http://ec.europe.eu/food/plant/pesticides/). However, three products (Blindar®, Bioten®, Remedier®)

Table 13.2 An updated list of potential BCAs against *Verticillium dahliae* in pepper

Potential BCA	References
Bacillus amyloliquefaciens SF82 and RS11	[40]
Bacillus pumilus (Astona®)	[41]
Bacillus spp.	[42]
Bacillus subtilis	[43]
Bacillus subtilis ZO4	[40]
Bacillus velezensis (Botrybel®)	[41]
Funneliformis mosseae (formerly *Glomus mosseae)*	[37][a]
Fusarium oxysporum f. sp. *lycopersici* CECT 2715	[37][a]
Fusarium oxysporum Fo47	[37][a]
Glomus deserticola	[37][a]
Penicillium rubens(formerly *Penicillium oxalicum*) PO212	[44]
Pseudomonas fluorescens	[43]
Pythium oligandrum (Polyversum®)	[37][a]
Pythium oligandrum (mixture of 5 isolates)	[37][a]
Rhizophagus intraradices (formerly *Glomus intraradices*)	[37][a]
Trichoderma asperellum B35	[37][a]
Trichoderma asperellum ICC012 + *Trichoderma gamsii* ICC080 (Blindar®, bioten®, Remedier®)	Ministerio de Agricultura, Pesca y Alimentación (https://www.mapa.gob.es/es/agricultura/temas/sanidad-vegetal/productos-fitosanitarios/fitos.asp, last visit: 16th November 2019)

[a]The original references can be found in this excellent review

based on the same mixture of *Trichoderma asperellum* ICC012 and *Trichoderma gamsii* ICC080 (two fungal BCA registered in the EU) are commercialized to manage Verticillium wilt in several crops, including pepper (Table 13.2). Other two products based in Bacillus strains (Botrybel® and Astona®, Table 13.2) were commercialized some years ago in Spain to control Botrytis diseases according to a former regulation of biostimulants that was repealed. We assayed in our laboratory these two products against Verticillium wilt in pepper and they were partially effective [41].

In any case, it is always important to know how a BCA works in the corresponding plant-pathogen interaction. In the next section the different mechanisms of action of BCAs will be reviewed.

13.3 Mode of Action of BCAs (Direct and Indirect)

The mechanisms of action of BCAs can be divided into direct antagonistic effects on the pathogen or indirect responses triggered in the host [46]. Both direct and indirect mechanisms can occur at the same time in some BCA, with a synergistic or additive effect. These mechanisms are summarized in Fig. 13.3, and will be

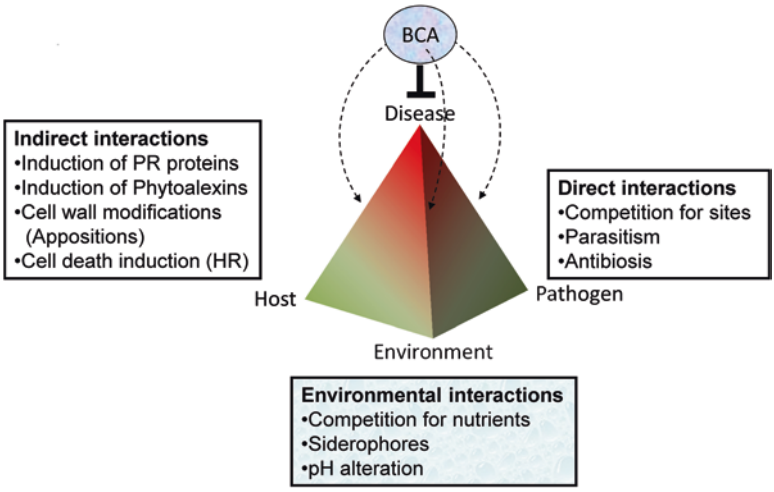

Fig. 13.3 Mode of action of BCAs. BCAs reduce disease incidence by acting upon the three corner stones of the disease triangle; host, environment and pathogen. Direct interactions with the pathogen reduce the disease by antagonism with the pathogen through competition for infection sites, parasitism or antibiosis. Indirect interactions are interactions with the host plant to trigger defense responses against the pathogen including Pathogenesis related (PR) proteins and phytoalexins, as well as cell wall fortification and cell death induction. BCAs also interact with the environment making it less hospitable for the pathogen by competing for nutrients, producing siderophores that sequester iron and altering the pH

discussed below in more detail, specifically regarding biological control of Verticillium wilt.

13.3.1 Direct Antagonistic Interactions: Competition, Antibiosis and Parasitism

Competition, mentioned above, is part of the direct antagonistic interactions with the pathogen. Competition with BCAs at the root surface is the first layer against Verticillium wilt. The BCAs *Fusarium oxysporum*Fo47 is able to outgrow *V. dahliae* in the roots of pepper effectively protecting the plant from pathogen colonization [47]. Competition is one of the mechanisms of BCAs against Verticillium wilt and it is due to the fitness of the BCAs that allows it to grow faster and better utilize the resources.

Antibiosis is another weapon of BCAs and it is based on the secretion of toxic compounds. The endophytic bacterium *Bacillus halotolerans* Y6 from Verticillium wilt-resistant cotton possesses strong antagonistic abilities that inhibit *V. dahliae* spore germination and mycelial growth. This Bacillus secretes a β-glucanase (Bgy6) able to degrade key components of the pathogen [48]. There is a number of other toxic substances to *V. dahliae* that are secreted by BCAs. Phenol, 2, 4-bis (1, 1-dimethylethyl) secreted by Pseudomonas is a toxic compound to *V. dahliae* that allows this bacteria to reduce pathogen growth [49]. Other Bacillus strains use a pore-forming protein (TUBP1) that acts on *V. dahliae* producing leakage of intracellular material [50]. Bacillus also produces a number of lipopeptides, specifically Fengycin, which contribute to the antagonistic activity against *V. dahliae* [51]. *Pseudomonas fluorescens* 2–79 produces a dibenzo annulated pyrazine, phenazine, which is able to completely inhibit *V. dahliae* growth in the presence of glucose [52]. Lactobacillus strains producing high levels of lactic acid inhibit *V. dahliae* growth in vitro [53]. On the other hand, volatile organic compounds (VOCs) have been observed recently to be also involved in *V. dahliae* antibiosis. The non-pathogenic *Fusarium oxysporum* Fo12 produces VOCs that are toxic to *V. dahliae*. Furthermore, Fo12 is able to sense VOCs produced by *V. dahliae* and activate the production of new VOCs toxic to the pathogen [54]. The bacteria Streptomyces secretes substances that inhibit *V. dahliae* growth through a process that seems to target the structure of fungal cell membrane [55]. Additionally, RNA interference is a potential mechanism of biocontrol agents. Cross-kingdom RNA interference is a process known to happen between host and pathogen, therefore this process might be also possible between BCAs and pathogens. RNA interference has been observed to reduce *V. dahliae* infection by host induced gene silencing in cotton plants [56] which indicates that *V. dahliae* is able to up-take RNAi during infection. Antibiosis depends on the complex secretome of the BCAs since it can be induced by the presence of the pathogen, the host, or the environment.

Parasitism has been observed to be an integral component of Trichoderma and Bacillus against *V. dahliae*. *Trichoderma atroviride* is able to control *V. dahliae* through direct mycoparasitism. This effect has been observed to be partially due to degradative enzymes, like peptidases, [57]. *Trichoderma cyanodichotomus* exhibited moderate hydrolase activity of cellulase, chitinase, β-1,3-glucanase, and protease, which might be involved in mycoparasitism against *V. dahliae* [58]. *Bacillus cereus* is also able to control *V. dahliae* by secreting degradative enzymes as chitinases [59]. Viruses could also be included in this category. Mycoviruses from fungal plant pathogens can reduce the virulence of their fungal host and have therefore potential as biological control agents. Several viruses have been isolated from *V. dahliae* (VdPV1, VdCV1, VdRV1) that could potentially be used as BCA [60]. However, it has yet to be proven that these viruses reduce the pathogenicity of *V. dahliae*.

13.3.2 Indirect Interactions: Induction of Responses in the Plant

It has been reported that rhizobacteria are able to induce responses in the plant. Plant growth promotion was one of the responses induced by these bacteria. Because of this feature, they were called plant growth promoting rhizobacteria (PGPR). However, these bacteria were also able to induce the immune system of the plant. More recently it was observed that not only rhizobacteria but also some beneficial fungi are able to promote plant growth, and these fungi were defined as plant growth promoting fungi (PGPF). PGPR and PGPF have been observed to be able to protect plants against *V. dahliae*. For instance, Bacillus is able to protect pepper against *V. dahliae* [40] and *Pseudomonas fluorescens* PICF7 controls Verticillium wilt in olive and Arabidopsis [61]. Additionally, swimming motility and siderophore production by PICF7 are not necessary for Verticillium wilt control in olive or*A. thaliana*, but cysteine auxotrophy cause a reduction in the effectiveness of PICF7. Moreover, PICF7 controlled *Botrytis cinerea* infection in the Arabidopsis leaves if it is applied to the roots, which proofs the ability of this strain to induce systemic resistance [61]. Moreover, colonization of olive roots either by strain PICF7 or by *V. dahliae* triggers differential systemic transcriptomic responses, many of them associated with defense-related genes [39]. PICF7 induces transcripts of lipoxygenase, and genes involved in biosynthesis of phenylpropanoids, terpenoids and plant hormones in olive trees [62]. The PGPF *Penicillium rubens* PO212 protects against *V. dahliae* in pepper [44]. This protection seems to relay not only on direct reduction of the pathogen growth but also on the induction of defenses in the plant. When applied to the roots, PO212 is able to induce the plant immune system by means of pathogen-related (PR) proteins. The fungus *Fusarium oxysporum*Fo47 is also able to induce defenses in pepper that are effective to control *V. dahliae* [47]. The nature

of this response will be further discussed in the Sect. 13.5 for the specific case of Fo47.

The BCAs harbor MAMPs (microbe associated molecular patterns), whose recognition by the plant triggers the immune system. The underlying mechanism of the recognition has been the topic of many works and will not be discussed here [63]. Fractions of cultures of BCAs retain the property to induce the plant immune system indicating that the living organism is not necessary to induce the immune system [64]. However, these fractions might not induce the same responses as the living BCA. *Fusarium oxysporum* f. sp. *lycopersici* was able to protect pepper against *V. dahliae*, but its insoluble fraction did not protect even though it retained the ability to induce PR genes [65, 66].

The plant also might not only respond to the BCAs but also cast its recruitment. The entomopathogenic fungus *Beauveria bassiana* EABb 09/16-Su has shown to produce substances that completely inhibit *V. dahliae* growth [32]. Wounding that simulated herbivory attack caused a retention of Beauveria inocula in the rhizosphere at 30 days after inoculation. Therefore, it is probable that plant defense response changed as a result of wound stress, causing the apparent recruitment of Beauveria in the rhizosphere [67]. It is unknown whether damage caused by *V. dahliae* also produces such a response.

13.3.3 The BCA Responds to the Presence of the Pathogen

The BCA *Paenibacillus polymyxa* Sb3–1 inhibited the *in vitro* growth of *Verticillium longisporum*. Both Paenibacillus and Verticillium produce volatile and soluble substances as a reaction to each other VOCs, and these VOCs were important to modulate their interaction and therefore the antagonistic response [68]. This role of VOCs as signaling molecules is different from the works on Fusarium were VOCs are described as toxic molecules, even though such a signaling role is not discarded for some of the Fusarium VOCs [54].

13.3.4 The BCA Inhibits Plant Responses Necessary for the Pathogen

On the other hand, BCAs do not only activate but also deactivate responses in the host. It was observed that the development of *V. dahliae* disease requires the ethylene-activated transcription factor EIN3. The beneficial endophytic fungus *Piriformospora indica* significantly reduced the development of *V. dahliae* disease [11]. *P. indica* inhibited the growth of *V. dahliae* in a dual culture on PDA agar plates and pretreatment of Arabidopsis roots with *P. indica* protected plants from *V. dahliae* infection. The *P. indica*-pretreated plants grew better after *V. dahliae*

infection without activating stress hormones and defense genes in the host, which indicated that *P. indica* deactivated the Ethylene pathway necessary for *V. dahliae* to cause the disease. Furthermore, *P. indica* represses *V. dahliae*-induced gene expression and *V. dahliae*-induced phytohormone accumulation in the shoots [11].

13.3.5 The BCA Responds to the Presence of the Plant

The process of interaction with the plant involves a reprogramming of the BCA physiology in order to activate certain pathways necessary for the colonization. This requires that BCAs recognize the plant to be able to trigger such a response. However, not only the genes necessary for the colonization are activated but also the production of substances that do not seem to play a role in the colonization process. In the presence of the host (*A. thaliana*) the BCA *Streptomyces lividans* increases the production of prodiginines (tripyrrole pigments toxic to *V. dahliae*) which do not seem to play a role in the colonization process [69].

Additionally to the response produced in the BCA, *V. dahliae* is also able to sense the host. *V. dahliae* express different genes depending on whether the host is susceptible or not [70]. More genes are activated in *V. dahliae* coming into contact with susceptible cultivars of the host coding for niche-adaptation, pathogenicity, virulence and microsclerotia development. The interaction with the plant seems to reprogram not only the BCA but also the pathogen.

13.4 Fusaria Strains as BCAs

Fusarium is a genus of fungi that are globally distributed in soil and organic substrates, and includes several plant pathogen species and *formae specialis* [71]. A first approach to use Fusaria to control plant diseases was to use non-host species or races that lead to an incompatible interaction with a particular cultivar of the host plant [65, 72]. However, non-pathogenic Fusaria have also been described, and it has been proved that they play a role in suppressiveness of some soils [73]. For that reason, non-pathogenic isolates have been tested to check if they have effects on plant diseases as BCAs. Their mode of action can be different depending on the strain, and can include any of the mechanisms explained in the previous section of this review.

The number of non-pathogenic Fusaria isolates that are effective against plant diseases is continuously increasing, and they act against several plant pathogens (in many cases pathogenic Fusaria). There are excellent reviews about this topic [73, 74], but the knowledge in the field is increasing in the last years. One of the promising non-pathogenic Fusaria as BCA is *Fusarium oxysporum* Fo47 [73, 74]. In Table 13.3 is shown a list of the reported plant-pathogen interactions in which Fo47

Table 13.3 List of plant/pathogen interactions in which Fo47 has been reported to confer protection to the disease

Host	Pathogen	References
Tomato	*Fusarium oxysporum* f. sp. *lycopersici*	[75]
Tomato	*Fusarium oxysporum* f. sp. *radicis-lycopersici*	[76]
Chickpea	*Fusarium oxysporum* f. sp. *ciceri*	[77]
Flax	*Fusarium oxysporum* f. sp. *lini*	[78]
Pea	*Fusarium oxysporum* f. sp. *pisi*	[79]
Carnation	*Fusarium oxysporum* f. sp. *dianthi*	[80]
Asparagus	*Fusarium oxysporum* f. sp. *asparagi*	[81]
Watermelon	*Fusarium oxysporum* f. sp. *niveum*	[33]
Cotton	*Fusarium oxysporum* f. sp. *vasinfectum*	[33]
Cucumber	*Pythium ultimum*	[82]
Pepper	*Verticillium dahliae, Phytophthora capsici*	[83]
Eggplant	*Verticillium dahliae*	[33]

was effective. In the next section we will review the advances in the study of this strain.

13.5 *Fusarium oxysporum* Fo47 as a BCA of Verticillium Wilt

The BCA *Fusarium oxysporum* Fo47 has been studied for several years. Research done on Fo47 has elucidated some of the complexities of its mechanism of action. This mechanism has been divided into two main processes, direct interactions with the pathogen and indirect interactions. Both interactions have been described broadly in Sect. 13.3. Fo47 direct antagonistic interactions with the pathogen consist mainly of competition for inoculation sites and nutrients. The biological fitness of Fo47 allows it to colonize efficiently different ecological niches displacing low competitive pathogens. On the other hand has been established that Fo47 induces defense responses in the host plant even though it colonizes only the dermis and outer cortex of the root [47, 84]. The mechanism of action of Fo47 is discussed below following these two main categories.

13.5.1 Biological Fitness of Fo47

The biological fitness of the BCA Fo47 allows it to efficiently compete in the soil with the microbial community. Fo47 is able to colonize the soil in a wide range of temperatures and humidity [85]. Fo47 takes advantage of these properties to displace pathogens through competition. The load capacity of the soil is higher if there

is no microbial community present meaning that Fo47 would be established in higher densities. Nonetheless, Fo47 is able to establish in relatively high densities in a variety of soil types and in the presence of microbial communities.

The roots are a more hospitable niche that the bulk soil and therefore most of the competition seems to occur at the root surface. Fo47 is also able to efficiently colonize the plant root [47, 84]. Fo47 is able to colonize the root much faster than the pathogen *V. dahliae* reducing significantly the presence of the pathogen in the root. However the pathogen was not excluded from the root surface and some pathogen could still grow alongside the BCA [47]. When inoculated together in PDA plates it was observed a reduction in growth of *V. dahliae* but under the microscope it was not observed any interaction between the hyphae of both fungi. The higher biological fitness of Fo47 in PDA and in the root surface allows this fungus to grow faster than *V. dahliae* without excluding it from the medium. However, similarly to Fo47, other pathogenic strains of *F. oxysporum* are also able to efficiently colonize the root of the host. Fol8, a pathogenic *F. oxysporum* strain, is controlled by Fo47 only if this pathogen is applied in lower inoculum concentrations than the BCA [86]. At least a 50-fold inoculum excess of Fo47 was necessary to successfully control the pathogen *F. oxysporum* f. sp. *radicis-lycopersici* in tomato [87]. The biological fitness of both strains is similar and therefore efficient competition is only achieved by Fo47 if it is present in higher inoculum concentrations. Similarly to *V. dahliae*, pathogenic strains of *F. oxysporum*are not excluded from the roots by Fo47, therefore indicating that competition occurs for nutrients rather than for infection sites.

13.5.2 Induced Resistance: Fo47-Induced Resistance

Fo47 is able to colonize the outer cortex of the root [47, 84]. However, plant defense reactions stop Fo47 from colonizing deeper tissues. Fo47 elicited early physiological responses in flax cell cultures such as transient H_2O_2 production and Ca^{2+} influx. Other pathogenic strains of *F. oxysporum* (e.g., Foln3) also induce such responses on the host but they are less intense. Moreover, Fo47 induced stronger host cell death than the pathogenic Foln3 [84]. These strong responses to Fo47 lead to a Fo47-induce resistance (FIR) that protects the plant against pathogens.

The oversimplified view of induced resistance either as ISR (systemic induced resistance) or SAR (systemic acquired resistance) does not allow to correctly describe the response triggered by Fo47 in its host. Classical ISR was defined by the response triggered by PGPR. These bacteria induce a systemic response in the plant that is dependent on the plant hormones jasmonic acid and ethylene. A phenomenon denominated as priming was also considered a hallmark of ISR [88]. Duijff et al. [89] observed that the response induced by PGPR (*P. fluorescens* WCS417r) did not activate PR-1 or chitinase expression prior to pathogen inoculation while Fo47 did. This implies that even though both BCAs were able to protect tomato against Fusarium wilt exclusively through induced responses in the plant, such responses were different. Treatment of plants with combinations of *P. fluorescens* and Fo47

showed greater protection than any of the BCAs applied alone which indicates that they trigger complementary mechanisms of action [90].

Similarly to ISR, priming is an important part of FIR. It has been observed that several genes (a chitinase CHI3, a glucanase GLUA, and PR1a) were primed in tomato roots treated with Fo47 and challenged with the pathogen Fol8 [91]. However, priming triggered in FIR is tissue specific since in tomato cotyledons GLUA and PR1a were induced directly without the need of pathogen challenge. Moreover, analysis of the xylem sap of tomato treated with Fo47 showed that a PR5 protein (NP24) was induced by this BCA [92]. In pepper also the expression of a chitinase gene (*CaCHI2*) and a gene (*CaSC1*) involved in the biosynthesis of capsidiol, the major phytoalexin of pepper, is primed in stem but not in roots during FIR against *V. dahliae* [83]. However, not all components of FIR are primed, Fo47 is also able to induce *CaBPR1* before pathogen challenge in pepper [83]. PR1 induction is characteristic of SAR but the response to Fo47 also triggers early production of JA-Ile (jasmonate-isoleucine) in pepper roots, a hormone associated with the ISR [47]. Recently, FIR has been observed to be independent of the major plant defense hormones (ethylene (ET), jasmonic acid (JA), and salicylic acid (SA)) in tomato against Fusarium wilt [93]. On the other hand, it has been observed that signaling and biosynthetic genes for ET, SA and JA were differentially expressed in pepper and tomato after Fo47 treatment (Veloso and Díaz, in preparation). Furthermore, JA-Ile and SA were observed to be produced in the roots of pepper after Fo47 inoculation [47]. Caution has to be taken when using models like SAR or ISR since they have been fitted to Arabidopsis. The marker gene for SA in Arabidopsis, *PR1*, is induced by SA in this plant, but its pepper ortholog (*CaBPR1*) is induced not only by SA but also by ET and JA [94]. Moreover, in tomato two PR1 orthologs (*LePR1a* and *LePR1b*) are both dependent on SA and ET [95]. In summary, FIR is dependent on the host plant were it has been activated and it might different in some of its processes.

13.5.3 How Does Fo47 Produce FIR?

Random amplified polymorphic DNA (RAPD) analysis indicated that Fo47 cluster together with other biocontrol strains of Fusarium [96]. This indicates that these biocontrol strains share genetic material that is linked to the biocontrol capacity. This does not mean that all *F. oxysporum* biocontrol strains produce FIR. *F. oxysporum* biocontrol strains Fo47 and CS20 did cluster together but while Fo47 protected pepper from *V. dahliae*, CS20 did not (Veloso data not published) indicating that the mechanism of biocontrol between Fo47 and CS20 is not the same. Trouvelot et al. [97] produced Fo47 mutants affected in their biocontrol activity using transposition of the Fot1 element. They observed the loss of Fo47 biocontrol activity against the pathogen *F. oxysporum* f. sp. *lini* in flax. These mutants were not affected in their in vitro growth or in their competitiveness in soil compared with wild-type strain Fo47. Similarly, a non-pathogenic mutant of *F. oxysporum* f. sp. *melonis* lacks the

ability to protect muskmelon against the virulent parental strain of *F. oxysporum* f. sp. *melonis* [98]. The non-pathogenic *F. oxysporum* f. sp. *melonis* was neither affected in its growth *in vitro* nor in its capacity to penetrate into the roots. This argues against competition being sufficient on its own to control *F. oxysporum* f. sp. *lini* in flax or *F. oxysporum* f. sp. *melonis* in muskmelon.

L'Haridon et al. [99] identified genes involved in biocontrol activity of the strain *F. oxysporum* f. sp. *melonis* 24 (Fom24). They challenged tomato cells with germinated microconidia of Fom24, protective on tomato, and its mutant rev157 which had lost its protective capacity. Most of the sequences identified for Fom24 were coding for proteins of unknown function. However, the number of genes differentially expressed was not very high. It is unknown whether the biocontrol capacity is a truncated part of the virulence mechanism or they are totally independent mechanisms.

Pathogenicity can be restored in Fo47 by inserting genetic material from the pathogen *F. oxysporum* f. sp. *lycopersici*. Ma et al. [100] obtained the recombinants by inserting artificial drug resistance genes in the donor genetic material and in the recipient host fungi. The acquisition of chromosome 14 transformed Fo47 into a pathogenic strain in tomato. This might lead to the idea of a broken pathogenic mechanism that was complemented by the genes contained in chromosome 14 and therefore restored pathogenicity. This hypothesis is also supported by the fact that inserting chromosome 14 into *F. oxysporum* f. sp. *melonis* was not sufficient to make this strain pathogenic in tomato. The process of horizontal chromosome transfer is largely unknown but it has huge implications on genetic diversity and plasticity of Fusarium strains and it attracted the interest of many researchers over the last years. This process will not be discussed here and neither the process by which pathogenicity is complemented since they have been covered in other recent works [101, 102].

Why inserting chromosome 14 from *F.oxysporum* f. sp. *lycopersici* into *F. oxysporum* f. sp. *melonis* does not make this strain pathogenic to tomato but it does if inserted in Fo47? Additionally to tomato, Fo47 can also be made pathogenic to cucurbits (cucumber, melon and watermelon) by inserting genetic material from the pathogen *F. oxysporum* f. sp. *radicis-cucumerinum* [102]. Therefore, it does not seem like Fo47 has some specificity to tomato that *F. oxysporum* f. sp. *melonis* lacks. It has been observed that non-pathogenic strains of Fusarium have usually less active transposable elements (Veloso, data not shown). The activity of the transposons has been related with virulence in Fusarium strains. Transposases were more active in a virulence-enhanced variant of the pathogen *F. oxysporum* f. sp. *cucumerinum*. This variant was obtained after four cycles of infection on a resistant cultivar of cucumber. The virulence along with the transposase activity significantly increased over consecutive rounds of infection in a resistant cultivar [103]. Fo47 has lower number of transposable elements that virulent strains, yet it is potentially transformed into pathogenic while other pathogenic strains cannot be transformed into pathogens of other hosts. The genetic stability that the reduced transposable environment of Fo47 provides might be necessary for the integration of the genetic material obtained by horizontal gene transfer. Transposons produce small RNA than

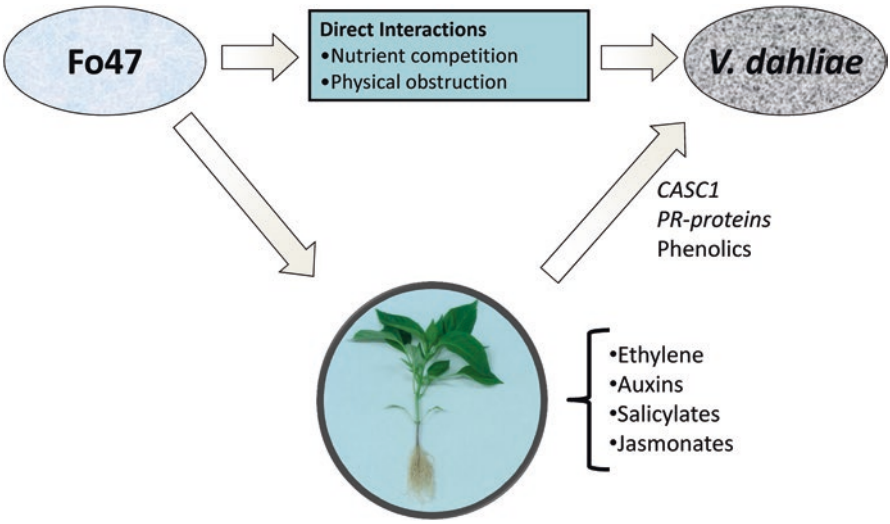

Fig. 13.4 The response observed for Fo47 in pepper against *V. dahliae* involves: First, direct interactions between Fo47 and the pathogen that putatively rely on competition for nutrients and/or physical obstruction. Second, an induction of responses in the plant that involves several hormones, namely ethylene, auxins, salicylates and jasmonates, and that produces the increase in *CASC1* expression, PR-proteins and phenylpropanoids. These two responses act together to reduce the *V. dahliae* infection and the subsequent symptoms. However this response is different to the one observed in tomato and comparisons with other species, as Arabidopsis, have to be made with caution

act as virulence factors modulating signals on the host [104]. It is unknown if transposable elements affect FIR. Further experiments are necessary to fully understand the mechanism by which Fo47 triggers resistance in the host.

13.6 Conclusions

In the case of the tripartite interaction pepper-*V. dahliae*-Fo47, our present knowledge allow us to draft a working model (Fig. 13.4), that should be completed in the future, and maybe it is not applicable to other Fo47 interactions (e.g., tomato-*Fusarium oxysporum* f. sp. *lycopersici*-Fo47). In any case, this and other BCAs deserve extensive studies to, after knowing how they work, achieve a reliable and effective disease management.

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Chapter 14
Fungi as Biological Control Agents of Plant-Parasitic Nematodes

M. R. Moosavi and R. Zare

14.1 Introduction

In recent decades, concerns about the environmental hazards of using chemical nematicides and limited alternative crops for rotation have led to the development of biological control agents as a component of crop protection. Biological control is now a key strategy used for controlling pests worldwide. Eilenberg et al. [1] defined biological control (or biocontrol) as follows: "The use of living organisms to suppress the population density or impact of a specific pest organism, making it less abundant or less damaging than it would otherwise be".

Four basic strategies can be used in biological control (*i*) Introduction: which is considered as a classical technique whereby an exotic helpful organism is introduced into a new region and become fully established. This strategy is usually used against introduced pests that have no indigenous antagonists. (*ii*) Augmentation: in this method, laboratory-bred individuals can be released to compensate the inefficiency of present microbial agents. The inadequate level of control can be driven by low number of native natural enemies. (*iii*) Inoculation: when an indigenous antagonist is not present or an introduced one cannot survive permanently, an inoculative release is made at the beginning of planting season. This process may need to be repeated for each following crop. (*iv*) Inundation: in this technique the mass culture of a pathogen is carried out for urgent use at critical periods when rapid suppression of pest population is necessary [2].

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Biological control agents have an important effect in the regulation of plant-parasitic nematode populations. Though numerous organisms including fungi, bacteria, viruses, nematodes and other invertebrates show antagonistic activity against plant parasitic nematodes [3] their significance in suppressing nematode population is not the same [4]. Various aspects of biological control of nematodes using microbial control agents have been already reviewed by many authors [5–15].

The developmental process of progressing and commercialization of biological agents include the isolation and identification of microbial agents associated with plant-parasitic nematodes especially in suppressive soils (a soil that completely suppresses nematode reproduction); examination of their potential ability in controlling nematodes; mass production; formulation and application. Considering legal and commercial aspects is also vital in the ultimate success of microbial control agents [16, 17]. Investigating nematode-suppressing soil demonstrated that their controlling activity is due to egg-parasitizing fungi, generalized fungal antagonists, mutualistic fungal endophytes, rhizobacteria and obligate parasitic bacteria [18]. Comprehending the mechanisms of suppressiveness can be useful in plant-parasitic nematode control by helping in manipulating these mechanisms.

Lots of natural enemies attack nematodes and decrease their populations, but the number of organism which could be employed for biocontrol is restricted. In other words, many soil types all around the world show biological control activity but their effect on nematodes can vary from insignificant to complete suppression. In this section the biological control of plant-parasitic nematodes by fungal agents will be emphasized according to recent research progresses.

Fungal biological control is an exciting and rapidly developing research area and there is growing attention in the exploitation of fungi for the control of nematodes. The relationship between nematodes and fungi that infect them has been the subject of widespread mycological studies. Our information about fungal biological control agents has originally been based on the voluminous and detailed work by Charles Drechsler [19–23]. Different aspects of fungal biological control of nematodes have been reviewed by many authors [2, 18, 24–27]. Hallmann et al. [28] classified these fungi into three large groups: nematophagous fungi, saprophagous fungi, and endophytic fungi; however, we do not follow this classification here. We consider all nematode parasitic and antagonistic fungi as: (*i*) nematophagous fungi and (*ii*) endophytic fungi, and their taxonomy and mode of action are briefly shown in Table 14.1.

14.2 Nematophagous Fungi

The term “nematophagous fungi” refers to a diverse group of fungi which colonize and parasitize nematodes for exploitation of nutritious substances. Some of them are obligate parasites of nematodes, but the majority is facultative saprophytes [27]. They are usually regarded as soil inhabitant [29], however, they can be found in aquatic environments [30]. The zygomycetous fungi are completely reliant to water

Table 14.1 Taxonomy of some nematode parasitic and antagonistic fungi and their infection mechanism

Fungal group	Phyllum	Anamorph	Teleomorph	Infection structures
Nematophagous fungi				
Nematode-trapping	Zygomycota		*Stylopage*	Adhesive hyphae
			Cystopage	Adhesive hyphae
	Ascomycota	*Arthrobotrys*	*Orbilia*	Adhesive networks
		Dactylellina	*Orbilia*	Adhesive knobs and/or nonconstricting rings
		Drechslerella	*Orbilia*	Constricting rings
		Gamsylella	*Orbilia*	Adhesive branches or unstalked knobs
	Basidiomycota	*Nematoctonus*	*Hohenbuehlia*	Adhesive "hour-glass" knobs
Endoparasitic	Oomycota		*Myzocytiopsis*	Zoospores
	Chytridiomycota		*Haptoglossa*	"Gun cells", injection
	Blastocladiomycota		*Catenaria*	Zoospores
	Ascomycota	*Harposporium*	*Podocrella*	Ingested conidia
		Drechmeria	?	Adhesive conidia
		Hirsutella	*Ophiocordyceps*	Adhesive conidia
	Basidiomycota	*Nematoctonus*	*Hohenbuehelia*	Adhesive spores
Egg- and female-parasitic	Oomycota		*Nematophthora*	Zoospores
	Ascomycota	*Pochonia*	*Metaordyceps*	Appressoria
		Purpureocillium	*Cordyceps*	Appressoria
		Lecanicillium	*Cordyceps*	Appressoria
Toxin-producing	Basidiomycota		*Pleurotus*	Toxic droplets
			Coprinus	Toxin, "spiny structures"
Endophytic fungi				
Balanciaceous	Ascomycota	*Neotyphodium* spp.	*Epichloë*	Unknown[a]
		Ephelis	*Balansia*	
Nonbalanciaceous	Ascomycota	*Acremonium* spp.	?	Unknown[a]
		Nonpathogenic *F. oxysporum*	?	Unknown[a]
		Colletotrichum	?	Unknown[a]

(continued)

Table 14.1 (continued)

Fungal group	Phyllum	Anamorph	Teleomorph	Infection structures
		Phomopsis	?	Unknown[a]
			Glomus spp.	Unknown[a]
	Basidiomycota		*Piriformospora*	

[a]see text for possible mode of action

film that encircles soil particles for finding the nematode host by their swimming zoospores. Nematophagous fungi were first described in the late 1800s and afterward many scientists have contributed to illuminate various aspects of this fascinating group of fungi [27].

Nematophagous fungi are found in most fungal taxa like Ascomycetes (anamorphic Orbiliaceae and Clavicipitaceae), Basidiomycetes (Pleurotaceae), Zygomycetes (Zoopagales), Chytridiomycetes and Oomycetes [31]. It is suggested that the nematophagous habit evolved from lignolytic and cellulolytic fungi in different fungal taxonomic groups, as an adaptation to conquer competition for nutritious substances in soil [32].

The ecology of nematophagous fungi has been extensively reviewed [33–35]. Soil and various organic substrata, especially dung [36, 37] are appropriate sources for these fungi. Nematophagous fungi usually prefer organic soils; however, they can reproduce in nearly all types of soils because of their few nutritional and vitamin requirements [38].

The ability of nematophagous fungi in rhizosphere occupation is very critical for influencing on their biological control capability. Many nutrients leak from plant roots into rhizosphere as "root exudates" that greatly influence the rhizosphere ecosystem. Microbial components of rhizosphere could modify these nutrients and enhance the growth of root and shoot of plants, or could uptake them and increase their population. Here the nematophagous fungi, like other micro-organism, could be affected and could be effective. They can parasitize their nematode host, while also can be parasitized by other myceliophagous species [27]. Combining the existing non-destructive techniques to analyze dynamic of biotic component of the rhizosphere is tempting. Adjustment, manipulation or genetical engineering of the rhizosphere resource exchange could be very important for modifying the behavior of nematophagous fungi, which in turn influence their capability to control root diseases [27].

The majority of nematophagous fungi are facultative parasites, while some of them are obligate parasites of nematodes [28]. The facultative parasites can infect nematodes through producing structures which trap migratory stages of nematodes, producing specialized adhesive spores, or by means of developing appressoria on specialized hyphae that can penetrate through the nematode cuticle or eggshell. Some fungal species can produce special attacking devices which are like a sharp knife and cut the nematode cuticle [39].

Obligate parasites can initiate infection using their spores. The spores may ingest, germinate in nematode's digestive system and breach through its wall, or may adhere to the nematode cuticle and penetrate directly [33]. Many biotic and abiotic factors make introduction of nematophagous fungi to soil problematic [27].

A molecular technique is devised for investigating rhizosphere. In this method a series of genetically engineered bacteria are used whose reporter genes (like *gfp* and *lux*) are jointed with promoters that are induced by some rhizosphere conditions (like starvation, contaminants, quorum sensing). These bacteria are called "bioreporter bacteria" and studying the engineered biocontrol bacteria make a high augmentation in our rhizosphere knowledge [40].

Nematophagous fungi are divided into four groups according to their mode of action against nematodes: (*i*) nematode-trapping (= predacious or predatory fungi), (*ii*) endoparasitic, (*iii*) egg- and female-parasitic and (*iv*) toxin-producing fungi [41]. It is demonstrated that nematophagous fungi can produce extracelullar enzymes that may be important in their parasitism. Some of these enzymes are characterized (Table 14.2).

Table 14.2 Extracellular enzymes isolated and characterized from different nematophagous fungi (modified from Lòpez-Llorca et al. [27])

Enzymic group	Fungal group	enzyme name	Origins	References
Serine proteases	Nematode-trapping fungi	PII	*A. oligospora*	[42]
		Aoz1	*A. oligospora*	[43]
		Mlx	*Arthrobotrys microscaphoides*	[44]
		Ds1	*Arthrobotrys shizishanna*	[45]
		spr1	*Monacrosporium megalosporum*	[46]
	Endoparasitic fungi	Hasp	*Hirsutella rhossiliensis*	[47]
	Egg-parasitic fungi	P32	*M. rubescens*	[48–50]
		VCP1	*P. chlamydosporia*	[51, 52]
		PL	*P. lilacinum*	[53]
		Ver112	*Lecanicillium psalliotae*	[54, 55]
Serine carboxypeptidase		SCP1	*P. chlamydosporia*	[56]
Chitinases/chitosanases	Egg-parasitic fungi	CHI43	*M. rubescens*	[57]
		CHI43	*P. chlamydosporia*	[57]
		pcchi44	*P. chlamydosporia*	[58]
		–	*P. lilacinum*	[59]
Phospholipase	Egg-parasitic fungi	PLD	*P. lilacinum*	[60]

14.2.1 Nematode-Trapping Fungi

14.2.1.1 Introduction

As the name of this group implies, they are soil-borne fungi that entrap moving stages of nematodes using trapping structures of various shapes and sizes. These fungi are not host specific and could trap all soil-dwelling nematodes. To date approximately 380 nematode-trapping species have been identified [61]. Different fungal species produce one or more types of different trapping devices. These structures can vary from simple fungal hyphae covered with sticky secretions (*Stylopage* spp.) to much more complex structures. They can be adhesive branches, simple loops, two-dimensional, or three-dimensional networks. Adhesive three-dimensional nets, the most common type of fungal traps, are constructed when the loops create a three-dimensional configuration (e.g. *Arthrobotrys oligospora, A. superba, Dactylella pseudoclavata*). Other groups of trapping fungi produce adhesive spores (*Meristacrum* spp.) or adhesive knobs (*A. haptotyla, Nematoctonus* spp.). Lateral branches of vegetative hyphae create non-constricting rings which entrap the entering nematodes by wedging around their body. Constricting rings (*Arthrobotrys dactyloides, Monacrosporium doedycoides*) are the most specialized trap, which has three cells that swell quickly and hold the entering nematode tightly [9, 12, 27, 61, 62].

In addition to predation, many fungal species of this group can also grow saprophytically. The trapping process can be divided into several steps including attraction of nematode, prey recognition, trap production, entrap the prey or adhesion to traps, penetration into nematode and colonize the host [63].

Trapping structures may differ even within a genus, for example *Nematoctonus robustus* produce adhesive knobs exclusively on hyphae, *N. leptosporus* exclusively on germinated conidia, and *N. angustatus* on both hyphae and conidia [64].

Some disadvantages like complexity in the establishment in the soil, their limited capturing activity and above all non-specific trap of plant-parasitic nematodes reduce their potential in biological control. Some *Arthrobotrys* species have been formulated and applied under specific conditions, but the results were inconsistent [12].

It was proved that trapping fungi also have the ability of secretion antimicrobial and nematicidal compounds like linoleic acid (*A. oligospora*, *A. conoides*) or pleurotin (*N. robustus*, *N. concurrens*). The production of linoleic acid was positively correlated with the number of traps formed [65].

14.2.1.2 Taxonomy

Nematode-trappers form a heterogenous group of fungi whose members belong to taxonomically different fungal phyla (Ascomycota, Zygomycota and Basidiomycota) [61]. Recent changes in Article 59 of ICN [66] put the systematic of fungi in a state

of flux. For now, priority is more important in nomenclature than sexual morph. Therefore, unified name should be chosen for the different possible existing names for various states or morphs of non-lichen-forming *Ascomycota* and *Basidiomycota* [67].

The teleomorphs of most nematode-trapping species are located within *Orbilia*, and their type of trapping apparatus arranged their taxonomic position [68]. These fungi were also classified according to their genetic data as follows: *Arthrobotrys* (adhesive three-dimensional networks), *Dactylellina* (stalked adhesive knobs and/or non-constricting rings), *Drechslerella* (constricting rings) and *Gamsylella* (adhesive branches and unstalked knobs) [69]. Other trap-producing species are *Stylopage* and *Cystopage* (belong to Zoopagales of Zygomycota) and *Nematoctonus* (belong to Pleurotaceae of Basidiomycota) [15].

According to phylogenetic studies, Orbiliomycetes is a monophyletic class diverging from Pezizomycotina at the initial phases of evolutionary history [70, 71]. It seems that passive predation (producing adhesive structures) and active predation (producing constricting rings) branched off about 246 million years ago and main steaky devices evolved about 198–208 million years ago [72].

14.2.1.3 Ecology

Despite recent advances, our knowledge about growth and development of trapping fungi in soil, particularly the factors which cause the switch from a saprotrophic to a parasitic phase, is not sufficient yet [12]. Formation of trapping structures is a key sign of shifting lifestyle from saprotrophic to predatory and can be triggered in response to various inducers such as amino acids, small peptides, nematode extracts, abscisic acid and ascarosides [73]. Ascarosides are small molecules produced by many soil inhabitant nematodes and can act as inducer of trap production [74].

The number of nematode-trapping species present in a specific soil and their population densities can considerably be different according to many biotic and abiotic variables. The most effective factors on distribution of nematode-trapping fungi are nematode host density, soil nutrients, moisture, pH and heavy metals [34, 75]. The highest densities are usually found in fall and in the upper 30 cm of soil [76].

A total of 54 nematophagous fungi were isolated and recognized from Scotland. The nematode-trapping fungi included 16 species while endoparasites included 15 species. *Arthrobotrys gephyropaga* and *Drechslerella brochopaga* among nematode-trapping and *Harposporium anguillulae* among endoparasites had the highest incidence [77] but in Irish sheep pastures 29 nematophagous fungi were isolated of which 12 were nematode-trapping and 17 were endoparasitic. In Ireland *Cystopage lateralis*, *Stylopage hadra*, *Drechmeria coniospora* and *Meristacrum asterosperum* had the highest incidence [78]. The following species were reported from Kenya: *Arthrobotrys dactyloides*, *A. oligospora*, *A. superba*, *Acrostalagamus obovatus*, *Dactyllela lobata*, *Harposporium aungulilae*, *H. liltiputanum*, *Haptoglosa heterospora*, *Monacrosporium asterospernum*, *M. cianopagum*, *Myzocytium* spp.,

Nematoctonus georgenious and *N. leptosporus* [79]. Recently two new species of *Dactylellina* were isolated and described in china. These new nematode-trapping fungi were *D. sichuanensis* and *D. varietas* which entraped nematodes by both adhesive knobs and non-constricting rings [80].

Application of chopped organic amendment [38] and glucose [81] to soil could increase the activity of nematode-trapping species and consequently increase the number of free-living and microbivorous nematodes. Probably organic amendments stimulated population densities, however, similar population densities of trapping fungi were found in plots with and without organic amendments [82, 83]. The effect of abscisic acid (ABA) and nitric oxide (NO) on the nematode-trapping fungus *Drechslerella stenobrocha* AS6.1 were tested and demonstrated that the trap development and nematode-trapping capability of *D. stenobrocha* were increased by ABA but decreased by NO [84].

It is apparent that the trapping fungi need a carbohydrate source for their proliferation but other factors, like those which cause fungistasis are also important in their abundance and trophic state in soil [12]. It is hypothesized that *Orbilia* species, the teleomorph of *Arthrobotrys* species, that are weak wood decomposers [85], support the fungi with carbon and energy sources, while nematode cadavers act as an important supply of nitrogen [32]. It is illustrated that predaceous behavior of *A. oligospora* can be controlled either by physiologically active compounds (amino-acids or vitamins) present in nematodes or by nitrogen sources [86, 87].

The majority of nematode-trapping fungi colonizes the bulk soil and waits until the passing nematodes contact them. Some fungi increase their trapping chance by producing secondary attractive compounds for nematodes, like *A. superba* which attract J2 of *Meloidogyne* species [28]. Others grow in rhizosphere, which give them superior predatory activity to trap plant-parasitic nematodes on their way toward the roots. For example, *A. oligospora* found more abundance in rhizosphere of tomato and barley plants because of its chemotropical attraction to the root tips [88]. Plant species obviously influence on rhizosphere and external root colonization. The highest incidence and diversity of nematode-trapping fungi is seen in association with pea rhizosphere [89]. Tomato roots are successfully colonized by *Dactylellina ellipsospora* and *D. dactyloides* in a pot experiment [90].

Various fungi have different efficacy in trapping and parasitizing nematodes. It is shown that *A. dactyloides* is more efficient in trapping *Meloidogyne graminicola* than *Dactylella brochopaga* and *Monacrosporium eudermatum*. Some nematode-trapping fungi are good antagonists but trap few nematodes, while others are efficient in capturing nematodes but do not establish well in soil. This subject limits the potential of this group as microbial control agents [28]. However genetic engineering is a promising tool that hopefully may improve the efficacy, establishment and survival of nematode-trappers.

14.2.1.4 Mode of Action

Attraction of the host is the first step in infection of nematode, which includes nematode host chemotaxis towards fungal hyphae or traps [91, 92]. It is not completely clear that what compounds are involved in chemotaxis [88, 93]. Trap-bearing mycelia were more attractive to nematodes than mycelia without trap structure. It can be concluded that attractive molecules are secreted mainly from mycelia hosting traps [63]. It has been suggested that attractive compounds resembled to nematode sex phremones or food signals [94].

Formation of different trapping structures can be stimulated by environmental, chemical and tactile stimuli [27]. Many studies showed that the presence of nematodes or some specific organic compounds (like amino acids, peptides, nitrogenous compounds, nematode extracts, abscisic acid and ascarosides) trigger the formation of trapping structures [73]. Trapper fungi may recognize nematode host signal molecules via their cell-wall receptors. For instance, *Arthrobotrys oligospora* possess a glycoprotein (AoMad1) on its cell wall surface which contribute in nematode recognition [95]. It seems that several signal cascades such as the mitogen-activated protein kinase (MAPK) cascade, the G-protein-coupled receptors (GPCRs) or small GTPases are involved in trap formation in consequence of changing lifestyle from saprophytic to parasitic in *A. oligospora* [96]. It is also demonstrated that the presence of competitor organisms and the level of nutritious substances are important in changing the trophic state from saprotrophic into parasitic [97, 98].

Nematophagous fungi adhesives commonly include proteins and/or carbohydrates [99, 100]. Treating the steaky layer with pronase emphasized that adhesive polymers in *A. oligospora* possess proteinaceous nature [101]. A nematode recognition role is suggested for N-acetylgalactosamine-specific lectin of *A. oligospora* in adition to its binding ability to carbohydrate ligand presented on nematode cuticle [102]. Carbohydrates that cover the surface of nematodes play an important role in both recognition phase of lectin binding and nematode chemotaxis [103, 104]. When a nematode touches *A. oligospora* traps, the amorphous sticky materials on the surface of the traps change to a fibrillar appearance [105]. Nematode infection triggers a signaling cascade in fungi resulting in penetration and colonization of the nematode [106]. We know a little about the signaling cascades. During trap formation, expression of the genes that are involved in construction of the trapping devices of *Dactylellina haptotyla* accompanied with those involved in fungal morphogenesis, was demonstrated [107]. The same results were reported for an entomopathogenic fungus, *M. anisopliae* [108].

The trapping devices are usually constructed on mycelium, while they may also be formed directly on germinating conidia [98] with variation among different taxa. For example, *A. dactyloides* has a greater ability for conidial trap production than *A. superba* and *A. oligospora*. Fungistasis and competition for nutritious substances can cause conidia to form traps directly, and live as parasites [28].

Sudden inflation of three cells which form the constricting ring after being touched by a nematode, result in capturing the prey (Fig. 14.1). The mechanism by which the inflation of the cells starts and ring closure happens in less than 0.1 s is

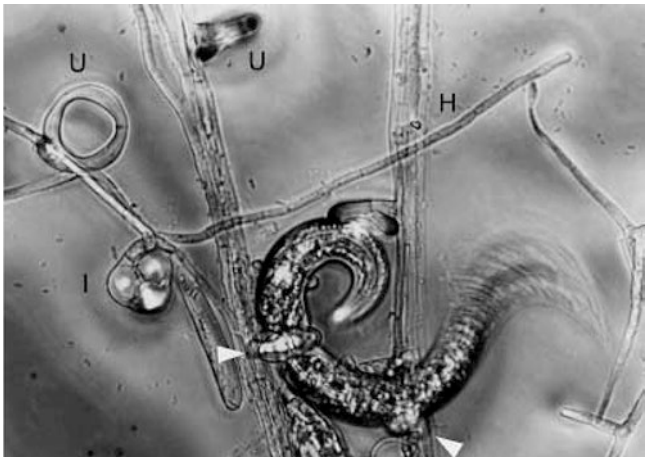

Fig. 14.1 A nematode is entrapped by two constricting rings of *Arthrobotrys dactyloides* (arrowheads). I: Inflated ring; U: non-inflated ring; H: hyphae (from Chen et al. [109])

not clear. Mild heat, pressure and Ca^{2+} can also stimulate the swelling of the cells *in vitro* [27]. The signaling pathways that took place in ring closure were examined and a model is suggested. According to that finding the nematode entrance exerts a pressure on the ring followed by activation of G-proteins. Consequently, cytoplasmic contents of Ca^{2+} increases in ring cells, calmodulin activates and at last the water channels open. Quick entrance of water via those channels make the cells inflate and entrap the prey. Calmodulin could regulate a key step in the signal transduction pathways after being activated by an increase in Ca^{2+}, because the ring closure was inhibited by calmodulin antagonists [109].

The nematode cuticle mostly consists of proteins, hence proteolytic enzymes (Table 14.2) may be important for penetration. *A. oligospora* produce a serine protease, named PII, which has been characterized, cloned and sequenced. This enzyme belongs to the subtilisin family and its expression is enhanced by the presence of proteins, especially those of nematode cuticle [110].

Aoz1, a PII homologue, is another serine protease secreted by *A. oligospora* [43]. Penetration of *A. oligospora* to nematodes was inhibited when serine protease inhibitors were applied [100]. Other nematode-trapping fungi can also produce serine proteases. *Arthrobotrys microscaphoides* produces Mlx and *Arthrobortys shizishanna* secretes Ds1 both with a high homology to the *A. oligospora* serine proteases [44]. A putative serine protease gene (*spr1*) was cloned and characterized from *Monacrosporium megalosporum*, whose predicted protein sequence was similar to PII and Azo1 from *Arthrobotrys oligospora*. The fungus has a single copy of this gene [46]. Similar enzymes have also been purified and characterized from the egg-parasitic fungi *Purpureocillium lilacinum* [53], *Pochonia chlamydosporia* [52], and *Lecanicillium psalliotae* [55].

14.2.2 Endoparasitic Fungi

14.2.2.1 Introduction

Most of these fungi are obligate parasites and poor saprotrophic competitors in soil, but usually have a broad nematode host range. These obligate parasites live their whole vegetative life cycle inside their infected hosts [12, 27]. Endoparasitic fungi infect vermiform plant-parasitic nematodes using their spores (conidia or zoospores). The spores can be ingested by the nematode which germinate in the intestines (mostly the esophagus or mastax), or adhere firmly on the nematode cuticle when the nematode passes the fungus. The spore contents are inserted into the nematode by means of a narrow penetration tube, apparently with some mechanical pressure [29, 111]. Then an internal mycelium produces, and finally penetrates the cadaver to sporulate on its surface [31]. Some endosparasitic fungi produce zoospores that swim toward the nematode, attach to the cuticle usually around the natural orifices, and then encyst. The encysted zoospores penetrate the host body via those natural openings and start their vegetative growth. Afterward the hyphae develop some sporangium containing zoospores [12].

14.2.2.2 Taxonomy

We know a little about the actual taxonomy and phylogeny of this group of fungi. They are found in Blastocladiomycota (zoosporic *Catenaria anguillulae*), *Harposporium* (teleomorph: *Podocrella*), or *Drechmeria* [27]. In higher order classifications, posteriorly uniflagellate fungi (or chytrids) have been placed into three phyla: Blastocladiomycota, Chytridiomycota and Neocallimastigomycota. In this classification *Catenaria* spp. were transferred to Blastocladiomycota instead of previously accommodation under Chytridiomycota [112–117]. The teleomorph of *Nematoctonus* (basidiomycetous *Hohenbuehelia*) contains both nematode-capturing and endoparasitic fungi [118].

Drechmeria was segregated from *Meria* [119] and its similarity with the Clavicipitaceae has been proven [120]. Some species demonstrate a continuum between the genera *Harposporium* and *Hirsutella*, developing two kinds of spores with the related kinds of conidiogenesis [121, 122]. According to a comprehensive phylogenetic study, six genera were protected in Ophiocordycipitaceae including *Drechmeria*, *Ophiocordyceps*, *Tolypocladium*, *Purpureocillium*, *Harposporium*, and *Polycephalomyces* [123].

The most distinctive nematophagous verticillium-like genera are *Haptocillium* (formerly *Verticillium*) species with adhesive conidia that stick to free-living nematodes [31]. Two endoparasitic fungi (*Acrostalagmus bactrosporus* and *A. obovatus*) with adhesive spores were transferred to the genus *Haptocillium* [124]. However, recent phylogenetic studies suggested that *Drechmeria* and *Haptocillium* should be merged and *Drechmeria* an older name is protected over *Haptocillium* [123].

Hirsutella species are insect, mite and nematode pathogens and most of them are synnematous. More than 70 species exist in this anamorphic genus, which are associated with *Ophiocordyceps* as teleomorph. The phylogeny of 47 isolates of *Hirsutella* was investigated by analyzing sequences of *tef1*, *rpb1* and 18S rDNA and generated six distinct groups. However little correlation was observed among molecular grouping and morphological or host characters [125]. A common nematode parasite of this genus is *H. rhossiliensis* [31].

14.2.2.3 Ecology

Reports of endoparsitic fungi from different countries indicate a nearly cosmopolitan distribution, but a few species are either tropical or temperate. They were mostly described in the United States and Canada [31]. They were also reported from Ireland [75] New Zealand [126], El Salvador [127], and from plants and soils in the maritime Antarctic [35, 128, 129]. *Drechmeria coniospora*, *Drechmeria balanoides*, *Harposporium anguillulae*, and *Hirsutella rhossiliensis* were also isolated infrequently in Central America [130].

Maximum densities of *Harposporium anguillulae* are usually found in March and June in Swedish agricultural soil, where going down to 30–40 cm, while *Hirsutella rhossiliensis* strongly declines after 20 cm [31] Densities of *H. rhossiliensis* in a Swedish agricultural soil peaked during September–November [76] Population densities of these endoparasites specifically declined after fallow periods [76].

Nematode endoparasites were usually found in deciduous and conifer litter, old dung, moss cushions, and decaying vegetation [75]. Addition of farmyard manure to agricultural soil increased the population of endoparasites [131]. Though nematodes are attracted toward *Drechmeria coniospora*, *Drechmeria balanoides*, and other endoparasites colonies [91, 132–135], they also can defend themselves gainst endoparasitic fungi. Upregulation in *C. elegans* antimicrobial peptides (AMPs) coding genes was reported after being infected with *D. coniospora*. These peptides usually destruct microorganisms cell-membranes from them NLP-31 had the strongest activity against *D. coniospora* [136]. No homologue gene for the *nlp* family has been detected in *Meloidogyne incognita* [137], but similar AMPs should exist in phytonematodes [96]. A transcriptome study was conducted on *D. coniospora* and its genome was completely sequenced. The fungus simplified necessary genes for saprophytic trophic lifestyle while modulated entomopathogenic genes and developed nematopathogenic genes [138]. The amount of glycoside hydrolase enzymes was greatly reduced that may lead to decreased ability of *D. coniospora* to adapt to diverse environmental conditions [139].

In contrast with *Hirsutella rhossiliensis* whose conidia are infectious only while attached to a conidiophore, the conidia of *Drechmeria* species are equally infectious after liberation, and could bind to a rather wide range of nematode species. Consequently when *D. balanoides* applied as a suspension of conidia and hyphal fragments had a much greater effect than *H. rhossiliensis* in controlling *Ditylenchus*

dipsaci and promoting growth of clover, both under gnotobiotic conditions [140] and in pot cultures [141]. According to these authors, *D. balanoides* has low saprotrophic ability and does not survive in the soil for prolonged periods without added nematodes. *Drechmeria* is known to parasitize several nematode species, and with records to date, each *Drechmeria* species appears to have a limited degree of nematode host specificity [124]. Several species of nematodes such as *D. dipsaci*, *Globodera rostochiensis* and *Panagrellus redivivus* were inoculated with the fungus. Conidia adhered to all species but some of them were removed while the nematode moved through a layer of wet sand. Colonized individuals produced different quantities of conidia that were approximately 16,000, 11,700, and 840 for the above nematode species in the order mentioned [142]. *Drechmeria bactrosporum, D. obovatum* and *D. balanoides* all abundantly produce conidia on a bacterivorous nematode, *Plectusi* sp. (Fig. 14.2) [124].

Drechmeria balanoides was also recorded on dead needle of *Pinus densiflora* in Tsukuba, Japan [143]. The difference between phytophagous and bacteriophagous nematodes is of great ecological importance in relation to endoparasitic and other nematophagous fungi. The host relation hypothesis proposed for endoparasitic fungi [144] is incompatible with the many reports of relatively little host specificity. The free-living stages of the same nematodes can be parasitized by different array of taxa, mainly *Drechmeria* and *Hirsutella* [31].

Additional examples of extensively studied endoparasitic fungi are *Drechmeria coniospora*, *D. balanoides* and *Nematoctonus* spp. [12]. Comparing with

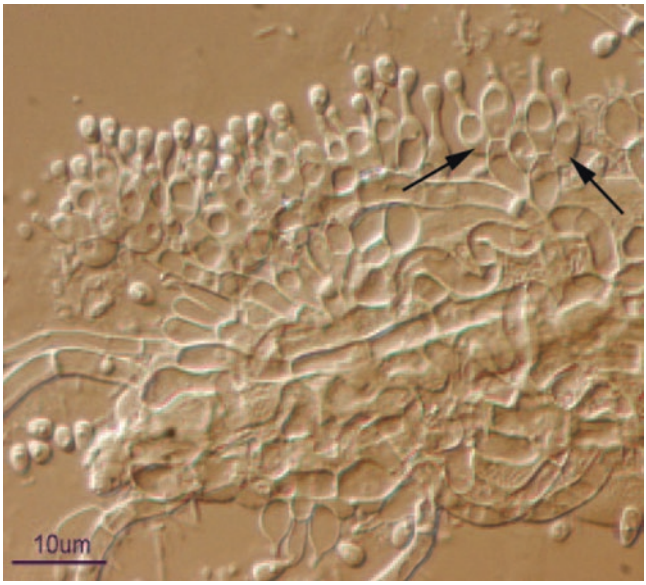

Fig. 14.2 Sporulation of *Drechmeria obovatum* occurring externally on hyphae constructed on head end of a nematode, arrowheads show some proliferating conidiogenous cells (from Glockling and Holbrook [124])

nematode-trapping fungi, such endoparasitic fungi are more amendable to practical application [76].

Hirsutella rhossiliensis was able to decrease nematode invasion, and therefore nematode populations, of *Meloidogyne javanica*, *Heterodera avenae*, *H. glycines* and *Criconema xenoplax* were decreased. It also successfully infected several other species of *Heterodera, Ditylenchus destructor*, *Meloidogyne hapla*, *Pratylenchus penetrans*, *Anaplectus granulosus*, and even larvae of *Globodera rostochiensis* [145].

In an *in vitro* experiment, *H. rhossiliensis* killed *Ditylenchus dipsaci* in 4 days, and juveniles of *M. incognita* in 2 days [146]. *Hirsutella rhossiliensis* is considered responsible for rapid fluctuations of *C. xenoplax* populations in peach orchards [9, 147, 148]. Without nematodes as a food source, the population of *H. rhossiliensis* in soil dies out [149, 150]. *Hirsutella rhossiliensis* (18 isolates), *H. minnesotensis* (8 isolates) and *H. vermicola* (3 isolates) were compared for their nematode parasitism. Most isolates of *H. rhossiliensis* and *H. minnesotensis* parasitized higher percentages of the cyst nematodes (*Heterodera glycines* and *H. avenae*) than the four non-cyst nematodes (*Meloidogyne hapla*, *Bursaphelenchus xylophilus*, *Heterorhabditis bacteriophora*, and *Steinernema carpocapsae*). *Hirsutella vermicola* had weak or no ability for parasitizing the six assayed nematode species [151]. The complete sequence of mitochondrial genome of *H. rhossiliensis* [152] and *H. minnesotensis* [138] was identified. This data can be of help in comprehending the distribution and genetic diversity.

The conidia of *Hirsutella* species are infective only when they are attached to the phialide [153], and furthermore, conidial germination can be greatly affected by soil fungistasis [147, 154]. Consequently, the species seems less suited for biological control than species of *Drechmeria* [141]. No growth was observed below pH 5 on an agar pH gradient [155]. Except for isolates originating from Hoplolaimidae that grew more slowly, other different isolates had uniform characters of nematode pathogenicity. The Hoplolaimidae originated isolates had larger conidia and were less pathogenic toward nematodes than isolates from other nematode hosts [156]. The fungus produced 78–124 conidia from a colonized individual J2 larva of *Meloidogyne hapla* and caused a 50% decrease in J2 penetration of lettuce roots [157]. Patel et al. [158] succeeded to produce inoculum of *H. rhossiliensis* in liquid culture stirred in 5-L containers. *Hirsutella minnesotensis* is the second nematophagous species parasitizing the J2 of the soybean cyst nematode, *Heterodera glycines* [159]. The entomopathogenic species of *Hirsutella* did not attach to nematodes with their conidia and therefore had no controlling effect [146].

Hirsutella rhossiliensis is frequently seen in association with nematode populations and there are several reports on its suppression effect on populations of *H. schachtti* [160, 161], and potato cyst nematodes [162]. One worthy species for further investigation is *H. rhossiliensis* [12] with an obligate parasite lifestyle, make its population density related to population of its host nematode [150]. Contrasting with encouraging results of controlling nematodes by *H. rhossiliensis* in greenhouse and laboratory assays [59, 163–165] the fungus did not decrease the population of cyst and root-knot nematodes in a number of field trials [83, 166]. Because of the

fungus inconsistent results, a better understanding of its ecology and population dynamics after being introduced into soil is critical for fungus successful use as an inundative commercial biocontrol agent.

A real-time PCR assay was developed to quantify the *H. rhossiliensis* [167] and *H. minnesotensis* [168] in soil. The results showed that the quantity of *H. rhossiliensis* DNA (according to real-time PCR) decreased over time (rapidly in the first 17 days and gradually for the succeeding 42 days), regardless of eggs or J2 of *H. glycines* as inoculum [169].

It is demonstrated that *H. rhossiliensis* could not decrease the *M. javanica* population on tomato over the long time [166], however, a related fungus, *H. minnesotensis*, was considered to have the ability of decreasing *M. hapla* population between 61 and 98% [170, 171].

Drechmeria coniospora can kill its host within 24 h and produce 5000–10,000 conidia on each infected nematode [31]. Application of 106 conidia per 250-cm^3 pot or 1000 living infected *Panagrellus redivivus* as vectors could regulate *Meloidogyne incognita* in sterile or unsterile soil [172]. About 70% of nematodes which were inoculated with the conidia of *D. coniospora* retained attached condia after 16 h, with young ones being preferentially infected [173]. Positive correlation between reduction in spore adhesion and the nematode age increment has already been reported for *Pasteuria penetrans* [174].

Adding organic amendment to soil can indirectly increase the population densities of the fungus by stimulating bacteriophagous nematodes. Application of *D. coniospora* as a biological control agent is not considered feasible, because its population density had not increased adjacent to plant roots; meanwhile the fungus has narrow host range that usually does not include the plant-parasitic nematodes [173, 175, 176].

14.2.2.4 Mode of Action

Because of wider mouth openings of bacteriophagous nematodes which facilitate conidial ingestion, this group of nematodes are much more prone to parasitism by endoparasitic and nematode-trapping fungi. Chemical factors are responsible for the attachment of *Drechmeria* conidia to specific parts of the body [31]. The processes of *D. coniospora* conidiogenesis and penetration into nematode cuticle were illustrated by light- and electron-microscopy (Fig. 14.3) [177, 178].

Drechmeria coniospora secretes collagenase before and during penetration [177]. The fungus occupies the pseudocoelum of the nematode without colonization of the internal organs. Nematode can ingest the conidia, but no germination is seen in intestine [179]. Thus direct penetration of conidia through cuticle is the only way of infection.

Drechmeria coniospora attracts susceptible nematodes [133, 134]. The fungus develops teardrop-shaped conidia coated with a sticky mucous-like layer containing radiating fibrils [180, 181]. Conidia of the fungus stick to the nematode chemosensory organs, specifically in the mouth region and in male anal region of certain

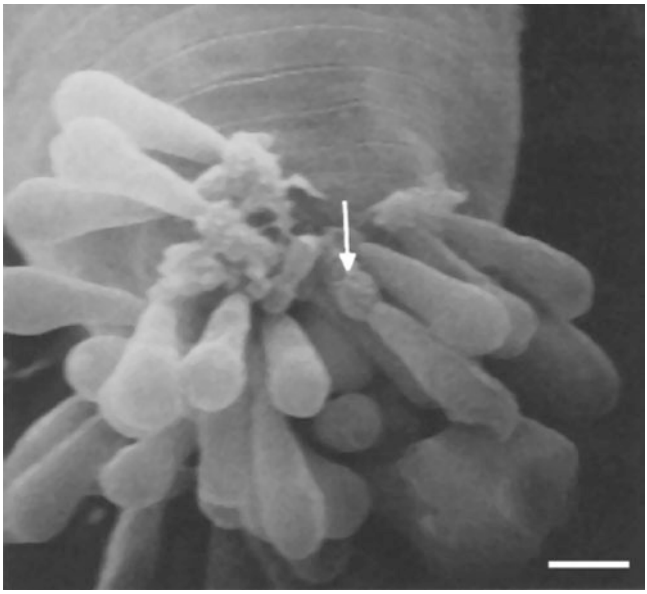

Fig. 14.3 Adhesive conidia of *Drechmeria coniospora* on the head of a nematode. *Arrow* Adhesive bud of conidia, *bar* 2 μm [177]

species. Infected nematodes lost their ability to respond chemotactically to all attraction sources [182] and they were no longer attracted by colonies of the fungus. The site-specific adhesion of conidia was demonstrated for bacteriophagous, a few plant-parasitic (*Meloidogyne* and *Aphelenchus*), and animal-parasitic nematodes while there were no specific binding for some plant-parasitic nematodes like *Pratylenchus*, *Ditylenchus* and *Criconemella* species [176]. Conidia of *D. coniospora* can adhere to the chemosensory organs of root-knot nematodes but do not penetrate and colonize the nematode. Application of the fungus resulted in decreasing root galling in tomato roots, emphasized on involvement of chemotactic interference [172]. There are similar reports for insect-parasitic species (*Neoaplectana* and *Heterorhabditis*) [183] and *Acrobeloides* [173] where conidial adhesion occurs without any penetration.

It seemed that sialic acid-like carbohydrate (acetyl-neuraminic acid) which localized in head and tail regions, involve in binding to a lectin that located on the parasite's conidia. Treatment of spores with sialic acid and treatment of nematode with lectin Limulin reduced adhesion [182, 184]. Pronase treatment of the *Caenorhabditis elegans* also prevents adhesion of the conidia, but the nematodes regenerate the lost protein material after 2 h in Tris buffer [179]. The adhesion is also suggested to be mediated by sensilla exudates [179].

Adhesive on the conidial surface of *D. coniospora* always keeps its fibrillar appearance [105]. The fibrillar layer is dissolved in Pronase E. Infection was inhibited by Chymostatin (a protease inhibitor), suggesting the involvement of

chymotrypsin-like proteases in the infection process [93]. After the binding of conidia of *D. coniospora* to nematode cuticle, an infection vesicle is developed within the cuticle layers [185, 186].

It is likely that a motile nematode previously colonized by *D. coniospora*, can be trapped by a second nematophagous fungus as well, however, penetration of *Arthrobotrys oligospora* to a *D. coniospora* colonized nematode is inhibited and its hyphae are often killed when placed adjacent to those of *D. coniospora* [111].

Genomic analysis of *D. coniospora* demonstrated a core set and specific genes required for nematode parasitism. Less than 2% of *D. coniospora* genome consists of transposable elements. The genome contains many gene sets effective on detoxification and bearing oxidative stress. Diverse groups of transcriptional factors are detected in *D. coniospora* that regulate many existing protein kinases [187]. The transcriptional factors usually play a role in controlling the expression of virulence genes [188]. *Drechmeria coniospora* secretes a new peptaibiotic family with antibiotic and nematicidal activities. It seems that DcNRPS1 is responsible for producing peptaibiotic and the accompanied genes is responsible for endoing enzymes involved in regulation of the transcription, biosynthesis and transportation. The peptide may be of help in killing the nematode host and/or prevent other microorganisms to colonize the nematode corpse [189].

The uniflagellate zoospores of *C. anguillulae* attract toward natural openings (mouth, anus, excretory pores, etc.) of nematodes and after contacting with cuticle, show an amoeboid movement before encystment happen. A cell wall coated with a sticky material cover the encysting zoospores, and the flagellum is withdrawn. A penetration peg is developed from the encysted zoospore which breaches the nematode cuticle and usually invades and digests the nematode cuticle within 24 h. Then the hyphae develop some sporangium containing zoospores that can infect new nematode hosts after releasing. Ability to parasitizing nematode eggs is also reported for *C. anguillulae* [190].

Hirsutella rhossiliensis is a typical endoparasitic fungus of nematodes. It produces adhesive spores that attach to and penetrate the cuticle of passing nematodes [154]. The conidia are infectious if only they are attached to the phialides [153] and one conidium is generally enough to infect a nematode. When the fungus penetrates its host, the nematode will be totally colonized, and within a few days the new infectious conidia will be produced [191].

One neutral serine protease [192] and more recently a new extracellular alkaline protease (Hasp) [47] has been described from *H. rhossiliensis*. This enzyme was purified, cloned and examined against nematodes. Hasp could kill the juveniles of the soybean-cyst nematode (*Heterodera glycines*) after purification [47]. *Hirsutella minnesotensis* was transformed successfully via *Agrobacterium tumefaciens*-mediated transformation to produce fluorescent proteins. The morphology and pathogenicity of transformants was similar to those of wild type. The conidia penetrated into passing nematodes between 12 and 24 h post-inoculation (hpi) for second stage juveniles of *C. elegans* and 12–32 (hpi) for second stage juveniles of *H. glycines*. The whole body consumption of *C. elegans* and *H. glycines* and production of new conidia was last about 156 and 204 hpi, respectively [193].

14.2.3 Egg- and Female-Parasitic Fungi

14.2.3.1 Introduction

In contrast with the numerous migrating nematodes, some plant-pathogenic nematodes spend the majority of their life cycle inside plant roots or on their surface in cysts and/or in root knots. These sedentary stages persist in the soil and serve as a selective substratum for fungal colonization by egg parasites. Many opportunistic soil fungi have been isolated from the eggs, cysts and sedentary females that lay their eggs in gelatinous matrices, such as *Meloidogyne* spp. and *Tylenchulus semipenetrans* [3, 12, 31, 194, 195]. Generally, egg and cyst parasitizing fungi are more numerous than those infecting females [196]. This group of fungi uses appressoria or zoospores to infect their hosts [27]. The parasites of egg and sedentary stages have attracted more attention because of their high potential in biological control of economically important nematodes. These fungi that can saprotrophically survive well in rhizosphere, are relatively easy to mass-culture and are more effective in infecting because their host is sessile (eggs, developing juveniles and females).

Among all nematode parasitizing fungi, comparatively few have been considered as promising biocontrol agents [25], and of these the most frequently isolated fungi are *Pochonia chlamydosporia* and *Purpureocillium lilacinum* [25, 197–201]. Species of *Pochonia*, *Purpureocillium*, *Drechmeria*, and *Hirsutella* are among the most favorable biocontrol agents against plant-parasitic nematodes [7, 9, 29, 147, 202–205].

14.2.3.2 Taxonomy

The egg-parasitic fungi previously accommodated under the genus *Verticillium* were transferred to the genus *Pochonia* according to both morphological and molecular characters [206, 207]. The teleomorphs of *Pochonia* species belong to *Metacordyceps* [208]. *Pochonia* species mostly produce dictyochlamydospores or at least some irregularly swollen hyphae. The production of dictyochlamydospores was mostly used to characterize *Diheterospora*, but this is an unreliable character for distinguishing species of this genus, because they are absent or scanty in some species, while similar structures also occur in species of *Rotiferophthora* and *Drechmeria* [207].

Their species can be more or less easily distinguished on the basis of conidial shape and the position and abundance of dictyochlamydospores [206, 207]. *Metapochonia* is a new genus segregated from *Pochonia* whose macrospores are real resting spores. In this genus, dictyochlamydospores are scarsely present and they are typically submerged in the agar. The nematophagous species of *Metapochonia* are *M. suchlasporia*, *M. goniodes* and *M. rubescens* [209].

According to the newest taxonomic studies of *Pochonia*, the genus is considered as a dimorphic anamorph genus and its asexual state can be split to A-anamorph and

B-anamorph. A-anamorph produces ameroconidia (aseptate spore) while B-anamorph produces stalked dictyochlamydospore [210]. However, it is believed that more work is required before adopting the dimorphic terminology [211].

Although all *Pochonia* species could parasitize *Meloidogyne javanica* eggs [212] and considered as the best egg colonizers, but other species like *Purpureocillium lilacinum* and *Lecanicillium lecanii* are also effective in egg parasitization [27]. Phylogenetic analysis of complete mitochondrial genome of *Lecanicillium saksenae* find a close phylogenetic relationship between the fungus and *Lecanicillium muscarium*. However a conserved part of the mitogenome (the *trnC* between *cob* and *cox1*) in *L. saksenae*, *P. chlamydosporia* and *Acremonium implicatum* was absent in *L. muscarium* mitochondrial genome [213]. It should be also noted that a part of the genus *Lecanicillium* is recently combined in *Akanthomyces* and the current name of *L. muscarium* is *Akanthomyces muscarius* [214].

In addition to the mentioned facultative parasites in the Hyphomycotina, other fungi belonging to Oomycota (*Nematophthora gynophila* and an undescribed lagenidiaceous fungus) and Chytridiomycota (*Catenaria auxiliaris*) are also reported as obligate parasites of cyst nematode females [7].

14.2.3.3 Ecology

Purpureocillium lilacinum is abundant and active in subtropical and tropical areas [12], while *Lecanicillium* is mainly found in tropical areas [207]. *Lecanicillium* is reported from W. Indies, Dominican Republic, Peru, Jamaica, USA, Sri Lanka, Indonesia, Iran and Turkey [215–217]. *Nematophthora gynophila* is prevalent in soils of northern Europe infested with cereal cyst nematode and usually occurs together with *P. chlamydosporia*, and both are involved in declining of this pest species [2]. *Pochonia chlamydosporia*, *P. bulbillosa*, *Purpureocillium marquandii*, *P. lilacinum*, and *P. carneus* were isolated from *Ascaris* eggs buried in soils in the Czech Republic, Pakistan, Afghanistan, and Cuba. *Pochonia* spp. and *P. lilacinum* rapidly infected and killed the eggs [218]. *Pochonia chlamydosporia* is one of the most cosmopolitan species, but its *Metacordyceps* teleomorph is so far known only from slug eggs in the tropics [219]. *Pochonia chlamydosporia* is the major egg pathogen of *Heterodera* species in all European and American countries examined [9, 147, 220]. The species is also found as an efficient parasite of *Meloidogyne* root-knot nematodes [198, 221–223].

Metapochonia suchlasporia was a rather common fungus in central and northern Europe [31] especially on *Heterodera* cysts in Denmark, Sweden and the Netherlands, [224–227] while *P. chlamydosporia* is more restricted to young cysts in these countries [207].

Some species, such as *Purpureocillium lilacinum* and *Pochonia* spp. are presumably not influenced by antimicrobial activity of the matrices produced by root-knot [228] and cyst nematodes [2]. These fungi are more abundant on galled roots infected by *Meloidogyne* spp. than in the rhizospheres of healthy roots [229], and their isolates have been collected from a broad range of cyst and root-knot

nematodes with a worldwide distribution [2]. Two distinct barriers impede the infection of nematode eggs by fungi, the eggshell and the cuticle of the second stage larvae within the egg [28], therefore, immature eggs are more prone to parasitism than those containing larvae (Fig. 14.4). *Pochonia chlamydosporia* colonizes dead eggs of *Heterodera* more efficiently than live ones, with a trophic favorite of young stages, before the embryo development is completed [230].

Many experiments illustrated that egg-parasitic fungi are preferably inhabited at rhizosphere [26, 231] and inside roots [232]. Plant species influence the growth of *P. chlamydosporia* [231], and nematode parasitism may help support the long period maintenance of the fungus in soil [2, 233]; although the fungus is more effective when applied on poor hosts for the nematode, than when applied on fully susceptible crops. Inoculation of the nematode poor host plants prior to a susceptible host could assist the fungus to construct a high level of population density that could manage the nematode efficiently [31]. It seems that the fungus could not provide a sufficient nematode control by itself and must be integrated with other managing measures [222, 234, 235]. All egg and female parasites grow willingly on artificial media and some produce resting spores which serve as a survival stage in soil [2].

An important consideration in commercial use of biocontrol agents is their survival after exposure to chemical pesticides and their viability in response to environmental condition [17]. *Pochonia chlamydosporia* was grown on PDA medium containing abamectin, captan, imidacloprid + thiodicarb, thiamethoxam, carbendazim + thiram, fludioxonil, pyraclostrobin + thiophanate methyl + fipronil, difenoconazole and fipronil; but the growth of the fungus was suppressed by all agrochemical used. However, when the experiment conducted in pots, *P. chlamydosporia* could tolerate agrochemicals, decrease *M. incognita* populations and establish in the soil and rhizosphere [236]. The survival of *P. chlamydosporia* propagules reduced by 90% at 150 min after it was sprayed on soil surface. Relative humidity above 61%, air temperature between 19 and 29 °C, soil temperature between 25 and 35 °C and irradiance between 1172 and 2126 μmol of photons m^{-2} s^{-1} impose a negative exponential effect on the viability of *P. chlamydosporia* over the time [237].

Different isolates of *Pochonia* spp. differ in virulence [26], ability to colonize the root epidermis and cortex [88], dictyochlamydospore production [207], and saprophytic ability [238] while all of these characters are considered important for the use of the fungus as a biocontrol agent.

Dictyochlamydospores are usually used as an inoculum to introduce and to establish the fungus in the soil and rhizosphere. There are some biological (dilution plating on a selective media) and molecular (PCR, real-time PCR, and RFLP) methods which are developed to screen the presence, abundance and activity of the fungus in the soil, rhizosphere and nematode egg masses [239]. Isolates that obtained from cyst nematodes have greater ability to parasitize the cyst nematode eggs than isolates recovered from root-knot nematodes, and therefore it is suggested that the fungus has host preference [12, 212].

It is now demonstrated that *P. chlamydosporia* has the ability to live as a multitrophic and symbiotic fungus in response to different hosts, substrata and environmental conditions [240, 241]. The genus (*Pochonia*) has the ability to survive in its

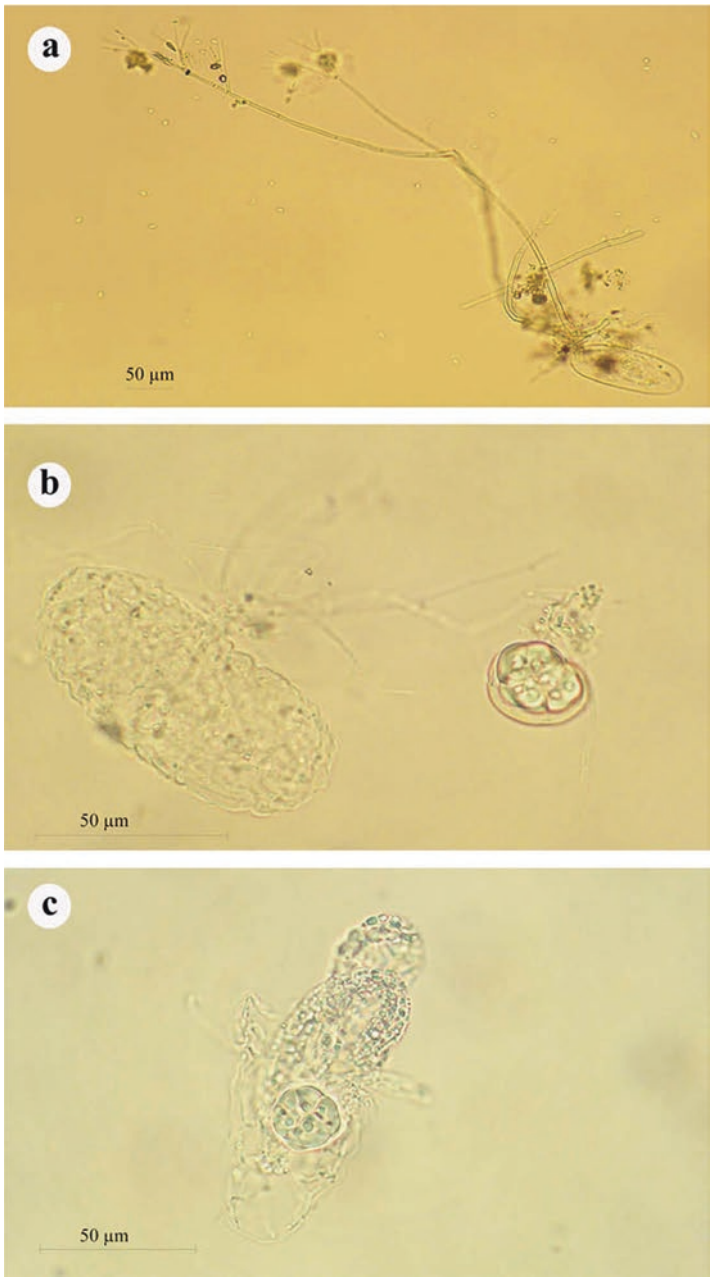

Fig. 14.4 Parasitized eggs of *M. javanica* infected by *Pochonia chlamydosporia* var. *catenulata* (**a**) condiophore on an egg, (**b** and **c**) dictyo-chlamydospore associate with an immature and mature infected egg [212]

saprophytic mode within the soil when both the plant and nematode hosts are absent [242]. We know a little about the factors that cause switching trophic state from saprotrophs to parasites. In contrast with more abundance of *P. chlamydosporia* in organic soils, its antagonistic activity may be no greater than in a mineral soil [12]. The fungus can be formulated and introduced as fungal hyphae and conidia, but dictyochlamydospores are the most popular form of inoculum. Single application of 5000 dictyochlamydospores per gram soil of vegetable crops in tropical soils provided sufficient control of root-knot nematodes but in Europe the results have been less satisfactory [12].

The ecology of *P. chlamydosporia* has been the subject of many studies [29, 198, 221, 222, 224, 225, 227, 229, 243, 244]. Among all species of *Pochonia*, *P. chlamydosporia* var. *chlamydosporia* has been studied extensively as a potential biocontrol agent against nematodes [26, 222]. Its teleomorph, *Metacordyceps chlamydosporia*, has been found on slug eggs in tropical countries [31]. There are also some reports on the ability of other members of this genus like *P. c.* var. *catenulate* [239, 245], two varieties of *M. suchlasporia* [224, 225], *M. rubescens* [48, 246], *P. bulbillosa* and *P. globispora* [212], parasitizing nematode eggs. Managing of cyst nematodes and root-knot nematodes by this fungus in greenhouse and microplot trials has been repeatedly reported [197, 200, 201, 212, 247, 248]. *Pochonia chlamydosporia* is also ovicidal to the large roundworm, *Ascaris lumbricoides* [249] and slug eggs [250, 251].

In peanut fields, *Meloidogyne arenaria* was more frequently parasitized by *P. chlamydosporia* than *Heterodera glycines* [197]. In contrast with many successful trails of the potential application of *Pochonia chlamydosporia* against plant parasitic nematodes [9, 198, 201, 205, 222, 252], its first use of conidial suspensions was failed [253].

Lòpez-Llorca and Duncan [246] illustrated the colonization of *Heterodera avenae* by species of *Pochonia* using SEM. *Pochonia chlamydosporia* can be effectively integrated with the nematicide Aldicarb. The nematicide mostly prevents initial nematode injury, while the fungus part causes a long-term protection. Aldicarb did not influence the activity of the fungus and resulted in a better control of *M. hapla* than treatment with Aldicarb or *P. chlamydosporia* alone [247].

The suppression effect of separate and combined application of *Metapochonia bulbillosa*, *P. chlamydosporia* var. *catenulata*, *P. chlamydosporia* var. *chlamydosporia*, *Lecanicillium aphanocladii* and *Trichoderma harzianum* against *M. javanica* was tested in laboratoary and greenhouse. Combined application of fungi enhanced the egg infection rate significantly. No treatment could suppress the nematode as the same level as cadusafos nematicide (96%), however the integrated use of *P. chlamydosporia* var. *catenulata*, *Lecanicillium aphanocladii* with *P. chlamydosporia* var. *chlamydosporia* (85%) or *Trichoderma harzianum* (83%) controlled the nematode satisfactorily [254]. But, combined application of *P. chlamydosporia* and *P. lilacinum* had no superior effect in colonizing *M. enterolobii* eggs in vitro compared to their solo application [255]. Integrated use of *T. longibrachiatum* with cadusafos could decrease the required concentration of chemical nematicide in controlling *M. javanica* on zucchini plants. The optimal concentration for the best plant

control and least nematode reproduction assigned as 1.7 mg a.i./kg soil of cadusafos and 10^8 conidia/ml suspension of the fungus [256].

Combined application of fungal biocontrol agents and plant defence inducers is a phytonematode management strategy under study [257]. However, it seems that *P. chlamydosporia* (IRAN 1212 C) could stimulate the innate plant defence and change the concentration of zucchini defence enzymes maybe due to its endophytic characteristic [258]. Differential ability was reported for various isolates of *P. chlamydosporia* in inducing plant-dependant systemic resistance. Various host types also showed different response to presence of the fungus. Only two isolates out of five could stimulate systemic resistance in tomato but not in cucumber against *M. incognita* [259].

Using an isolate of *P. chlamydosporia* var. *catenulata* in combination with crop rotation notably reduced nematode populations in soil following a tomato crop [239]. The fungus can also be introduced in combination with an arbuscular mycorrhizal symbiont, *Glomus desertorum*. Their combination added to tomato nursery seedlings resulted in more efficient control. In this experiment 68% of eggs parasitized, while *P. chlamydosporia* alone could parasitize 52% of eggs [260]. Application of *P. chlamydosporia* together with chopped leaves of *Azadirachta indica* (neem) acted synergistically in reducing both gall index and nematode population in tomato in pot experiments [261]. Applying the combination of *P. chlamydosporia*, *Trichoderma harzianum*, and *Glomus mosseae* significantly controlled *Heterodera cajani* on pigeon pea [262]. The consistency of such approaches needs extensive assessment.

Trichoderma harzianum was more effective against *M. javanica* on kidney bean in sandy loam or loam soil but not in clay loam soil. It is suggested that *T. harzianum* may induce plant defence in sandy loam soil [263]. In the sandy loam or loam soil amended with 2% organic matter (leaf litter), *Trichoderma longibrachiatum* exhibited its maximum efficacy and could decrease the nematode reproduction rate the same as the chemical nematicide [264]. Soil type could affect the ability of *P. chlamydosporia* in soil colonization and in controlling *M. incognita* where the fungus exhibited more activity in sandy soil more than clayey soil [265].

The biology of *P. chlamydosporia* and its potential for biological control of cyst and root-knot nematodes has been reviewed [222, 235]. The fungus proliferates in calcareous loams and organic soil in England and could survive at least 3 month after application, however, different isolates varied significantly in their survival ability and also in proliferation in different soils [157, 202, 244]. The fungus prefers peaty sand soil rather than loamy sand or sand in tomato plots infected with *M. incognita*; however in sandy loam microplots a 90% control of *M. hapla* could be achieved, only if the temperature did not exceed 25 °C [247]. Its optimal pH for growth was 6, but some could grow even at pH 3 [155]. Control of *H. schachtii* was directly related to the quantity of young infected females but not to the number of colonized cysts. Effective control will achieve only if few egg produced and many of them were colonized [203]. In tomato soils, fluctuations in population densities of *M. incognita* and *P. chlamydosporia* followed each other, and supplemented soil with *Meloidogyne* species cause a population increscent of the fungus [31]. The

fungus can naturally decline the nematode populations [266] and partial sterilization of the soil with 38% formaldehyde destroyed the nematode-decline effect according to killing fungus population [62].

It was observed that addition of wheat bran to alginate pellets was essential for the successful and consistent establishment of the fungus [244], while de Leij and Kerry [201] had found that adding dictyochlamydospores and hyphal fragments without any extra food base would result in the best establishment. Supplemented the inoculum with an energy producing base could enhance competition from the residual microflora that may cause an adverse effect on survival and multiplication of *Pochonia* [267]. In the other hand, it seems that the fungus (especially hyphae and conidia inocula) essentially need an energy source for its establishment in mineral soil [268]. The comprehension of the tritrophic system is important for a successful application [269].

Approximately 10^3–10^4 CFU of the fungus per gram soil usually suppresses the cyst nematodes, and dictyochlamydospores are regarded as more efficient than alginate-bran pellets [234]. Up to 43% of egg masses of *M. hapla* were parasitized when 5000 dictyochlamydospores were added to each cm^3 of soil, but no effect was observed on lettuce weight, root galling, or egg production [157]. When the population of nematode is high, a successful biocontrol could not be expected [270]. The fungus could produce about 5×10^6 dictyochlamydospores per gram of sand-barely bran medium mixture [62]. On the condition that extra nutrients were added, fungus strongly proliferated in soil [270]. Some isolates of *P. chlamydosporia* reproduce in the rhizosphere of appropriate host plants without any unfavorable effects on the plant [201, 234, 269, 271]. Longer contact time between *P. chlamydosporia* and *M. javanica* in the soil prior to transplanting resulted in more nematode control [272].

For a successful exploitation of *P. chlamydosporia* as an efficient biological control agent, not only a detailed information of the molecular mechanism involved in infection of the nematodes is required, but also of ecological concern is the population dynamics of the fungus in the rhizosphere [26]. Devising a biological control strategy, it is vital to understand the dynamics of *P. chlamydosporia* in relation to the nematode populations; however, interpreting the basic information of such an approach is difficult due to difficulties in quantifying the fungus in the rhizosphere and to the lack of a simple relationship linking fungal abundance to its activity [28]. Quantification of filamentous fungi is not easy since they are not composed of single, simple-to-quantify units of nearly the same size. Like other fungi, *P. chlamydosporia* has several life stages that comprises of multicellular hyphae and dictyochlamydospores mixed together with unicellular conidia. Consequently, understanding a robust association of fungal biomass with nematode numbers, which practically relates to nematode infection and control, is problematic because any component of fungus quantified could result from any of the life stages, even the resting stages which not certainly contribute in nematode colonization at the time of estimation [28].

Several methodologies are in hand for such examinations, including selective plating, immunological and PCR-based techniques [273]. The

dictyochlamydospores could be extracted and enumerated from soil [274]. Media for the selective isolation and quantification of *P. chlamydosporia* were firstly devised by Gaspard et al. [229, 243] that was a chitin-rose bengal agar with 50 mg/L benomyl. de Leij and Kerry [201] and Kerry et al. [244] recommend cornmeal agar (Oxoid) with 37.5 mg carbendazim, 37.5 mg thiabendazole, 75 mg rose bengal, 17.5 mg NaCl, 3 mL Triton X-100, and antibacterial antibiotics; and Moosavi et al. [212] used Shrimp-Agar medium with 3 g shrimp shell powder, 17 g agar, 37.5 mg carbendazim, 37.5 mg thiabendazole, 17.5 mg NaCl, 3 mL Triton X-100, and 200 ppm each streptomycin sulphate and penicillin.

Kerry and Crump [275] described a quantification method for diseased eggs of *Heterodera* species. Egg parasites of cyst nematodes could also be quantified by a standard technique in which the cysts were extracted from soil and then were crushed, afterward their contents were reincorporated into the original soil sample and colonization activity on newly produced eggs is then assessed [276]. Several methods were devised to quantify the ability of the fungus in colonizing the plant rhizosphere in sterile and unsterile soil [271]. Specific primers for the β-tubulin gene of *P. chlamydosporia* are developed to detect the fungus on infected plant roots [273, 277]. It is documented that the most precise explanation of fungal dynamics in the soil could only be achieved by combining culture- and PCR-based techniques together rather than using either method alone [278].

Metapochonia suchlasporia was particularly successful in colonizing eggs and exhibited more chitinase and protease activities [225, 227]. The fungus secretes several extracellular enzymes (especially protease, chitinase and collagenase) that serve as virulence factors involving in pathogenicity [279]. Tikhonov et al. [57] demonstrated that *M. suchlasporia* always secretes higher volume of enzymes compared with *P. chlamydosporia*. Further production of enzymes may be the key factor for this species that must be considered, while scanty dictyochlamydospore production [207] can be a disadvantage for this species which affects its dispersal and survival in the soil. Comparing with *P. chlamydosporia*, this fungus has also a lower minimum and optimum temperature for growth [31] that could limit its commercial application in many countries with warm climate (including the Mediterranean). As optimum growth temperature for this variety is measured (18–21 °C [207]) this fungus can be a good candidate for temperate and cool regions. The most effective isolate of *P. chlamydosporia* (*V. chlamydosporium*) tested by Irving and Kerry [230] was also infectious at 5 °C and should probably be identified as *M. suchlasporia*.

The ex-type strain of *Metapochonia rubescens* was isolated from eggs of *H. avenae* [48, 219, 246]. The fungus showed optimal growth at pH 6, but generates red pigments on acidic media [155] that when was extracted in chloroform/methanol had nematicidal effect on potato cyst nematode, *Globodera rostochiensis* [280]. This fungus parasitized eggs of *Heterodera* and *Globodera* species *in vitro*, and as demonstrated in TEM photographs, developing appressoria and penetration hyphae with an interior infection bulb [281].

Describing *P. globispora* in the genus *Pochonia*, Zare and Gams [282] have anticipated the potential of this species as a possible biocontrol agent of nematodes. *Metapochonia bulbilosa* is usually recovered from forest soils [207], but apart from

its isolation as an ovicidal species from *Ascaris* eggs in Pakistan and Afghanistan [218], its association with nematodes had not been sufficiently known [31]. Moosavi et al. [212] reported effectiveness of the last two species in colonizing *M. javanica* eggs.

Metapochonia goniodes was originally observed on a species of *Bunonema*, but the mode of entry into the nematode could not be established [21]. Recently only two isolates of this species have been available, and these were originally not directly associated with nematodes [31].

Purpureocillium lilacinum is another facultative parasite that has been employed as a biological agent for the control of plant-parasitic nematodes. The fungus potential for human pathogenicity (ocular and cutaneous infections, onychomycosis, sinusitis, and deep infections in immunocompromized patients [283]) seems to preclude its practical application, but some genetic differences were found between human-pathogenic isolates and the nematode parasites [31]. *Purpureocillium lilacinum* and the similar *P. marquandii* were isolated from eggs of *Ascaris lumbricoides* exposed in soils in the Czech Republic, Pakistan, and Cuba [218], and both fungi also penetrated, colonized and killed eggs of *Toxocara canis*, the canine roundworm [284]. Therefore *P. lilacinum* might also be applied as a biological control agent against animal helminths in vivo [31].

Purpureocillium lilacinum is a multitrophic fungus, which can live as a parasite on its host, a saprophyte in soil and an endophyte of plant root [285]. The fungus has a broad geographical distribution and was first observed in association with nematode eggs [286], and like *P. chlamydosporia*, it is principally regarded as an egg parasite (Fig. 14.5). Eggs of *Meloidogyne incognita* and *Globodera pallida* were efficiently colonized in Peru [287]. List of the sensitive nematode species to *P. lilacinum* were named in an extensive review [6]. Early examinations using *P. lilacinum* as a biocontrol agent were encouraging [6]; however, isolates known to be parasitic on nematode eggs and present at high population levels were unable to control

Fig. 14.5 *Purpureocillium lilacinum* conidiophores arising from an infected *M. javanica* egg

root-knot nematodes [199, 288]. Many factors such as ecological components in connection to the establishment ability of the fungus in soil [288] and genetic factors important in determining levels of pathogenicity [3, 289] can be involved in this inconsistency.

Pathogenicity of different isolates of *P. lilacinum* to nematodes varies greatly [9]. Their pathogenicity was somewhat correlated with their UV resistance, that can also similarly be seen in grouping made by random amplified polymorphic DNA (RAPD) [290]. There is an inconsistency between greenhouse assays and those that were conducted in the field [252]. *Purpureocillium lilacinum* have also been used joined with organic resources like oil cake, leaf residues and seeds [25, 291] but as usual, reliable control of nematodes has been difficult to achieve.

An attempt was done to select low cost substrate for spore production of a nematicide strain of *P. lilacinum*. Coffee husks, cassava bagasse, and defatted soybean cake were utilized as substrates, and sugarcane bagasse was used as support. The products obtained by solid-state fermentation were tested for their nematicide activity against *M. incognita* in pot experiments containing *Coleus* as host plant. After 2 month the best results were achieved with defatted soybean cake, which showed almost 100% reduction in the number of nematodes, while the reduction with coffee husk was 80% and with cassava bagasse was about 60% [292].

The use of oxamyl 2 weeks before and during transplanting gave similar results to the commercial product containing *P. lilacinum* but superior to soil solarization [293]. However, integration of other usual controlling methods with application of *P. lilacinum* commonly resulted in better phytonematode control. Concurrent application of *P. lilacinum* and *Pseudomonas fluorescens* suppressed *M. hapla* on carrot more efficiently compared to their solo application [294]. *Meloidogyne incognita* in tomatoes was successfully controlled by integrated application of chemical (floupyram) and biological (*P. lilacinum* as BioAct WG) nematicides [295]. Inducing plant immunity system by defence hormones in integration with biological control enhance nematode suppression. The best tomato growth and lowest *M. javanica* reproduction was achieved when jasmonic acid and *P. lilacinum* were applied at 1.5 mM and 40.51×10^6 conidia ml^{-1} suspension, respectively [296]. Contrary, application of *P. lilacinum* as BioAct WG on resistant tomato-cucumber plants resulted in no significant increase in *Meloidogyne incognita* suppression [297].

The fungus has the ability to produce antibiotics (leucinostatin and lilacin) and chitinolytic enzymes [223]. Production of a serine protease which serves as a crucial component in disintegration of the eggshell is also documented [53, 298]. Decrease in *Rotylenchulus reniformis* population in tomato in India was accompanied with *P. lilacinum* population increment, which causes the level of control comparable to that by carbofuran [299]. In some *Meloidogyne*-suppressive soils in California, it seemed that *P. lilacinum* play an unimportant role in managing the nematode population [243]. Unlike positive correlation between population densities of *P. lilacinum* and *P. chlamydosporia*, no correlation was seen with *Meloidogyne incognit* [229]. Adding 10 or 20 g of fungus-colonized wheat kernels per a 76 cm diam microplot at planting time (even better with an additional treatment 10 days before planting) gave good protection against *M. incognita* and increased tomato

yield significantly [300, 301]. Application of *P. lilacinum* in potato fields of Peru provided a lower galling index due to *M. incognita* than nematicide treatments [287], and the introduced fungus sufficiently established with a single application [302]. Different selective media have been devised for monitoring *P. lilacinum* by Mitchell et al. [373] PDA each liter contained 10 g NaCl, 50mg pentachloronitrobenzene, 50 mg benomyl, 1 mL Tergitol NP10, and antibacterial antibiotics, Cabanillas and Barker ([300]: PDA with dichloran and oxgall together with antibacterial antibiotics), and Gaspard et al. ([243]: chitin-rose bengal agar with 50 mg/L iprodione).

Economically production and formulation of filamentous fungal control agents remains problematic [303]; however, recent progresses in technology have made it possible to produce extremely concentrated formulations that can easily and successfully be used on a field scale [304–307]. The highest number of microsclerotia was produced when *P. lilacinum* was cultured in liquid medium containing 0.2 g/l of ferrous sulphate. The produced microsclerotia were more viable, more resistant to stress condition and more pathogenic to *M. javanica* compared to those of *P. lilacinum* conidia [308]. *Purpureocillium lilacinum* strain 251 was developed as a commercial product in Germany (BioAct® WG) and South Africa (Pl Plus®) for cyst and root-knot nematode management. They are applied as dispersible granules for application in water [12, 307, 309].

Species of *Lecanicillium* are mostly entomogenous or fungicolous [207]. *Lecanicillium psalliotae* (once found in a cyst of *Globodera rostochiensis*) and *"Verticillium" leptobactrum* (mainly in *Heterodera* eggs) were occasionally isolated from nematodes [226]. Recently a few species of the genus *Lecanicillium* have been transferred to *Akanthomyces* [214]. *Verticillium lecanii* (= *Akanthomyces muscarius*) was also rarely observed as parasites of cysts and eggs of *Heterodera* and *Meloidogyne* species [196, 221]. An insect isolate of "*Verticillium lecanii*" was examined for its *in vitro* controling ability of *Globodera pallida*. After 2 months, eight isolates (most of them probably *L. muscarium* and one *L. longisporum*) out of 14 isolates could sufficiently colonize the eggs [310]. When *L. lecanii* was added to monoxenic soybean cyst nematode cultures, it successfully colonized the cyst and female; and reproduced in gelatinous matrix but no egg penetration was observed. Hence its antagonistic activity against soybean cyst nematode was attributed to chemical secretion [311]. *Lecanicillium lecanii* appears to be specific to soft scale insects, Coccidae or Lecanidae [207]. *Lecanicillium muscarium* can efficiently control *Meloidogyne incognita* on tomato [312] and okra [313]. Increase in initial *L. muscarium* conidial concentration upto 10^6 per ml resulted in lower *M. incognita* final population but more tomato foliage growth [314].

14.2.3.4 Mode of Action

The eggshell of nematodes is composed of three distinct layers and mostly consists of protein and chitin, which organized in a microfibrillar and amorphous structure [315]. These layers are an outer vitelline layer, a chitin layer and an inner

lipoprotein layer [316]. Penetration to the eggshell of nematode occurs from an appressorium, a specialized penetration peg or lateral branches of mycelium [27]. Chitinases and proteases play an important role during eggshell penetration, and leads to disintegration of eggshell layers [317, 318]. The fungi which can produce more extracellular enzymes (especially protease, chitinase and collagenase) are considered much more effective in infection of nematode eggs [279], and it is demonstrated that fungi differ in their ability to degrade nematode eggshells, and infection process can be affected by the nematode host [298, 317] Maybe the emanated signals from the egg influence fungal growth and development, and penetration of the eggshell [319].

The genome of *P. chlamydosporia* [320] and *P. lilacinum* [321] has been sequenced. The pathogenicity of *P. lilacinum* is under the influence of many genes, which code different hydrolytic enzymes including proteases, carbohydrate esterases and glycoside hydrolases [321]. The genome of *P. chlamydosporia* encodes for many hydrolytic enzymes, particularly proteases and glycosidases from different protein families. The highest number of glycoside hydrolase enzymes was counted in *P. chlamydosporia* compared to the number of enzymes in other nematophagous fungi such as *M. anisopliae*, *A. oligospora* or *Trichoderma atroviride* [241]. The same occur with chitin modifying enzymes [322]. Contrary, *P. chlamydosporia* genome presents fewer enzymes necessary for degrading plant cell-wall such as cellulases, xylanases and pectinases [241].

The infection of nematodes and their eggs by various nematophagous fungi follows a similar, general pattern [27]. *Pochonia chlamydosporia* is regarded as a parasite of females and eggs of cyst and root-knot nematodes, and develops branched mycelial networks that form appressoria on the eggshell [12, 198, 246, 323].

Contact of the hyphae with the eggshell is the first step in penetrating nematode eggs by *M. rubescens*, followed by developing an appressorium covered with an extracellular material or adhesive. The extracellular material contains a protease (P32) that can be immunologically detected [27]. Deducing from labeling of the adhesive on the appressoria of *P. chlamydosporia* and *M. rubescens* with the lectin Concanavalin A, a glycoprotein nature with mannose/glucose moieties is suggested for that sticky material [324]. The fungus penetrates the nematode eggshell from the appressorium by means of both mechanical and enzymatic components. As the nematode eggshell mainly contains chitin and proteins [316], proteases, chitinases and lipases play an important role during eggshell penetration; however their penetration involvement have not as yet been examined to the same extent and detail [48, 57, 318]. Eleven isolates of *Pochonia chlamydosporia* that were kept in Rothamsted Research Station culture collection were selected and their ability in producing chitinases, esterases, lipases and serine protease (VCP1) were quantified and compared. The isolates were chosen so that they had different hosts, substrata and geographical origins. The results demonstrated that significant differences in enzyme production could be seen between different isolates, time of growth and the amounts of enzymes produced. No significant relationship were observed between trophic phase (parasitic or saprobic) and enzyme activities of the isolates, suggesting that switch in trophic phase is more complex and depended on several factors

[325]. *Pochonia chlamydosporia* can degrade chitosan, an antifungal compound that severely affects plant pathogenic fungi, but not nematophagous and entomopathogenic fungi. It is demonstrated that the most abundant extracellular secreted proteins of *P. chlamydosporia* grown with chitosan as main carbon and nitrogen sources, involve in carbohydrate or protein degradation and egg penetration [326]. An endochitinase gene (pcchi44) [58] and a new serine carboxypeptidase (SCP1) genes [56] were isolated, identified and cloned from *P. chlamydosporia*. About 34 secondary metabolites were identified from *P. chlamydosporia* that mainly belong to alkaloids, phenolics, pyranones and resorcylic acid lactone. The so far known secondary metabolites secreted from other species of *Pochonia* are about 105 substances including β-carotene-type neurosporaxanthin, cyclodepsipeptides, dahiane type diterpenoids, linear lipopeptide, nonaromatic polyketides, pentanorlanostane triterpenoids, phenol-terpenoid hybrids, polycyclic aromatic compounds, polyhydroxylated pyrrolizidine and verticillin-type diketopiperazines [327].

Serine proteases were purified and characterized from *Metapochonia rubescens* [48], and *M. suchlasporia* [281]. Involvement of the enzyme in pathogenicity was suggested by its immunolocalization in appressoria of the fungus [49].

Pochonia chlamydosporia secretes the VCP1 protease that involves in hydrolyzing eggshell proteins of *Meloidogyne* species but not those of *Globodera* [317]. Thickness of the egg shells of *Globodera* are approximately twice as those of *Meloidogyne* which cause more resistance to disintegration [281]. A chymoelastase-like protease is also produced by *P. chlamydosporia*, which has the ability of hydrolysing host nematode proteins *in situ* [52]. Subtilisin-like proteases are the most important classes of extracellular enzymes that different isolates could have up to four isoforms of them. The enzymes decompose the proteins of their nematode hosts and are very important in fungal pathogenicity [298]. The similarity of the enzyme with that secreted by *Metarhizium anisopliae* is demonstrated [51].

It is also demonstrated that a serine protease and chitinases that are effective in degrading the eggshell, and a nematotoxin, phomalactone, which secreted by *P. chlamydosporia* enhance the pathogenicity [12, 317]. Further studies using Enterobacterial Repetitive Intergenic Consensus PCR (ERIC-PCR) revealed that different isolates of *P. chlamydosporia* produce a range of different proteases, and that the difference in the enzymes perhaps relates to the different ecological niches occupied by each fungus [298, 328]. Transformation of *Lecanicillium attenuatum* by introducing the Pr1A-like cuticle-degrading protease (*Cdep1*) gene (originating from *Beauveria bassiana*) increased protease activity of *L. attenuatum*. Egg hatch inhibition of *Heterodera glycines* and its J2 mortality was increased by 17–76% 2–14 days and 43–152% 1–13 days after incubation in cell-free fungal culture filtrates, respectively [329].

ERIC-PCR generated data using in phylogenetic analysis illustrated that the different isolates of the fungus were related to its host from which the isolate had been obtained [330]. Comparison of the similarity of amino acid sequences between proteases from different nematophagous fungi showed a high level of conservation, with only minor insertions and deletions [331]. Minor variation in amino acid sequence may influence substrate utilization and host preference [51] that has been

documented in VCP1 proteases from different isolates of *P. chlamydosporia*. Substitution of an alanine by a glycine in the S3 substrate-binding region of VCP1 confers enzymatic activity against eggshells of *Meloidogyne* [330].

Purpureocillium lilacinum is a well-studied antagonist of some nematodes like *Radopholus similis* and *Tylenchulus semipenetrans*, but most research has carried out on the infection of *Meloidogyne* spp. and *Globodera rostochiensis* eggs [12]. The suppression effect of the fungus was recently reported on *Pratylenchus thornei* [332]. This fungus has been extensively evaluated for reducing nematode damage to a range of crops and its application usually caused a significant nematode control.

Purpureocillium lilacinum secrete a serine protease and several chitinases that involve in drastic degradation of the eggshell structure [333, 334]. A very similar mode of egg penetration is seen in fungi that are distantly related. After successful host recognition, they attach to host surface and penetrate into it enzymatically and/or physically (via appressorium). It has been demonstrated that many fungi, even those lacking the ability to produce appressoria, possessed appressorium-forming genes [335]. Phylogenetic analysis of a chitinase gene from *P. lilacinum* with those from mycoparasitic, entomopathogenic and nematophagous fungi illustrated such similarity that it has been hypothesized that probably the gene was acquired by gene transfer from bacteria [336].

All zoospore producing species develop resting spores that survive in soil when their host is absent. These fungi colonize the female nematode and prevent cyst formation. Life cycle of *N. gynophila* is completed within 5 days at 13 °C in the cereal cyst nematode [337]. The zoospores need flooded soil for motility, and therefore nematode infection is limited to periods following rainfall. It is difficult or impossible to culture the obligate parasites *in vitro*, therefore their commercial prospect is ambiguous [338].

14.2.4 Toxin-Producing Fungi

14.2.4.1 Introduction

The toxin-producing fungi secrete a toxin that immobilizes the nematodes before penetration of hyphae through the nematode cuticle [27]. The toxin producers found in nematophagous Ascomycota [339], nematophagous Basidiomycota or non-nematophagous fungi [340].

Toxin-producing fungi secreted a number of compounds *in vitro* that may have nematicidal or nematostatic traits. The *in vivo* role of such compounds is usually not well known but *Pleurotus ostreatus* produces droplets of a potent toxin that quickly immobilizes nematodes [341] and has the structure of trans-2-decenedioic acid [342].

The known nematicidal compounds produce by nematophagous Ascomycota are members of “oligosporon, 4′,5′-dihydrooligosporon, talathermophilins A and B, phomalactone, aurovertins D and F, paeciloxazine, a pyridine carboxylic acid

derivative, and leucinostatins" [339] while "Thermolides A and B, omphalotins, ophiobolins, bursaphelocides A and B, illinitone A, pseudohalonectrins A and B, dichomitin B, and caryopsomycins A–C" are produced by nematophagous Basidiomycota or non-nematophagous fungi [340].

Finding that basidiomycetous *Pleurotus* and *Coprinus* have some species (like *P. ostreatus* and *C. comatus*) that produce toxin [341, 343], it is suggested that the nematophagous habit may be more widespread among Basidiomycota than previously thought [27]. It has been recently demonstrated that *Pleurotus eryngii* produces toxins, which are effective against *Panagrellus* sp. larvae. The fungus was able to decrease the number of intact eggs of *M. javanica* by producing chitinases and proteases [344]. Species-specific interactions has been reported between *Pleurotus* species and bacterial-feeding nematodes as some species/isolates of tested nematodes survived exposure to different *Pleurotus* species [345].

Antibiotic (nematicidal and antifungal) activities have been demonstrated for *Drechmeria coniospora*, *Harposporium anguillulae* [33], *Lecanicillium*, *Purpureocillium lilacinum* [6], and *Pochonia* [298]. *Purpureocillium lilacinum* can secretes acetic acid that paralyzes juvenile nematodes [346] and a toxin with nematocidal properties [347]. Some bioactive compounds have been isolated from *in vitro* cultures of *P. chlamydospora* [2] and *Metapochonia suchlasporia* [155], but their role in soil is poorly understood [2]. Because of the rapidity with which the nematode embryos were killed, it is suggested that *Catenaria anguillulae* secreted toxins [2]. Several oligosporin antibiotics have been described from *A. oligospora* [348].

Finding new compounds with nematicidal activity and analyzing their synthesizing pathways is a new field to be exploited. It is feasible to use the genetically modified organism in biocontrol after those pathways are fully understood at the molecular level [349].

14.3 Endophytic Fungi

Some fungi grow within plant tissue but do not cause lesions or other disease symptoms and are referred to as endophytes. These organisms can be mutualistic if they defend the plants against herbivores or pathogens and parasites. *Acremonium* spp. may secrete general toxins that influence on grazing mammals and herbivorous insects, and induce plant root modification which decrease nematode feeding and reproduction [350]. Endophytic fungi are recently considered as an important component of integrated pest management [351], however, there is evidence that at least one of them (*Epicoccum nigrum* a leaf endophyte of *Populus trichocarpa*) is pathogen facilitator [352].

Endophytic fungi consist of a rather heterogeneous group which can be divided into the balanciaceous endophytes (= grass endophytes) and the nonbalanciaceous endophytes according to their phylogeny and ecology. The balanciaceous endophyte members are ascomycetous fungi from genera *Epichloë* and

Balansia whose anamorphs are *Neotyphodium* and *Ephelis*, respectively [353]. The nonbalanciaceous endophytes are mainly the nonpathogenic ascomycetous fungi (*Alternaria*, *Colletotrichum*, *Fusarium*, *Trichoderma*, *Guignardia*, *Leptosphaerulina*, *Nigrospora*, *Phoma*, *Phomopsis*, *Xylaria* and *Acremonium*) [353, 354] and less frequently from basidiomycetous fungi [355]. A number of endophytic fungi are nonpathogenic isolates of ordinary plant pathogens like *Fusarium oxysporum* that during *in vitro* tests secreted metabolites, which were toxic to *Radopholus similis, Meloidogyne incognita* and *Pratylenchus zeae* [356, 357]. Even though this fungus can decrease the numbers of nematodes developing in roots, its mode of action is not clear.

The mechanisms involved in controlling phytonematodes by endophytic fungi is not well-understood but is probably multifactorial [358]. This can be due to toxin production; competition for space and resources in the roots; alteration of the physiological state of root tissue; colonization of feeding cells to the detriment of nematodes [2, 359]; direct attack, kill, paralyse or repel phytonematodes; produce plant hormones; interfere with finding plant roots; inducing plant resistance; or enhance plant tolerance [358]. Other possible mechanisms are promoting plant yield, distributing of soil nutrient, and increasing plant tolerance to abiotic stress and drought [360]. *Neotyphodium* spp. in the leaves of grasses may also rely on a toxic secretion mechanism to lessen nematode infestations in roots [2]. There was little evidence for systemic growth of endophytic fungi within leaves but none was found in support of systemic growth within plant host [361].

Arbuscular mycorrhizal (AM) fungi are the well-known plant root associated endophytes which have mutualistic relationship with more than 80% of all plant species [362]. These fungi are obligate symbiotic parasites of plants that have been widely reported to enhance the growth of nematode-infected plants and, in some cases, to decrease nematode infestations [363–365].

Plant growth enhancement is happened by improving plant access to nutrients, particularly phosphorus, and especially under conditions of poor nutrient availability. These fungi also assist access to and uptake of water and alleviate heavy metal toxicity [2].

AM fungi could suppress pathogens with both biotrophic and necrotrophic lifestyle by direct or indirect effects [366]. Direct involving mechanisms are mainly competition for food and space while indirect mechanisms include plant-mediated effects [362]. Roots were shared as a resource for food and space between plant parasitic nematodes and AM fungi. According to proximity in tissue, more reciprocal effects were expected between AM fungi and endoparasitic nematodes. Plant-mediated effects of AM fungi on phytonematodes comprise enhancing plant tolerance, stimulating plant defence and changing in root diffusates resulted in modified rhizosphere interactions [362]. However, the different mechanisms are interrelated and biological control achieved by involving more than one mechanisms [367]. Migratory endoparasitic nematodes were the only group whose numbers were greater on AMF-infected plants [368]. For example, inoculating wheat by AM fungi increased its susceptibility to *Pratylenchus neglectus* by repressing the

production of root defence metabolites related to plant resistance. However, presence of AM fungi still supply the host plant with nutritional benefits [369].

Role of AM fungi in suppressing nematode damage to plant and in decreasing nematode densities in the soil has been illustrated in many cases, although most of them include *Meloidogyne* species. Nematode multiplication rate can be reduced if plant roots are colonized by AM fungi before nematode invasion. The greatest decreases in nematode infestations usually occurring in roots extensively occupied by the fungus before the nematodes invade [2]. Production and secretion of root diffusates may be interfered or some nematotoxic compounds can be produced by AM fungi, however their exact mode(s) of action is not well understood [12]. The efficacy of endophytes depends on the plant colonized and the species of troublesome nematode [2].

Endophytic fungi, especially AM fungi are produced commercially as crop-growth enhancers. They have the advantage that they can be applied as seed treatments, and then they will multiply rapidly and colonize the rhizosphere and plant roots. This can result in protecting the plants from nematode invasion [370]. The level of nematode management can be satisfactory, although the effect of different isolates of the same species can differ distinctly in suppressing nematode damage [12]. Active isolates of *F. oxysporum* against *R. similis* are being developed and undergoing field trial on banana plantations in Central America and East Africa [12]. However, it is speculated that induced resistance has an important role in the interaction, but the modes of action are poorly understood [12].

Some nematophagous fungi have the ability to colonize plant roots as a probable survival strategy [27]. It is demonstrated that *P chlamydosporia* and *M. rubescens* endophytically colonize barley roots [56]. The plant defense reactions were probably induced by nematophagous fungi, but these never prevented root colonization. The nematode-trapping and toxin-producing fungi cause necrotic areas on roots at their initial stages of colonization, but were never later observed, even when the fungi proliferated in epidermal and cortical cells. It seemed that monocotyledon plants extensively colonized by nematophagous fungi resulted in producing abundant mycelia, conidia and chlamydospores [27]. The egg parasite fungi, like *Pochonia* spp., that grow as endophytic fungi may have higher chance to parasitize eggs of economically important endoparasitic nematodes (like cyst and root-knot species) inside the roots and to decrease succeeding spread and roots infection by the second generation of juveniles. Some structures similar to trapping devices were seen in epidermal cells colonized by *A. oligospora*, which can use to entrap newly hatched juveniles escaping the roots. The ability to colonize plant roots by nematophagous fungi is a novel area of research that deserves in-depth investigations [27].

The endophytic root colonization potential of different groups of nematophagous species was investigated recently. The egg-parasite *P. chlamydosporia* and the toxin-producing *Pleurotus djamor* had the ability to endophytic colonization of barley roots. The nematode-trapping species *A. oligospora, D. dactyloides*, and *N. robustus* were all also capable of similar root colonization. Only the endoparasitic fungi *H. rhossiliensis* and *Nematoctonus pachysporus* were not capable of endophytic root colonization [88, 371, 372]. The fungi penetrated into plant cell

walls of epidermis and cortex cells by means of appressoria, and developed inter- and intracellularly. It was the first time that appressoria formation was seen in *A. oligospora* [88, 371].

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Chapter 15
Control of Pepper Powdery Mildew Using Antagonistic Microorganisms: An Integral Proposal

César Guigón López, Héctor Adrián García Ramírez, and Laila Nayzzel Muñoz Castellanos

15.1 Introduction

15.1.1 Pepper Crop

Pepper belongs to the genus *Capsicum* which are native to South, Middle and part of North America, they have been cultivated for thousands of years; pepper is an important crop in the world and have been used as spices, fruit and medicine. There are about 30 species of plant in the genus *Capsicum*, however only five species are cultivated on large scale, *Capsicum annuum* is the most cultivated [1].

Around 36,092,631 tons are produced around the world, being China and Mexico the main producing countries [2]. In México the pepper crop has a social, economic and productive importance since at present it satisfies 100% of the national requirements and export 29.79% of its production to countries like United States and Canada [3]. However, phytopathogen microorganisms are responsible for causing devastating diseases in the field that represents the loss of plants or harvest, therefore causes significant economic losses. The most important microorganisms that affect the pepper crop worldwide are *Phytophthora capsici*, *Rhizoctonia solani*, *Fusarium solani* and *Leveillula taurica* (pepper powdery mildew), all these causal agents cause millions of annual losses every year [4–6].

Particularly, the powdery mildew of pepper is becoming an increasing problem in pepper production areas, because may lead to a decrease in production yield. In the

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field, it is very difficult to predict and control epidemics due to the intercellular growth of the fungus [7]. The chemical control is difficult and expensive, the genetic control is not enough, so new alternatives are sought in the area of biological and genetic control.

15.1.2 Pepper Powdery Mildew

Pepper powdery mildew is caused by the fungus *Leveillula taurica* (Lév.) G. Arnaud (anamorph: *Oidiopsis taurica* (Lév.) Salmon) is a serious disease affecting the leaves of pepper, however it can affect other economic importance crops such as tomato, eggplant, onion, cotton, cucumber, etc. Is one of the most damaging diseases in the field and greenhouses [6]. *L. taurica* belong to the Ascomycetes, to the Erysiphaceae family whose life cycle involves a sexual (teleomorphic) and asexual (anamorphic) state with a complex life cycle. The chasmothecia hibernate in the soil or in infected leaves, later the asci protrude from the chasmothecial wall to expel the ascospores, these germinate in a new leaf where they form an appressory to penetrate the epidermal cells walls where the haustoria are formed, later the hyphae develops in the surface of the tissue of the host where the conidiophore will be formed and conidia on the surface of the infected leaves [8] (Fig. 15.1a, b).

Its growth and reproduction dependent on living hosts therefore cannot be cultured *in vitro*, these characteristics represent a challenge to be used as experimental systems [9]. The development of the disease is related to favorable conditions for the fungus growth; the germination of the conidia occurs in a range of 10–37 °C and can withstand temperatures of 40 °C, the optimum temperature of colonization of the leaf is 15–25 °C and the highest germination ranges are given in a humidity relative from 75% to 85% [10]. Under these favorable conditions, the fungus reproduces rapidly and the spores can germinate and infect the plant tissue in less than 48 h, secondary infections occur due to the release of conidia by wind affecting mainly the leaves, however fruits may be affected. The primary symptoms of the disease is the presence of yellowish or brownish discoloration, the edges of the infected leaves wind upwards, in the lower part of the surface the white fungal colonies are observed (Fig. 15.1c). Affected leaves are detached prematurely causing defoliation, reduction of the photosynthetic capacity and exposing the fruit to sunburn [11] (Fig. 15.1d).

For the prevention and control of the powdery mildew, it is important to have an early detection and intensive management plan in the greenhouse, since once the infected pepper leaves are detected it is very difficult to control, besides, it is probable that many more leaves are already infected, however they do not present symptoms [12]. Early infections have a loss of 30% of the total production, compared with late infections [6, 13]. However, the disease can occur at any time throughout the season if environmental conditions are favorable, severe infections early in the season can result in heavy yield losses, it is more severe on old leaves just prior to the fruit set [11].

Fig. 15.1 Powdery mildew caused by *Leveillula taurica* in wild plant *Proboscidea parviflora* (**a**) and in the abaxial surface of "jalapeño" pepper leaves (**b**). Yellow lesions by powdery mildew in "jalapeño" pepper plants (**c**) and severe plants defoliation caused by pepper powdery mildew in "jalapeño" pepper (**d**). All photographs of the northern Mexico

15.2 Current Methods for Powdery Mildew Control

In recent years, the control of the powdery mildew is based on the use of chemicals with systemic and non-systemic fungicidal activity, because powdery mildew is an aggressive and destructive disease and satisfactory control without the use of fungicides is unlikely [14]. The fungicides of the strobilurins family like Kresoxim-metil, azoxystrobin are the most successful in the last decade and they have been used to powdery mildew control. Other fungicides from different families such as; propiconazole, mancozeb, tebuconazole, cyproconazole and sulfur salts, are also used for the control of the disease, obtaining significant effects in the field [15–17].

Due to health risks, environmental contamination and the resistance of the fungus to agrochemicals, scientists around the world are searching for new alternatives for the control of powdery mildew, of which the use of different genera of bacteria such as *Bacillus* and *Pseudomonas* and different genera of fungi such as *Trichoderma*, *Ampelomyces*, *Metarhizium*, *Sporothrix*, among other microorganisms have been tested as biocontrol agents for powdery mildew [18–20].

The formation of varieties resistant to powdery mildew provides another alternative for the control of the disease, recent studies provide information about new sources of resistance to pepper powdery mildew that can be used in breeding programs to be incorporated into commercial species, however cultivars resistant are not available for all crops [21, 22].

In addition, new biotechnological tools such as CRISPR-Cas9 have recently been used to introduce targeted to the alleles that encode for the mildew resistance locus proteins, the results provide methodical information for the genetic improvement of crops [23].

15.2.1 Problems Associated to Chemical Control

As previously mentioned, until now chemical control has been used for the control of powdery mildew due to its relative effectiveness, however, the constant use of fungicides involves exposure of the chemical to the environment, particularly if it persists in the soil or reaches the aquifers where it can cause an impact on terrestrial and aquatic ecosystems [24]. On the other hand, the use of pesticides have been associate to health damage including pathological changes at the molecular, cellular, tissue, organ and system levels, particularly in the immune, nervous, reproductive and endocrine systems [25]. The damage caused to health depends of different factors such as the period and level of exposure, the chemical to which it is exposed, the damages caused may be short-term, for example: eye and skin irritation, headaches, dizziness and nausea, or may cause chronic as: cancer, asthma, diabetes, among others [25, 26].

Another problem associated to the use of fungicides is the risk of inefficiency in medium and long term, because the fungus has a high potential to develop mechanisms of resistance. Systemic fungicides are generally more susceptible to the development of resistance, because they have a mechanism and a single site of action, which means that only one metabolic pathway of the pathogen is affected, for example, the strobilurin bind to the protein subunit of the cytochrome bc1 complex of the electron transport chain inner mitochondrial membrane, which inhibits cellular respiration of the fungus [27]. However, strains resistant to azoxytrobin and kresoximmethyl have been reported, which was attributed to a single point mutation in the cytochrome b gene [28, 29].

Due to all these problems associated with the use of pesticides, particularly fungicides, there is strong pressure from some organizations for the prohibition of them, therefore it is likely that in the future agricultural fungicides will be banned in the States United. Due to this, other alternatives to the use of chemicals have been investigated, with the objective of reducing the use of fungicides and the damages associated with their use, an example of this is the use of microorganisms such as bacteria and fungi as biocontrol agents that are able of inhibiting the growth of the pathogen or promoting the plant resistance.

15.2.2 Biocontrol Alternative

Commercial products based on microorganisms tend to reduce the use of synthetic pesticides, due to the interest on increasing the use of biological control since they do not induce the development of resistance by pathogens, in addition to acceptance by the society of the dangers associated with the use of synthetic pesticides and the contribution to food safety, chemical products have reduced their production by 2%, while biopesticide production has increased by 20% [30].

Additionally, by the problems of chemical control, biological control offers a more environmentally friendly alternative with health, since it reduces the chemical content in the environment and in the fruits, for that reason the study and the use of biological control agents for pepper powdery mildew has increased in recent years, so that some antagonistic fungi such as *Ampelomyces quisqualis* and *Pseudozyma flocculosa*, have resulted in the development of commercial products [31]. Also recent studies aim to test different species of microorganisms as biocontrol agents, as well as their mechanisms of action; results show that the use of biocontrol agents such as *Trichoderma harzianum* and *Bacillus subtilis* have a significant effect in the reduction of the disease [19]. Another work shows that fungi *T. asperellum* and *Metarhizium anisopliae* are usually effective in the control of the powdery mildew in only some types of pepper [20]. In some cases combinations of biological control agents with chemical fungicides have been carried out, such is the case of the combination of *Pseudomonas fluorescens* and azoxystrobin, results show that the dose of the fungicide was reduced by 50% [32]. The combined use of biocontrol agents such as *T. harzianum*, *T. viride*, *Bacillus subtilis*, *P. flourescens* and *Saccharomyces cerevisiae* and chemical resistance inducers like calcium chloride, potassium monohydrogen phosphate, potassium bicarbonate, saccharin, ascorbic acid, chitosan humic and folic acid have been suggested as easy, safe and cost-effective control methods [33]. The results of the studies with biological control agents around the world demonstrate a significant effect in the control of pepper powdery mildew, which provides an effective and safe alternative for the management of the disease, however there are still mechanisms of action to be investigated, as well as future microorganisms that demonstrate having an effective activity for the control of the disease.

15.3 Fungi as Biocontrol Agent of Powdery Mildews

There are several fungi (about 40 species) found between the biological control agents of the powdery mildew. Among these, *A. quisqualis* and *Trichoderma* spp. are may be, the best studied and the more used commercially. However, there are other fungi mycoparasitic or antibiotic producer that can be good alternative. Some of these fungi are described below.

15.3.1 Ampelomyces quisqualis

Ampelomyces was one of the first mycoparasites to be studied in detail and was also the first fungus used as a biocontrol agent of plant pathogen fungi. This mycoparasite has now become one of the most advanced in terms of commercial development of a fungal biocontrol agent for plant pathology. Even though from ecological perspective, it seems there are a number of biotic and abiotic factors that do not seems favorable for the activity of *Ampelomyces* against powdery mildew fungi in the field, the widespread, natural occurrence of these fungi in pepper crops (unpublished data) suggest that in some region (as northern Mexico) it is possible. Therefore, we cannot leave aside the alternative of using this mycoparasite in a biocontrol program of the pepper powdery mildew.

The fungal genus *Ampelomyces* is considered to be major intracellular mycoparasite of Erysiphales species worldwide. Commonly, *A. quisqualis* found attacking *Erysiphe, Sphaerotheca, Podosphaera, Uncinula* and *Leveillula* [34, 35]. In the field, the hyphae and pycnidia of *Ampelomyces* commonly develop inside the hyphae, conidiophores, conidia and ascomata of the Erysiphales [36]. Pycnidia vary in shape depending upon the fungal structure in which they are formed. Inside pycnidia are the conidia, cylindrical to spindle-shaped, and occasionally curved and two-spotted [37].

Species recognition in the genus is problematic because a cultural and morphological similarity with other fungal species. Moreover, researcher think it is not reliable to use morphological characteristics for species delimitation in the genus, so from 17 species in *Ampelomyces*, just *A. quisqualis* is widely accepted. This specie, the most important biocontrol species of the genus, presents an important variation between strains and has been recognized in them the existence of physiological forms [37]. Thus, because several factors have effects on the morphological and cultural characteristics, some researchers think that a screening program based on *in vitro* growth rates is not the right way to select *A. quisqualis* strains for use in biocontrol programs. Other authors suggest that selection of good candidates can be supported by their evaluation of the high sporulation rate in culture and the high mycoparasitic activity [38].

Currently, most species of the genera have been described based mainly on their mycohosts and plant hosts. The existence of some degree of mycohost specialization in the strains of the mycoparasite can have important implications and some precautions are needed [37, 39, 40]. This, for example, can explain the satisfactory or unsatisfactory efficacy, reported for *A. quisqualis* in different works [41, 42]. However, Kiss [31] found that mycoparasitic activities of the eight strains isolated from six different powdery mildew species, did not depend on their mycohost species of origin. In similar way, Angeli and co-workers [39] tested the mycoparasitism of 24 strains of *A. quisqualis* against powdery mildew in strawberry, grapevine and cucumber plants under controlled conditions. All strains were effective against the powdery mildews. However, there were important differences the grapevine and the

cucumber powdery mildews, they were generally more susceptible than strawberries powdery mildew.

In addition, accurate identification requires of analyses of the internal transcribed spacer (ITS) region of the nuclear ribosomal RNA gene (nrDNA) and actin gene (act1) [43]. Even more, molecular analyses of ITS region and act1 have revealed considerable genetic diversity and suggested that ITS groups could be related to the host fungus [43–45]. Angeli et al. [39] perform a phylogenetic analysis of 28 different *A. quisqualis* strains isolated from different species of Erysiphaceae collected in different countries and possessing different ITS rDNA sequences. They classified these strains into different genetic groups, which generally correlate with the fungal host of origin and morphological and growth characteristics. A very interesting molecular analysis, based on the ITS rDNA and actin gene sequences, conclude that a high genetic diversity and some degree of mycohost specialization are present within *A. quisqualis* complex composed of at least four distinct clades [43]. Also, that the specie might have coevolved through the processes of specialization and adaptation.

It seems that mycoparasitism is the principal mechanism by *A. quisqualis* control powdery mildew, as the presence of toxins has not been detected, acts without producing antifungal compounds and destroys the invaded powdery mildew colonies only slowly, in 5–10 days [46]. This mycoparasitic activity is characterizing by invasion and destruction of host cytoplasm, that is, a rapid degeneration of cell contents [34]. *A. quisqualis* grow slowly, *in vitro* its radial growth rate on Czapek Dox agar plus 2% malt extract is 0.5–1.0 mm d-1 at 23 °C. Growth and sporulation was enhanced by high relative humidity and temperature of 25 °C. Under high humidity conditions, conidia germinate in 10–20 h and the emerging hyphae can penetrate the hyphae and conidia of powdery mildew. Mycoparasite continue growing internally and produce their intracellular pycnidia in 5–8 days. These pycnidia, conidia and resting of hyphae initiate the life cycle of the mycoparasite in the spring [46]. Powdery mildews colonies and conidia also stimulate the germination rate and tube elongation of *A. quisqualis*. In powdery mildew colonies the mycoparasite conidia and picnidia are formed in microcyclic conidiofores.

Although this activity is slow, it is important because it suppresses the sporulation rate of its fungal hosts, and the infected plants regain vigour after the mycoparasite has killed the pathogens. Thus, the benefic effect goes further, it is know that the parasited powdery mildew mycelium produce less damage on the infected plant. Moreover, it was observed that cucumber plants regained vigour, chlorophyll content and also CO_2-fixation in the infected cucumber leaves as so much as in uninfected controls [34].

Extracellular lytic enzymes play a role in the degradation of the powdery mildew hyphal walls during penetration. Mycoparasitic activity of the different *A. quisqualis* strains positively correlated with the *in vitro* activity of cell wall degrading enzymes β-1,3-glucanase, protease and chitobiase [39]. The levels of enzymatic activity differed between genetically different strains. It is to draw attention that, all the strains tested displayed chitobiase activity, but no endochitinase activity was detected. Angeli et al. [39] also showed a significant positive correlation between

chitobiase and protease activity with intra-hyphal formation of pycnidia and the inhibition of conidiation in vivo. The inhibition of germination of *L. taurica* conidia by effect of chitinolytic and glucanolytic enzymes has been shown in other studies [20].

However, because *A. quisqualis* destroys mildew colonies slowly, this may be a strong limitation of its use against *L. taurica* in peppers. In a sensible pepper hibrid, cultivated in arid environment, powdery mildew epidemics usually reach high growth rate [20]. This high growth rate may be an impediment for biocontrol agent efficiency as was shown in grapevine when the mycoparasite do not control grapevine mildew in this conditions [47].

A. quisqualis has been used only in a few cases in pepper powdery mildew. Different of other powdery mildew that grow only on the leaf surface, part of lifecycle of *L. taurica* in peppers takes place inside the leaf, forming haustoria through which they feed on the plant. *A. quisqualis* significantly reduced the accumulation of hyphal biomass inside the leaf tissue at temperatures 20–25 °C [42]. Also, biocontrol agent reduces germination and cumulative germ tube length on leaves of sweet pepper. This is important as *L. taurica* conidia germinate and their germ tubes grow on the leaf surface until they encounter a stomata through which they can enter the plant. This effect on conidial germination on leaves suggest a new mode of action of this mycoparasite. Under the temperatures 15–25 °C *A. quisqualis* did not affect disease when applied alone, but preparation with mineral oil AddQ significantly suppressed the disease in sweet pepper at 25 °C. It seems that it reduces the colonisation of hyphae in the leaves. In greenhouse, the mycoparasite suppressed the disease on leaves at three plant heights and reduced leaf coverage over the whole plant. In commercial greenhouse and severe disease conditions, *A. quisqualis* + AddQ treatments were not reduced disease levels. In field experiments to evaluate the leaf coverage by *L. taurica* and thallus viability in plants of two cultivars in two minimal night temperature environments, the biocontrol agent reduced both two variables at 120 days after planting and at 13 and 20 °C. Temperatures >18 °C enhance the activity under field. Kumar et al. [12], found *A. quisqualis* (20 g l^{-1}, four sprays at 15-days intervales) reduced powdery mildew severity in two different cultivars and accordingly, treated bell pepper plants increased their yields (ton/ha). Moreover, use of mycoparasite combined with neem oil was more effective than chemical fungicides alone. *A. quisqualis* can combined with other biocontrol agents for best performance. In strawberries, combined with *T. harzianum* and *B. subtilis*, were able to reduce powdery mildew (*P. aphanis*) incidence in the greenhouse and in tunnel-protected strawberries [41].

15.3.2 Trichoderma *spp.*

The genus *Trichoderma* comprises the imperfect phase of *Hypocrea* (Ascomycota, Order Hypocreales). The genus has the following characteristics: rapid growth in culture medium; dispersed, floccose, or tufted compacts; size and shape of the

various conidia; and coloring of conidia varying from green to yellow, or hyaline chlamydospores, sometimes present; well-defined conidiophores and conidia formed at the phyalid ends of differentiated hyphae, tending towards mass aggregation [48]. Because its great metabolic capacity and its aggressively competitive nature, species of *Trichoderma* are fungi with a wide and diverse ecological niche. There are present in a several different soil or foliar habitats and can be a dominant species in the microbiota present [49, 50].

In the field of biocontrol, *Trichoderma* species are knowing as a mycoparasites [51, 52], antibiotic producers [53, 54] and inducer of systemic resistance in plants [55, 56]. These fungi posse different ecological advantages over others fungi, so its use in agriculture are bigger than other fungal species. Particularly, its use as biocontrol agent is widely knowing against some of the more important plant pathogens in the soil or in the foliage parts of the plants, including those causing powdery mildews of pepper.

Of the overall mechanisms of action of *Trichoderma* spp. for plant diseases biocontrol, we can be highlighted some of them, that may be the most important in a biocontrol of the pepper powdery mildew. Here is important to take into account that two mechanisms are considered the most important ways to control powdery mildews by fungal antagonists, mycoparasitism and antibiosis.

Mycoparasites can attack pathogen just when infection already stablished [31], so a certain level of disease has to be tolerated. Also, it is important to mention that when sporulation rate of the pathogen is high (as in *L. taurica*), the mycoparasites cannot stop their spread, but can follow the spread of the disease and reduce its severity and damage to the crop. Antibiosis, meanwhile, can kill powdery mildew colonies rapidly by plasmolysis of their cells. Hydrolytic enzymes (chitinases and β-glucanases) and other antifungal compounds were consigned as causing collapse of the cells of powdery mildews [31].

15.3.3 Mycoparasitism

Is the ability to parasitize other fungi, and is a complex process involving four distinct stages: First, chemotropic growth, in which a chemical stimulus attracts the antagonistic fungus. Then, specific recognition, mediated by lectins on the cell surface of both the pathogen and antagonist. Immediately, attack and coiling of *Trichoderma* around the host hyphae. Finally, secretion of lytic enzymes that degrade the host cell wall [57]. During the process of mycoparasitism, *Trichoderma* secretes cell wall-degrading enzymes that will degrade the cell wall of the host fungus. This will then release oligomers, activating the expression of genes involved in mycoparasitism [52]. Evidence for this recognition comes from studies on transcriptomics, which show the induction of enzyme genes before actual contact with the pathogens [58, 59]. Lysing of the cell wall of phytopathogens is mainly done by glucanases, chitinases, and proteases [60, 61]. Other enzymes that degrade smaller polymers, such as β-1,6 glucanases, β −1,3glucanases, and mannosidases, may be

involved in the complete and effective degradation of the cell wall of plant pathogens by *Trichoderma* spp. [60, 62].

The β-1,3glucanases, catalyze the hydrolysis of the β-1,3glucan chain, a polymer composed of D-glucose residues bound in a β-1,3 configuration. They are cleaved into the following compounds: Exo-β-1,3-glucanases, which sequentially hydrolyze β-1,3 glycosidic bonds at the non-reducing end of the glucan molecule, releasing glucose as the end product. Endo-β-1,3- glucanases that randomly cleave -β-1,3 bonds along the polysaccharide chain by releasing small oligosaccharides, with glucose as the final product [51]. Synergistic action occurs between these two enzymes, with different modes of action to degrade β-glucans [58, 63].

Chitinase also are important to mycoparasitism. The chitinolytic system of *T. harzianum* and *T. atroviride* presenting a complex system of more than six chitinolytic enzymes [64], that include endo and exochitinases. Endochitinases cleave the chitin molecule internally in chitotetraose, chitotriose and diacetylchitobiose. Exochitinases are subdivided in chitobiosidases and N-acetyl-D-glucosaminidases. Chitobiosidases catalyze the progressive liberation of diacetylchitobiose and N-acetyl-D-glucosaminidases hydrolyze diacetylchitobiose in monomers of N-acetylglucosamine [65].

Proteases can also participate in the mycoparasitism by *Trichoderma*, destabilizing the cellular integrity of the phytopathogen [66] and inactivating the enzymes produced by pathogens during the plant infection process [67]. The genes of some serine endopeptidases (p8048, ss10) [67, 68] and aspartic proteases (papA, p6281) [69, 70] seem to be involved in the control of some plant pathogens. Nonetheless, studies on protease characterization, isolation, and/or cloning are lower than those related to chitinases and β-1,3-glucanases.

15.3.4 Antibiosis

This mechanism involved the production of secondary metabolites with anti-fungal activity. The term “secondary metabolites” refers to a group of different natural chemical compounds possibly related to survival functions, such as competition against microorganisms, symbiosis, metal transport, differentiation and antibiosis [71]. The *Trichoderma* spp. produce these antifungal compounds that diffuse in substrate, and prevent other species from encroaching. Antibiosis against powdery mildews, causing complete plasmolysis of their cells in 24–48 h [72].

If, as mentioned above, the competitive ability mediated by production of antifungal compounds, is one of the most important interaction between fungal species, it can explain the successful use of *Trichoderma* to control pepper powdery mildew [20, 73]. The control of *L. taurica* by *Trichoderma* has been based on the use of cultivated filtrates. Culture filtrates of *Trichoderma* spp. contain a mix of different enzymes related to hydrolysis of fungal cell wall and secondary metabolites with antifungal activity [20, 74]. The composition of the filtrate depends on the incubation time [74] and can include over 200 types of antibiotics [75]. The application of

this mixture of enzymes and metabolites, resembles the activity of *Trichoderma* when it colonizes the plant tissue to prevent the arrival of another competitor microorganism. Among *T. viride, T. harzianum, T. hamatum, T. longiflorum* and *T. koningii*, filtrates of *T. viride* were the most effective with a control rate upper of 90% [76]. The combination of *T. viride* and *T. harzianum* filtrates reducing conidial germination of *L. taurica* in the greenhouse and the field and controlled infections [73]. Use of filtrates of *T. asperellum* reduced the *L. taurica* conidia germination, inhibition did not occur until 30 h post-incubation. The greatest inhibition occurred with extracts of 10 days of incubation, which coincided with a highest enzymatic activity [20]. This information about effect of filtrates on conidia germination is very important if it is consider that conidia are essential in over summering and in secondary spread of powdery mildew. So, an antagonist capable of suppressing or denaturing of conidia will be required for areas where high dissemination rate occurs [77]. In addition, Woo et al. [75] indicate that *Trichoderma* culture filtrates contain macromolecules and low molecular weight compounds that induce strong changes in cytosolic Ca^{2+} level in isolated plant cells and may cause programmed cell death. This cell death can limit the movement of biotrophic pepper powdery mildew.

In addition, strains of the genus *Trichoderma* are well-known producers of volatile organic compounds (VOCs) some of which are widely reported to have antibiotic and immunosuppressive activities as well as less desirable phyto- and mycotoxic activities [78]. Some secondary metabolitos of *Trichoderma* exert an antimicrobial effect at high doses but act as microbe-associated molecular patterns (MAMPs) and as auxin-like compounds at low concentrations [79]. The VOCs isolated from the several *Trichoderma* species shown a wide range of biological activities. It has been mentioned approximately 479 of VOCs that have been reported and those occurring as simple hydrocarbons, heterocycles, aldehydes, ketones, alcohols, phenols, thioalcohols, thioesters and their derivatives, including, among others, benzene derivatives, and cyclohexanes [78]. In *Thichoderma*, VOCs have been studied as a mechanism of action for the inhibition of phytopathogenic microorganisms [80]. Some results demonstrate the ability of *T. harzianum* to produce VOCs capable of inhibiting the growth of *F. oxysporum* up to 40% [81]. On the other hand, a greater inhibition of the growth of *Sclerotinia sclerotiorum* and *Sclerotium cepivorum* is reported, due to the production of VOCs by *T. longibrachiatum* [82]. The production of these compounds is related to the antagonistic effect of different etiological agents that cause different diseases (*Fusarium oxysporum, F. solani, Rhizoctonia solani, S. rolfsii, S. sclerotiorum, Colletotrichum capsici, Helminthosporium oryzae, Alternaria brassicicola, A. alternata, A. brassicae, A. solani*) [83, 84]. In addition to the direct effect on pathogens, VOCs have been shown to have a positive effect on plants. Kottb et al. [85] reported changes such as increased trichome numbers, accumulation of defense-related compounds such as H_2O_2, anthocyanin, camalexin, and increased expression of defense-related genes. *Arabidopsis thaliana* in interaction without contact with *T. asperellum*, also demonstrated a dual function of 6-pentyl-α- pyrone improved its resistance to the phytopathogenic fungi *Botrytis* and *Alternaria* and reduction of spore germination of *Alternaria* [85].

The production of VOCs could be a control mechanism of pepper powdery mildew, however there are no experimental works that demonstrate the activity of VOCs on the fungus *L. taurica*, therefore this mechanism cannot be ruled out for future research, since VOCs play an important role in the antagonistic effect of *Trichoderma* on different phytopathogenic fungi.

15.3.5 Competition for Space or Nutrients

Some fungal species may compete by simply occupying all of the tissue available at a particular site on the plant. When plant tissue surface has a limited number of infection sites, abundance of a fungal biocontrol species provide the protection of the tissue [86]. The competition for resources is also prevalent among fungi. This come about when one specie depletes available nutrients to the detriment of other species, or when one specie blocks another species from access to nutrients. For the biological control of pepper powdery mildew, this competition implies that biocontrol agents are the first colonizing species and possess characteristics such as wide dispersal and rapid spore germination, mycelial growth and metabolism [86]. In this sense, it has been widely documented that the success of the strains of *Trichoderma* as biocontrol agents is due to several characteristics that have made it an ubiquitous genus present in any habitat and at high population densities [49]. Of which properties, for pepper powdery mildew biocontrol, we can highlight their high reproductive capacity, ability to survive under very unfavorable conditions, efficiency in the utilization of nutrients and capacity to modify the environment. Moreover, *Trichoderma*'s metabolism gives it advantages of the great capacity to mobilize and take up environment nutrients, due to ability of to obtain ATP from the metabolism of different sugars, such as those derived from polymers wide-spread in fungal environments: cellulose, glucan and chitin among others, all of them rendering glucose [87]. Components of the glucose metabolism, include assimilation enzymes and permeases, together with proteins involved in membrane and cell wall modifications. The glucose transport system efficiency may be crucial in competition. The high affinity glucose transporter, Gtt1, of *T. harzianum* CECT 2413 is only expressed at very low glucose concentrations. Gtt1 helps the CECT 2413 to compete in very poor nutrient environments [87].

15.3.6 Induction of Resistance in Plants

Microorganisms are also able to stimulate host plant resistance to disease through the production and secretion of elicitor molecules. Systemic acquired resistance (SAR) and induced systemic resistance (ISR) in plants to various diseases could be achieved by different infectious agents and in various plant parts. The research in plant immunity induced by beneficial microorganisms suggests that *Trichoderma* spp. are potent inducers of ISR in plants [88]. When plants immune system detects

motifs or domains with conserved structural traits typical of entire classes of microbe trigger pathogen- or microbe-associated molecular patterns (PAMPs or MAMPs, respectively) [56]. MAMP-triggered plant responses are elicited rapidly and transiently. Effective *Trichoderma* strains produce a variety of MAMPs, of which can be highlight xilanase, cellulase, cerato-platanins, swollenins, endopolygalacturonase, alamethicin, trichokonin, 6-pentyl-α-pyrone, harzianolide and harzianopyridone [56]. Increase of total phenol content is one of the first stage of defense mechanism. The *Trichoderma* isolate PB 22 showed most potential for the sheath blight of rice (*Rhizoctonia solani*) suppression, which was related with high total phenol content [89]. In *Arabidopsis thaliana,* the activated defense signaling pathway elicited by *T. virens* and *T. atroviride* depends on the amount of conidia inoculated, involves salicylic acid and/or jasmonic acid and was characterized by accumulation of hydrogen peroxide and camalexin in leaves, while *T. virens* produced also indole-3-carboxaldehyde which affect plant development [90]. Finally, these effects in plants reduced de severity of symptoms by *B. cinerea*.

In pepper, some *Trichoderma* isolates were endophytic on roots in which delayed disease development by *P. capsici* and induced strong and divergent defense reactions [91]. The induction of pepper gene expression depended on the *Trichoderma* isolate, as was the case of multiple lipid transferase protein (LTP)-like protein family, which works in *Trichoderma*-induced resistance to infection by *Phytophthora* spp.

15.3.7 Inducing System-Wide Changes in Plants

Recent studies suggest a novel mode of action for the biocontrol agents, as the selective compensation of the impact of a pathogen on the plant. *Trichoderma* species are widely recognized for the wide range of possibilities they offer to improve plant growth conditions. There is a lot of scientific information that documents the characteristics of *Trichoderma* as a biocontrol agent, biofertilizer, soil bioremediator, etc. Recently, new reports have emerged that document other magnificent opportunities offered by *Trichoderma* to mitigate crop damage derived from the action of phytopathogenic agents. In relation to the pepper powdery mildew attack, for its biocontrol we can highlight some innovative research that offers additional opportunities to those already described. The mechanisms described below may mark the direction of new research for the study of the *Trichoderma*-pepper-*L. taurica* interaction to confirm its potential and advantages to improve the biological control of pepper powdery mildew.

One of the most devastating symptoms and signs related to powdery mildew is the loss of photosynthetic capacity caused by the presence of fungi in leaves, foliage yellowing and defoliation [92]. For this reason, recent information about the ability of some *Trichoderma* species to induce important system-wide changes in plants is interesting [93]. Inducing system-wide changes in plants gene expression requires that microbes located in the plant produce chemical signals which are transmitted to the rest of the plant, thereby altering the crop's phenotype [93, 94]. Moreover, applying only the symbiont-associated molecular patterns (SAMPs) induces the

changes in plant phenotype. Harman et al. [93] describe how the roots of the cultivated plants when being colonized by *Trichoderma*, develop the up-regulation of genes and pigments that improve the photosynthesis of the plants. Also, describe that certain *Trichoderma* strains activate biochemical pathways that reduce ROS to less harmful molecules.

Nitric oxide (NO) is a gaseous bioactive molecule that has been established as a major signal in animals and recently, its importance in plants emerged from pioneering works on plant responses to pathogens [95]. NO is important for regulation of numerous plant processes, such as growth and development, stomata function, adaptation to low or elevated temperatures, salt and water stress, and also in the induction of defense responses, [96, 97]. The ubiquitous involvement of NO in plant physiology regulation makes it one of the pivotal messengers of signaling networks. During pathogen infection and in addition to the production of reactive oxygen species, plants also generate NO (and NO-derived molecules such as S-nitrosothiols (SNOs) and peroxynitrite) as a defense mechanism [98]. This process involves a cascade of signals affecting a broad spectrum of molecules, which some authors have called a "process of plant immunity". The suppression of NO would reduce its levels inside the plant, thus limiting the defense responses and allowing the root colonization by symbiotic microorganisms [99].

Related to plant pathogen infection, NO is rapidly produced during the incompatible interactions with biotrophic pathogens as the pepper powdery mildew. Its production is generally associated with plant cell death and hypersensitive response [95]. The analysis of the above information allows us to establish hypotheses that the NO involved control of pepper powdery mildew with *Trichoderma* and propose future experiments that demonstrate the hypotheses proposed. If, as previously mentioned, *Trichoderma* culture filtrates can cause programmed cell death, and NO production is generally associated with this plant cell death, spraying pepper plants with the culture filtrates, programmed cell death limits the biotrophic movement of *L. taurica* in the leaves and the quickly NO production induce defense genes, such as pathogenesis-related 1 (PR1) gene and phenylalanine ammonia lyase [95]. On the other hand, it is currently known that *Trichoderma asperelloides* manipulates the production of NO from the host [97]. NO is a virulence factor of *Fusarium oxysporum*. Evidence from several experiments suggests that *T. asperelloides* can suppress NO production and block the elicitation of NO by *F. oxysporum,* by impacting the expression of some genes that are reported to respond to NO. It will be interesting to establish if similar mechanisms can be carried out when *T. asperellum*, or another species, is applied to the foliage of pepper plants for the control of *L. taurica*.

15.3.8 Other Powdery Mildew Antagonistic Fungi

In addition to *A. quisqualis* and *Trichoderma* spp., other biocontrol agents have shown biocontrol efficacies against different powdery mildew. Guigon et al. [20] successfully employed *Metarhizium anisopliae* strain Ma70 against *L. taurica*, even

though we did not have previous information on its use for powdery mildews biocontrol. In the field, the results when using this antagonistic fungus are seriously affected by the genotype of pepper. The greatest reduction in the incidence and severity of the powdery mildew were lower when a landrace genotype (arbol pepper) was used. The precise mechanisms by which *M. anisopliae* prevent the establishment of powdery mildew remain unclear, but antibiosis seems to be most likely.

In greenhouse tomato, *Acremonium alternatum* reduced infection by *L. taurica* [100]. The effect was not due to direct parasitism, the effect was systemic and was presumably due to induction of host resistance by substances released for conidia when were killed, and the effect depended upon leaf age and level of infection.

Cephalosporium sp. and *Paecilomyces farinosus* also have been reported as potential biocontrol agent of *L. taurica* in peppers [31].

Other producing pycnidia fungi, in addition to *A. quisqualis*, are also hiperparasite of powdery mildews. Hence the importance of accurate identification of the antagonist in studies to detect and select candidates to be biocontrol agents. *Phoma* species are frequently confused with *Ampelomyces*. Separating species is through studies of their morphology and life cycle and ribosomal DNA internal transcribed spacer (ITS1) sequence analysis [101]. These authors showed that *P. glomerata* can colonize and suppress development of powdery mildew and may have utility as a mycoparasitic agent.

Lasiodiplodia theobromae is another fungus reported as a mycoparasite of a powdery mildews in different plants [102]. Applications of *Penicillium oxalicum* suppressed powdery mildew on different genotype of strawberries, under controlled and field conditions [103]. Different species of *Lecanicillium* have been reported as mycoparasite of *Sphaeroteca fuliginea* [104]. Seventy-two hours were enough for hyphae of *S. fuliginea* had collapsed and were encircled by *L. muscarium* and at this time primary sporulation was observed [105].

15.4 Bacteria as Biocontrol Agent

Research on bacteria as biocontrol agents has been studied for years, although new sources of bacteria with some biotechnological potential are still being investigated, the genera of *Bacillus* and *Pseudomonas* are of the most used and researched, so that numerous commercial products based on these microorganisms have been used as a biopesticide for different types of diseases.

Bacillus is the most exploited genus in the agricultural area with 85% of bacterial products due to its ability to produce endospores which makes its formulation and application more efficient in the field, also offers metabolic diversity and different mechanisms to carry out biological control of different pests and diseases [106]. *B. thuringiensis* is the most commercialized biocontrol agent in the world, however there are other species such as *B. subtilis*, *B. pumilus*, *B. licheniformis* and *B. amyloliquefaciens*, which have been implemented in commercial formulations for the control of various diseases of fungal origin.

Biological control based on bacteria represents a challenge for future research, because very few genera have been studied as biocontrol agents for powdery mildew, however, there are several studies aimed at the use of biocontrol agents of some genera of bacteria for different powdery mildews (pepper, cucumber, tomato).

15.4.1 Bacillus *spp.*

Bacillus spp. are aerobic bacteria, sometimes facultative anaerobic, they are gram-positive bacillus with flagellar movement. Undoubtedly the genus *Bacillus*, is one of the most used biocontrol agents due to wide use for the control of pests and diseases, *B. thuringiensis* was the first to show potential as a biocontrol agent due to the discovery of Cry proteins. From this fact different species of *Bacillus* have been widely studied to reduce the incidence of agricultural diseases, in addition species such as *B. thuringiensis* have been used successfully as an effective product for pest control [107]. The genus *Bacillus* comprises a long genetic biodiversity and is present in a wide variety of environmental conditions; from seawater to soils of different types and conditions. One advantage of the genus *Bacillus*, it is its ability to produce spores in adverse environmental conditions, these give it the ability to resist high temperatures, extreme pH, lack of nutrients or water [108]. This features facilitates industrial management and its field application.

As in many other diseases, some strains of *B. subtilis* have been studied for the control of powdery mildew, obtaining a control of the disease similar to fungicide azoxystrobin and with the products based on *Ampelomyces* and *Lecanicillium*. In addition, *B. subtilis* did not significantly improve by adding other control methods such as fungicides, mineral oil or mycoparasites, the biological treatment resulted in a sporulation decrease of about 90%, this effect is attributed to the ability of the bacterium to survive on the leaf surface, probably as stable cell aggregates, forming colonies that colonized the surface of the leaf following epidermal cell junctions, in which a sort of fibrillar material connected bacterial cells to each other and to the leaf surface, furthermore to antagonistic interactions between the bacterium and the causative agent [109].

Other studies show that treatments with *B. subtilis* at a concentration of 0.4 gr/L, have a good control of the powdery mildew of zucchini statistically similar to that obtained by azoxystrobin or penconazole, however the combined use of fungicides and *B. subtilis* gives better results than individual use of these, the results suggest a synergistic effect between the fungicide and the biocontrol agent *B. subtilis* [110].

Some filtered cultures from different microorganisms have been used to control powdery mildew, including *B. pumilus* filtrates. These treatments were more effective than the fungicide, and the combination of penconazole and the filtrate further raised the effectiveness of the treatment under field conditions. Through analysis of the filtered culture, eleven volatile compounds belonging to the family of the aldehydes, esters, alcohols and fatty acids were detected. Results suggest that the mixture of the chemical compounds present in the culture filtrate acts synergistically as a fungicide. Therefore, the use of this mixture and fungicides may decrease the amount of fungicide applied [111, 112].

It is known that *B. subtilis* is a producer of molecules with antifungal and antibiotic activity, so that the use of filtered *B. subtilis* cultures has been studied for the control of powdery mildew under greenhouse conditions. The results suggest the production of fatty acids and/or their derivatives with antifungal activity such as n-hexadecanoic acid (palmitinic acid), 2- heptanal, 2-octenal and octadecanoic acid (oleicacid) [113]. On the other hand, a strain of *B.amyloliquefaciens* isolated from greenhouse soil was tested as a greenhouse biocontrol agent, the results showed that both the bacteria and the substances obtained by fermentation in broth have an efficiency of up to 95% and play a role important in reducing the onset of the powdery mildew, an effect attributed to the induction of systemic resistance, as well as the secretion of substances that inhibit the germination of fungal spores by the plant [114].

There are some mechanisms by which *Bacillus* protect plants from diseases caused by pathogenic microorganisms, these mechanisms can be divided into:

(a) Direct mechanisms. The production of compounds with direct effect on the pathogenic microorganisms. These metabolites can be antibiotics such as cyclic lipopeptides, lytic enzymes such as chitanase, glucanases and proteinases, unregulated waste products, ammonia, carbon dioxide, hydrogen cyanide [115–118].
(b) Indirect mechanisms. Competition for space and/or nutrients, leachate/exudates, siderophores production, induction of systemic resistance to the plant through the production of phytohormones and molecular patterns [119–121].

The strain *B. amyloliquefaciens subsp. plantarum*, which has also been used in commercial products as a biocontrol agent in agriculture, produce different secondary metabolites related to a total of 11 gene clusters, this represents that more than 9% of the genome is related to the synthesis of antimicrobial metabolites [122, 123]. Studies show that the antifungal activity is due to the activity of lipopeptides synthesized by non-ribosomal peptide synthetases (NRPSs) or hybrid polyketide synthases and non-ribosomal peptide synthetases (PKSs/NRPSs) [124]. Lipopeptides are giant molecules composed of a cyclic peptide linked to a β-hydroxy or β-amino fatty acid chain [125, 126]. Recent studies demonstrate the relationship between antifungal activity and the production of cyclic lipopeptides, so that the production of iturin and phenicin enhance in the presence of some phytopathogens [127]. Lipopeptides with fungicidal activity such as iturins, fengicins and surfactins act in the fungal cytoplasmic membrane, induce pore formation, osmotic imbalance and death of the microorganism [128]. Additionally, multiple mechanisms that alter intracellular calcium homeostasis, energy metabolism and RNA processing have been reported [116]. It has been shown that lipopeptides such as surfactins are involved in the formation of a stable biofilm when the interaction between *B. subtilis* and *Arabidopsis* roots occurs, the surfactin expression capacity reduces the biofilm formation ability, colonization range and the biocontrol capacity of the disease caused by *Pseudomonas syringae* [129]. Biofilm production could be involved in the biocontrol mechanism for powdery mildew, however scientific studies are needed to support it.

Although lipopeptides have been the most studied metabolites, there are other compounds such as lytic enzymes, which are involved in the degradation of the cell wall of phytopathogenic agents, mainly in fungal agents. It has been shown that chitinases and β-glucanases are responsible for the degradation of the main polysaccharides that make up the fungal cell wall [130]. Studies show that *B. subtilis* has the ability to produce β-1,3-glucanase capable of inhibiting the growth of phytopathogenic fungi such as *Pyricularia grisea* and *Rhizoctonia solani* [131]. Likewise, the production of new proteins with antifungal activity by different strains of *Bacillus*, are being investigated as possible biocontrol mechanisms [132].

Some studies show that volatile organic compounds (VOCs) produced by *B. amyloliquefaciens* inhibit the growth and germination of *Fusarium oxysporum* spores, these compounds play a very important role in the survival of the pathogen short and long distance [133]. In addition, VOCs significantly inhibited motility traits, the production of antioxidant enzymes and exopolysaccharides, biofilm formation and colonization of tomato root by *Ralstonia solanacearum* [134].Therefore VOCs are also crucial in the effectiveness of biological control with *Bacillus*. Additionally, VOCs are related to the promotion of plant growth and induction of ISR in different greenhouse and field conditions in tomato, pepper, melon, watermelon, tobacco, *Arabidopsis* sp., cucumber, etc. [135], this due to the increase in photosynthesis and the endogenous content of gibberellins, auxins, cytokines and decrease in ethylene levels [136].

The induction of systemic resistance to diseases using microorganisms, particularly bacteria such as *Bacillus*, have been extensively studied due to the production of compounds that function as chemical signals (elicitors) such as lipopeptides, phytohormones and VOCs that induce the activation of PR genes (pathogenesis-related) which code for PR proteins with antimicrobial activity [137] or induce the production of secondary defense metabolites such as flavonoids, phytoalexins, auxins or glucosinolates [138]. Likewise, the PR2 gene encodes a β-1,3 glucanase and PR3 encodes a chitinase, it should be noted that this induction was performed by VOCs of *Bacillus* sp. [139], In addition, structural changes in the cell wall have been reported through the accumulation of lignin [140]. In other studies, plants treated with *B. amyloliquefaciens*, has resulted in a significant increase in the production of superoxide dismutase, peroxidase, polyphenol oxidase and phenylalanine ammonia lyase, in addition the increase in salicylic acid and the expression of the PR-1 gene was demonstrated, which resulted in the induction of ISR to powdery mildew [114].

15.4.2 Pseudomonas *spp.*

Pseudomonas spp. It is a gram-negative, aerobic bacterium that is found in a wide variety of agricultural soils, due to its adaptability to grow in the rhizosphere, this bacterium is usually interesting since it has many different characteristics that make it suitable for use as an agent of biocontrol and plant growth promoter. Although

Pseudomonas lacks the ability to produce spores which complicates the formulation of commercial products, however there are other advantages such as: the ability to grow rapidly in medium culture for obtaining large-scale inoculum, rapid use of root exudates, adaptability to different environmental stress conditions, among others [141]. The success of biocontrol depends on multiple factors, including the ability of microorganisms to survive in the rhizosphere, compete with the microbiota of the environment and at the same time, protect the plant from infection, so *Pseudomonas* is interesting for its study, due to its ability to colonize the rhizosphere in high density, compete with other microorganisms, as well as the production of secondary metabolites with activity against fungi and bacteria [142].

Although *Pseudomonas* is known as a biocontrol agent for pathogens associated with root diseases, nevertheless some strains of this genus have been used for the control of leaf diseases; particularly for the powdery mildew from which a moderate reduction of the disease has been obtained; result a percentage of incidence between 20% and 26.6% due to the application of *P. fluorescens* in greenhouse conditions, also the application of this bacterium combined with azoxytrobin improves its effectiveness, in addition to reducing the amount of fungicide applications [33, 142]. The advantage of using a bacterium as a control agent is the combination with a fungicide such as azoxystrobin, since due to the mechanism of action of the chemical it does not alter the growth of bacteria, in fact it is able to tolerate concentrations of 300 ppm, of such that in the field a 96.03% reduction of the disease has been obtained using a dose of 250 mL/ha of azoxystrobin in combination with a strain of *P. fluorescens*, instead using azoxystrobin and the strain separately, 88.04 are obtained and 70.48% reduction of the disease [143]. Additionally it can be mentioned that this same combination produced greater yield in such a way that it was increased by 243% with respect to the control. Although it cannot be said that *P. fluorescens* is the best control agent for powdery mildew, compared to other microorganisms, better results are obtained in the field, even in some studies better effects have been reported than some *Trichoderma* strains. This due to its potential for disease control and its ability to protect seeds and roots from fungal infections, thanks to its ability to combine with fungicides and its multiple mechanisms of action, which is not limited to an antagonistic effect on the pathogen [144]. However, in other studies *Trichoderma* has a better effect than *P. fluorescens*, this is because different environmental conditions favor one microorganism more than the other, or the microbiota found in the environment is different and therefore the results vary from one medium to another [73]. In addition, some species of *Pseudomonas* are known to be producers of secondary metabolites, of which phenazine-1-carboxylic acid (PCA) was tested for the control of different diseases of chili pepper including powdery mildew. Field results show a reduction in the incidence of pathogens, the effect is comparable with chemical fungicide treatments such as chlorothalonil, hexaconazole and mancozeb. These results are directed to the use of PCA as an antifungal compound, which could decrease or replace the use of chemical products [145].

Pseudomonas, it has high competitiveness for space, nutrients and protection for biotic and abiotic factors [146], so that the effectiveness of pathogen control depends

on different factors such as mobility, chemostasis response, among others [147]. Another factor is the ability to produce siderophores under iron-limiting conditions, it is known that the production of siderophores not only plays an important role in plant growth promoting, but also in the biocontrol of pathogens, since they deprive other iron microorganisms therefore the survival of the pathogens is reduced [148]. The genus *Pseudomonas* is characterized by the production of compounds such as; 2,4-diacetylphloroglucinol (DAPG), phenazine-1-carboxylic acid (PCA), pyrrolnitrin (Prn), hydrogen cyanide (HCN) and pyoluteorin (Plt) [141, 149–151], in addition that some strains of *P. fluorescens* are capable of synthesizing a wide diversity of compounds with antimicrobial activity, they can also produce other growth promoting compounds such as indoleacetic acid, salicylic acid and pyochelin [150].

Due to these characteristics, some strains of *Pseudomonas* have been study models for the identification of genes involved in the production of secondary metabolites, so that the gacS/gacA system has been identified, which is an environmentally sensitive regulator necessary for production from DAPG [152]. Through new molecular tools have been identified genes involved in the synthesis of these compounds; phenazines are pigments produced by some *Pseudomonas*, more than 100 different phenazines have been described, of which PCA, phenazine-1-carboxamide (PCN), 2/hydroxyphenazine and 2-hydroxyphenazine-1- carboxil acid have been associated with biocontrol [153]. The phenazines biosynthesis genes are clusters highly conserved, a locus of seven genes phz A-G, are responsible for the synthesis of the first derivative of the phenazine called PCA. Phenazine diversity results from the enzymatic modification of PCA, these enzymes are encoded by the operon genes PhzO, PhzH, PhzS, and PhzM. Gene activation for phenazine production is regulated by quorum sensing, in the case of *P. chlororaphis* and *P. fluorescens*, the phenazine operon is coupled to the regulatory genes phzI and phzR [154, 155]. Pyrrolnitrin (Prn) is a secondary metabolite produced by numerous species of *Pseudomonas* which has been investigated for its activity against a wide range of Ascomycetes and Basidiomycetes, however it has been shown to be ineffective for use in agricultural fields because to its instability under sunlight [156]. Subsequently, improvements have been made to the structure, first a substitution was made in the chlorine in the pyrrole ring to increase stability, in addition the activity was increased when finding the derivatives 4-(2,3-dichlorophenyl)pyrrole-3carbonitrile and 4-(2,2-difluoro-1,3-dioxol-4yl)pyrrole-3-carbonitrile known as fenpiclonil and fludioxonil respectively, both compounds were developed as agricultural fungicides [157].

Like *Bacillus*, *Pseudomonas* has the ability to induce ISR in plants. In experiments related to the induction of resistance to powdery mildew of the vine caused by *Uncinula necator*, the application of *P. fluorescens* resulted in an increase in gene products peroxidase, phenol oxidase and chitinase [158]. Similarly, the application of *P. fluorescens* induces an increase in chitinase and β-1,3-glucanase levels in chili plants, in addition to the accumulation of phenolic substances and PR proteins, in chili plants infected with *L. taurica*, this response suggests a systemic resistance induced by treatment with *P. fluorescens* [142].

15.4.3 Other Bacteria

Although *Bacillus* and *Pseudomonas* are the most studied bacteria genera as biocontrol agents for diseases, there are other bacteria that have been investigated for their possible application, for example, some strains of *Streptomyces* have been studied, results show that the strains provide significant protection against powdery mildew, the effect was improved by performing treatments in a shorter period to inoculation of the pathogen, which suggests a short durability of the bacteria in the environment [159].

Likewise, endophytic actinomycete fermentations have been used, treatments inhibited spore germination, mycelium expansion and an increase in the activity of defense enzymes on the leaf was observed [160]. Some strains of *Serratia marcescens* reduce disease levels up to 59% in greenhouse conditions, applications were repeated after 4 days when the colonies of powdery mildew started to appear, field results show that the combination of biocontrol agents with K_2SiO_2 reduces the disease by 10–70% however, lower temperatures and high humidity were necessary for optimal performance [161].

On the other hand, there are reports where some strains of *Streptomyces* induce a ISR through the jasmonic acid/ethylene-dependent pathway, in addition some related transcripts related to tryptophan biosynthesis, phenylalanine and phenylpropanoid were enhanced and the activity of phenylalanine ammonia lyase increment, the results suggest similar effects with *Pseudomonas* [162].

In spite of the little information generated in other species of bacteria and its use as a biocontrol agent against powdery mildew, there is a wide field of study in the search for novel microorganisms capable of inhibiting the pathogens growth, inducing systemic response in the plant or some other mechanism of action.

As can be seen, microorganisms offer a sustainable alternative to protect crops of agronomic importance from phytopathogenic microorganisms, as well as from chemicals that in large quantities have an adverse effect on human health, the environment and also promotes the resistance of pathogens to fungicides. Bacteria are an option for use as biocontrol agents, because they are easy to handle in the laboratory, as well as their large-scale reproduction, they also offer a metabolic diversity that helps the biological control of diseases in plants, for which reason studies of different mechanisms of action and genetic expression are necessary to increase the knowledge and effectiveness of formulations used in the field.

15.5 Biocontrol and the Phyllosphere Microbiome

The importance of the microbiome of the phyllosphere in biocontrol and the promotion of plant growth has been suggested for years. This microbiome, includes bacterial, viruses, archea or fungi communities, which have important effects on host biology as they are highly active and influence their metabolism and physiology.

Beneficial roles include nitrogen fixation, protection against pathogens and the production of plant hormones [163]. Therefore, there is a possibility that the leaf microbiota can be used to reduce the use of agrochemicals to control leaf pathogens [20].

However, as mentioned before, little is known about the interactions between parasitized fungi, their mycoparasites and the variety of organisms, present in plant foliage [164, 165]. Moreover, biocontrol in the phyllosphere has been visualized as a strategy that involves the biocontrol agent, the pathogen and the plant as a trifactorial relationship, we need to change this perception. In a broader ecological approach, the relationship is really multi-trophic relationships, in which there are a number of biotic factors that do not seem favourable for the activity of antagonists against powdery mildew fungi in the field [34].

The phyllosphere is biologically rich and diverse, and is an environment highly dynamic and strongly affected by the different factors of the environment (e.g. rain, temperature, radiation, water availability, relative humidity, dew) [166]. Thus, for its establishment, the introduced biocontrol agents have to tolerate a very hostile environment and have to compete with the resident phyllosphere microbiota for nutrients (scarce nutrients often) and space [167, 168]. This nutrient availability and resident microbial communities, vary considerably in dependence of multiple factors such as plant age and environmental conditions as fluctuating temperatures, relative humidity and free water, atmospheric gases, light and radiation, wind and pollution [167]. In addition, the phylloplane is affected by cultural practices, particularly by crop protection treatments. Microbial populations in the phylloplane are influenced by leaf exudates, leaf surface chemicals in leachates and exogenous substances [169]. Because all these factors change all the time, the establishment of the biocontrol agent introduced is inconsistent as the effectiveness against the target pathogens is inconsistent.

The presence of other microorganisms in the leaf can influence the presence of powdery mildew, however, also the presence of powdery mildew can affect the microbial communities [170]. This is possibly through dedicated or multifunctional secreted effector proteins.

The presence of powdery mildew has been related to the microbial species richness and diversity in the leaves, when these variables are high the colonization of powdery mildew is weak, while these indexes decrease host plants are highly colonized [171]. In the pumpkin (*Cucurbita moschata*)-powdery mildew system, it was confirmed that the richness and diversity of the foliar fungal microbiome correlates with the severity of the powdery mildew infection. However, caution is needed in that regard, as increasing the richness of strains is not always beneficial. For example, increasing the richness of strains within the *P. fluorescens* biocontrol species can cause community collapse and the consequent loss of plant protection [172]. In this particular case, antagonistic interactions increased disproportionally with species richness, mutual poisoning between competitors lead to a 'negative complementarity effect' and a complete loss of plant protection.

The transient modification of the plant microbiota after biocontrol agent application were identified. In pepper, the effects of biocontrol agent on the microbiota in the phyllosphere have been different from those of the roots. The spraying of

B. thuringiensis has no effect on bacterial and fungal biomass in the phyllosphere, but the structure of the microbial community was significantly affected [171]. In contrast, application of *P. corrugata* CCR80 and *Chryseobacterium indologenes* ISE14 to roots of pepper modified the bacterial and fungal community composition, but this effect was temporal for fungal community and only for 80 days [173]. On the other hand, applications of *Trichoderma* to the phyllosphere can affect the microbiota. The spraying of *T. harzianum* T22 in strawberry foliage modified the composition and diversity of fungal populations.

Other novel approach are the studies with the intention of designing the leaf microbiome or the phyllosophere with the intention to biocontrol some pathogens in this habitat [163]. These studies highlight the complex and fluid interactions between plant, pathogen, biocontrol agent, microbe community, and the environment. More research is needed to define involved mechanisms and whether these can be useful in the biocontrol of powdery mildew. In this sense, it is known some mechanisms appear to be the excretion of phytohormones or enhancing the activity of microorganisms in the phyllosphere [174]. Another novel approaches are the high-throughput sequencing or next-generation sequencing, that give a much better and more detailed picture of microbiome evolution [175]. Their impact on the development of next-generation bio-products is indisputable hence they are considered an important engine of innovation towards sustainable agriculture. They provide a holistic view of microbial community composition and functions and can identify changes in the microbiome that went unnoticed using the more conventional techniques [176]. A good review of biocontrol in the microbiome era is present by [175].

15.6 Biocontrol Inside the Integrated Diseases Management

The information described for biological control agents, brought out the possibility of use these microorganisms along with fungicides and others control alternatives (as botanicals), in integrated diseases management schedules for sustaining pepper production while ensuring food safety.

The use of biocontrol agents in sanitation treatments can become a part of an integrated disease management strategy of pepper powdery mildew [38], however this is a less exploited strategy to control it. *Ampelomyces* destroy completely powdery mildew chasmothecia [38], this effect against chasmothecia perform better compared to their effects on the conidial stages. So, late-season application of *A. quisqualis* can reduce the overwintering inoculum that initiates the disease epidemic in the following year [177, 178]. However, it should be taken into consideration that chasmothecia were only parasitized by *Ampelomyces* when these were still immature, this means that timing of the *Ampelomyces* treatments in autumn should be based on the monitoring of the development of chasmothecia in the plants. For it, epidemiological models are helpful. Recently, a model was developed to predict the time-course of development of the *E. necator* chasmothecia [179–181].

In Mexico, efforts have been made to generate this type of models on the epidemic development of *L. taurica* in tomatoes and chili [182]. However, there is still no applicable model.

There have been several investigations on the establishment and survival of biocontrol agents in the phyllosphere under controlled conditions, which in most cases revealed a quick decline of the biocontrol agent populations in the phyllosphere. It was shown that the survival of several *T. harzianum* strains on strawberry leaves considerably declined within 3 days under greenhouse conditions [183]. Likewise, populations of *T. atroviride* SC1, which were applied to greenhouse grown strawberries, rapidly declined from approx. 3×10^5 to 1×10^1 CFU mm^{-2} leaf area within 7 days [184]. Under high-tunnel greenhouse, *T. asperellum* and *M. anisopliae* establishment in two different types of pepper plants ranging from 1 to 5×10^4CFU [20]. The CFU decreased slightly 30 days after inoculation, as compared with 15 days and fungal persistence was not affected by pepper type. Accordingly, biocontrol agent applications usually have to be repeated to compensate for their rapid decline in the phyllosphere [167]. Here arises the question about the way biocontrol agent are used, this has been one of the most frequently asked questions. The *Trichoderma* applications were made at interval of 20 days [20], or at 7–10 days and even at twice in a week [77]. Thus, there is a possibility of achieving higher level of powdery mildew control by *Trichoderma* by decreasing the interval between two consecutive sprays from 20 days to about 3–4 days. There are several approaches to improve biological control. By using biocontrol strains with increased tolerance to the hostile conditions in the phyllosphere [185]. By improved formulations to protect the biocontrol agents from UV radiation or facilitate their adherence to plant surface [186]. The manipulation of the growth conditions during the fermentation process already affects the accumulation of endogenous compounds (e.g. sugars) in the propagules, which might result in increased tolerance of the biocontrol agents to environmental stress in the phyllosphere (e.g. protection from desiccation) [168].

How was commented before, if conidial germination rate is related to differences in virulence against powdery mildew, pre-activation of the biocontrol agent conidia is a good strategy. The preliminary initiation of conidial germination in nutrient solution prior to application, might reduce the conidia's germination time at the target site under suboptimal temperature conditions and, thereby, improve their efficacy [187]. This was reported for *T. harzianum* against *B. cinerea* under field conditions.

The combined use of biocontrol agents represents another promising approach to overcome inconsistent efficacies in the phyllosphere. It was suggested that improved disease suppression arose from distinct ecological requirements [188], distinct habitats [189] and different modes of action of the biocontrol agents [190]. However, most of the investigations on simultaneous use of they were performed under controlled conditions. Therefore, the suppression of the pepper powdery mildew must be tested and improved in field conditions. Furthermore, it is worth to note that also antagonistic effects between biocontrol agents might occur and should be considered as well. Foliar spray with biocontrol agents can have a considerable activity against pepper powdery mildew [19]. *T. harzianum* and *B. subtilis* showed significant reduction in diseases incidence. *T. viride*,

P. fluorescens and *S. cerevisiae* showed moderate reduction. However, it was not determined their effect as combined treatments. Studies with mixtures of commercial products based on the biocontrol agents *B. subtilis, T. harzianum* T 22 and *T. atroviride*, resulted in reduced suppression of *B. cinerea* in strawberries as compared to some single microorganism treatments [191, 192].

Integration of biocontrol with other methods is recognized from many times ago [193]. Thus, treatments that combined microorganisms with cultivars resistance, plant extracts (resistance inducers), additives (as glycerol, tween) and chemicals fungicides (at low rates), have been tested in recent years. There are reports that the antagonistic fungi give better control on partially resistant cultivars than on susceptible cultivars [193]. Landrace pepper genotype (arbol pepper) and hybrid pepper genotype (jalapeño "Grande" pepper), both with different resistance level have been combined with the antagonistic fungi *T. asperellum* and *M. anisopliae* [20]. This time, fungi give better results in arbol cultivar than in jalapeño pepper. Integrating *Bacillus* spp., *B. chitinosporus*, *B. pumilus, B. subtilis* and *B. thuringiensis*, plus inducer resistant chemicals as chitosan, humic acid, oxalic acid, salicylic acid and cow's skim milk, reduced conidia germination of *L. taurica* [194]. Elad et al. [10] combined the use of *A. quisqualis, T. harzianum*, neem extract, sulphur and shade net to control of powdery mildew and increase yields in sweet pepper. Spraying pepper plants with the *B. thuringiensis*, chitosan and cow's skim milk, resulted the best combination for reduction of pepper powdery mildew severity. After test different chemical fungicides, biological agents and plant extracts, Kumar et al. [144] select Dinocap, *A. quisqualis* and neem oil to integrate an integrated pepper powdery mildew management package. This system allowed Dinocap to be used only once, therefore, it seems an economically viable and ecological alternative.

Combined *Pseudomonas fluorescens* and azoxystrobin induced systemic resistance to *L. taurica* [32]. The effect was related to induction of activity of enzymes peroxidase, polyphenol oxidase, phenylalanine ammonia lyase, β-1,3 glucanase, chitinase, catalase and to the increase of total phenols. Use of culture filtrates of *T. viride, T. harzianum* and *P. fluorescens* combined with dipotassium hydrogen orthophosphate and plant extract *Azadirachta indica* also was recommended to integrated management of *L. taurica* in pepper [73]. When pepper is grown in a greenhouse, the alternation of sulfur, *T. harzianum* + JMS Stylet oil, *A. quisqualis* + AddQ oil and neem are effective in controlling pepper powdery mildew [42]. However, its effectiveness varies when the environment changes. In the greenhouse, the climate can affect the development of powdery mildew and, at the same time, promote the activity of the biocontrol agents and make a pathogen more vulnerable to these control agents. A novel and robust approach to integrated pest management in peppers is presented by Dogan et al. [195]. They designed a Microbial-based Production System (MPS) that consider only microbial-based products/materials for control of invertebrate pest and disease in greenhouse-grown peppers. With MPS there were less key pest and more natural enemies, but did not fully prevent the damage caused by powdery mildew, nevertheless, promoted lower disease ratings in two growing periods. These production systems are healthier and environmentally friendly and may also be applicable to the pepper crops grown in open field conditions.

15.7 Conclusions

Pepper powdery mildew is a serious constraint in the production of pepper since it causes significant losses. This disease has proven to be a complex problem of difficult solution for which the chemical control options are not efficient enough or are restricted for environmental and public health considerations. The use of biological agents for its control has not been as effective, so their consistent use requires developing integrated management strategies. Scientific information is currently available on the capacity of some microorganisms (fungi and bacteria) whose potential can be exploited if their ecophysiological skills, their particular needs and the limitations of each of them are taken into account. Particularly, fungi such as *Trichoderma* spp. and *A. quisqualis*, bacteria such as *B. subtilis* and *Pseudomonas* spp., have been efficiently evaluated for the control of pepper powdery mildew in different environments and growing conditions, as well as in different types of formulations. The results are encouraging, especially if the microorganisms are combined with each other and/or combined with other ecorational strategies which have also been evaluated with good results. There are other less studied microorganisms may be good alternatives as *M. anisopliae, P. glomerata, Cephalosporium* sp., *A. alternatum, P. farinosus, Streptomyces* sp. and *Serratia marcescens*. There is wide information that supports the integrated use of these microorganisms in combination with mineral or essential oils or plant extracts that potentiate their activity as biocontrol agents. The use of microorganisms in combination with low doses of chemical fungicides has also been a good strategy. The design of strategies should also consider that the environment of the phyllosphere is complex. The biocontrol agents must be able to integrate into the microbiome of plants where, in addition to *L. taurica*, various microorganisms are present that form a complex and intricate network of interactions of which the biocontrol must be part. Finally, we must consider that leaving aside the use of synthetic chemical fungicides will take time and that for the moment we are in need of using them under a rational scheme. This dependence will remain as long as we can select more efficient biocontrol agents, capable of integrating into the microbiota of the phyllosphere and performing the work for which they have been selected.

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Chapter 16
Molecular Mechanisms of the Interactions Between Nematodes and Nematophagous Microorganisms

Juan Li, James Borneman, Paul Ruegger, Lianming Liang, and Ke-Qin Zhang

16.1 Introduction

The global economic loss caused by PPNs in agriculture is estimated to be more than 157 billion US dollars each year [1]. The high impact of these nematodes on world agriculture is because of their wide distribution and their ability to attack every species of agronomic plants [2]. For decades, traditional methods to control PPNs have mainly relied on chemical nematicides, though crop rotation and resistant crop cultivars have also been used. However, currently available chemical nematicides can cause severe environmental problems and harm to human health, so most of them have been withdrawn from use. Moreover, the effectiveness of crop rotation is limited in some cropping systems due to PPNs often wide host ranges and/or long-term survival capabilities. Furthermore, the high genetic diversity within/among nematode populations can limit the effectiveness of nematode-resistant crops due to the limited resistant mechanisms of most current crop cultivars. Consequently, global crop production remains under heavy threat of PPNs. There is therefore an urgent need to find novel, environmentally friendly, and effective management strategies to control PPNs.

For decades, scientists have been interested in developing environmental-friendly biological control agents to control the population of PPNs. Thus, as the natural enemies of nematode, nematophagous microorganisms have attracted significant

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attention. Up to now, several nematophagous fungi have been developed as nematode biological control agents, such as the nematophagous fungi *Paecilomyces lilacinus* and *Pochonia chlamydosporia* and nematophagous bacteria from the genus *Bacillus* [3–5]. Although they show great promise in antagonizing plant-parasitic nematodes, the practical application of nematophagous fungi is still limited, partly due to their relatively low effectiveness and inconsistency in agricultural and forest environments. We and others posit that elucidating the molecular mechanisms underlying the interactions between nematophagous microorganisms and nematodes will be crucial for the development of highly effective biological control agents, which in turn can be used to create effective strategies to control PPNs.

Recently, with advances in biotechnology, several genetically engineered nematophagous fungi with higher pathogenicity have been created [6, 7]. For example, Åhman et al. enhanced the virulence of the nematode-trapping fungus *Arthrobotrys oligospora* by introducing additional copies of the endogenous cuticle-degrading protease PII gene in its genome [8]. The engineered *A. oligospora* strain produces higher number of infection devices and it showed an increase in its speed of capturing and killing nematodes compared to the wild-type strain [6]. Yang et al. transferred a cuticle-degrading serine protease gene *ver112* from one nematophagous fungus *Lecanicillium psalliotae* to another (*P. lilacinus*) through restriction-enzyme-mediated integration transformation. One of the resulting transformants, *P. lilacinus-112*, showed higher nematicidal activity against *Panagrellus redivivus* and *Caenorhabditis elegans* than the wild-type strain [9]. The success of these engineered fungi provides another strategy to enhance the effectiveness of nematophagous fungi [9, 10], which should be greatly facilitated by obtaining a better understanding of the molecular basis of microbe-nematode interactions.

16.2 Introduction of Nematophagous Microorganisms

Nematophagous microorganisms are those fungi or bacteria with the capacity to capture, parasitize or paralyze nematodes at all stages of their life cycles such that they control the populations of plant- or animal-parasitic nematodes.

16.2.1 Nematophagous Fungi

There is great interest in using nematophagous fungi as biological control agents against PPNs. These organisms are also good models for performing adaptive evolutionary research. Nematophagous fungi are broadly distributed in a wide range of habitats. Up to now, more than 700 nematophagous fungi and related organisms have been described, and they belong to diverse phylogenetic groups, including the Ascomycota, Basidiomycota, Blastocladiomycota, and Oomycota (Table 16.1). According to their mode of action, nematophagous fungi can be divided into

Table 16.1 Classificationsof nematophagous microorganisms

Fungi/ Bacteria	Types	Taxonomy	Species
Fungi	Nematode-trapping fungi	Ascomycota	*Dactylellina haptotyla*
			Drechslerella stenobrocha
			Arthrobotrys oligospora
			Arthrobotrys musiformis
			Arthrobotrys musiformis
			Arthrobotrys superba
	Endoparasitic fungi	Ascomycota	*Harposporium cerberi*
			Harposporium anguillulae
			Drechmeria coniospora
			Haptocillium balanoides
			Hirsutella rhossiliensis
			Hirsutella minnesotensis
		Basidiomycota	*Nematoctonus concurrens*
			Nematoctonus haptocladus
		Blastocladiomycota	*Catenaria anguillulae*
			Catenaria auxiliaris
		Oomycota	*Haptoglossa heterospora*
			Myzocytiopsis vermicola
			Myzocytiopsis enticularis
	Egg- and female-parasitic fungi	Ascomycota	*Pochonia chlamydosporia*
			Paecilomyces lilacinus
			Lecanicillium psalliotae
			Lecanicillium lecanii
			Hyalorbilia oviparasitica
		Oomycota	*Nematophthora gynophila*
	Toxin-producing fungi	Basidiomycota	*Pleurotus ostreatus*
			Coprinus comatus
			Stropharia rugosoannulata
Bacteria	Opportunistic parasitic bacteria	*Bacillus*	*Bacillus thuringiensis (Bt)*
			Bacillus sp. RH219
			Bacillus laterosporus
			Bacillus nematodida B16
	Rhizobacteria	*Pseudomonas*	*Pseudomonas fluorescens*
			Pseudomonas protegens
			Pseudomonas chlororaphis
	Endoparasitic bacteria	*Pasteuria*	*Pasteuria thornei*
			Pasteuria nishizawae
			Pasteuria usgae
			Pasteuria penetrans

nematode-trapping fungi, endoparasitic nematode fungi, egg and cyst parasitic fungi and toxic fungi [11].

Nematode-trapping fungi are species which can capture nematodes by producing specialized trapping structures such as constricting rings, adhesive knobs, adhesive networks, adhesive columns, and non-constricting rings. In general, these fungi are saprophytic in soil. However, when they sense the presence of their nematode prey, these fungi can switch their lifestyle to become predatory via producing these specific trapping devices [11]. It has been proposed that the carnivorous feature of nematode-trapping fungi probably emerged 400–520 million years ago [12, 13]. According to their taxonomic classification, nematode-trapping fungi all belong to a monophyletic group in the order Orbiliales, Pezizomycotina, Ascomycota. Traditional taxonomy of nematode-trapping fungi have mainly relied on the morphology of their conidia, without consideration of the morphology of their trapping structures, resulting in species with similar trapping devices being classified into different genera [14–16]. However, recent studies based on the rRNA ITS region and/or18S rRNA gene sequences have suggested that trapping devices are more informative in taxonomic classifications (e.g., [17–19]). In 1999, Scholler et al. suggested that nematode-trapping fungi can be classified into four genera: (i) *Dactylellina* characterized by stalked adhesive knobs but also including the species characterized by non-constricting rings and stalked adhesive knobs, (ii) *Gamsylella* characterized by adhesive branches and unstalked knobs, (iii) *Arthrobotrys* characterized by adhesive networks and, (iv) *Drechslerella* characterized by constricting rings [18]. In 2005, based on more refined DNA sequencing, Li et al. suggested that the species in genus *Gamsylella* should be transferred to either *Arthrobotrys* or *Dactylellina* [19]. Moreover, Li et al. hypothesized that trapping devices might have evolved from adhesive knobs into two lines: one that lost the adhesive function and evolved to form constricting rings and the other that retained the adhesiveness and evolved to form three-dimensional networks [19]. In 2007, Yang et al. proposed an evolutionary pathway for five types of trapping devices based on comprehensive phylogenetic analysis [20]. They suggested that the initial trapping structure evolved along two lineages with one developing into constricting rings and the other into adhesive traps. Among adhesive trapping devices, the adhesive network separated from the others early and evolved at a steady but slow rate. The adhesive knob evolved through stalk elongation with the final development being non-constricting rings [20]. Also, based on the characterization of the trapping devices, they classified nematode-trapping fungi into the genera *Arthrobotrys*, *Drechslerella* and *Dactylellina*, with *Arthrobotrys* being characterized by adhesive networks, *Dactylellina* by stalked adhesive knobs and/or non-constricting rings, and *Drechslerella* by constricting-rings [19]. With the development of next generation DNA sequencing technologies, the genomic sequences of three nematode-trapping fungi have been obtained: an adhesive network-forming species *A. oligospora*, an adhesive knob-forming species *Monacrosporium haptotylum* and a constricting ring-forming species *Drechslerella stenobrocha* [21–23]. These genome sequences can provide valuable information enabling a much deeper understanding of the biology of nematode-trapping fungi. Beside the aforementioned nematode-trapping

fungi, there are two Basidiomycete fungi, *Coprinus comatus* and *Stropharia rugosoannulata*, that have the ability to produce specialized nematode-attacking devices named "spiny ball" and "acanthocyte", respectively [24, 25]. These sharp structures cause damage to the cuticle of nematodes, resulting in leakage of the interior contents of the nematodes, suggesting that mechanical forces can also function as a virulence factors [24, 25].

Endoparasitic fungi are the nematophagous species that infect nematodes with conidia or zoospores. So far, more than 100 nematode-endoparasitic fungal species have been reported [11]. All of them can produce a large number of adhesive conidia, and these mature conidia can rapidly germinate and form an adhesive bud that adheres to the cuticle or to the sensory structures in the head or vulva regions of nematode [26]. For example, *Drechmeria coniospora* can deliver up to 10,000 conidia inside a single nematode via the secretion of collagenase and chymotrypsin-like proteases that enable penetration into the nematode; the resulting fungal hyphae can digest the nematode into corpses, and form new conidia within 3 days [27, 28]. Beside *D. coniospora*, *Hirsutella rhossiliensis* is another typical endoparasitic fungus that produces adhesive spores that attach to and penetrate nematodes. Similar to *D. coniospora, H. rhossiliensis* also delivers large amounts of conidia after it penetrates a nematode, and a serine protease extracellular alkaline protease is one of the enzymes that helps this fungus penetrate the cuticle of nematodes [29, 30].

Egg-parasitic fungi are a group of microbes which can form a specialized penetration peg named appressoria or lateral mycelial branches that infect nematode eggshells [31]. Taxonomic classification shows that the egg-parasitic fungi included species from the genera *Pochonia*, *Paecilomyces*, *Lecanicillium, Hyalorbilia* and *Nematophthora*, which are closely related to many entomopathogenic fungi such as *Metarhizium* spp., because eggshells of both nematodes and insects mainly consisted of chitin. Thus, there might be similar infection mechanisms between the entomopathogenic and egg-parasitic nematophagous fungi. This group of fungi have been considered as promising biocontrol agents because they can survive as saprobes in the rhizosphere, and are effective in infecting eggs, developing juveniles and females of nematode [32]. It has been reported that egg- and female-parasitic fungi can produce extracellular hydrolytic enzymes such as chitinases and proteases that degrade nematode eggshells [33, 34]. Among all of the egg- and female-parasitic fungi, *Pochonia chlamydosporia* is the most extensively studied species. This fungus can parasitize both females and eggs of cyst and root-knot nematodes [35]. Their secreted extracellular hydrolytic enzymes, such as serine protease VCP1 and chitinases*pcchi44*, are effective at degrading the eggshell [34, 36]. *Clonostachys rosea* (syn. *Gliocladium roseum*) is another widely distributed facultative saprobe in soil [37]. It has shown strong nematicidal activities against various nematodes, such as *Caenorhabditis elegans*, *Panagrellus redivivus* and *Bursaphelenchus xylophilus*. Two extracellular serine proteases (Lmz1 and PrC) have been isolated from *C. rosea* and identified as important factors in its pathogenicity [38, 39].

Toxin-producing fungi produce toxins to immobilize nematodes [31]. Up to now, more than 200 compounds with high nematicidal activities have been identified from more than 280 fungal species, and their diverse structures mainly belong to

alkaloids, peptides, terpenoids, macrolides, oxygen heterocycle and benzo compounds, quinones, aliphatic compounds, simple aromatic compounds, and so on [40, 41]. About 60% of these nematicidal compounds have been novel molecules, which therefore hold considerable commercial potential for controlling PPNs. Up to now, although no major commercial product based on nematode-toxic fungi and the compounds they produce have been developed, several nematicidal substances have been patented and tested in agronomic situations. For example, the thermolides A − F (1–6) identified from a thermophilic fungus *Talaromyces thermophiles* has displayed potent nematicidal activity similar to commercial avermectins [42].

16.3 Nematophagous Bacteria

At present, numerous bacteria with the ability to suppress a wide range of nematode species have been identified, and they belong to several genera including *Bacillus*, *Pseudomonas* and *Pasteuria*. Based on their infection modes, those nematophagous bacteria can be classified into obligate parasitic bacteria, opportunistic parasitic bacteria, rhizobacteria, parasporal Cry protein-forming bacteria, endophytic bacteria and symbiotic bacteria [43]. Here, we only describe several extensively studied bacteria.

Bacillus thuringiensis (*Bt*) is an ubiquitous spore-forming bacterium which produces proteinaceous protoxin crystals (called crystal proteins or Cry proteins) during sporulation [44]. The Cry proteins show specific toxin activity on caterpillars, beetles and nematodes but do not affect vertebrates; thus, *Bt* has been described as an ideal biopesticide in organic agriculture [45, 46]. Up to now, three families of Cry proteins (Cry5, Cry6 and Cry55 family) have been found to exhibit potent activities against the larvae of nematodes with Cry5B being the most extensively studied [46–48]. It can interact with specific receptors located on the apical side of gut epithelial cells of nematodes. This interaction results in the formation of lytic pores, lysing these epithelial cells which leads to nematode death [49, 50]. The Cry6Aa2 protein causes different effects including growth inhibition, reduced brood size and abnormal motility of *C. elegans* [48]. In 2014, Yu et al. administered both Cry6A and Cry5B proteins to nematodes, which provided a highly effective strategy to PPNs [51]. Several *Bt* strains have had their genomes sequenced, providing a strategy to discover novel Cry toxins. For example, Latsenko et al. identified three Cry-like genes belonging to the Cry21 family from the genome sequence of *Bt* strain DB27 [52]. Since *Bt* strains appear to be harmless to humans and other mammals, and their pathogenic activity targets a narrow range of insect or nematode species, they have considerable potential as biological control agents for NPPs [53].

Among *Bacillus* spp., *Bacillus nematocida*(B16), which was isolated from a forest soil sample in Yunnan China, exhibits high toxic activity against the nematode *Panagrellus redivivus* [54]. This bacterium lures nematodes to their death by a "Trojan horse" mechanism [55]. At first, B16 releases 2-heptanone and other volatile organic compounds that mimic the bacteria that are the nematode's food source.

After being eaten by the nematodes, B16 can colonize in the intestinal of nematode, and then secrete several toxic factors such as extracellular alkaline serine protease Bace16 and a neutral protease Bae16, which digests components of the host intestinal tissue, eventually killing the host. This "Trojan horse" mechanism expands the already diverse suite of pathogenic mechanisms used by bacteria [55]. B16s mode of action has been reported to be regulated through a quorum sensing (QS) system, which enables bacterial cells to sense each other and instigate density-dependent responses [56]. A subsequent investigation has revealed that the ComP-ComA system, a conserved QS system in the genus *Bacillus*, is involved in the pathogenesis of B16 [57]. Recent research has also suggested that the G protein-coupled receptor (GPCR, STR-2) on the AWC neuron of the nematode is the receptor of the 2-heptanone, and the resulting the signal is transmitted through PLC and cGMP pathways [58].

Pasteuria penetrans, is a gram-positive bacterium that can colonize more than 300 nematode species, including the majority of important PPNs and free-living nematodes [59, 60]. Phylogenetic analysis suggests *P. penetrans* is closely related to *Bacillus*, and that this bacterium has evolved from an ancient symbiotic bacteria associated with nematodes [61]. The endospores produced by *P. penetrans* can adhere to the cuticles of second-stage juveniles (J2) of the nematodes, and then the endospores can produce an infection peg that penetrates the nematode cuticle. The carbohydrate ligands, which are collagen-like fibers and gelatin-like proteins on the surface of the endospore, are thought to play critical roles in endospore attachment [62–64]. The terminal region of the infection peg extends into the pseudocoelom of nematodes, branches dichotomously, and produces a mycelial ball or microcolony to kill the nematodes [65]. *Pasteuria penetrans* can cause significant decreases in the reproductive capacity of nematode females. Although *P. penetrans* shows promise as a biocontrol agent, this bacterium is still difficult to be cultured in the laboratory. The genome sequence of *P. penetrans* and genomic comparisons may facilitate a better understanding of *P. penetrans*-nematode interactions, enabling this bacterium to be incorporated into an effective strategy to manage NPPs at an agronomic scale.

Pseudomonas spp. are gram-negative bacteria that can use a wide variety of compounds as energy and carbon sources. These *Pseudomonas* species exhibit different lifestyles, with some of them being saprophytes while others are plant pathogens or opportunistic human pathogens. Up to now, there have been several species of *Pseudomonas*, including *Pseudomonas aeruginosa*, *P. fluorescen, P. protegens*, and *P. chlororaphis*, which have been shown to be capable of suppressing diverse soil microbes, including fungal, bacterial, viral, and oomycete pathogens, as well as some PPNs. Although *Pseudomonas'* hosts are highly diverse, ranging from plants to mammals to nematodes, their virulence factors and pathogenesis strategies are similar. At present, *Pseudomonas aeruginosa* PA14 has been used to construct a pathogenesis model (*C. elegans-P. aeruginosa*), which has been used to determine molecular host-pathogen interactions [66–68]. *Pseudomonas aeruginosa* strain PA14can kill *C. elegans* through either the toxin-mediated "fast killing" mode or the cell-growth-mediated "slow killing" mode [67]. In the "fast killing" mode, PA14

kills *C. elegans* within 4–24 h by low-molecular-weight diffusible toxins called phenazines. However, in the "slow killing" mode, PA14 kills *C. elegans* within 2–3 days by the accumulation of the bacteria in the intestine. In addition, another *P. aeruginosa* species (PAO1) can cause the "red death" phenomenon observed in *C. elegans* [69]. At least three pathways (the phosphate signaling/PhoB pathway, the MvfR-PQS pathway, and the pyoverdin iron acquisition system) are involved in the "red death" response, which requires a red colored PQS + Fe^{3+} complex [69]. It has been reported that the QS system is also an important mechanism that regulates *the* pathogenesis of *P. aeruginosa* infection [70]. Although the extracellular protease AprA and several secondary metabolites such as 2, 4-diacetylpholoroglucinol (2,4-DAPG) have shown significant biological activity against PPNs, the molecular interactions between *Pseudomonas* and PPNs remain poorly understood [71, 72].

16.4 Molecular Mechanisms of Nematophagous Fungi

16.4.1 Attraction and Recognition

In general, the infection process of nematophagous fungi mainly consists of attraction/recognition, adhesion, penetration and digestion [73]. These processes can involve biochemical, physiological and morphological interactions between nematophagous fungi and nematodes. Nematophagous fungi can produce certain volatile organic compounds (VOCs) to attract nematodes. For example, the endoparasitic fungus *Esteya vermicola* can continuously produce VOCs, including monoterpenes (α-pinene, and β-pinene) and the terpenoid camphor, to attract the pinewood nematode (PWN) *Bursaphelenchus xylophilus* [74]. Interestingly, those VOCs are similar to the volatile compounds emitted from the pine tree host of PWN, suggesting that *E. vermicola* mimics the scent of the host to attract PWN [75]. The nematode-trapping fungus *Monacrosporium rutgeriensis* can produce three substances to attract *Panagrellus redivivus*. In 2017, Hsueh et al. isolated several VOCs from *A. oligospora,* and one these compounds, methyl 3-methyl-2-butenoate (MMB), can trigger strong sex- and stage-specific attraction of several *Caenorhabditis* species, suggesting that nematode-trapping fungi have evolved to use olfactory mimicry to attract its nematode prey.

Although we know that fungi can secrete compounds that attract nematodes, how fungi recognize nematodes and initiate morphological transition remains unclear. It has been proposed that nematophagous fungi can recognize nematodes via chemicals produced by nematode. For example, Pramer and Stoll demonstrated that "nemin" produced by the nematode *Neoaplectana glaseri* can cause a morphogenetic switch and trap formation in *Arthrobotrys conoides* [76]. Up to now, researchers still haven't determine the completed chemical composition of nemin. Some components include amino acids and polypeptides, which can also regulate the production of traps. Recently, many species of nematodes were shown to constitutively secrete a family of small molecules named ascarosides. Initially, ascarosides were identified as a small molecular pheromone that regulates the development and

behavior of nematodes. Ascarosides can also trigger trap formation in *A. oligospora* and several related species producing three-dimensional adhesive networks [77]. Recently, Wang et al. found that *Stenotrophomonas maltophilia*, the food of some nematodes in soil, can release urea which activate the lifestyle switch of *A. oligospora*, which may assist in the population balance in the soil ecosystem [78]. Also, Liang et al. [79] found that nitrate and other nitrogen sources could stimulate *A. oligospora* to produce adhesive networks by deleting the adhesive protein coding gene *Aomad1*, suggesting that the presence of *AoMad1* may help fungi recognize nematodes.

16.4.2 Adhesive Proteins

Although nematophagous fungi can change their lifestyles to prey on nematodes around them, the production and quantity of trapping structures are highly associated with their efficiency to capture nematodes. Based on electron microscope studies, huge amounts of adhesive substances secreted by nematophagous fungi accumulated on the outside surface of traps or spores, which helps them to adhere to the nematode cuticle [80]. The major adhesive substances are extracellular fibrillary polymers [81]. However, the exact chemical components of these adhesive substances remain largely unknown. Adhesive proteins are also thought to play other roles including nutrient storage, trap development, nematode attraction and recognition and defense against competitors [82].

In 1979, Nordbring-Hertz and Mattiasson identified a group of carbohydrate-binding proteins (lectins) from adhesive traps [83]. Since then, lectins have been thought to be the most functionally important substances during fungal recognition of nematodes. However, a recent study showed that none of the seven putative lectin genes showed significant expression difference during nematode infection [23], and when the lectin gene (AOL_s00080g288) was deleted from *A. oligospora*, there was no affect on the fungal growth or its pathogenicity against nematodes [84]. These results suggested that the role of lectins in the pathogenesis of nematophagous fungi may have been overstated. Besides lectins, a large number of genes encoding cell-surface receptors, signal transducers and adhesion molecules in host-pathogen interactions have been identified in nematode-trapping fungal genomes, providing a more comprehensive understanding of adhesion in nematophagous fungi.

16.4.3 Extracellular Enzymes Involved in Pathogenesis

After capturing nematodes via trap devices and adhesive proteins, extracellular enzymes including serine proteases, collagenases and chitinases, can be subsequently secreted by fungi to breakdown the physical and physiological integrity of nematodes cuticles and eggshells, facilitating fungal penetration and colonization [85].

Serine proteases are the most studied extracellular enzymes secreted by nematophagous fungi. P32 was the first serine protease identified from the egg-parasitic fungus *Pochonia rubescens* (syn. *Verticillium suchlasporia*) [86]. Up to now, more than 20 serine proteases have been purified or identified from various nematophagous fungi, and all of them have shown the ability to degrade nematode cuticles with high efficiency. Thus, they also have been named as cuticle-degrading proteases [87–89]. Interestingly, the cuticle-degrading proteases from nematode-trapping fungi have different properties than those from nematode parasitic fungi. The proteases derived from nematode-trapping fungi are active at neutral pHs while the proteases from nematode-parasitic fungi are active at alkaline pHs [90]. Also, properties of their substrate-binding sites and their electrostatic surface potential distributions may be the reason why they show different catalytic and nematicidal activities [91]. Positive selection analyses have suggested that adaptive evolution may have played an important role in the evolution of the pathogenicity of cuticle-degrading proteases in nematode-trapping fungi [90]. The crystal structures of the two cuticle-degrading proteases - Ver112 from *L. psalliotae* and PL646 from *P. lilacinus* -showed that different amino acids of the substrate binding sites, such as the S1 and S4 pockets, contributed to their different hydrophytic activities [92]. In addition, nematophagous fungi, can contain multiple serine proteases. For example, 24, 17 and 32 putative serine protease encoding genes have been identified in *A. oligospora*, *D. stenobrocha* and *P. chlamydosporia* genomes, respectively [22, 23, 93]. For *A. oligospora,* only two of the 24 serine protease encoding genes (*P186*, *AOL_s00215g702*; and P12, *AOL_s00170g103*) were significantly up-regulated when the fungus was exposed to nematode extracts, suggesting the functional importance of these two proteases. Moreover, other factors such as nitrogen sources, environmental pH and/or other stress-inducing conditions may also affect the expression of serine proteases in nematophagous fungi [94–96].

Since the most abundant structural component of the nematode eggshells is chitin, it makes sense that egg-parasitic fungi often have large numbers of chitinases that can degrade the nematode eggshell during infection. The first chitinase purified from nematophagous fungi was Chi43 [34]. Up to now, one chitinase from the nematophagous fungus *C. rosea* has had its crystal structure analyzed [97, 98]. This work has suggested that the DXDXE motif and the catalytic residue Glu174 play important roles in chitinase activity. Although no chitinases have been purified from nematode-trapping fungi, 16 and 8 genes encoding putative GH18 chitinases have been identified from *A. oligospora* and *D. stenobrocha* genomes, respectively, suggesting that chitinases are also functionally important in nematode-trapping fungi [22].

Because the main component of nematode cuticles are collagens, nematophagous fungi have evolved to contain many collagenases, which enable them to digest these nematode cuticles. In 1980, Schenck et al. found that eight nematophagous fungi secreted extracellular collagenases with the ability to effectively hydrolyzing collagens [99]. In 2002, Tosi et al. found that all the species in the *Arthrobotrys* genus produced collagenases [100]. However, the definitive functions of these

enzymes has not been experimentally confirmed [23]. Moreover, besides the putative functional enzymes mentioned above, many gene families related to pathogenicity have also been found to have significantly expanded in nematophagous fungi. For example, a total of 147, 226 and 305 glycoside hydrolases (GHs) were predicted in *D. stenobrocha*, *A. oligospora* and *P. chlamydosporia* genomes, respectively [22, 23, 93]. The function of GHs is to degrade cellulose, lignocellulose, hemicellulose, xylans and other constituents of the cell wall [101], indicating that these GHs may also have important functions in nematophagous fungi. In addition, 2, 17 and 26CFEMcontaining proteins (an eight cysteine-containing domain) were identified in *D. stenobrocha*, *A. oligospora* and *M. haptotylum* genomes, respectively [21–23], and these proteins may function as cell-surface receptors, signal transducers, or adhesion molecules in host-pathogen interactions in nematophagous fungi [102].

16.4.4 Trap Formation

For most of the nematophagous fungi, they change their lifestyles from a saprophytic stage to a parasitic stage when they sense nematodes around them. For nematode-trapping fungi, the production of trapping structures indicates this transfer from saprophytic to parasitic lifestyle. These morphogenesis changes involve multiple signal transduction pathways including responses to environmental signals that lead to these cellular changes. For most fungi, G proteins are the major class of sensors involved in important biological processes [103]. In nematophagous fungi, the G-protein signal transduction pathway is essential for trap formation. For example, the formation of constricting rings in *Arthrobotrys dactyloides* is stimulated by the G-protein activator and the associated increase in intracellular Ca^{2+} concentrations [104]. Beside the G-protein signal transduction pathway, the other important fungal signal transduction pathways likely involved in trap formation include glycosylphosphatidylinositol-specific phospholipase C, mitogen-activated protein kinases (MAPKs), serine/threonine protein phosphatase 2A, calcyclin binding proteins and Ca2+/calmodulin-dependent protein kinase, because they have been shown to be upregulated during trap formation [23, 93].

Trap formation is a complex process. These special morphological structures represent a remarkable adaptation of these fungi to their environment. Molecular phylogenetic analyses have suggested that traps evolved along two major lineages, one with constricting rings and the other with adhesive traps including three-dimensional networks, knobs and branches. Interestingly, lived nematodes are not the only factors capable of inducing trap formation. At present, several types of materials such as nemin, ascarosides, small peptides with a high proportion of nonpolar, aromatic amino acids or their amino acid constituents, and abscisic acid can also induce trap formation [23, 105–107]. Recently, two metabolites (paganins A and B) isolated from the nematode-trapping fungus *Arthrobotrys entomopaga* also have been shown to harbor the ability to induce the formation of adhesive knobs [108].

As early as 1984, Dowsett et al. showed that numerous cytosolic organelles named electron dense bodies fill trapping cells, but they are not present in vegetative hyphae [109]. For most of the dense bodies in the mature traps, their diameters are approximately 0.35 μm, and they are enveloped by a single-layer membrane that is approximately 9 nm thick. Because the dense bodies remain intact during aging, it was thought that these organelles were independent of trap development [110]. Electron microscopy revealed that the dense bodies degraded through an autophagic process, finally turning into vacuole-like structures [110, 111]. Although scientists believed that the formation of dense bodies was likely related to peroxisome biogenesis [112], the exact biochemical properties and biogenesis mechanism of dense body remains to be elucidated.

Recently, researchers have been able to identify the molecular mechanisms of trap formation by using genomic and/or transcriptional data. Up to now, several nematophagous fungi have had their genomes sequenced, including the nematode-trapping fungi *A. oligosporus* [23], *M. haptotylum* [21], *Drechslerellastenobrocha* [22] and *Duddingtonia aflagrans* [113]; as well as the egg-parasitic fungi *Purpureocillium lilacinum* [114] and *Pochonia lamdosia* [93]; as well as the endo-parasitic fungi *Hirsutella minnesotensis* [115] and *D. coniospora* [116]. The application of genomics, proteomes and transcriptomes has greatly increased our understanding of the molecular mechanisms of these special types of fungi, including the transition from saprophytic to parasitic lifestyles, trap formation and pathogenesis. For example, at least 90 genes related to translation, amino acid metabolism, carbohydrate metabolism, cell wall biosynthesis, cell division and membrane biogenesis have been found to be up-regulated at the early stage of trap formation, and a putative model describing how adhesive networks are formed in the fungus *A. oligospora* was proposed by Yang et al. [23]. In this model, the signal transduction pathways that appear to be activated by nematodes as well as the resulting downstream cellular processes that lead to trap formation - such as expression of specific genes, post-translational modifications, amino acid metabolism, carbohydrate metabolism, energy conversion, and cell wall and membrane biogenesis- have all been proposed [23]. Moreover, the genes related to rapid cell growth, intracellular signal transduction and protein degradation may also lead to the formation of trapping structures of *D. stenobrocha* [22].

In addition to the aforementioned genes identified by omics data, gene knockouts have also been used to causally link specific genes to trap formation. For example, knocking out the*atg8* gene, an gene related to the autophagic pathway in fungi, led to a decrease of trap formation in *A. oligospora* [117]. In addition, the absence of a malate synthase (*Mls*) gene *AoMls* led to decreased trap formation as well as significant reductions in conidiation and the utilization of fatty acids and sodium acetate [118].

16.5 Nematode Response to Pathogen Attack

16.5.1 Physical Barriers and Avoidance Behavior

Nematodes have evolved sophisticated systems for defense against pathogen attack. Their first line of protection is their multi-layered cuticle [119]. Also, the pharynx within the nematode mouth as well as the intestine provide important physical barriers as the pharynx can break open bacteria while the intestine contains many hydrolytic proteins (e.g. lysozymes, lipases, lections and some proteases) that can kill bacteria and other microorganisms [120].

In addition, the sophisticated chemosensory system of nematodes plays an important role enabling the nematode to recognize and avoid chemical, physical and biological cues from dangerous pathogens. At present, most studies of this avoidance behavior have depended on the model nematode, *C. elegans*. Numerous pathogens have been used to observed the response of *C. elegans* including *B. thuringiensis* [121], *P. aeruginosa* [67], *Salmonella typhimurium* [122], *Serratia marcescens* [123] and *Microbacterium nematophilum* [124]. Signaling from toll-like receptor 1(*TOL-1)* and insulin/insulin-like growth factor-1 (IGF-1) receptor DAF-2 play essential roles in nematode avoidance of pathogenic bacteria [121, 123]. Moreover, the G protein-coupled receptors in several olfactory neurons are also involved in mediating the avoidance behavior in *C. elegans* [123, 125, 126].

16.5.2 Innate Immune Responses

Although the majority of signal pathways involved in innate immune responses of various parasitic nematodes are similar to those found in *C. elegans*, there are still many differences. For example, some immune effectors such as lysozyme, C-type lectins and chitinases are much less abundant in *Meloidogyne* spp. than in *C. elegans* [1]. Also, because the endoparasitic PPNs spend the majority of their life cycle inside plant root tissues and feed on the cytoplasm of root cells, they are not exposed to a diverse suite of microbes, making many of the antibacterial and antifungal genes – such as the antibacterial genes (*abf* and *spp*) and the antifungal *nlp*, *cnc, fip* and *fipr* gene families – unnecessary [1, 127]. Conversely, *Meloidogyne* spp. have two times as many fucosyltransferase-encoding genes as *C. elegans*. These fucosyltransferases in the plant-parasitic nematodes might help root-knot nematodes escape detection by pathogens [128]. All the aforementioned differences occur between *C. elegans* and plant-parasitic nematodes, *C. elegans* is still the most effective way to study innate immune responses to microbial pathogens.

For *C. elegans*, both the epidermal and intestinal innate immune responses are important for defense against microbial pathogens. Several innate-immune signaling pathways can be activated during the pathogen infection, including the p38 Mitogen activated protein kinase pathway, the DAF-2/DAF-16 insulin like

signaling pathway, and the ERK (extracellular signal regulated protein kinase) MAPK pathway. The p38 MAPK pathway, which functions via the NSY-1/SEK-1/PMK-1 axis, plays a key role in both the epidermal and intestinal innate immune responses in *C. elegans* [129]. The p38 MAPK pathway protects against infection by upregulating secreted immune response molecules, including C-type lectins, lysozymes and antimicrobial peptides [130]. In infection of *D. coniospora*, the G protein-coupled receptor (GPCR) DCAR-1 together with its ligand 4-hydroxyphenyllactic acid (HPLA), activates the G12α protein GPA-12 [131]. Then, GPA-12 which acts upstream of protein kinase Cδ, in turn activates TIR-1 through phospholipase Cβ EGL-8 [132]. Then, TIR-1, the highly conserved Toll/IL-1 resistance (TIR) domain protein, activates PMK-1which then turns on the MAPK pathway during both bacterial and fungal infection, which leads to the upregulation of the aforementioned innate immune responses [133]. One of these responses is the expression of the antimicrobial peptide nlp-29. Nlp is a gene cluster that encodes 6 antimicrobial peptide molecules. A recent study has further demonstrated that the NSY-1/SEK-1/PMK-1 p38 MAPK pathway can also be activated by the Gqα protein EGL-30 through β EGL-8 after infection of the pathogenic bacterium *P. aeruginosa* [132, 134]. Concerning the downstream signaling molecules of the p38 pathway, two conserved transcription factors, ATF-7 and SKN-1, have been to be upregulated during bacterial infection [135, 136]. However, to respond to *P. aeruginosa* infection or to the pore-forming toxins produced by human pathogens, including *Staphylococcus aureus*, *Streptococcus pyogenes* and *Aeromonas hydrophilia*, the endoplasmic reticulum unfolded protein response (UPR) functions as the downstream signal of the p38 MAPK pathway [137, 138].

The second major cascade involved in nematode innate immunity against microbial pathogens is the DAF-2/DAF-16 signaling pathway. DAF-2, which is the ortholog of the mammalian forkhead box O (FOXO) transcription factor, is a negative regulator of DAF-16. Reduction in the DAF-2 signaling cascade can result in the dephosphorylation of DAF-16, then leads to nuclear translocation and transcriptional activation [139, 140]. Although *daf-2* mutants are more resistant to several pathogenic bacteria than wild type worms, its pathogen-resistant phenotype can be completely abolished by mutations in *daf-16*, indicating that DAF-16 plays an important role in innate immunity against bacteria [141]. DAF-16 is not normally activated during bacterial infection in wild type worms because *daf-16* mutants, with or without *daf-2* mutations, exhibit similar sensitivity to pathogenic bacteria as wild type worms. However, DAF-16 is required for worm survival upon infection by nematophagous fungi such as *D. coniospora* and *C. rosea* [142]. After fungal infection, EGL-30-Ca^{2+}-DUOX-1-ROS-CST signaling regulates DAF-16, which is functionally independent of the DAF-2 insulin-like signaling pathway.

The third major signaling cascade involved in *C. elegans* innate immunity is the ERK (extracellular signal regulated protein kinase) MAPK (ERK/MPK-1) pathway. When the gram-positive bacterium *M. nematophilum* colonizes the rectum and post-anal cuticle of *C. elegans*, it induces a pronounced swelling of the surrounding hypodermal cells. The ERK cascade reduces this tail swelling response, thereby protecting *C. elegans* from severe constipation caused by the bacterium [143].

Interestingly, the ERK/MPK-1 pathway also plays a role during *P. aeruginosa* (PA14) infection of *C. elegans*. The ERK signaling pathway functions to activate autophagy to protect worms against cellular damage triggered by *P. aeruginosa* infection [143]. Interestingly, autophagy can increase the longevity of nematodes infected by *P. aeruginosa* by reducing the necrosis of intestinal cells [144].

16.6 Conclusions

Although our understanding of the molecular interactions between model nematodes and nematophagous microorganisms has greatly increased over the last decade, there is still an enormous amount of work to be done. With the advances in molecular methodologies as well as the increasing availability of omics data from both PPNs and their interacting microorganisms, we expect considerable progress will be made over the next few years. For decades, researchers have mainly focused on understanding the mechanisms of interaction between specific nematophagous microbes and nematode species. However, both the nematodes and nematophagous microbes live in a complex soil ecosystem. Thus, there are also many interactions among these other organisms that need to be understood. We posit that increases in our understanding of all of these molecular interactions will provide key findings that enable the development of effective, biologically-based, strategies to control PPNs.

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Part IV
Market and Commercialization

Chapter 17
Trends for Commercialization of Biocontrol Agents (Biopesticide)

Catherine Regnault-Roger

17.1 Introduction

The need to implement an agricultural system taking into account sustainable development has fostered many initiatives to develop alternative methods in order to reduce the use of chemical synthetic pesticides for plant protection. Among these alternatives, the use of Biocontrol Agents (BCAs) or biopesticides has aroused increasing interest because of their ecological advantages. So far exists nowadays a societal claim for friendly environmental plant protection from people in several developed countries and also from National Authorities. Two examples illustrate these considerations:

(i) the increasing demand for organic products from consumers. Stores and supermarkets enhance now their range of products and organic products are offered in the best location on the shelves (ii) a Round Table name "Grenelle Environnement" took place in 2007 in France bringing together the government, local authorities, trade unions, business and voluntary sectors to draw up a plan of action of concrete measures to define the key points of public policy on ecological and sustainable development issues. According to agriculture, it was decided to increase the share of organic agriculture to 20% by 2020, and to halve the amount of chemical pesticides. Thus, the alternative methods to the uses of synthetic pesticides are focused and among them, the uses of BCAs (biopesticides). This situation follows re-registration procedures for Plant Protection Products (PPP) which occurred in several developed countries during the two last decades as a consequence of

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improvement of scientific knowledge about biological and toxicological data and environmental concerns.

So, it is now the moment to estimate which room there will be for Biocontrol Agents (biopesticides) within Plant Protection Products market in the next future. After having defined what are BCAs and biopesticides, this chapter is questioning about the commercialization of BCAs which represents a small part of the PPP global market, the technical and the plant protection challenges, as well as some registration considerations.

17.2 Biocontrol Agents (BCAs) or Biopesticides: A Concept in Evolution

Plants could not have survived in the course of their evolution without acquiring characteristics which enabled them to reproduce and defend themselves. The study of the numerous compounds they harbour has contributed to a better understanding of the framework of interspecific relationships between them and bio-aggressors. Following these observations, several strategies are been developed in order to limit or eradicate these bio-aggressors, and some involve biological based-products, also called biopesticides or biocontrol agents (BCAs).

There are several definitions of biopesticides as to which kind of organisms or chemicals emitted by organisms should be considered to be a biopesticide: micro-organisms, arthropod predators or parasitoids only or including also transgenic products, semiochemicals, botanicals [1]. Will Hintz, animator of the Biological Working Group of Canadian Weed Science Society (CWSS), initiated a forum on CWSS website to debate about the definition of biopesticides. It highlighted that the first strategies were based on the uses of biopesticides defined to be living organisms for "the planned use of natural enemies to suppress or reduce the populations of a pest until it no longer represents a problem" [2]. Van Driesche and Bellows [3] specified "the use of parasitoids, predators, pathogens, antagonists or competitive populations to suppress a pest population". Completing the definition reported from Smith [4] "the use of biological agents which, directly or indirectly, are able to control pests or weeds", Hintz [5] indicated that "Biopesticides can be interpreted as plant-derived compound substances (or in a broader definition of all organism substances) having a protective effect on plants. Biopesticides can be found in nature or chemically synthesized".

In the same mind, the US EPA (Environment Protection Agency) states that biopesticides are derived from "natural materials as animals, plants, bacteria, and certain minerals". They fall into three classes of compounds: (i) microbial pesticides including bacteria (among the most used microbial pesticides are subspecies and strains of *Bacillus thuringiensis*, or Bt), fungi, virus or protozoan as the active ingredient, (ii) Plant-Incorporated-Protectants (PIPs) that are "pesticidal substances that plants produce from genetic material that has been added to the plant…The

protein and its genetic material, but not the plant itself, are regulated by EPA", (iii) biochemical pesticides that are "naturally occurring substances that control pests by non-toxic mechanisms". This definition mentions that "conventional pesticides, by contrast, are generally synthetic materials that directly kill or inactivate the pest. Biochemical pesticides include substances, such as insect sex pheromones, that interfere with mating, as well as various scented plant extracts that attract insect pests to traps" [6]. To complete these categories, EPA specified the properties of biopesticides. They are "usually inherently less toxic than conventional pesticides"; they are specific i-e. they "affect only the target pest and closely related organisms, in contrast to broad spectrum of conventional pesticides"; "they are effective in very small quantities and often decompose quickly", and "when they are used as a component of Integrated Pest Management (IPM) programs, biopesticides can greatly decrease the use of conventional pesticides, while crop yields remain high".

Regarding the EPA biopesticide definition, it has to be emphasised that the category of PIPs is not recognised by the European Union (EU) as biopesticides because PIPs are transgenic compounds. In Europe, Genetically Modified Organisms (GMOs) fall under EC Directive 2001/18/EC, which requires risk assessment, labelling, and public information on GMOs, although all pesticides of any kind are under Regulation EC/1107/2009 "concerning the placing of plant protection products on the market" completed by Directive 2009/128/EC "establishing a framework for Community action to achieve the sustainable use of pesticides".

The current term uses in UE is not biopesticides but Biocontrol Agents (BCAs). Why BCAs and not biopesticides? Following a discussion within the REBECA Botanical Working Group, two reasons were given: (i) biopesticides which name is built by adding "bio" to "pesticide" evoke the bad marketing position of pesticides because of ecological hazards, and (ii) it is necessary to control pests but not eradicate them for better environmental balance. REBECA is the acronym for "Regulation of Biological controls Agents". It is one of the sources that popularized the name of Biological controls Agents (BCAs) as a new concept. REBECA belonged to the Sixth Framework Program of the EU and set in 2004. It was defined as "a task force to review current legislation, guidelines and guidance documents at Member State and EU level and compare this with similar legislation in other countries where the introduction of new biopesticides has proven to be more successful." To carry out this program, a coordination action gathered industrials, regulators, academic and researchers to make brain storming and to bring out proposals for a balanced regulatory environment which could lead to better access to biopesticides for growers and farmers. Another approach popularized the Biocontrol Agents concept: the book of the British Crop Protection Council (BCPC) edited by Leonard G. Copping which illustrated this evolution. BCPC decided to produce a book name "*The Biopesticide Manual*" in 1998 as "a companion of the long established *the Pesticide Manual*" (which is published over 40 years by BCPC), as said Van Embden [7]. This decision derived from the successes, reviews and sales for biologically based products. *The Biopesticide Manual* was covering in detail the biological effects observed with the products and indicated when it was known the modes of action of commercialized products.

The classification of the products changed with the editions. The main changes for the sections of the products were between the first and the second editions (respectively 1998 et 2001):

– The "Living system" section including baculoviruses, bacteria and fungi, Protozoa and nematodes changed into "Micro-organisms".
– The "Natural Products" section kept its title and included the substances (chemicals) derived from micro-organisms and higher plants as well.
– The "Insect Predators" section became "Macro-organisms" to clarify the classification because some of the organisms classified into this section were not necessarily insects. Parasitoids and predators belong to this section.
– The "Pheromones" section changes into "Semiochemicals" to be more accurate and included chemicals used in mating disruption, lure and kill or insect monitoring strategies.
– A section "Genes" gathered the genes that have been used to transform world crop to confer tolerance to herbicide application or resistance to attacks by viruses or insects.

Another significant change was coming with the third edition (2004). The title of the book became "*the Manual of Biocontrol Agents*" that illustrated the international change in attitude in the pest management. Van Lenteren [8] explained that "the initial trend occasionally to select non-chemical strategies for crop protection seen during the 1980s and 1990s has turn into the official pest management policy in many countries". This book contained the fourth sections indicated previously, but the fifth one "Genes" became an "Appendix", then disappeared in the 2009 edition because a new book, a world compendium entitled *GM Crops Manual* was edited by Copping in November 2010 (216 p). After Copping's retirement, Dr. Roma Gwynn continued the work and edited the *Manual of Biocontrol Agents* (5th edition published in 2014).

These considerations show that, whatever the name of biopesticide or BCAs, lots of efforts were devoted to developing the Biocontrol products.

17.3 The Development of Biocontrol Agents during the Two Last Decades

Because of more requirements for health and environmental safety, re-registrations procedures in several developed countries severely cut down the number of chemical active ingredients for PPP. In EU, more than 50% of active substances were not included in Annex I. Therefore, the reduction of the choice of chemical pesticides enhances the interest for BCAs.

Following the sections defined in the "*Manual of Biocontrol Agents*" which are now currently accepted, the number of BCAs increased from 175 products (including natural products, semiochemicals, micro-organisms and macro-organisms) in

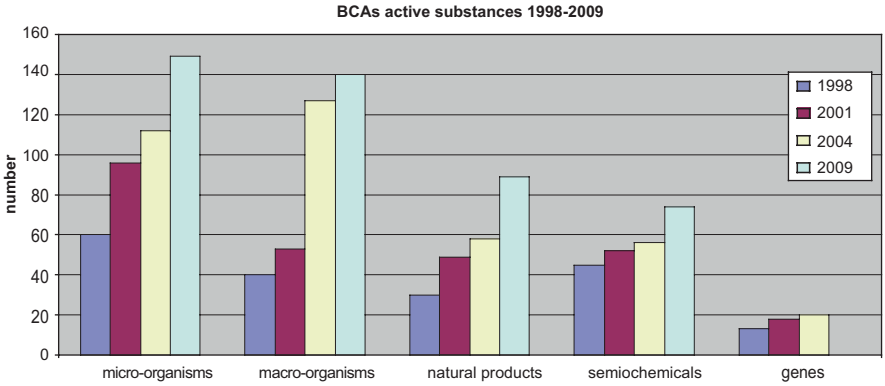

Fig. 17.1 Development of the number of BCAs active substances available for commercialization during last decade (according to L.G. Copping, The Biopesticide Manual (1998, 2001) and The Manual of Biocontrol Agents (2004, 2009))

1998 to 250 in 2001, 353 in 2004 and 452 in 2009. Consequently, the number of products available over 10 year is multiplied by 2.58. All products increased but not in the same way (Fig. 17.1).

Many new entries are noted for micro-organisms and macro-organisms more than for semiochemicals and natural products. Macroorganisms products are 3.5 higher in 2009 than 1998 and in the same period micro-organisms multiply by 2.5. These two categories represent now 64% of the entries. This development is constant but accelerates during the five last years. In the same time, natural products stay at the same levels around 18–20% and semiochemicals (pheromones) regress a little to down to 16%. Several arguments explain this situation: (i) the different kinds of sectors for which the BCAs products are used and the high-value crops they produce, (ii) the registration restrictions for some BCAs natural products which do not present all the guarantees for safety uses. The ratio benefits/risks is not the same for all BCAs as the following illustrations through examples will show it.

17.3.1 Micro-organisms

This category includes baculoviruses, bacteria and fungi, Protozoa and nematodes. Some micro-organisms are known to be pathogens for human, animal and plant pathogens, and to produce toxins but they also synthesize antibiotics. However, in contrast to chemical pesticides, micro-organisms BCAs (MBCAs) have a history of safe use.

Bacteria The most popular species used as MBCAs is undoubtedly *Bacillus thuringiensis* or Bt, a widespread Gram-positive, soil-dwelling bacterium. During sporulation many Bt strains produce crystal proteins (proteinaceous inclusions),

called G-endotoxins. There are many crystal producing Bt strains that do not have insecticidal properties but several have insecticidal action. Thousands of strains are divided in 70 sub-species. Some of them were selected because of the toxic properties of Cry protein for Lepidoptera caterpillars (*Bacillus thuringiensis kurstaki* Btk), for Diptera (*Bacillus thuringiensis israelensis* Bti), or Coleoptera (*Bacillus thuringiensis tenebrionis* Btt). Presently 13 strains of Bt are currently used in agriculture. They are commonly used as a biological alternative to a pesticide. The target site of G-endotoxins is the insect midgut. The cells of the midgut become paralyzed and the normal digestion by the insect is disrupted. Cry inserts into the insect gut cell membrane, forming a pore. The pore results cell lysis. Because of the mode of action of G-endotoxins involving very precise receptors, Bt develops a strong specificity.

Spores and crystalline insecticidal proteins produced by *B. thuringiensis* have been used to control insect pests since the 1920s. Valent Biosciences (ex-Abott) in Illinois, USA, is historically the first company which developed the Bt in formulation. The second manufacturer was Certis (California, USA) [9]. Bt is now used as specific insecticides under trade names such as Dipel® and Thuricide®. Because of their specificity, these biopesticides are regarded as environmentally friendly, with little or no effect on humans, wildlife, pollinators, and most other beneficial insects.

In fact, Bt occupies an important position of the sales of biopesticide global market because of controlling a wide range of harmful insect species by the different strains of Bt [10]. Bt subsp. *aizawia* (Bta) controls Lepidoptera like *Heliothis*, *Spodoptera*, *Helicoverpa*, *Pieris* species and also *Ostrinia nubilalis* (Hûbner) (European corn borer, ECB) or *Plutella xylostella* (L.). Bt subsp. *tenebrionis* are used against Colorado potato beetle (*Leptinotarsa decemlineata* (Say)). Bt is largely used to protect tomatoes cultivation, orchards and fruit trees (apple, plum, pear, cherry, peach, apricot, almond), rice, cabbages, red fruits, nuts, aromatic plants, vineyard, *Allium* spp. (garlic, onions, leek). Beside these agricultural uses, Btk is also used to protect forest against pest insects (*Thaumetopoea processionea*, *Lymantia dispar*). Formulations of Bt are also widely used by domestic gardeners and commercial growers.

However, not all caterpillar pests are equally susceptible to Bt. Bt is effective against ECB if it is applied just as the larvae are hatching. Bt formulations for use against Colorado potato beetle may vary in effectiveness [11]. But these observations are not general. Experiences were conducted to test the efficiency of Dipel® (Btk) and Xen Tari® (Bta) against *Helicoverpa armingera* to protect tomatoes and no significant difference was noted between the Bt and chemical reference [12]. However, the resistance of the insects is one of the problem induced by the wide use of Bt in formulation. Some moth species, including some populations of diamond back moth, have evolved resistance to the Bt variety *kurstaki* toxins [13, 14]. One of the solutions is to alternate the use of Bt strains. Duchon Dorris and Armengaud [12] observed that the uses of Dipel® (Btk) and Xen Tari® (Bta) alternatively on vine, vegetable cultivations or cotton avoided resistance phenomemon.

Baculoviruses are pathogens that attack insects and other arthropods. They are usually extremely small and are composed of double-stranded DNA that codes for genes needed for virus establishment and reproduction. The majority of baculoviruses used as biological control agents are in the genus *Nucleopolyhedrovirus*. These viruses are excellent candidates for insecticidal applications because of their specificity [15]. They do not show negative impacts on plants, mammals, birds, fishes, and more generally on non-target insects. However, the high specificity of baculoviruses is also cited as a weakness for agricultural uses, because of the narrow spectrum of its activity. They are used to protect vegetable crops, tomatoes, cabbages, orchards (apple, pear, walnut and plum), cotton, corn and also forest habitat. Their targets are for example Lumber Gypsy moth *Lymantria dispar,* Beet armyworm *Spodoptera exigua*, Tobacco budworm *Helicoverpa zea,* or *Spodoptera littoralis.* Some companies continue to explore the expansion and development of agricultural-use viral insecticides and the effectiveness of insecticide "cocktails" consisting of environmentally friendly chemical agents and baculoviruses [16].

Insect-Pathogenic Fungi Some insect species are particularly susceptible to infection by naturally occurring insect-pathogenic fungi. These fungi are very specific to insects, often to particular species, and do not infect animals or plants. Because fungi penetrate the insect body, they can infect sucking insects such as aphids and whiteflies that are not susceptible to bacteria and viruses. Infected insects stop feeding and die relatively rapidly. Their bodies could be enveloped but not always, by fungal mycelium from different colours which gave them the aspect of an embalmed body of mummy. Several fungal species have potential as microbial insecticides and, in some countries, are commercially available in formulations that can be applied using conventional spray equipment. Several isolates of *Beauveria bassiana* are commercialized (isolates GHA, 447, 74,040 Bb 147, HF 23) under several trade names Mycotrol® (Mycotech), Organiguard® (Emerald BioAgriculture) etc. They are used in a wide range of targets to control grasshoppers, locusts, for uses on ornamentals, non-food crops in greenhouses, and to manage house flies, aphids, thrips, and spider mites. Another fungus *Beauveriabrongniarti* was commercialized under the name of Betel® [17].

17.3.2 Macro-organisms

The macro-organisms section involves insects and mites that are parasitoïds or predators for other insects and also phytophagous insects and mites that can be used for weed control, in glasshouses, interior spaces and in outdoor agriculture.

Insect Parasitoids develop on or within a single insect host and ultimately killing it. The immature parasitoid feeds on body fluids and organs of the pest. The parasitoïd is a natural enemy of the insect pest. Most beneficial insect parasitoids are Hymenoptera (wasps) or Diptera (flies). They are specialized in their choice of host

and only attack a specific life stage of one or several related species. The life cycle and reproductive habits of beneficial parasitoids can be complex. Only the female searches for host and eggs are laid most of the time in or on the host. Different parasitoid species can attack different life stages of insect host. Adult parasitoids are usually more susceptible to pesticides than their hosts but immature parasitoids will usually die if their host is killed. Because of this specificity, biological control by parasitoids is a success story. The most emblematic example is the use of trichogramma against *Ostrinia nubilalis* (ECB). As an example, the French cultivation of maize protected by these parasitoids enhanced from 30,000 ha in 1997 to more than 150,000 ha in 2018. Additional information to educate growers for good practices in using the BCAs product including *Trichogramma brassicae* (TR16+® or Pyratyp Opti®) is provided by the company Biotop™. Other species of trichogramma are also developed. To face the invasive species from South America *Tuta absoluta* which was coming in France through the Mediterranean area, a BCA product has been developed based on *Trichogramma achaeae*, a parasitoïd identified in Spain for its efficacy on the pest [18]. A new product named Trichotop®-TA with this parasitoid is commercialized [19].

Insect Predators can be found in almost all agricultural and natural habitats. They are arthropods and they prey on insects and mites. They include beetles, true bugs, lacewings, flies, midges, spiders, wasps, and predatory mites. Some predators are specialized in their choice of prey, others are generalists. They kill or consume many preys and they attack immature and adult preys as well. Each group may have a different life cycle and habits and some of them are useful natural enemies of insect pests and they play a role in the suppression of the pests. Predators are used with success to control pests in glasshouses. The Chrysopidae *Chysoperla carnea* (Stephens), the Coccinellidae *Hippodamia convergens* (Guérin) and the Cecidomylidae *Aphidoletes aphidimyza* (Rondani) control aphids *Myzus persicae* (Sulzer), *Macrosiphum euphorbiae* (Thomas), *Aphis gossypii* (Golver) and *Aulacorthum solani* (Kaltenbach). The acarids *Amblyseius cucumeris* and *A. degenerans*, the bugs *Orius laevigatus*, *O. insidiosus* et *O. majusculus* control thrips on pepper *Capsicum* spp. [20]. The management of thrips by acarids was the most popular to protect cut flowers and flowerpots on more than 65 ha of French glasshouses in 2005 (Bertrand and Trottin, personal communication).

Unfortunately, if some predators are very useful insects, others prey on other beneficial insects as well as pests. *Harmonia axyridis* (Pallas), the Chinese ladybird, is one of the insects used as predator of aphids. It was introduced in North America, then in Europe (Benelux) and rapidly became an invasive species, killing native ladybird larvae in the country it invaded. It is a polyphagous voracious predator and puts biodiversity in danger. In U.K., a national survey website was launched to monitor its spread [17].

Weed Feeders Insects can control weeds by feeding on seeds, flowers, leaves, stems, roots or by transmitting plant pathogens, which will infect plants. The weed-feeding natural enemies develop several qualities that are used for controlling pests

in agriculture but also exotic plant species which have been introduced to new locations around the world. They are specific to one plant species and have a negative impact on plant individuals and the population dynamics of the target weed. They are prolific and good colonizers. Consequently they become widespread in all habitats and climates that the pest weed occupies. Shelton [11] was successful in controlling nodding thistle (Canada; Kansas, U.S.), ragwort (British Columbia, Canada; California and Oregon, U.S), klamath weed (Ontario, Canada; California, Oregon and southeast Washington, U.S.) alligator weed (Florida, Louisiana, and Texas, U.S.), and water lettuce (Florida). He underlined that using biological agents for weed control is beneficial because "once a population of biocontrol agents is established, minimal effort is required to conserve it" and therefore it is less expensive compared to herbicidal sprays.

17.3.3 Semiochemicals

According to the definition of OECD [21], semiochemicals (SCs) are chemicals emitted by plants, animals, and other organisms – and synthetic analogues of such substances – that evoke a behavioural or physiological response in individuals of the same or other species. They include pheromones and allelochemicals.

- Pheromones are semiochemicals by excellence and numerous researches were conducted to evaluate the role they play for social insects. They produced by individuals of a species that modify the behaviour of other individuals of the same species (i.e. an intraspecific effect). Pheromones are chemical mediators which are emitted by individual of a species to give indication to others about territory and movement, aggregation, mating, oviposition and nest-building, sexual maturation, alarm etc. [22].
- Allelochemicals are semiochemicals produced by individuals of one species that modify the behaviour of individuals of a different species (i.e. an interspecific effect). They include allomones (emitting species benefits), kairomones (receptor species benefits) and synomones (both species benefit). Plant allelochemicals are involved into plant defence as well as pollination. Because of their origin, plant allelochemicals could also be considered into botanicals.

Most SCs are volatile because their molecular weight is light. The volatility gives to this chemical signal an advantage for communication because it can travel long distances in the wind.

The specificity of the pheromones and their properties stimulated investigations on their potential for pest control. For over 20 years, several reviews and books focused on this topic [23–29]. The pheromones are used to lure insects and trap them following three main approaches: (i) detection and monitoring; (ii) mating disruption; (iii) attract and kill (or lure and kill) mass-trapping capture.

The principle of the use of insect pheromones for detection and monitoring is to attract insects to the trap in order to determine their occurrence in the field. Most

often, the trap bait contains a female sex pheromone to attract males into the traps. Consistent trapping protocols are essential to have relevant information for identification of the insects, the evaluation of insect populations and year to year comparisons. This monitoring gives very useful information for decision making on insecticide treatments in the fields, to survey and sample low density populations.

The mating disruption approach involves confusing males by placing several point sources of female sex pheromones in the field. The male follows false trails and expends mating energy in pursuit of artificial pheromone sources. Consequently, the reproduction of the targeted population is reduced.

The attract-and-kill mass trapping is based on formulations containing a combination of pheromone which attracts the insect, and an insecticide that kills it. According to Flint and Doane [30], damage to the target species was very limited, but success was reported against the Chinese tortrix *Cydia trasias* (Meyrick) to protect Chinese scholar-trees *Sophora japonica* L.; damage to the trees was reduced by about 70% following control of three generations [31].

The efficiency of pheromones as BCAs is not the same for the three strategies. The detection and monitoring approach is certainly the most efficient because trapping insects is a tool for further insecticidal treatments for organic farming and classical agriculture as well. It is currently also used on a large scale for experimental or conventional cropping. Another application is the monitoring of insecticide resistance and distribution in a population, because of the difficulties of sampling by traditional methods [32]. According to Royer and Delisle [33], the use of pheromone traps to survey the density of an arrhenotoc species is inappropriate but is relevant to follow the change of geographical distribution.

The success of mating disruption strategy for control of insect pests depends on the quality of dispensers to deliver a homogenous emission of pheromone to achieve a sufficiently saturated area for male confusion and capture [34]. Mating disruption strategies has been developed with success in the forests of North America as well as in arboriculture and vineyard in Europe [35].

The efficacy of attract and kill mass trapping strategy for control of insect pests largely depends on the targeted species. The knowledge of the biology of the species (monogyny, polygyny, protandry) as well as the density of population, the surface to be protected and the position of the traps are essential to the success of this method [33].

Biocontrol by pheromones is not as well developed as it could be. There are several reasons for this situation: the quality of pheromone formulations, the motivation of the agricultural producers and the cost of treatments:

– Most insect sex pheromones are multi-component with precise ratios of components which may be expensive to manufacture. The current commercial formulations of pheromones do not always sufficiently mimic the natural chemical blends pheromones from females. One difficulty is that the chemical signal changes according to the geographical distribution of insect species [36] and to season generation renewal [37]. Consequently, a comparison between a virgin

female in a trap versus a commercial pheromone showed the superiority of the insect [34].

– Another point to temper this approach is the high level of constraints for the farmer. Pheromone pest management needs the installation of many traps in which it is essential that the diffusers allow a regular and sufficient release of the pheromone. This also requires constant monitoring of the plots where the traps are distributed, and these plots must be isolated from external contaminations using reinforcement of the treatments at the edges of the treated area. This requires increased vigilance by the farmer to monitor the phases of development of the various parasites to avoid the phenomena of resurgence.
– The cost of the products (insect sex pheromone formulations and traps) is another factor that restricts this approach for insects of economic importance.

Except the current use for monitoring pests during the crop, the use of pheromone is relevant situations when conventional pesticides are not operating or when the environmental conditions (forests with high trees, arboriculture) do not facilitate the use of conventional pesticides. It is also appropriate for high crops values. In France, the biocontrol of grapevine moths is conducted by mating disruption method in Champagne in the past 20 years to protect this prestigious vineyard. Today Champagne has 9000 ha (42% of this area) protected by RAK® (BASF) for the 2010 season. Within entire France, only 4% of vineyards are protected by the mating disruption method. The company BASF explains that the particular professional organization of the growers into a Joint-Trade Organizational Committee on Champagne Wine (CIVC: Comité Interprofessionnel du Vin de Champagne) makes possible the management of this biocontrol method because wine growers are working together. The company underlines that "In Champagne, all the ingredients are there for the technique to work: a structured wine-producing network, motivated wine growers opting to "fight as a community" rather than to work individually, and a single joint-trade organization fighting for a common cause and participating in technical follow-up" [38].

17.3.4 Botanicals and Natural Products

This category of compounds includes a widespread range of compounds or extracts with very various properties. Beside plant extracts like Ryania or Sabadilla which contain a mixture of several alkaloids as active ingredients, plants essential oils, rosemary and clove oils, jojoba oil etc. are found and also plants allelochemicals (thymol, linalool) or laminarine, an algal extract. Micro-organism derived compounds with insecticide properties (streptomycin, spinosad), yeast extract hydrolysate and Actinomycete derived herbicide (bilanofos) or fungicide and bactericide (kasugamycin) are classified within [17]. Botanicals and plant derived compounds take up for 60% of these compounds.

The botanical extracts come from fractionation of the plant by various processes and their composition varies depending on the botanical sample, the experimental conditions and the physicochemical properties of the compounds. Thus, the extracts from the same plant are not only complex but also the molecular composition is very variable from one extraction to another. Moreover, the complexity of the plant metabolism results in a large number of molecules. Mendelsohn and Balick [39] estimated more than 500,000 plant allelochemicals.

For decades, the use of botanicals was more focused on the control of insects than other plants organisms. They are repellent, antifeedent, antinutritional, or neurotoxic. More generally, they affect the biotic potential of parasites and pests. Plant extracts and allelochemicals act also on a broad diversity of species like nematodes, phytopathogene micro-organisms (fungi and bacteria), as well as other species plants (allelopathy). In recent years, the improvement of knowledge of plant resistance mechanisms against bio-aggressors underlined that plants allelochemicals play an essential role in plant defence. Phytoalexines are low molecular-weight compounds of a non-proteinaceous nature, mainly belonging to polyphenols, terpenoïds and polyacetylenes. They are synthesised de novo in response to biotic or abiotic stresses and participate in plant induced resistance. Others, for example diferulates, are involved in the mechanical and biochemical barrier that constitutes the wall of maize grain [40, 41].

Consequently, the potential of plant allelochemicals and botanicals for plant protection could be used in two alternative strategies. The first one aims at reinforcing the protection of the plant using traditional soaps with formulation including plant allelochemicals or plant extracts as active ingredients. It is the oldest use which has been made of plant extracts and allelochemicals. The second one aims at reinforcing the plant defence by developing its own mechanisms through allelochemicals. It is more recent and probably less risky than the first one.

17.3.4.1 Botanicals in Formulations

The commercialized pesticide soaps and specialties including plant allelochemicals and botanicals can be used in both organic and conventional agriculture depending on the formulation.

Plant allelochemicals and botanicals are still not used in plant protection to their full potential. Before the second World War four main groups of compounds were commonly used: nicotine and alkaloïds, rotenone and rotenoïds, pyrethrum and pyrethrins, and vegetable oils. Some of them had several inconvenient properties because of their toxicity on non-target species (nicotine) or the instability of the molecules (pyrethrum). As a consequence, the use of these substances decreased with the commercialisation of chemically synthesised insecticides which moreover were easier to produce and handle and were less expensive. But as a result of the many demonstrations of the ecological hazards of synthesised insecticides, there was renewed interest in the 1970s for botanicals. Research on neem (*Azadirachta indica*, Meliaceae) illustrated this renewed interest. In fact, botanicals as products of

metabolism resulting from species co-evolution exhibit many advantages like selectivity, specificity, biodegradability [42].

However, during the whole twentieth Century, only a limited number of botanicals or plant allelochemicals were used for crop protection. Isman [43] indicated that only a few botanicals and plant extracts are currently commercialised. Four sub-stances are mainly used, pyrethrum, rotenone, Neem, and essential oils, followed by nicotine, ryania and sabadilla for minor uses. Several factors hamper the industrial development of insecticide formulations containing plant compounds [1, 44, 45]. Beside economical and commercial considerations such as availability of the raw material and its accessibility, or standardization and refinement of plant commercial products, the toxicity of plant extract compounds on non-targeted species is not negligible. Although they are natural, all products are not necessarily safe for people and for the environment. The current claims that plant protecting products or BCAs should not posed unreasonable risks to people or the environment, means that the evaluation of these compounds meet today's most stringent standards of scientific knowledge. In this context, the risk assessment for botanicals and plant allelochemicals have to be evaluated by taking into account their toxicological nature and their ecological advantages, as well as the exposure scenario linked to the current use of the formulated end products. As an illustration, let us consider the most commercialized botanicals.

Nicotine was one of the first molecules used as an insecticide since the use of aqueous extracts of tobacco against the sucking-piercing insects of cereals was mentioned in 1690. But the active molecule of this plant, nicotine, was isolated only in 1828 and later synthesized in 1904 [46, 47]. This very stable alkaloid in its levogyre form is neurotoxic for insects, mammals and birds. It is an acetylcholine mimic, interfering with the transmission of signals in nerves. The LD_{50} of nicotine is 50 mg kg^{-1} for rats but 3 mg kg^{-1} for mice. A dose of 40–60 mg can be a lethal dosage for adult human beings through paralysis of respiratory muscles and doses as low as 1–4 mg can be associated with toxic effects in some individuals. Nicotine is neither an initiator nor a promoter of tumours in rodents, but it is also toxic for birds. Today, some countries like China or Bolivia use nicotine to protect rice cultivation (by immersing the stems of tobacco in the plantations) and potato fields (spraying) [48]. In the USA, nicotine registration is a restricted pesticide use only in greenhouse for ornamentals against adult whiteflies, aphids, and thrips, since May 2008, because of risks for applicators both during and after application, for people who might be exposed to nicotine residues in treated greenhouses, and for consumers of plants from treated greenhouses [49] The Commission of the European Communities decided to not include nicotine in Annex I because it was not demonstrated "a safe use with respect to operators, workers, bystanders and consumers" [50].

Pyrethrum is presently the botanical the most sold on the market. It results from a powder obtained by crushing dried flowers of daisies belonging to the family of Asteraceae: *Chrysanthemum* spp.*, Pyrethrum* spp.*, Tanacetum* spp. *Chrysanthemum cinerariaefolium* Benth & Hook was first used in Europe in the 1800s against lice

and flies [51]. Other species of *Chrysanthemum, C. roseum, C. tamrutense* and *C. carneum* also contain significant amounts of pyrethrum. Pyrethrum or pyrethrins is a mixture of six esters pyrethrins They are very toxic and act very quickly on insects and have low to moderate toxicity towards mammals Toxicity is mentioned for non-targeted species, especially fish, invertebrate and bees. However, its great instability in light, air and moisture considerably reduces risks related to its use. Despite its high production cost, it is a natural insecticide that is currently widely used (1000 tonnes of pyrethrum are sold every year with about 90% being used in non-agricultural sites in USA) [52]. It is recommended for the control of flying and crawling insects and arthropods and mites on fruits, field crops, ornamentals, greenhouse crops and house plants as well as stored products, domestic and farm animals. It is normally applied in combination with piperonyl butoxide, a synergist that inhibits detoxification [17]. Pyrethrins were included for uses as insecticide only in Annex I of Council Directive 91/414/EEC in December 2008. It entered in force in September 2009 until August 2019 [53]. But EPA [52] concludes that pyrethrins are eligible for reregistration provided mitigation measures. Because pyrethrum is registered for use in agricultural, residential, commercial, industrial and public health sites in USA, these mitigations focus on the restriction for (i) using the end-products in specific places (e.g. nursing homes, hospitals, schools etc.), and (ii) on the method of application of the end-products and the protection equipment required for applicators, and (iii) the number of application for agricultural use in relation to the season and the pest pressure. This example demonstrates that the most popular botanicals must be used cautiously.

Rotenone is widespread in Fabaceae (ex Papilionaceae) growing in Asia (*Derris* spp.) and in America (*Lonchocarpus* spp.). Rotenone is one of oldest insecticides used all over the world. The use of crushed roots of Fabaceae to catch freshwater fish by native populations of South America was mentioned as early as 1665 [54]. The active ingredient belongs to flavonoïds. Rotenone inhibits cellular respiration and energy metabolism at the level of the mitochondrial respiratory chain. Harmless for warm-blooded animals, it is very active against cold-blooded animals such as amphibians, fish and reptiles. Although some accidents were reported with enzymatic inhibition, rotenone was regarded for a long time as being moderately toxic for mammals. Cases of chronic toxicity leading to kidney and liver damage were noted, and it was also found to be carcinogenic for rodents [55]. More recently a link between rotenone and Parkinson's disease was hypothesised [56]. Rotenone used alone is not toxic for bees but is lethal in combination with pyrethrum [17]. Rotenone is now classified in the highest category of toxicity [57]. Until recently, rotenone was used in organic agriculture alone or associated with other ingredients such as pyrethrinoïds, synergist (piperonyl butoxide), sulphur or copper to control a wide range of arthropod pests including aphids, thrips, moths, beetles and spider mites. However, following the regulatory update 46/2007 within the frame of Directive 91/414/EEC and EC Decision (2008/317/EC) published on 10 April 2008, because of a lack of required information, the rotenone substances should not be included in the Annex I to Directive 91/414/EEC and consequently was withdrawn

from the European Union plant protection products market at the date of October 10th 2009 [58]. Nevertheless, rotenone has been granted essential use in the UK, Italy and France until 2011 on fruit trees, ornamentals and potatoes only. This derogation was limited to professional users with appropriate protective equipment. The uses of rotenone were also restricted in the USA for livestock, residential and homeowner use, domestic pet uses, and all other uses except for piscicide uses. Consequently, rotenone was registered to be applied directly to water to manage fish populations in lakes, ponds, reservoirs, rivers, streams, and in aquaculture, to eliminate completely or partially undesired fish species in the treatment area [57].

Neem is extracted from *Azadirachta indica* A. Juss which is native to arid regions of India. The ability of the oil to repel pests has been known for thousands of years. The oil has also been used on skin and medicinally. Neem is a part of the traditional practices in India. It is a mixture of more than 100 limonoid compounds including azadirachtin, salannin, and nimbin and their analogues. All these compounds act differently and numerous effects of Neem on insects have been reported. Salannin causes repellence and feeding deterrence, while azadirachtins are the only compounds that have a significant activity as inhibitors of insect growth [58]. This results from an inhibition of the synthesis of ecdysteroids with, as a consequence, a disruption of moults and of the reproductive cycle of the insect. Neem oil was classified by EPA [59] in class IV (no significant mammal toxicity). It had a mild (minimal) effect on skin sensitisation and eye irritation but was not cytotoxic and mutagenic according the test of Ames. However, Kleter et al. [60] reported that, according to Boeke et al. [61], some unknown hazards with new extraction methods would produce toxic effects of the Neem extract in mice and guinea-pigs with gastro-intestinal spasm, hypothermia and death with 200–400 mg kg^{-1} of leaf extract. Neem and azadirachtin were recently suspected to be endocrine disruptors but with contradictory results [62–64].

In relation to its environmental impact, Neem is sensitive to light and degrades in water [44]. Consequently, it has limited persistence in the environment. The half-life of azadirachtin A after spraying on leaves of tomato or potato was 1 day [65]. A study on six aquatic organisms (crayfish, shrimps, mosquitos' larvae, water fleas) concluded that the risk values of azadirachtin and neem-based insecticides (Neemix™ and Bioneem™) did not exceed the criteria. No ecological hazard was likely to result from their use [66] or from the forest pest management application on aquatic macroinvertebrates [67]. Azadirachtin acts on a wide range of insects: balsam fir sawfly *Neodiprion abietis* (Harris), thrips, leaf miners, aphids, caterpillars, pine false webworms. It deters certain insects, such as locusts, from feeding, and it interferes with the normal life cycle of insects, including feeding, moulting, mating and egg laying.

Tested on over 300 species, it has been effective on 90% of susceptible species with a large variability of DL50 [68]. Recommended by the National Research Council of "Tree for solving global problems" [69], Neem is considered by some experts to be a remarkable BCA [70, 71]. However, the development of this

insecticide is hampered by: (i) cultivating the plant on a commercial scale; (ii) extraction of the active ingredients; (iii) development of persistent formulations and shelf life [72]. According to Kleeberg and Ruch [73], the standardization of Neem seeds extracts, which show a large variation of azadirachtin content, is one of the key factors to enhance the commercialization of Neem products. Neem and azadirachtin are currently registered in several countries. In USA, azadirachtin was first registered in 1985 [56] and the Clarified Hydrophobic Extract of Neem Oil, a naturally occurring compound in 1995 [74] and in European Union azadirachtin (Margosa extract) was approved in June 2011 by the Commission of the European Communities until May 2024 [75].

Essential Oils (EOs) know a strong development because they have wide range activities against bacteria, virus, fungi, weeds and also insects (insecticide, antifeedant or repellent). Grieneisen and Isman have counted more than 1300 published articles per year devoted to botanical research efforts since 2012 [76], with 20% focused on EOs including numerous reviews [77–83]. EOs have many industrial applications since a long time. They have been used since ancient times as cosmetics and pharmaceuticals, also since the last century in fine chemistry and aromatics for the food industry and until today they still play a major role in our daily life. Moreover, the plant protection in agriculture is now one very important focus of EOs activities. They develop activities as antipests (insects, fungi, bacteria, nematodes and virus), as herbicides and for weeds control, as compost to improve the conditions of the soil, and some of them have an effect in post-harvest disease control [84].

It appears from these examples of the main botanicals that have been available on the market over the last decades that the situation is complex and that only a few compounds used in insecticide formulations really appear to have a future as BCAs.

17.3.4.2 Plant Extracts and Allelochemicals Enhancing Inducted Resistance

The stimulation of plant induced resistance involves not only plant allelochemicals, but also other microbial, fungal or mineral molecules. Among the elicitors, plant polyphenols are strongly implicated in these mechanisms [85]. The elicitors currently identified are mainly of microbial origin but plant extracts from *Hedera helix* L., *Salix alba* L., *Viscum album* L., *Alchemilla vulgaris* L., *Reynoutria sacchalinensis* (F. Schmidt) were identified as inducers of resistance against Fire Blight of apple and of Cotoneaster watererii [86]. *Reynoutria sacchalinensis* induced phenolic phytoalexins. Marketed under the name of Milsana® (KHH Bioscience), it is used particularly in North America for the protection of ornamental plants like roses and begonias, and also against various Oïdium of vegetables and fruit [87]. *Macleaya cordata* extract registered under the name of fungicide Qwel® (Camas Technologies Inc), induces increased amounts of polyphenolic phytoalexines and also SAR (Systemic Acquired Resistance) [17]. Another plant extract, *Trigonella foenum*

graecum L., marketed under the name of Stifénia®, was approved against the vine oïdium [88]. Plant inducers act on a very broad spectrum of plant species and fungal and viral pathogens as well, whilst the expression of their efficacy can be cultivar dependent. In the same context, studies highlight that the physiological stage of the treated plants plays a significant role in the expression of the stimulation of plant defence; for example, Stifénia® whose use is recommended before flowering. Elicitors to be efficient must be used at a receptive physiological stage of the plant.

The limit of this technology is probably the incomplete control of disease (20–85%) or non-significant results under field conditions because the expression of induced resistance is influenced by environmental conditions, genotype and crop nutrition. However, the stimulation of natural plant defence will provide a useful and practical approach to be used in association with fungicides, by decreasing the frequency and amount of chemical treatment [89].

This overview underlines that BCAs provide interesting approaches to decrease the widely uses of synthetic pesticides in plant protection. Is it any consequence on the PPP market?

17.4 BCAs (Biopesticide) Market Outlook

17.4.1 A Strong Increase Since the Last Decade

The biocontrol market was evaluated in 2005 for 670 million USD that represented 2.5% of global pesticides market (26.7 billion USD). The total market for global and synthetic pesticides was valued at 37.86 billion USD for 2009 [90]. Pesticides industry was expecting a $45 billion global market for 2014 with a global demand for pesticides rising 2.9% annually and reached57.5 billion in 2018. It is projected to register a CAGR of 3.4% during the forecast period (2019–2024). The herbicides accounted for the largest share of 42.7% in the market, followed by fungicides and insecticides with 28.4% and 24.3% in 2018 [92].

Compared to this situation, the biopesticide market remains small even if it increased substantially (2.5% of global pesticides market in 2005, but 6.4% in 2018). The last evaluation of the biocontrol market revenue was $3.7 billion in 2018, and during the forecast period 2018–2023 this market will consider growing with a healthy CAGR of 9.86% [91]. North America is the leader for using biocontrol products following Europe including Russia. These continents are considered to be high growth rate according to their well-established and mature agricultural markets. They are followed by the mid growth rate countries such as China and Japan, Pacific and South East Asia including Australia and New Zealand. The last group is represented by Africa, Middle East and South America which develop a low biocontrol market [92]. However, the Latin America biocontrol market was valued at USD 423.7 million in 2015, and is projected to grow at a CAGR of 16.5% to reach USD

1050.7 million by 2021 [93]. Consequently, the demand for BCAs is rising steadily in all parts of the world.

The surface area for organic agriculture increases in several countries. In Western Europe, they lay over than 7287 million ha compared to total agricultural surface area of 178 million ha (4%) in 2007; then 10 years later, in 2017, it was evaluated 12.6 million hectares of agricultural land in the EU-28. This corresponds to 7% of the total utilised agricultural area of the EU-28. It represents an increase of 25% between 2012 and 2017. The countries with the highest shares of organic land were Austria, Sweden and Estonia. Organic farming is quite developed in Austria with 11.5% (372,000 ha) in 2007 and 23.4% of total utilised agricultural area in 2017, followed by Estonia (19.6%) and Sweden (19.2%), then by Italy (14.9%), Czechia (14.1%), Latvia (13.9%) and Finland (11.4%) [94].

17.4.2 Key Points for Developing BCAs

Biocontrol Agents will develop not only through the enhancement of organic farming but also through Integrated Pest Management (IPM). The strong increase coincides with the growth of control of biological pest in the sector of high-value crops like vegetables in greenhouses, vineyard, tree and fruit farming. The example of vineyard and apple trees biocontrol in Germany is quite representative. The organic farming lays on 4% of surface area for vineyard but IPM using Bt and pheromones totals up to 60% of the surface area for this cultivation. For apple, organic farming cultivates 8% vs. 45% for IPM using baculoviruses and pheromones [95]. IPM using the relevant tools for controlling pests in a friendly environmental approach, will undoubtedly be the future for BCAs.

Many BCAs were developed at the beginning by SMEs and occupied what it is called a “niche market”. But now major companies are interested by this sector. Since 20 years, a ballet of merging-acquisitions has occurred between leader companies of PPP market and numerous SMEs or start-up societies of the field of biotechnologies and biocontrol. They were acquired by these major companies which contribute to stimulate the biopesticide market in the next years.

Huge progress in manufacturing, transportation and conservation of BCAS were done during the ten last years. Because BCAs include into formulations living organisms or natural substances which require particular conditions (e.g. temperature as cold chain), they need to be manipulated with care and caution. These special conditions are now integrated into the quality and traceability processes of manufactures.

Regulation is another point that could be improved to develop BCAs. Before they can be used as plant protection products, BCAs have to be registered. In the registration process, the risk assessments associated with their properties and their uses have to be evaluated. These risks are linked to the toxicity on the organisms and populations, as well as the exposure. Potential hazards for humans (operators, bystanders, consumers), wildlife and the environment (fate in air, soil and water,

non-target organisms including the routes to which they are exposed) must be identified and evaluated depending on the uses of the end-products. The regulation is not the same for macro-organisms, micro-organisms, semiochemicals and natural products, neither the regulation rules in UE, USA, Canada, Australia and New-Zealand [96]. For example, in USA, registration procedure is under code 40 CFR (Code of Federal Regulations) of application of FIFRA (Federal Insecticide, Fungicide and Rodenticide Act). The Registration Eligibility Decision (RED) of EPA details clearly the risk assessments linked to uses and exposures. Under considerations based on experiments and reliable data, many natural products are now considered to be minimum risk pesticides (40CFR 152.25f). They are listed (list 25b) and are exempt from the requirement of FIFRA. In EU, a new regulatory framework was established in 2009. The Directive 2009/128 / EC, has implemented a new regulatory framework for sustainable use of pesticides (PPP) in order to have to better address the health and environmental concerns of society. It gives priority to integrated pest management. Regulation (EC)1107/2009 introduced the concept of low-risk active substances or plant protection products (Articles 22 and 47). The globalization of the commercial exchanges, of which BCAs (biopesticide) world market is a part, strongly needs a harmonization of the regulatory rules at an international level.

17.5 Recent Lessons Through a Case Study

In France, very active public policies push for the development of biocontrol products. Over the past decade, public Authorities have decided to promote a plant protection approach for reducing the use of synthetic plant protection molecules. Consequently, the biocontrol market is presently expanding in this country.

17.5.1 A New Law for Developing BCAs

Following the Directive 2009/128/EC, several action plans named “Ecophyto” were implemented at the French level with the purpose to have a progressive reduction in the use of synthetic plant protection products (PPP). The adoption of a new law called “Avenir pour l’Agriculture” of October 13, 2014 followed. This law intends to promote an agro-ecological approach and in the same time to develop biocontrol products on the market. This last point resulted of a parliamentary mission conducted by the MP (deputy) Antoine Herth. The conclusions of this mission led to the 2011 report titled: “Bio-control for Crop Protection, 15 Recommendations to support Green Technologies” [97].

17.5.2 Biocontrol Market: A Dazzling Development for the Last 4 Years

As the result of this foresighted policy, the biocontrol products left a "niche market", conquering market shares with double-digit growth (+25% between 2015 and 2016) [98]. This situation leads today to a market 110 million Euros with an increase of 18% in the agricultural sectors (named Agri) and 54% for gardens, green spaces and infrastructures (also named JEVI) (Fig. 17.2). This strong increase in JEVI was the result of the Ecophyto II plan which banned the uses of "non-organic" phytosanitary products in public gardens since 2017 (and 2020 for all public spaces).

The latest assessment indicates that the biocontrol market today represents 5% of the plant protection market in this country. The ambition of the International Biocontrol Manufacturers Association (IBMA) in France is to reach by 2025 the 15% mark that means to multiply by 3 the current sales of biocontrol products. A survey of the Company ADquation Marketing Studies specialized in agricultural studies in France" (http://www.adquation-em.fr/fr) was conducted in June 2017. It showed that microorganisms, which account for 10% of the market for biocontrol products, grew by 29%; macro-organisms that account for 15% of sales increased by 12%; chemical mediators (mainly pheromones) increase by 14% with a market share of 18% and natural substances occupying 57% of the French market are displayed with +33% (Fig. 17.3).

The IBMA pointed out three actions to develop the sector: (i) promoting innovation through the development of research; (ii) informing for raising awareness of existing solutions; (iii) training to learn how to use these new products; and (iv) encouraging supportive public policies such as regulatory reform to streamline the approval procedures for these products. This last issue has been demanded for several decades by the Biocontrol Industry, which claims that these substances of natural origin are part of everyone's daily life (for example, garlic extract or orange essential oil).

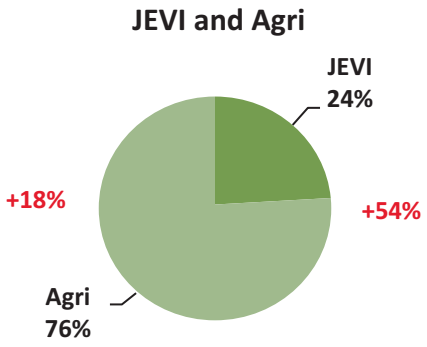

Fig. 17.2 Percentages of biocontrol products in agriculture (Agri) and JEVI (gardens, green spaces and infrastructures) according the survey of ADquation Marketing 2017

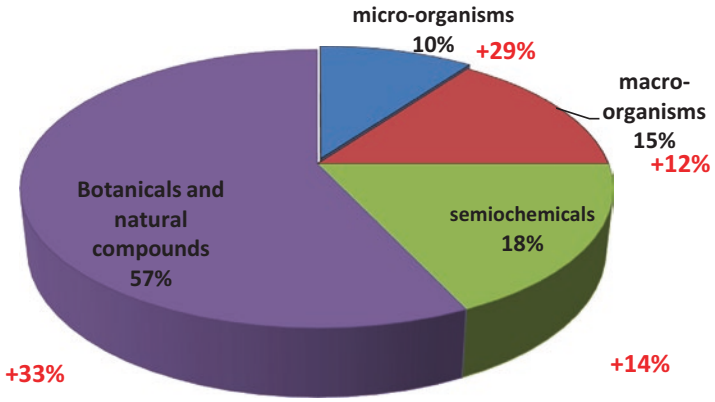

Fig. 17.3 Growth of Biocontrol products in France expressed as a percentage of sales of IBMA France members (according to the survey of ADquation, 2017) Marketing studies published in June 2017

17.5.3 Clues for Developing Biocontrol Products

As mentioned above (6.4.2.), the production of these active substances requires special know-how. Several technological locks that previously prevented the development of biocontrol products have indeed received solutions over the past ten years. Improvements of special conditioning, handling and storage conditions required by the biological organisms or biological substances, and of the standardization of finished products were noticed. That is why the number of biocontrol product solutions based on macro-organisms and micro-organisms has been growing strongly in recent years.

These technical improvements facilitate the implementation of biocontrol devices in the plots and have reduced the performance of the tasks by operators. The cost of the work associated therewith consequently decreased. However, the implementation of biocontrol solutions is generally more expensive (not only the labour but also prices of raw materials and active substances) than conventional treatments of plots. For example, the sexual confusion (pheromone) biocontrol method which covers more than 50% of the Swiss and German vineyard is subsidized, both countries granting financial aid to offset costs.

But already other innovations appear that will upset the current conditions of use of biocontrol products. Trichogramma releases by drones in maize crops have been applied successfully to areas in South western France, and the treated surfaces was multiplied by a factor of 10 in two years between 2015 and 2017 (Delos personal communication). This example shows that the costs associated with the use of biocontrol products will undergo in the coming years profound changes related to the techniques and tasks to be performed.

Developing the BCAs market in a coherent manner requires the pursuit of a rigorous scientific approach, based on advances in knowledge to promote

agro-ecological, scientific and technological agriculture. According to this consideration, is it therefore reasonable to encourage by derogations or hazardous authorizations, because they are "natural", the placing on the market of BCAs based on plant extracts whose active ingredients are known for their endocrine disrupting properties or which is advised to spread considerable doses to have the required efficacy (e.g. a garlic nematicide extract recently approved for doses of 25 kg/ha)?Developing biocontrol products to reduce the use of synthetic plant protection products makes sense only if they lead to significant progress in protecting the environment and preserving human and animal health. It is not because they are natural that biocontrol products could be used without risk. Some of them could be toxic like Boldo or Pennyroyal essential oils with inappropriate use [78]. Biocontrol products, whether natural extracts, microorganisms or macro-organisms should be subject to the same treatment that synthetized PPP for commercialization but with protocols linked to their specificity. Preserving plant health through biocontrol therefore presupposes not only promoting public and private research in this area and encouraging the technological progress made by agribusiness, but also applying a rigorous biomonitoring approach.

This outlook on biopesticide market gives us hope that a strong reflection is now conducted to develop the BCAs. This would lead to a better use of chemical and non–chemical pesticides in a combined action in order to strengthen sustainable agriculture.

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