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Ravi S. Singh
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Genetic Manipulation of Secondary Metabolites in Medicinal Plant

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Preface

Secondary metabolites are diverse chemical compounds with a wide range of bioactivities, and their presence in certain organism, including plants, is crucial. Understanding their biosynthesis, storage, transport, function, and elicitation are some of the important aspects for their genetic improvement. Since secondary metabolites in plants are naturally synthesized in low amounts, unlike primary metabolites, and mostly extracted from wild species, their production is commercially costly and, in most cases, a non-viable option in contrast to extraction from cultivated ones. Hence, medicinal plants are being intensively researched globally, and technologies are being developed to harness these plants for secondary metabolite production. Genetic manipulation is one such area where conventional and biotechnological approaches have been attempted for improving desired traits, including the yield of secondary metabolites in medicinal plants. Furthermore, advances in sequencing technology, genomics, molecular biology, transgenic technology, and gene editing have hastened and simplified the tasks of deciphering genes/regulatory elements/proteins and elucidating their functions.

This book entitled *Genetic Manipulation of Secondary Metabolites in Medicinal Plant* is prepared with updated information on the genetic manipulation of secondary metabolites and related aspects in medicinal plants.

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Chapter 1
Conventional Approaches Toward the Production of Bioactive Compounds from Medicinal Plants

Anjani Kumar, Kanchan Bhamini, and D. N. Singh

Abstract Bioactive compounds in plants are basically secondary plant metabolites. These bioactive compounds are formed within the medicinal plant via the primary metabolic pathway and are not essential for its normal growth and development. Bioactive compounds found in medicinal plants have a lot of phytoceutical properties; these bioactive compounds protect plants from several foreign particles and are utilized for cell signaling. Apart from this, these bioactive constituents are used to prepare valuable products such as medicines, perfumes, flavorings, pesticides, coloring agents, etc. Therefore, medicinal plants are more popular among the people because of their applicability to the treatment of common as well as chronic diseases. Molecular biology as well as biotechnology shows a very significant role in the field of pharmaceutical compound. Nowadays, there are various important biotechnological as well as molecular biology tools available like micropropagation, embryo culture, anther culture, and fermentation based tools that have enabled in vitro production of bioactive compounds. Micropropagation is widely exploited to enhance the production of bioactive compound, but it is costly. So, the bioreactor based production of pharmaceutical constituents in medicinal plants through the culture of plant cells is not feasible. Elicitation will also act as a tool for the enhancement of pharmaceutical compounds in medicinal and aromatic plants. This paper provides a detailed description of the various conventional modes of producing bioactive compounds for better understanding and summarizes their potential to help evaluate their suitability and economic feasibility.

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1.1 Introduction

Medicinal plants are generally used to treat common as well as chronic diseases, because they are rich in bioactive compound. Nowadays, the incidence of disease is very high due to the changing lifestyle of people; therefore, it is necessary for the development of their particular medicines. Currently, several synthetically derived medicines are used for the treatment of various diseases which are not economically feasible as well as cause opposing effects (O'Donovan et al. 2019). All synthetically produced medicines have the potential to cause adverse effects, and it can manage the disease for a few times as well as sometimes intensify the patient's conditions. Therefore, adopting alternative approaches to completely excludes the diseases through applying comparatively safer, more effective, and more affordable techniques. However, medicines are categorized into two broad groups: one comes under synthetically produced medicines, and other belongs to the naturally formulated medicines. Synthetically produced drugs are less common due to their side effects; however, natural based drugs always give a better result (Karimi et al. 2015; Tewari et al. 2019). Organic based as well as naturally derived medicines and herbs are more preferred by people for the purpose of medications. Hence, the development of naturally produced drugs is significantly increased due to their popularity. The medicines obtained from medicinal plants are the big challenges that are observed in the pharmaceutical industries (Li and Lou 2018). The importance of organically as well as naturally derived products is due to the phytocompounds present in the medicinal plant. The phytoceutical compounds in plants are constituents produced by plants that have many medicinal properties. The distinctive bioactive compounds in medicinal plants are synthesized as secondary plant metabolites. The biosynthesis of major secondary plant metabolites and the interaction between different primary metabolic pathways are depicted in Fig. 1.1. This is observed that more than 80% of the people around the world generally use plant based medicines to cure primary healthcare needs (WHO 2019). The efficacy of medicinal plants is highly dependent on the pharmaceutical components contained therein. The area of bioactive compounds is not limited to treating the diseases. However, this medicinal plant provides a commercial value to human welfare, such as shikonins and indigo applied for dye purposes, to induce flavoring vanillin and capsaicin are used, fragrances are also induced through rose and lavender oils, caffeine and nicotine used as stimulants as well as nicotine and piperine highly used as insecticidal purposes. Currently, progresses in the field of natural plant product, phytomedicine and bioassays, have directed to the discovery of numerous bioactive molecules that are being used as phytoceuticals, nutraceuticals, cosmeceuticals, and other purposes. These plant-derived natural metabolites are the main home of numerous medicines, which are directly or indirectly used as modified/copied synthetic product. Moreover, wide cultivation of medicinal plants totally depends on many factors such as selection of

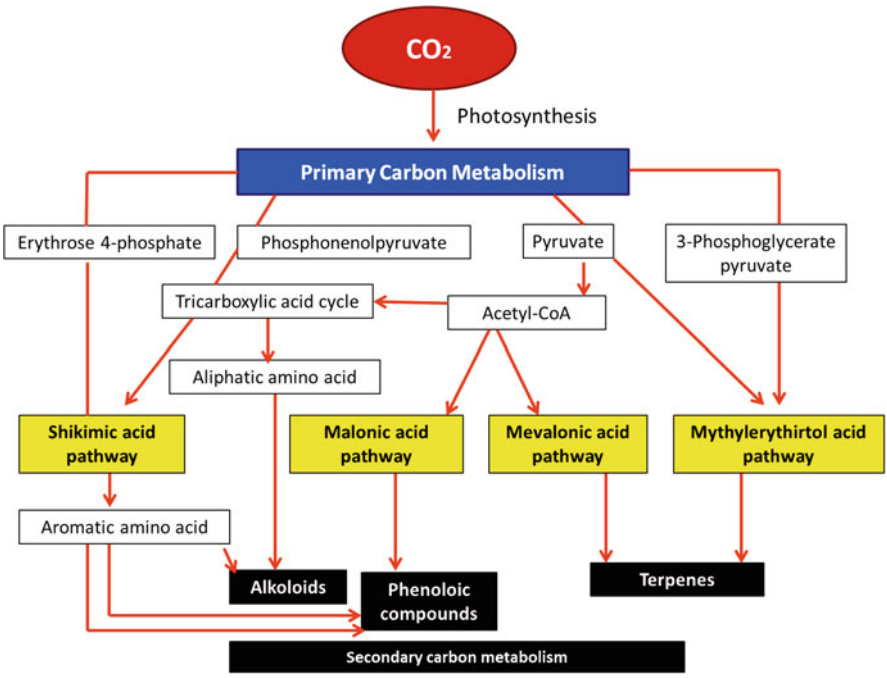

Fig. 1.1 Outline for the biosynthetic pathways related to biosynthesis of secondary metabolites as well as their interrelationships with primary metabolism in plants. (Adapted and modified from Uwineza and Waśkiewicz 2020)

suitable seeds or propagating materials for vegetative propagated crop, and decent cultivation practices, as well as appropriate management approaches such as fertilization, irrigation, and weeds, pests, and diseases control should be accepted to attain a higher yield with good quality of the raw material. The position of biologically active compounds in medicinal plants depends on the cultivation practices, traditional plant breeding approaches, novel molecular biology techniques, and biotechnological methods. Hence, it is very important to advance the numerous cultivation operations in that way to yield plant based products to meet the demand and supply for the medicinal industries around the world. Application of chemical fertilizer also increases the yield as well as overall plant based compound in medicinal and aromatic plants. However, higher application of this chemical based fertilizer causes severe problem in plant as well as environment. One of the big demerits of these chemical based fertilizers is their prices; small scale farmers could not purchase fertilizers due to their cost. Apart from this, modern biotechnology as well as molecular biology approaches has been highly applied for the making of bioactive compounds. But this is very expensive and practically not feasible for small scale growers. As we know that, bioactive compounds are not involved for the usual growth and development process of plant. However, this compound protects plant from pathogens as well as foreign particles. These secondary metabolites are used to

making food additives, flavors, and other valuable industrial materials for human welfare. Secondary metabolite is produced within the plant via the primary metabolism pathway. Plant cell culture is also used for the production of secondary metabolites, but it faces several biotechnological boundaries. However, the producing plant secondary metabolites in very low quantity through plant cell culture. Currently, the production of bioactive compound by means of transgenic hairy root culture is most demanding tool for plant tissue culture. Current achievement in the area of molecular biology specifies that transcription factors (TFs) are new molecular tools utilized for plant metabolic engineering that enhance the production of bioactive compounds. The treatment of plant as well as plant cells with biotic or abiotic elicitors enhances secondary metabolite production from cell cultures. Moreover, lots of elicitors have been reported which stimulate the defense mechanism of medicinal as well as aromatic plants in the plant tissue cultures. There are various types of elicitors used to enhance the production of bioactive compounds like plant growth regulators, stress hormones, chitosan, microbial extracts, and physical stresses. Elicitors are the molecules which increase the level of bioactive compound from the medicinal plant, and these elicitors are categorized into biotic as well as abiotic (Kumar et al. 2018a, b). Here enlisted a very important example for induction of bioactive compounds through elicitors. The expression pattern of three genes of the MYB transcription factor (SbMYB1, SbMYB2, and SbMYB3) was considered in response to elicitors (MeJ, SA, and SNP) in *Selaginella bryopteris*. The response was evident in the expression of MYB genes and flavonoid production. Out of the three MYB genes studied, SbMYB2 was found to be very reactive to identified elicitors (MeJ, SA, and SNP) of the flavonoid pathway (Kumar et al. 2018a, b). The other example is also related to the medicinal plant (*Selaginella bryopteris*). Transcriptome analysis was done on medicinal plants such as Sanjeevani (*Selaginella bryopteris*) in frond as well as root for understanding the regulation of flavonoid biosynthetic pathway. It was reported that genes responsible for flavonoid biosynthesis were upregulated in the frond compared with root. Additionally, this in silico expression data was well-known through RT-PCR based analysis and exhibited expression of most of these genes in the frond involved in biosynthesis of flavonoids. These recognized putative genes could be valuable to accelerating the bioactive yield of flavonoids in *Selaginella bryopteris* (Singh et al. 2018). Apart from this, it is essential to adopting reliable agricultural operations such as organic farming, intercropping, and the use of PGPRs as biofertilizers to improve plant growth as well as development. Moreover, modern biotechnology encompasses the traditional biotechnology techniques. However, there will not be the future scope of modern biotechnology without involvement of conventional biotechnology. In case of conventional biotechnology, use of natural organisms to make food as well as additional valuable products; which is used for human, while novel biotechnology includes the manipulation of genes and living tissues in a controlled environment to produce new cell. Here mentioned some conventional breeding techniques, such as hybridization, selective breeding, and mutagenesis, are mainly utilized in conventional biotechnology program. Apart from this, additional tools including selective breeding method, food processing, and plant tissue culture are

directly involved in the production of phytoceutical compound. The genetic diversity of microorganisms and the improvement in the field of genetics prolonged the prospective of outdated biotechnology. Conventional biotechnology denotes to the old methods of using living organisms to yield new products or change foods as well as useful products for human welfare. Conventional biotechnology has great efficiency and accuracy in the making of bioactive compounds. Modern techniques have the same foundation; however, it refers to biotechnological techniques for the manipulation of genetic material in a disease-free condition. Traditional strategies are simpler, faster, and more cost-effective compared with biotechnology based methods like genome editing, genetic transformation, metabolic engineering, and synthetic biology. Generally, these factors clearly signify the significance of the role of medicinal plants as well as different methods to monitoring their active secondary compounds. Several methodologies are involved in the selection procedure for bioactive compounds and every step carried out through different conventional and nonconventional measures for the production of bioactive compounds. However, here we will discuss only conventional methods for the enhancement of phytoceutical compounds from medicinal plants.

1.2 Medicinal Plants

Medicinal plant is a general term for plants utilized for treating several chronic diseases. There were several parts of medicinal plants such as roots, stem, leaf, and flower also utilized directly for treatment of various diseases. In Table 1.1, several important medicinal plants are enlisted to clear understanding of the usefulness and knowing their importance. In the past, doctors were not available for curing patient; at that time, home therapies are the best sources for curing the health problems. Various common diseases such as respiratory infections, gastrointestinal infections, skin diseases, wound healing, hypertension, constipation, anemia, menstrual problems, malaria, jaundice, as well as fever have been treated through medicinal plants (Khalkho et al. 2015; Mussarat et al. 2014). There were lots of information about medicinal plants as well as their bioactive value reported in our Vedas and scriptures. Apart from this, increasing population is the major factor hindering the availability of medicinal herbs. Technologies have come up with many techniques to the conservation of the medicinal plants, so that they can be exploited to remedy many terrible diseases. *Selaginella bryopteris* (L.) Baker, commonly known as "Sanjeevani-like plant," has traditionally been used as a medication for several human health complications for centuries in India (Kumar et al. 2018a, b). Various phytoceutical compounds are isolated from medicinal plant, but due to the increasing population, several medicinal plants come under endangered zone. Micropropagation techniques have been used to minimize these problems, and maintaining the biodiversity of medicinal and aromatic plants is done through the use as well as development of bioactive compound through different in vitro culture techniques. As everyone knows, intact plants have a very little amount of

Table 1.1 Important medicinal plants with their use in numerous diseases

S. no.	Botanical name	Common name	Application
1	*Abroma augusta*	Ulatkambal	Diabetes, arthritis, and dysmenorrhea
2	*Abrus precatorius*	Jequirity bean	Used for remedy of tetanus as well as to prevent rabies
3	*Abutilon indicum*	Mallow	Broadly used as a conventional medicine as a blood tonic for ulcers as well as vaginal problem
4	*Acacia arabica*	Babool	Used as wound curative, oral care, as well as restrict blood loss
5	*Acacia catechu*	Kadirkasth	Useful to maintain blood pressure, dysentery, diarrhea
6	*Achyranthes aspera*	Prickly chaff flower	For curing asthma, dropsy, and cough as well as used for dog bite, snakebite, etc.
7	*Aconitum ferox*	MeethaVish	Remedies for fever
8	*Aconitum heterophyllum*	Atees	Used for the treatment of patients with urinary infections, diarrhea, and inflammation
9	*Acorus calamus*	Bach	Treatment for neurological, gastrointestinal, respiratory, metabolic disorders
10	*Adhatoda vasica*	Vasaka	Blood disorders, heart troubles, asthma, fever, jaundice
11	*Aegle marmelos*	Bael	It is anti-inflammatory in nature and helps to cure ulcer, diabetes, respiratory problems, and cancer
12	*Alangium salvifolium*	Ankol	For treatment of diarrhea, snakebite, inflammation, piles, as well as skin diseases
13	*Albizia lebbeck*	Shirish	Used to prevent scabies, lung ailments, and piles problem
14	*Alhagi camelorum*	Yavasa	Flowers are used as remedy for piles problem and to relax from constipation
15	*Allium cepa*	Onion	To treat gastrointestinal disorders, asthma, and bronchitis
16	*Allium sativum*	Garlic	To maintain high blood pressure and protect against illness, like common cold
17	*Aloe barbadensis*	*Aloe vera*	Used for skin related problem like itching and to treat acne
18	*Alpinia galanga*	Kulanjan	Flatulence, dyspepsia, vomiting, seasickness
19	*Alstonia scholaris*	Chitvan	Fever, skin ulcers, enhancing lactation
20	*Amomum subulatum*	Badi Elaichi	Bronchitis, asthma, appetizer, digestant
21	*Amorphophallus campanulatus*	Jimikand	Dysentery, piles, hemorrhoids
22	*Anacyclus pyrethrum*	Akarkara	Common cold, sore throat, and fell relaxed from digestive problem
23	*Ananas comosus*	Pineapple	Remedy for arthritis, helps digestion, pain relieving capacity
24	*Andrographis paniculata*	Kalmegh	Used for the treatment of diarrhea, cold and fever, jaundice

(continued)

Table 1.1 (continued)

S. no.	Botanical name	Common name	Application
25	*Aquilaria agallocha*	Agaru	Remedy for digestion problem and asthma, increases blood circulation
26	*Areca catechu*	Betel palm	Used to treat abdominal cavity as well as killing worms
27	*Argyreia speciosa*	Vridhadaru	Treatment of swelling, arthritis
28	*Asparagus racemosus*	Shatavari	Relief from gastric problem, nervous related problem
29	*Azadirachta indica*	Neem	Used to kill bacteria and fungi and for suppressing tumor and skin related problem
30	*Bacopa monniera*	Brahmi	Memory enhancer, anxiety, stress
31	*Barleri aprionitis*	Vajradanti	Cough, fever, jaundice inflammation, urinary infection
32	*Betu lautilis*	Bhojpatra	Wound healing, obesity
33	*Boerhaavia diffusa*	Punarnava	Anemia, liver diseases, wound healing
34	*Boswellia serrata*	Shalai Guggal	Joint pains, headache, diabetes
35	*Butea monosperma*	Palasha	Used as antiseptic agent, skin disease, relaxed from cough problem
36	*Cassia angustifolia*	Senna	Treatment of constipation, used for hair growth
37	*Cassia fistula*	Amaltas	Ulcers, antiseptic, wound healing, fever
38	*Celastrus paniculatus*	Malkagini	Muscle cramps, backache, osteoarthritis, paralysis
39	*Centella asiatica*	Mandukparni	Wound healing, treatment of various skin problems as well as relaxed from fever
40	*Cinnamomum tamala*	Tamalpatra	Treatment of dental problem, black spot on skin, and cough
41	*Cinnamomum zeylanicum*	Dalchini	Headaches, cough, influenza, relaxed from respiration problem
42	*Cissampelos pareira*	Patha	Ulcer, fever, asthma, cholera, treatment of wound
43	*Clerodendron serratum*	Bharangi	Remedy for malarial fever, respiratory related diseases
44	*Coleus barbatus*	Pathar Chur	Kidney stone, calculus
45	*Commiphora mukul*	Guggulu	Heart related problem, arthritis and paralysis
46	*Coriandrum sativum*	Coriander	Digestive, pain reliever, rheumatoid arthritis
47	*Costus speciosus*	Ketaki	Obesity, hyper-lipid anemia, diabetes
48	*Crataeva nurvala*	Varun	Kidney stones, bladder stones
49		Nagarmotha	Fever, diabetes, solar dermatitis

(continued)

Table 1.1 (continued)

S. no.	Botanical name	Common name	Application
	Cyperus rotundus		
50	*Desmodium gangetium*	Shalparni	Analgesic, anti-inflammatory
51	*Eclipta alba*	Vringraj	Used for hair growth, improve digestion related problem, dropsy disease
52	*Elettariacar damomum*	Elaichi	Nausea, vomiting, dry cough
53	*Embelia ribes*	Vai Vidanka	Skin disease, snakebite, helminthiasis
54	*Emblica officinalis*	Amla	Asthma, jaundice, constipation, eye disease
55	*Gloriosa superb*	Calihari	Remedy for skin related problem, kidney disorder, abortion, cholera, treatment of cancer
56	*Glycyrrhiza glabra*	Mulethi	Digestive disorders, ulcers, bronchitis
57	*Gymnema sylvestre*	Gudmar/ Madhunasini	Diabetes, hydrocil, anti-asthmatic
58	*Hemibismus indicus*	Anantamool/ sariva	Appetizer, carminative, aphrodisiac, astringent
59	*Holorheena antidy sentrica*	Kurai	Scabies, antipyretic, amoebic dysentery
60	*Lawsonia inermis*	Henna	It has antibacterial and antifungal properties, for skin and hair related problem
61	*Mentha pipertia*	Peppermint	Used as mouth freshener, toothpastes, extensively used for fresh breathing, anxiety
62	*Mesua ferrea*	Nageswar	Treatment for asthmatic attack, skin burning problem, vomiting and piles
63	*Mucuna pruriens*	Velvet beans	Remedy from constipation and dropsy; increase intelligence level
64	*Ocimum sactum*	Basil	It is a natural immune booster, treatment of fever and cough, good for diabetes patient
65	*Piper aborescens*	Pepper	Relief from digestion problem, good for hair thickness, used in weight loss
66	*Piper longum*	Pippali	Remedy from cold and cough
67	*Plumbago zeylanica*	Chitvan	Arthritis, dysentery, skin diseases, intestinal disorder ailment, liver disorder
68	*Plumbago zeylanica*	Chitrak	Skin diseases, nervous related problem
69	*Ranwolfia serpentina*	Sarpa Gandha	Maintain blood pressure, anxiety, diabetes
70	*Saraca indica*	Ashok	Solve the problem of menstrual anomalies
71	*Selaginella bryopteris*	Sanjeevani	Constipation, fever, jaundice, and cancer
72	*Solanum nigrum*	Kakamachi	Skin diseases, asthma, fever, stomach related problem

(continued)

Table 1.1 (continued)

S. no.	Botanical name	Common name	Application
73	*Solanum xanthocarpum*	Kantakari	Asthma, anticancer properties
74	*Strychinos nux-vomica*	Kochila	Paralysis, helpful in wound related problem
75	*Swertia chiraita*	Chiraita	Used for liver problem, malaria, skin disease
76	*Terminalia bellerica*	Bahada	Cough, ulcer, liver problem, respiratory issue
77	*Terminalia chebula*	Harida	Digestive problem, ulcer, cough, kidney and liver related disease
78	*Tinospora cordifolia*	Giloe	Dengue fever, immunity booster, improve digestion, corona virus infection
79	*Tribulus terrestris*	Gokhur	Urinary related problem, aphrodisiac, digestive properties
80	*Trigonella foenum-graecum*	Fenugreek	Traditionally applied against respiratory infections
81	*Vetiveria ziziinoides*	Benachar	Treatment of ulcer, skin, vomiting
82	*Vincea rosea*	Sada Bahar	Used to cure diabetes and high blood pressure and have been applied as disinfectants
83	*Withania somnifera*	Aswagandha	Used for the treatment of asthma, stress, and cancer
84	*Zingiber officinale*	Ginger	Colds, nausea, arthritis, migraines, and hypertension

(Sources: modified and adapted from Swamy 2020)

biosynthesis of phytoceutical compounds; consequently, to accelerating the making of bioactive compound, elicitors are applied (Kumar et al. 2018a, b). Several types of elicitors have been reported to enhancing plant secondary metabolites; however, details about the study of elicitors are beyond this chapter. The current time has exhilarated the significance of herbal medicines. According to statistics, about 250,000 plant species have been identified, and 70,000 of them are characterized for their medicinal value (WHO 2019). Some countries such as India, China, and Africa are known as hotspots because of that maximum cultivation of medicinal plants. The Himalayan regions as well as Western Ghats of countries in Asia are the extreme places for medicinal plants (Joshi et al. 2016; Fathima et al. 2018). Important medicinal plants like basil, neem, *Aloe vera*, and turmeric are generally used for home for the treatment of several prevailing diseases (Behera and Mahalakshmi 2019). Therefore, medicinal plants are significant constituents of naturally derived medicines and have pharmaceutical properties. Some important and general medicinal plants reported for their pharmaceutical properties are listed in Table 1.1.

1.3 Medicinal Plants Are Enriched with Bioactive Compounds

Medicinal plants are the major sources of bioactive compounds which are used to cure several diseases. These plant based compounds are characterized into primary as well as secondary plant metabolites. Primary metabolites are directly involved in maintaining growth and several cellular functions. Meanwhile, secondary metabolites are enriched with several medicinal properties; therefore, it is utilized to remedy lots of common as well as chronic diseases. But there are some problems with secondary compounds, which are synthesized only in selective cells at a particular growth phase. The term "secondary" was first used by Kossel in the year 1891 which means primary metabolites occur in each living cell and secondary metabolites are found only suddenly. However, secondary plant metabolites are not involved to forming the basic molecular skeleton of the living organism. Table 1.2 shows classes of several different types of plant secondary metabolites (Verpoorte et al. 2002). As seen, in many time, different plant bioactive compounds provide medication in medicinal plants. Some very important examples of plant bioactive compounds with commercial relevance are mentioned in Table 1.3. Bioactive peptides are also very important constituents found in medicinal plants. Many bioactive peptides have to be produced in roots, leaves, stems, seeds, and flowers. They have the potential to be antioxidants, antimicrobials, immune modulators, anticancer agents, antimicrobials, and other valuable bioactivities (Salas et al. 2015).

Table 1.2 Classes of important secondary plant metabolites with important examples

Classes	Kinds	Examples
Terpenes	Sesquiterpenes	Limonene
	Monoterpenes	Farnesol
	Diterpenes	Taxol
	Triterpenes	Digitogenin
	Tetraterpenoids	Carotene
	Sterols	Spinasterol
Phenolic compound	Lignan	Lignan
	Tannins	Gallotannin
	Flavonoids	Anthocyanin
	Coumarins	Umbelliferone
Nitrogen- and sulfur-containing compounds	Alkaloids	Nicotine
	Atropine	Tropine
	Glucosinolates	Sinigrin

Table 1.3 Isolated products of bioactive compounds used in pharmaceuticals

Medicinal plant	Substance	Properties/applications
Aconitum napellus	Aconitine	Used to reduce fever, remedy from pneumonia
Atropa belladonna	L-hyoscyamine	Muscle relaxer, relaxed from ulcer problem, menstrual irregularities
Camptotheca acuminate	Camptothecin	Provide immune against tumor disease
Cannabis sativa	Tetrahydrocannabinol	Asthma, prevention in sexually transmitted disease
Catharanthus roseus	Dimeric Vinca alkaloids	Provide immune against tumor disease
Chondrodendron tomentosum	Tubocurarine	Helps in relaxing muscle
Cinchona pubescens	Quinidine	Relaxing from stomach problem
Coffea arabica	Caffeine	Used as stimulant and therapy against malaria
Colchicum autumnale	Colchicine	Headache, constipation
Crotalaria	Pyrrolizidine	Fever, scabies
Cytisus scoparius	Sparteine	Maintain blood pressure
Digitalis lanata	Digitoxin, digoxin	Heart therapy
Erythroxylum coca	Cocaine	Used as stimulant
Galanthus woronowii	Galantamine	Remedy for Alzheimer disease
Lycopodium clavatum	Huperzine	Remedy for Alzheimer disease
Papaver somniferum	Morphine	Heart related problem, digestion
Physostigma venenosum	Physostigmine	Remedy for Alzheimer disease
Pilocarpus jaborandi	Pilocarpine	Glaucoma treatment
Rauvolfia serpentina	Reserpine	Hypertonia treatment
Sanguinaria canadensis	Sanguinarine	Prevention from bacterial and viral disease
Strophanthus gratus	Ouabain	Heart related problem
Taxus brevifolia	Paclitaxel	Remedy from tumor disease

(Sources: modified and adapted from Geilfus 2019)

1.4 Major Areas of Bioactive Compounds

The major bioactive compounds are found in plants, which contain 80% of the secondary metabolites; however, these compounds occur in fungi, bacteria, as well as marine organisms (Bérdy 2005). The typical bioactive compounds in plants are found as secondary plant metabolites. These plant based secondary metabolites are mainly utilized to making valuable products for human welfare. These compounds are mainly utilized for the preparation of chemicals such as medicines, flavors, scent, pesticides, as well as dyes. Bioactive compounds like annins, terpenoids, alkaloids,

and flavonoids play important role against microbial infections. It was reported that approximately 8000 recognized phenolic compounds are synthesized with the help of shikimic acid pathway or from the malonate as well as acetate pathways (Rodney et al. 2000). Vinblastine is a type of alkaloid in the form of salts which is used for medicinal purposes, and it provides antitumor properties (Jordan and Leslie 2004). Apart from this quinine, which is the rich sources of antipyretic compound, it protects from malaria (Reyburn et al. 2009), as well as reserpine, which could be also used to maintain the high blood pressure. Alkaloids have lot of importance that helps in protein synthesis, and it also protects medicinal plants from animal and other biotic factors. Furthermore, alkaloids have potential to treat several diseases like cancer. In vitro experiments were done and found that the phenol have antimicrobial (Rauha et al. 2000), antiviral (Perez et al. 2003), anti-inflammatory (Santos et al. 2000), as well as vasodilatory actions (Padilla et al. 2007). This alkaloid has enormous properties which protect plant from several abiotic factors like drought, physical damage, or infections. UV radiation is mainly used to create mutation in plants. Due to induced mutation, the genetic material of living organisms are changed; therefore, the desired trait has been also changed. Most of the mutations are dangerous to plant so that the phenolic compounds play a very vital role to provide protection against UV radiation as phenylpropanoid pathway (Dietrich et al. 2004). Mainly the phenolic compounds have potential to guarding cells against oxidative stress through the searching of free radicals by hydrogen atom donation. Phenolic compounds have neuroprotective (Nichenametla et al. 2006), antifungal (Prats et al. 2007), anti-bacterial (Okunade et al. 1997), antiatherosclerosis (Tsuda et al. 1997), as well as anticancer potential (Olsson et al. 2004).

1.5 Production of Phytoceutical Compounds from Medicinal Plants

Pharmaceutical compounds such as secondary metabolites, isolated from medicinal plants, are used to prepare important medicines. This phytoceutical compounds are highly used in modern medicine to treat several chronic disease. There were several difficulties arising during the extraction of biologically active compound from medicinal plants. These bioactive compounds are synthesized in diverse parts of the plant. The level of bioactive compounds differs according to the species from which they are isolated and synthesized under stress condition. There were several stresses responsible for enhancing this bioactive compound such as attack of pathogen and different growth stage and development. Some of the important plant tissue culture techniques are available nowadays which help for obtaining phytoceutical compound. These important plant tissue culture methods used for obtaining bioactive compound are plant cell suspension culture, adventitious as well as hairy root culture. Plant tissue culture is a substitute method to getting significant bioactive compound production. The abovementioned approaches are manageable and

sustainable and overcome several embarrassments for the production of bioactive compound. Currently, a lot of attention has been given to research on hairy root culture for significant phytoceutical compound production. One of the important bacteria is directly related to hairy root culture for valuable bioactive compound production, which is *Agrobacterium rhizogenes* that initiate hairy disease in the root of plant. This hairy root has a lot of crucial drug properties. However, the production of bioactive compound through traditional method is based on different solvent as well as steam extraction. Novel biotechnological methods such as micropropagation and fermentation based method have facilitated in vitro production of phytoceutical compound. However, in this chapter, we will discuss only conventional method for secondary metabolite production. Some important systems that enhanced the bioactive compounds in medicinal plants are mentioned in subheadings.

1.5.1 Increased Levels of Bioactive Compounds Through Different Farming Systems

Nowadays, many people around the world prefer organic products. Therefore, worldwide demand for the production of organic foods has rapidly increased. Due to this, organic farming has become a real choice for farmers. Several studies have been conducted to knowing the enhancement of pharmaceutical products and reported that organic food have more amount of secondary metabolites, vitamin C, as well as dry matter (Ottesen 2010). This is also possible to increase bioactive compounds in medicinal plants through conventional approaches such as organic cultivation of medicinal crop, adopting different types of intercropping practices, root pruning, etc. Currently, several new biotechnological tools are available to increasing the bioactive compounds in medicinal plants, but here we discussed only traditional method. Furthermore, conventionally produced food have ample amount of vitamin A as well as proteins and nitrate. Several experiments have been performed on tomato and found that this crop is a full source of nutrients and phytoceutical compound with antioxidative compound which protect our body from foreign particles through vitamin, ß-carotene, lycopene, as well flavonoids (Caris-Veyrat et al. 2004). This is reported that organic nature of tomatoes had a considerably higher content of the quercetin, kaempferol, flavonoids, and naringenin compared with conventional mode of production (Mitchell et al. 2007). However, another experiment was also conducted on tomato crop and published in a review paper in the year 2007 by one of the participating scientists. Experiment revealed the phenolic compounds in organic crops to be 119% higher than in their conventional counterparts (Rembialkowska 2007). Furthermore, it was revealed that organic fruit as well as vegetables contain 40% more antioxidants and ample amount of vitamin C, iron, copper, and zinc (Medical News Today 2007). The production of bioactive compound is not limited only to tomato crop. Several research has also been done on different types of agricultural crops to know the increased levels of

bioactive compounds in different farming systems. Research interest is increasing in legume seeds that have spreading pharmaceutical industry in all over the world (Pacheco et al. 2017; Singh et al. 2017). Organic manure application has been proven to increase the physical as well as biological and chemical properties and regularly increase plant growth as well as yield due to the high level of organic matter content (Stephen et al. 2014; Mitran et al. 2017). Flavonoid, phenolic compound, as well as antioxidant activity are increased due to application of organic manure; however, this is not possible for inorganic fertilizers (Zeinab et al. 2013; Flieβbach et al. 2007). It was reported that phenolic content in organically produced apples has 19% higher phenolic compound compared with inorganically produced food (Wiebel et al. 2000). The organically grown strawberries have ample amount of phenolic compound; however, inorganically cultivated strawberries have less amount (Hakkinen and Torronen 2000). Furthermore, organic manure has potential to increase antioxidant activity as well as bioactive compounds such as flavonoid, phenol, beta-carotene, and lycopene contents (Dumas et al. 2003; Mohd et al. 2013). Apart from this, seeds harvested from fenugreek were intercropped with buckwheat and with the application of organic fertilizer enhanced the seed content of antioxidants and flavonoids (Salehi et al. 2019). These findings are of great interest for the pharmaceutical industry for fenugreek producers, particularly for semiarid regions. Thus, intercropping is a very important agronomical practice by which we could enhance the secondary metabolites in our genotypes. The interspecific interaction is possible through the intercropping operations as well as successfully utilizes environmental resources to increasing yield of medicinal plant. The commercial cultivation of *Atractylodes lancea* as a monocropping reduced yield due to their low survival rates. Therefore, intercropping is a real way to increase the survival as well as yield of *Atractylodes lancea*. Moreover, intercropping with *Zea mays*, *Calendula officinalis*, and *Tagetes erecta* increases the harvest of four volatile oils from *Atractylodes lancea* (Peng et al. 2021). It is reported that humic acid and vermicompost improve the chicory yield as well as phytocompounds such as the total contents of phenolics and flavonoids (Gholami et al. 2018). Intercropping as well as management practices such as fertilizer application have significantly increased the secondary metabolites in chicory root and fenugreek seeds (Garshasbi et al. 2021). The improvement in yield with secondary metabolites is observed during the intercropping of medicinal plants along with leguminous plant family. The constituents of thyme essential oil containing thymol, p-cymene, and γ-terpinene were increased under moderate as well as severe water scarcities in intercropping system treated with arbuscular mycorrhizal fungi (Machiani et al. 2021). The different intercropping practices with the application of organic fertilizers as well as biofertilizers characterize an actual approach to improve the whole seed and oil yields with the phytoceutical compound in dragon's heads and fenugreeks (Rezaei-Chiyaneh et al. 2021). Fertilizer system as well as different maturity stages greatly influenced the production of secondary metabolites in raspberries. Furthermore, oxidative stress enhances the defense mechanism as the level of secondary metabolites and antioxidant increases in raspberries (Frías-Moreno et al. 2021). The chemical composition of plants is affected by the abiotic as well as biotic

factors. Several abiotic factors like temperature, light, growing conditions, nutrients, and water have been well studied to know the impact on the profile of secondary metabolite, often resulting in increased production regarded as higher quality (Selmar and Kleinwachter 2013). The fruit raspberry contains high amount of polyphenols such as flavonols and anthocyanins. Some experiments have indicated that organic fruits hold more bioactive compounds than conventionally produced fruits. The organic produced samples contained significantly more dry matter, phenolic acid, and flavonoids, including myricetin, quercetin, luteolin, and quercetin-3-O-rutinoside (Ponder and Hallmann 2019).

1.5.2 Increased Levels of Bioactive Compounds Through Pruning Method

Before understanding the increased levels of bioactive compounds through pruning method, first of all, we have to know about the meaning of pruning. Pruning is a horticultural method that involves the selective removal of certain plant parts such as branches, buds, or roots that improve the overall development of plant (Clark and Matheny 2010; Zhang et al. 2018). On the basis of the season, pruning is classified into three categories such as spring, summer, and autumn pruning. On the other hand, pruning is divided on the basis of number of branches cut, and these are light cut, medium cut, heavy cut, as well as very heavy cut (Zhang et al. 2018). These horticultural operations have been reported to increase the number as well as leaf area in *Fraxinus mandshurica*, and it can also enhance the leaf biomass and root accumulation and promote the survival and growth of their seedlings (Yang et al. 2018). These horticultural operations are performed to enhance secondary metabolites in medicinal plants as well as other useful plants. Here some important reviews are compiled to get a better understanding about conventional approaches to increasing pharmaceutical products in plants. Furthermore, after pruning operations of *Diospyros melanoxylon* Roxb, healthy leaves improved more than fivefold ingredients of phenols as well as carbohydrates compared with non-pruned leaves (Mehta et al. 2020). These operations are not limited to medicinal plant only; it can also operate in trees for enhancing the phytocompounds. However, pruning operations can be also used in trees as well as shrubs for improving the biological yield of these crops. Remarkably, after pruning operations happen, fresh and dry weights increased. These above results were confirmed by quantitative real-time polymerase chain reaction, and scientist detected upregulation of flavonoid associated genes in leaves after pruning (Cao et al. 2022). These above consequences indicate that pruning stimulates leaf growth as well as bioactive compound accumulation in ginkgo seedlings. Moreover, inflorescence pruning in spiny coriander increases the leaf biomass without affecting the leaf levels of bioactive compounds with antioxidant activity (Campos et al. 2019). *Agastache rugosa* is a perennial plant; it is known as Korean mint which contains essential oils, flavonoids, and phenolic

compounds like flavone, tilianin, and acacetin, with sesquiterpenes, diterpenes, and triterpenes (Tuan et al. 2012; Zielinska and Matkowski 2014). These above phytocompounds show that 50% root pruning at 7 or 9 days before harvesting of the plant increased the concentrations of acacetin, rosmarinic acid, and tilianin and 30% root pruning at 5 days before harvesting improved in the levels of acacetin in these plants without disturbing plant growth (Lam et al. 2019). Root is very important plant organ hiding below the soil surface that supports the plant structure as well as supply of water and nutrient and assimilates storage. The regulation of root growth is one of the promising practices to boost the yield of citrus that is economically important and highly demanded fruit. There are different types of root pruning that are adopted for increasing the medicinal compounds including air root pruning and knife root pruning and also recently using root pruner machine mounted with a tractor. The root pruning is also a promising practice to overcome the alternate bearing of fruit tree, with citrus, by suppressing unnecessary growth and restricting the high fruit load during the on year and permitting better carbohydrate storage for the improvement of yield during the off season (Budiarto et al. 2019). The experiment was carried out to evaluate blackberry cultivars in respect of productivity as well as bioactive compounds and stated that among all the cultivars, the cultivar BRS-Tupy was superior in terms of productive characteristics; however, for constituents of anthocyanins and flavonoids, the cultivar Xavante stood out (Lugaresi et al. 2018). Usually it is seen that root pruning practices are applied for the overall progress in tree growth and their rhizosphere. Suitable selection of root pruning is an essential step for the effectiveness of bioactive compound (Du et al. 2012). Therefore, here we concluded that pruning would also help to enhancing the bioactive compound in several agricultural crops.

1.5.3 Increased Levels of Bioactive Compounds Through Traditional Breeding Methods

Many years ago, people have been searching new drugs in nature mainly in the form of medicinal plants. These important medicinal plants have phytoceutical properties which are used to cure several diseases. In addition, above 80% population all over the world believe that medicinal plant-derived compounds are used for improving health (Laloo et al. 2021). At the present time, many omics methods like genomics, transcriptomics, as well as metabolomics are used in medicinal plant for the production of phytomedicine. These omics founded studies helped in revealing the genes as well as proteins directly involved in the biosynthesis of significant bioactive compound (Mehta and Hasija 2018; Chakraborty 2018). Therefore, understanding of this gene-protein-metabolite mechanism with the help of omic technology can open up better openings for enhancing the production of important phytocompounds through conventional as well as modern plant biotechnology techniques. Approximately 72,000 species are used to prepare medicines (Schippmann et al. 2006). However,

a number of research have been carried out to the successful breeding of medicinal as well as aromatic plants by applying conventional as well as several modern breeding methods to achieve a major objectives (Pank 2006). The development of varieties from mixed population is a big task for plant breeder. Ample amount of genetic variation is found in medicinal plant, which is utilized by plant breeder for varietal development in a very short time with a simple selection method. Preservation of medicinal plants in the form of seed gene bank as well as field gene bank is a very important step for plant breeding. This preservation helps an abundant contribution for genetic improvement of medicinal plant through plant breeding method. Wild species, wild relative, and landraces have the important resources that contain higher genetic diversity compared with improved cultivars. A large number of medicinal plants around the world were developed from landraces or introduction materials through the process of mass, pure line, or clonal selection (Baydar 2005). In addition, many new cultivars of medicinal plants were produced through interspecific and intervarietal hybridization. Hence, we discussed here the enhancement of different types of phytocompounds in medicinal plants through the process of conventional plant breeding methods. There were several reports on conventional plant breeding method based secondary metabolite enhancement to be described here for clear understanding. Therefore, modern biotechnology is not only an option for increasing phytoceutical properties in medicinal as well as aromatic plants. The hybrid lavandin crossed between *Lavandula* × *intermedia* has more flower as well as essential oil compared to the other two types, and this has significantly constricted the agriculture of lavender (Baydar 2005). Apart from this, mutation breeding is a special breeding method that is used for genetic improvement of several agricultural crops. This breeding method is used to increasing bioactive compound in medicinal plant. Here collected are some useful information regarding induction of pharmaceutical properties in medicinal plant. Increase in the level of steroidal sapogenin in *Trigonella corniculata* with the application of dimethyl and diethyl sulfate has been reported (Jain and Agrawal 1987). Besides, *Papaver somniferum* variety which is nonnarcotic was produced through mutagenesis process (Kolakar et al. 2018). Ultraviolet radiation is highly used in mutation breeding program as a physical mutagen. Many varieties of agricultural crops have to be released through the process of ultraviolet radiation. Ultraviolet radiation is a part of the sunlight and reaches to the Earth surface. This solar radiation is categorized into three groups such as UV-A (310–400 nm), UV-B (280–310 nm), as well as UV-C (below 280 nm). Out of these three radiations, UV-B is the most active unit reaching the Earth surface. Despite the effect of UV-B on medicinal plants as well as aromatic plants, there were some interesting miracles being discovered. Increase in oil yield in basil plants through the exposures of UV-B radiation has been reported (Chang et al. 2009). Other studies also found that medicinal plants display the beneficial aspects in terms of increasing volatile oil and secondary metabolite production through ultraviolet radiation (Kumari et al. 2009b). Ultraviolet radiation also increased the ranks in tocopherol (Higashio et al. 2007), with ascorbic acid as well as flavonoids being important medicinal and aromatic plants (Kumari and Agrawal 2010). In several medicinal and fruit cultivars, anthocyanin accumulation is induced through the light

in the UV-B region with their wavelengths ranging between 280 and 320 nm, when applied in combination with solar infrared radiation (Arakawa 1988). Accumulation of terpenoid compounds at seedlings stage nearly five leaf growth in basil leaves has also been reported (Johnson et al. 1999). It has been observed that the variety mairei increased content of alkaloid "taxol" in *Taxus chinensis* exposed by increased UV-B radiation (Zu et al. 2010). In current years, some reports have been carried out to knowing the influence of enhanced UV-B radiation on hypericin concentrations and revealed that concentrations of this bioactive substance can be altered by UV-B (Germ et al. 2010). Another study was also carried out on *Cymbopogon citratus* and found enhancement in their medicinal value by procuring the higher content of z-citral in essential oil as well as enhancing their yield (Kumari et al. 2009a). Another breeding method used in the medicinal plants is the polyploidy. Polyploidy plays an important role to increase metabolic profile of medicinal and aromatic plants. Polyploidization can be achieved through many antimitotic agents such as colchicine, oryzalin, and trifluralin. Generally, the size of flowers, leaves, fruits, and seeds is increased due to polyploidy breeding. Artificial polyploidy also helps to enhance the vigor of specific plant parts and may be favorable when specific organs and biomass constituting the commercial product (Levin 2002). This breeding method has been used to enhance plant phytocompounds or to improve the metabolite profile. Biochemical analysis of numerous allopolyploid plant species suggests that their enzymatic capacity is greater than the parental individuals, and it is also rich in phenolic compounds (Levin 2002). Here presented is an overview of how polyploidy can be used to enhance metabolite production in medicinal plants. The effect of the polyploidy on biomass accumulation as well as the enhancement of bioactive compound was reported in *Echinacea purpurea* (L.) Moench. Moreover, this is observed that tetraploid plants had higher phenylalanine ammonia-lyase and cinnamate 4-hydroxylase activities compared with the diploid plants (Xu et al. 2014). Polyploidization influences the level of bioactive compound in medicinal plants described in Table 1.4. A lot of research has been carried out and found that polyploidy is also used to increasing bioactive compound in medicinal as well as aromatic plant. Polyploidy also enhances the secondary metabolites in the form of oil for the crop mint and lavender as well as clary sage (Jordanov et al. 1995; Moetamedipoor et al. 2022). Colchicine is highly used to induction of ploidy level. The morphine content of *Papaver somniferum* is enhancing through doubling the ploidy by the use of colchicine (Mishra et al. 2010). The autotetraploid of *Datura stramonium* and *Hyoscyamus niger* has many alkaloids in the form of scopolamine and hyoscyamine as compared with diploid plant (Berkov 2001).

1.6 Conclusion

This chapter describes the significance of phytoceutical compounds from medicinal plants as well as its categorization, development, and utilization. There are enormous necessities to making new phytoceutical compound to protecting against cancers,

Table 1.4 Consequence of polyploidization on the production of plant secondary metabolite

Plant species	Resultant ploidy level	Consequence on secondary metabolite production	Reference
Acorus calamus	4x	Above 95% increase in anticancer compound, i.e., beta asarone	Bertea et al. (2005)
Agastache foeniculum	4x	Enhanced the level of methyl chavicol metabolite content	Talebi et al. (2017)
Artemisia annua	4x	1.5-fold increased artemisinin content	Lin et al. (2011)
Datura stramonium	4x	52–152% alkaloid production increased	Berkov and Philipov (2002)
Dracocephalum kotschyi	4x	Level of methoxylated flavones rises	Zahedi et al. (2014)
Papaver sominferum	4x	Up to 50% enhanced morphine content	Mishra et al. (2010)
Solanum commersonii	4x	Level of phenylpropanoids increased	Caruso et al. (2011)
Camellia sinensis	4x	The concentration of polyphenols, catechins, and extractines increases and caffeine in two leaf shoots	Sardzhveladze and Kharebava (1990)
Cichorium intybus	4x	1.9-fold enhancement in phenolic; tenfold enhancent in chlorogenic acid	Ghotbi et al. (2013)
Linum album	4x	1.39- and 1.23-fold rise in podophyllotoxin production	Dixit and Chaudhary (2014)

cardiac disorders, pests, mosquitoes, infectious diseases, as well as immune disorders. It is observed that in vitro production of bioactive compound from medicinal plant is not the only method to enhancing the phytocompounds; however, here discussed are conventional approaches to getting secondary metabolites through different reliable farming systems, root pruning methods, as well as traditional breeding methods. The pharmaceutical constituents of medicinal plant have also increased through traditional breeding methods such as mutation breeding, polyploidy breeding, and hybridization. Moreover, in the present paper, several reviews are compiled to getting a clear understanding of conventional approaches toward production of bioactive compounds from medicinal plants. Therefore, further research is mandatory to understand the conventional mode of producing bioactive compounds.

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Chapter 2
RNA Interference: Novel Technique for Enhancing Secondary Metabolite Production in Medicinal Plants

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Abstract Plants are key sources of secondary metabolites. The medicinal property of plants is mainly due to the production of secondary metabolites. Human beings have been constantly trying to elevate the production of secondary metabolites in plants by various techniques, including hairy root culture, and RNA interference. The recent discovery of the technique called RNA interference has been found to be very efficient in manipulating plants for human needs. A number of genes control the existence of precursors and their transformation to necessary end products for the production of secondary metabolites in plants. But at times, undesirable compounds are produced which blocks the production of desired products. The production of such undesirable compounds can be prevented by RNA interference technology. This brief study gives a comprehensive idea of RNA interference and its application in augmenting secondary metabolite production in plants.

Keywords Medicinal property · miRNA · RNAi technology · Secondary metabolites · siRNA

2.1 Introduction

Human beings rely upon plants for a number of purposes. People use plants for satisfying their nutritional, domestic requirements. Natural flora has shown to be a highly helpful source for health improvement and the treatment of many diseases, and a variety of plant species are available that are still being used as treatments for various ailments in several regions of the world, including Asia, Africa, and South America (Duraipandiyan et al. 2006; Bolzani et al. 2012; Khalid et al. 2012). In

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excess of 35,000 species of plants are utilized for therapeutic reasons in different human societies across the globe (Lewington 1993), and for the first line of health care, roughly 80% of the population of the world relies on these conventional treatments, which frequently involve the extracts of plants (Sandhya et al. 2006). The unprecedented abundance of chemical diversity and natural products either as pure compounds or as homogeneous extracts of plants gives tremendous opportunities for novel drug discoveries, making ethnomedical research essential to the unveiling of new medications from indigenous medicinal plants (Yaseen et al. 2015).

The existence of secondary metabolites in plants is frequently thought to be responsible for the therapeutic and pharmacological effects of medicinal plants (Heinrich et al. 2004). Many of these secondary metabolites operate as protective substances against herbivores and diseases, while others work to draw pollinating agents and fruit dispersers, in slowing the development of surrounding rival plants, in providing plants with mechanical strength, or in absorbing damaging UV light (Ahmed et al. 2015). Plant secondary metabolites with documented therapeutic qualities include waxes, terpenoids, phenolics (including simple phenolics and flavonoids), alkaloids, and glycosides, as well as their derivatives. Nevertheless, this list is not exclusive to these compounds (Mustafa et al. 2017). Plant secondary metabolites have been reported to be efficient in treating various diseases, like atherosclerosis, cholesterol-related problems, coronary thrombosis, and bacterial and fungal infections. Some secondary metabolites have anti-inflammatory effects and exhibit antioxidant activity (Yagi et al. 2002).

The significance of plant secondary metabolites in the medical field demands their increased production. Plants cultivated in fields to produce secondary metabolites face challenges like lower yields and varying amounts caused by seasonal, regional, and environmental differences. For this reason, plant cell, tissue, and organ cultures have been demonstrated to be an appropriate substitute for the synthesis of secondary metabolites (Rao and Ravishankar 2002). Various methods have been developed recently for the synthesis of secondary metabolites and the accumulation of biomass, including elicitation, permeabilization, strain improvement, media and culture environment optimization, feeding of nutrients and precursors, biotransformation, and immobilization techniques. Moreover, several firms employ in vitro research, such as plant tissue culture and suspension cultures, for the commercial synthesis of secondary metabolites (Ghorpade et al. 2011). Large-scale production of secondary metabolites has been demonstrated by cell suspension cultures of various plants like *Berberis wilsoniae, Coptis japonica, Coleus blumei*, and *Lithospermum erythrorhizon* (Ellis 1988). Elicitors, which are substances that help in increasing the production or synthesis of specific compounds, can be used in extremely small concentrations for enhancing the biosynthesis of various secondary metabolites in plants (Radman et al. 2003). The compounds like pectin, chitin, cellulose, glucans, G-protein, and glycoproteins have been used as elicitors in plant tissue culture (Veersham 2004). Nanoparticles are yet another group of elicitors that have increasing application in the field of enhancing the secondary metabolite production in plants. Metallic, metallic oxide as well as carbon-based nanoparticles are commonly

used for this purpose. However, nanoparticles may result in phytotoxicity due to their effective absorption (Javed et al. 2022). Elicitation is a useful yet difficult technique that necessitates focused trial and error techniques (Singh 1990).

Another method for improving secondary metabolite production in plants is hairy root culture using the natural vector *Agrobacterium rhizogenes*. *A. rhizogenes* infects the roots, resulting in the formation of neoplastic roots that are genetically stable and has a high rate of growth even in hormone-free media (Pistelli et al. 2010). A number of reports have shown that hairy root cultures could aid in the production of secondary metabolites from dicot as well as monocot plants (Mukundan et al. 1997; Doran 2002; Rudrappa et al. 2005). Secondary metabolite production can also be augmented by biotransformation. The process of biotransformation involves changing the primary substrates into new substrates with new properties by using the appropriate enzymes or microorganisms (Ye et al. 2002, 2004, 2005). Many plant species have undergone biotransformation, such as the *Eucalyptus perriniana*, where carvacrol, thymol, and eugenol are transformed into glycosides (Shimoda et al. 2006), and the tobacco plant, where hyoscyamine is transformed into scopolamine through biotransformation (Moyano et al. 2007). Hence, biotransformation is a method for creating new active ingredients with various properties. Many secondary metabolites have been produced via genetic engineering of microorganisms and plants as well as in vitro recreation of metabolic pathways in plants (Vagner and Luzia 2014). Using a wide range of techniques to enhance these processes, significant progress has been achieved in the genetic engineering of microorganisms and plant cells to produce a variety of chemicals (such as isoprenoids, stilbenes or flavonoids) (Fazili et al. 2022). During the past two decades, remarkable progress has been achieved utilizing genetic engineering technologies to manipulate genes from various foreign sources and inoculating or introducing them into plants to bring about desirable features (Jagtap et al. 2011).

Research has shown that RNA interference (RNAi) is a mechanism used by all higher organisms to control gene expression. RNAi holds the possibility of improved accuracy and precision in plant breeding as RNAi technologies allow for the extremely specific downregulation of any gene's expression without impacting the expression of any other genes (Jagtap et al. 2011). A number of genes control the existence of precursors and their conversion to necessary end products for the production of secondary metabolites in plants. But at times, undesirable compounds are produced, which blocks the production of desired products. The production of such undesirable compounds can be prevented by RNA interference technology (Gomez-Galera et al. 2007; Borgio 2009). The effectiveness of RNAi for controlling multiple genes involved in the production of metabolites across a variety of tissues and developmental stages has long been acknowledged (Borgio 2009). For example, in transgenic opium poppies (*Papaver somniferum* L.), Allen et al. (2004) were able to inhibit the activity of codeinone reductase genes through DNA-directed RNAi, which led to the accumulation of precursor reticuline. The biosynthesis route for isoquinoline alkaloids, such as morphine, codeine, and berberine, in plants requires the precursor reticuline. Similarly, by using a hairpin-RNA-mediated RNAi technique to inhibit the expression of SQS (squalene synthase), a crucial enzyme of the

sterol pathway, the artemisinin content of *Artemisia annua* was increased. Artemisinin is an efficient antimalarial medication derived from *A. annua* L. It was found that some transgenic plants had a significantly higher amount of artemisinin than unaltered control plants, almost 3.14 times more (Zhang et al. 2009).

2.2 RNA Interference: History and Principle

The technique of dsRNA-mediated gene silencing known as RNA interference (RNAi) focuses on precisely degrading the mRNA cognate to dsRNA. In plants, animals, and fungi, RNAi-mediated gene silencing is called as posttranscriptional gene silencing, gene silencing, and quelling, respectively (Price and Gatehouse 2008; Nakayashiki and Nguyen 2008). Fire et al. (1998) coined the term RNA interference for the first time. The first report of an RNA interference phenomena was made by Napoli et al. (1990). Their research sought to ascertain if chalcone synthase, a crucial enzyme in the biosynthesis of flavonoids, was the rate-limiting enzyme in the biosynthesis of anthocyanins. The rich violet hue of petunias is a result of the anthocyanin biosynthetic pathway. Napoli et al. (1990) overexpressed chalcone synthase in petunias in an effort to produce violet petunias but instead got white petunias as a surprise. It was assumed that the inserted transgene was cosuppressing the endogenous CHS gene, since the levels of both endogenous and introduced CHS were 50 times lower than in wild-type petunias. Similar behavior was seen in *Neurospora crassa* in 1992, according to Romano and Macino (1992), who noted that the endogenous gene was quenched by the insertion of homologous RNA sequences. It was Guo and Kemphues (1995) who first reported RNA interference in animals. They observed the phenomenon in *Caenorhabditis elegans.* They observed that the Par-1 gene of *C. elegans*, which had a role in providing polarity to the organism, was degraded even though the hybridization of sense par-1 RNA to Par-1 transcript was not possible. Thus, they found that the repression of gene was possible with sense RNA as well. However, they could not elucidate the mechanism of mRNA degradation (Guo and Kemphues 1995).

Later, it was observed in *C. elegans* that gene silencing was caused by double-stranded RNA and not by single-stranded RNA, as proposed by Guo and Kemphues (Fire et al. 1998). They opined that degradation of mRNA by single-stranded sense RNA in *C. elegans* as proposed by Guo and Kemphues could have been due to the contamination of prepared RNA by double-stranded RNA that was due to the RNA polymerase activity of bacteriophages (Fire et al. 1998). So, they carried out the experiments after purifying the sense as well as antisense RNAs. The study was conducted on unc-22 gene of *C. elegans* that affects the function of muscles of the organism. They observed that dsRNA that was supplied to the medium decreased mRNA expression and suggested that RNAi might have a catalytic component. However, there was missing information as to how the degradation of mRNA was carried out by dsRNA even after this study also. The answer to this question was obtained after the study of Zamore et al. (2000). They observed that the

double-stranded RNA molecules were converted into short intermediate double-stranded RNAs called siRNAs or small interfering RNAs(siRNAs), which were then converted to single-stranded RNAs, which could base pair with mRNAs, thereby inducing their cleavage. Thus, siRNAs are the main inducers of RNA interference in organisms.

For RNA interference to occur, usually RNAs of approximately 20–30 nucleotides length are used. These are taken by Argonaute proteins, which lead the way to the degradation of target RNA by base pairing with target RNA and its sequence specific suppression. Based on the origin and biogenesis of siRNA or small RNAs or the target suppression mechanisms and biological roles, RNAi pathways can be of different types (Ketting 2011).

The structure of RNAs that act as substrates for small RNAs, or siRNAs, differs. They can be dsRNAs having blunt ends, RNA hairpins, (small or long having strict complementarity or less perfect cognacy), antisense or sense RNA or single-stranded RNA, which could be converted to double-stranded RNA with the help of the enzyme RNA-dependent RNA polymerases. The conversion of substrate RNA to small RNA is carried out by dicer or by miRNA or microRNA precursors or some other mechanisms that do not make use of dicer (Kim et al. 2009). The suppression of target mRNA can occur during transcription or after transcription. RNAi that occurs during transcription is commonly found in plants and very rarely found in animals. It includes methylation of DNA or suppression of modification of histones. RNA silencing after transcription (post transcription) may either occur by the endonucleolytic cutting of complementary RNA or translational suppression combined with breakdown of mRNA (Wassenegger 2005; Malik and Svoboda 2012).

2.3 Core Components of RNA Interference Machinery

The process of RNA interference is carried out by a variety of components, some of which act as initiators, effectors, amplifiers, and transmitters. For RNA interference to occur, the activities of the following components are necessary.

2.3.1 Dicer

Members of the RNase III family are one of the few nucleases that have dsRNA selectivity (Nicholson 1999). These nucleases cut dsRNAs at specific sites (Elbashir et al. 2001). An RNase III-like enzyme that Bernstein et al. (2001) discovered in a *Drosophila* extract was demonstrated to have the capacity to generate fragments with a size of 22 nucleotides, which is comparable to the size generated during RNAi. These authors demonstrated how this enzyme contributes to the beginning of RNAi. This enzyme was given the name Dicer (DCR) because of its capacity to break down dsRNA into consistently sized short RNAs (siRNA). Dicer possesses

four different domains, which include an amino-terminal helicase, a dsRNA-binding domain, two RNase III motifs, and a PAZ domain. PAZ domain consists of 110 amino acids and is found in proteins, such as Piwi, Zwille/Pinhead, and Argo. It also shares this domain with the RDE1/QDE2/Argonaute family of proteins, which were genetically associated with RNAi by separate investigations (Catalanotto et al. 2000; Tabara et al. 1999). It is believed that Dicer's tandem RNase III domains catalyze fragmentation of dsRNA (Agrawal et al. 2003).

2.3.2 Argonaute (AGO) Proteins

Argonaute proteins play an important role in RNA interference. All eukaryotic argonautes may be classified into three major families according to their structural characteristics and modes of action. They are AGO, WAGO (worm-specific argonautes, or secondary argonautes), and PIWI (P element-induced wimpy testis). Enzymes such as Dicer break down dsRNAs to miRNAs or siRNAs, which the AGO family proteins then bind with. This set of argonautes' function is crucial for the classical RNA interference, or the precise gene silencing by foreign double-stranded RNAs (Olina et al. 2018). AGO proteins are found in many eukaryotes, PIWI are found only in animals, and WAGo proteins are found in nematodes (Carmell et al. 2002; Zaratiegui et al. 2007; Meister 2013). Argonautes proteins have a well conserved and complex structure with four domains, namely, N terminal, PAZ domain, MID domain, and PIWI. L1 linker links N terminal and PAZ domains, while L2 links PAZ domain with MID domain. The binding of nucleic acid takes place in the space formed between the above lobes (Tolia and JoshuaTor 2007).

Argonautes with a complete tetrad are catalytically efficient and have the ability to break down RNA (Olina et al. 2018). A groove in the MID domain fits the guide RNA's 5′-terminal nucleotide (Frank et al. 2010). This nucleotide in short RNAs can be specially identified by various argonautes (Lau et al. 2001; Parker et al. 2005; Ghildiyal et al. 2008; Miyoshi et al. 2016). The 3′-end of guide RNA is bound to the PAZ domain (Lingel et al. 2003; Yan et al. 2003; Song et al. 2004). The N domain serves a supporting function in the target RNA cleavage as well as the RNA duplex unfolding process (Kwak and Tomari 2012; Hauptmann et al. 2013).

2.3.3 RNA-Dependent RNA Polymerase (RdRP)

The outcome of RNA interference and posttranscriptional gene silencing (PTGS) is powerful and intrinsic in nature. As a result, a mechanism has been postulated in which RNA-dependent RNA polymerases (RdRPs) are involved in both initiating and enhancing the knockdown effect. Plants that are transgenic or infected with viruses exhibit an increase of abnormal transgenic and viral RNAs. These abnormal RNAs may be recognized by the RdRP enzymes as templates, leading to the

production of antisense RNAs and dsRNAs that ultimately serve as the targets for sequence-specific destruction of RNA (Lindbo et al. 1993; Cogoni and Macino 1997, 1999; Depicker and Van Montagu 1997).

2.4 Mechanism of RNA Interference

RNA interference is basically carried out in two steps. The association of the RNA nucleases to a big double-stranded RNA and its cleavage into distinct 21–25 nucleotide RNA fragments (siRNA) constitute the first phase, also known as the RNA interference initiation step. These siRNAs join RNA-induced silencing complex (RISC), which is a multinuclease complex, in the subsequent stage, where it destroys the homologous single-stranded mRNAs (Agrawal et al. 2003).

2.4.1 Generation of siRNAs

Short interfering RNAs (siRNAs) have been found to be the most important molecules in initiating RNA interference. Bass (2000) anticipated the role of RNase III-type endonucleases in degrading dsRNA to siRNA for the first time by observing *E. coli* RNase III enzyme activity. The RNase III enzyme leaves a 3′ overhang of two nucleotides after staggered cuts are made on both strands of dsRNA. However, it was Tuschl's team who, for the first time, provided proof for the role of RNase III enzyme in RNA interference. They assessed the 21–23-nucleotide RNA sequences produced as a result of degradation of dsRNA in *Drosophila* cell-free system chemically. They demonstrated that the synthesized 21- to 23-nucleotide RNAs did not change the sugar-phosphate backbone but did include 5′-phosphate, 3′--hydroxyl, and a 3′ 2-nucleotide overhang (Elbashir et al. 2001). A target mRNA that is being frequently transcribed can be degraded for a considerable amount of time with just a few dsRNA molecules. Although there is a certain level of amplification when lengthy dsRNA is broken down into several siRNAs, it is insufficient to cause such continual mRNA degradation. RNA interference can be affected by mutations in RdRP genes, as a result of which RdRP may multiply siRNAs in the form of epigenetic agents, allowing their distribution throughout plants (Agrawal et al. 2003). The convincing role of RdRPs have been underlined by the studies conducted by various authors (Lipardi et al. 2001; Sijen et al. 2001).

2.4.2 Degradation of Target mRNA

The siRNAs produced, as mentioned above, are integrated into an argonaute (AGO)-family protein, and by exploiting the base-pairing homology of the siRNA, they give

AGO sequence selectivity that determines the mRNA to be targeted. The RNA-induced silencing complex (RISC), which is made up of the AGO protein and other proteins, is formed when siRNA or miRNA is incorporated. The target mRNA, in turn, may be cleaved or translationally silenced by the RISC. RNA-dependent RNA polymerase (RdRP) family protein can utilize a cleaved mRNA segment as a template for an additional round of dsRNA production, thereby beginning the second cycle of RNAi and generating secondary siRNAs (Fang and Qi 2016). Therefore, RNA interference is a cycle of mRNA degradation and siRNA production. As a result, mRNA translation is decreased, due to which protein production from mRNA translation is reduced (Hung and Slotkin 2021).

2.5 RNA Interference in Plants

The application of RNA interference in the field of plant biology has gained tremendous popularity due to the capability and efficiency of this process in inducing desirable characteristics and traits in plants (Jagtap et al. 2011). It is a natural gene regulation process that occurs in eukaryotes including plants (Kuo and Falk 2020). Plants follow two pathways of RNA interference based on siRNAs and miRNAs. siRNA-mediated pathway of RNA interference is initiated in the cytoplasm. This occurs when a target RNA, which can be an abnormal RNA or the RNA of virus (due to viral infection), is identified by the RNase III nuclease and is cleaved, resulting in the generation of siRNAs. Then, the RNA duplex is loaded onto Argonaute proteins, resulting in their degradation. However, miRNA-mediated RNA interference starts from the nucleus. Here, the primary miRNA produced in the nucleus is cleaved to produce the miRNA, which in turn is cleaved by the dicer like proteins to produce 22-nucleotide-miRNA duplex. This is transported to the cytoplasm, where the RISC comes into activity and degrades the target RNA (Kuo and Falk 2020). The technology of RNA interference can be exploited in plants for various purposes, like enhancing secondary metabolite production, increasing biotic and abiotic stress tolerance, etc. This can be done providing dsRNA exogenously to initiate RNA interference in plants (Das and Sherif 2020). Exogenous application can be done by injection, spraying, spreading, soaking root or seed, infiltration, and mechanical inoculation (Das and Sherif 2020). The technology can be used to change the architecture of plants, providing abiotic and biotic stress tolerance, for the elimination of allergens from plants, for the induction of male sterility in plants, for enhancing the production of secondary metabolites in plants, for the modification of color and scent of plants, for improving the nutritional quality of plants, for increasing the shelf life of fruits, for the removal of toxic compounds from plants, as well as for the development of seedless fruits (Jagtap et al. 2011). Table 2.1 shows the application of RNA interference in plants for inducing desirable traits in plants.

Table 2.1 Applications of RNAi in plants

Plant	Gene	Change incorporated by RNAi	References
Malus domestica	*hpRNA construct containing Mal d-1 inverted repeat sequence*	Removal of allergen Mal d1	Gilissen et al. (2005)
Oryza sativa L	Suppression of *OsGLU1* gene	Development of dwarf phenotype	Zhou et al. (2006)
Panax ginseng	Silencing of *DDS* gene	Decreased ginsenoside production	Han et al. (2006)
Petunia	Silencing of *PhPAAS* gene	Elimination of phenylacetaldehyde and 2-phenylethanol	Kaminaga et al. (2006)
Oryza sativa L	Suppression of *OsGA20ox2*	Development of semi-dwarf phenotype	Qiao et al. (2007)
Cucumis melo L. var. *cantalupensis* cv. Sun lady	*Coat protein gene*	Papaya ringspot virus type-W resistance	Krubphachaya et al. (2007)
Nicotiana tabacum cv. Samsun	Down regulation of *TA29*	Male sterility induction	Nawaz-ul-Rehman et al. (2007)
Triticum aestivum	Knockdown of *MLO*	*Blumeria graminis* f. sp. *Tritici* resistance	Riechen (2007)
Peanut	Suppression of *Ara h 2*	Elimination of allergen	Dodo et al. (2008)
Tomato	*hpRNA construct derived from PSTVd*	Potato spindle tuber viroid (PSTVd) resistance	Schwind et al. (2009)

2.6 Application of RNA Interference in Enhancing Plant Secondary Metabolite Production in Plants

The production of metabolites is regulated by various genes that are active within specific tissues or cell types (Verpoorte and Memelink 2002). Within the plant genome, there are approximately 20,000–60,000 genes, and a subset of around 15–25% of these genes is responsible for synthesizing secondary metabolites (Rastegari et al. 2019). Many compounds are still obtained from plants owing to the intricacy of their chemical structures, making them arduous to produce synthetically, and the expense associated with synthesizing chemicals is another significant challenge (Howat et al. 2014). Meeting the demand for these compounds often involves harvesting a substantial amount of plants from the wild, leading to depletion of their natural habitats (Samant et al. 2011). A possible technique to control the metabolite route is by RNA interference. For example, Zhang et al. (2015) reported that the production of phenolic acids in *Salvia miltiorrhiza* hairy root cultures can be significantly increased by using a combination of RNAi-mediated silencing of the chalcone synthase gene and treatment with salicylic acid. Likewise, Gong et al.

(2013) studied the impact of lycB gene silencing on carotenoid biosynthesis in *Haematococcus pluvialis*. Results revealed a substantial 99.4% elevation in lycopene content but a decrease in β-carotene content. In order to enhance the synthesis of plant secondary metabolites, several scientists have made numerous attempts to modify the plant genome. However, while many of these attempts have yielded unsatisfactory results, certain plants have demonstrated a response to RNAi by reducing the accumulation of plant metabolites (Han et al. 2010; Hücherig and Petersen 2013). In certain cases, RNAi has led to a decrease in certain secondary metabolites while promoting the accumulation of others (Fujii et al. 2007; Saema et al. 2015). These findings emphasize the importance of targeting specific phytochemicals and metabolic pathways in RNAi experiments aimed at manipulating plant secondary metabolites. Failure to do so may result in unexpected outcomes, highlighting the need for a precise approach when seeking to achieve desired results in plant modification. Table 2.2 shows RNAi-mediated gene-silencing efforts in medicinal plants and their effects.

2.7 Advantages of RNA Interference

The various advantages of RNA interference include the following (Kusaba 2004; Svoboda 2020).

1. It has an elevated degree of precision and efficiency, making it a popular and effective technique for examining gene activity.
2. With the help of RNAi, it is now possible to eradicate diseases and pests, incorporate unique plant features, and boost agricultural productivity.
3. The practical application of RNAi in agricultural plant genetic modification is possible.
4. It can be used to reduce the production of unwanted proteins by silencing unwanted genes.
5. It can be applied to enhance the production of substances in plants by downregulating or upregulating gene activity.
6. Even in plants with modest transformation efficacy, RNAi is an extremely successful knockdown method and is regarded to be helpful for genetic modification.
7. The technology has benefits above mutation-based reverse genetics in that it can controllably stifle transgene expression in multigene families.
8. RNA interference frequently function biologically in the control of endogenous gene expression, antiviral defense, and genome defense against transposable elements.

Table 2.2 RNAi-mediated gene-silencing efforts in medicinal plants and their effects

Plant	Genes/transcription factor subjected to RNAi	Effects of RNAi	References
Salvia miltiorrhiza	*Chalcone synthase*	Enhanced contents of phenolic acids hairy root cultures by combing the RNAi-mediated silencing of chalcone synthase gene with salicylic acid treatment	Zhang et al. (2015)
	Copalyl diphosphate synthase (CPS)	Decrease of tanshinones	Cheng et al. (2014)
Artemisia annua	*Pleiotropic drug resistance (PDR) transporter*	The level of β-caryophyllene was decreased in transgenic *A. annua* plants expressing AaPDR3-RNAi	Fu et al. (2017)
	1-Deoxy-D-xylulose-5-phosphate reductoisomerase (DXR)	Artemisinin contents in leaves were declined	Wang et al. (2018)
Ophiorrhiza pumila	Genes encoding *tryptophan decarboxylase (TDC) and secologanin synthase (SLS)*	Accumulation of camptothecin and related alkaloids, strictosidine, strictosamide, pumiloside, and deoxypumiloside was reduced in most TDC- and SLS-suppressed lines	Asano et al. (2013)
Lithospermum erythrorhizon	*LeEIL-1* (L. erythrorhizon EIN3-like protein gene 1)	LeEIL-1-RNAi repressed the expression of the above genes and significantly reduced shikonin production	Fang et al. (2016)
Vitis quinquangularis	*VqMAPKKK38*	Accumulation of stilbenes was almost abolished in RNAi-*vqmapkkk38* transgenic leaves	Jiao et al. (2017)
Nicotiana benthamiana	*Hydroxycinnamoyl transferase (HCT)*	Decrease in syringyl units and an increase in *p*-hydroxyphenyl units	Hoffmann et al. (2004)
Haematococcus pluvialis	Lycopene β-cyclase (*LycB*)	Increase in lycopene content by 99.4% while the β-carotene content was decreased	Gong et al. (2013)
Catharanthus roseus	Apoplastic peroxidase gene *CrPrx*	Results suggest a link between CrPrx and transcript regulation of key genes of monoterpenoid indole alkaloid biosynthetic pathway and alkaloid accumulation possibly	Jaggi et al. (2011)
Citrus	*CiMYB42* transcription factor	The overexpression of *CiMYB42* in sweet orange resulted in significant accumulation of limonin, whereas the downregulation of *CiMYB42* by RNAi resulted in a dwarf phenotype and less nomilin accumulation	Zhang et al. (2020)

(continued)

Table 2.2 (continued)

Plant	Genes/transcription factor subjected to RNAi	Effects of RNAi	References
Papaver somniferum	*Codeinone reductase (COR)*	The precursor alkaloid (*S*)-reticuline—seven enzymatic steps upstream of codeinone—accumulated in transgenic plants at the expense of morphine, codeine, oripavine, and thebaine. Methylated derivatives of reticuline also accumulated	Allen et al. (2004)
Taxus chinensis	*WRKY transcription factor, TcWRKY1*	Overexpression of *TcWRKY1* enhanced *dbat* expression in *T. chinensis* suspension cells, and RNA interference (RNAi) reduced the level of transcripts of *dbat*	Li et al. (2013)
Eschscholzia californica	Berberine bridge enzyme *(BBE)*	End products of isoquinoline alkaloid biosynthesis, such as sanguinarine, were considerably reduced, and reticuline was accumulated. Cells also produced a methylated derivative of reticuline, laudanine, which could scarcely be detected in control cells	Fujii et al. (2007)
Betula platyphylla	*Cycloartenol synthase (CAS)* and *β-amyrin synthase (β-AS)*	Betulinic acid production is positively regulated by suppression of the *β -AS* gene. The upregulation of *lupeol synthase gene (BPW)* and *β-amyrin synthase gene (BPY)*, as well as the conversion of 2,3-oxidosqualene to downstream products betulinic acid and oleanolic acid, can be significantly promoted by CAS interference	Yin et al. (2020)
Ocimum basilicum	*Coniferyl alcohol acyltransferase1 (CAAT1)*	Decreased levels of eugenol and accumulation of coniferyl alcohol and its derivatives	Dhar et al. (2020)
Panax quinquefolius	UDP-glycosyltransferase gene *Pq3-O-UGT2*	The levels of ginsenoside Rd., protopanaxadiol-type, and total ginsenoside were reduced	Lu et al. (2017)
Panax ginseng	Squalene epoxidase gene *PgSQE1* and *PgSQE2*	Interference of *PgSQE1* resulted in reduction of ginsenoside production. When *PgSQE1* was silenced in RNAi roots, there was a significant upregulation of *PgSQE2* and *PNX* (cycloartenol synthase), which led to an increase in phytosterol accumulation	Han et al. (2010)
	Dammarenediol synthase (DDS)	Reduction of ginsenoside production	Han et al. (2006)

(continued)

Table 2.2 (continued)

Plant	Genes/transcription factor subjected to RNAi	Effects of RNAi	References
Mentha x piperita	*Limonene-3-hydroxylase*	Suppression of the hydroxylase gene led to the buildup of limonene, which can account for up to 80% of the essential oil (as opposed to 2% in wild-type plants)	Mahmoud et al. (2004)
Coleus blumei	*Hydroxyphenylpyruvate reductase (HPPR) and rosmarinic acid synthase (RAS)*	Significantly diminished rosmarinic acid contents were seen	Hücherig and Petersen (2013)
Withania somnifera	*Cycloartenol synthase (CAS)*	Decreased level of withanolide content	Mishra et al. (2016)
	S-adenosyl L-methionine-dependent sterol-C24-methyltransferase type 1 (SMT1)	The transgenic RNAi lines had higher cholesterol content and lower levels of campesterol, stigmasterol, and sitosterol	Pal et al. (2019)
	Sterol glycosyltransferases (WsSGTL1)	Decrease in level of glycosylated sterols, withanolides (mostly withaferin A) were significantly more abundant, and withanoside V, the glycowithanolide of *W. somnifera*, was reduced	Saema et al. (2015)

2.8 Limitations and Disadvantages of RNA Interference

Even though RNA interference has various advantages, there are several disadvantages which include but are not limited to the following (Sledz and Williams 2005).

1. RNA interference removes the expression of genes in the whole organism and not in just a cell or tissue unless and until tissue specific knockouts are used.
2. An important constraint of RNA interference technology is the synthesis of a suitable and functional siRNA sequence.
3. Sometimes, even adhering to the suggested guidelines for siRNA production does not guarantee successful target gene repression or silencing.
4. The structure of siRNA, the sequence of siRNA selected, and the responsiveness of cell type to the absorption of siRNA are some of the critical factors that determine the success rate of this technology.
5. The half-life period of the protein or target message also needs to be determined for ideal gene silencing.

2.9 Conclusion

RNA interference (RNAi) is a biological process that involves the silencing of gene expression by targeting mRNA molecules (Pujar et al. 2023). RNAi has emerged as a powerful tool for manipulating gene expression and has been extensively used for functional genomics studies (Kubowicz et al. 2013; Paddison and Hannon 2002). In recent years, there has been growing interest in using RNAi to enhance the production of secondary metabolites in plants (Wagner and Kroumova 2008). One of the main strategies for enhancing secondary metabolite production is to manipulate the genes involved in their biosynthesis pathways. RNAi can be used to knockdown the expression of genes that act as inhibitors or repressors of secondary metabolite production in plants, thereby enhancing their accumulation (Saurabh et al. 2014; Bulgakov and Avramenko 2015).

Modern researchers are increasingly using RNAi to control the genes responsible for producing secondary metabolites. In order to assure that all members of a gene family may be silenced by RNAi, researchers must find a distinctive or conserved area of the target gene. Regrettably, a key obstacle is that many non-model plants involved in the synthesis of secondary metabolites lack comprehensive genomic information. Although RNAi requires 21–25 nucleotide homology to decrease gene function, one significant drawback is that it may potentially affect unintended targets (Scherer and Rossi 2003; Senthil-Kumar and Mysore 2011). Despite this drawback, scientists continue to use RNAi to pinpoint gene functions and boost the presence of desirable metabolites. RNAi-mediated gene silencing is a promising approach for enhancing secondary metabolite production in plants and microorganisms. By targeting key genes in metabolic pathways, RNAi can redirect metabolic flux towards the desired product, leading to increased yields and improved product quality. However, further research is needed to optimize siRNA or miRNA design and delivery methods and to overcome the challenges associated with off-target effects and delivery efficiency.

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Chapter 3
CRISPR/Cas9: A Novel Genetic Tool to Manipulate Plant Secondary Metabolite Pathways

Muthukrishnan Arun, Jayachandran Halka, and Kumaresan Kowsalya

Abstract The CRISPR/Cas9 gene editing method has brought a new era in genetic engineering and genome research. However, CRISPR/Cas9 system incorporation has changed gene editing methods due to its numerous alluring features such as high efficiency, simplicity, adaptability, and the capacity for multiplexing improvements. This system creates minor heritable mutations in the genome, which is significant for elucidating secondary metabolite pathways in medicinal plants. Scientists are increasingly focusing on mining critical genes in metabolic pathways and developing novel synthetic approaches in order to boost the production of potent compounds. However, the use of CRISPR technology in medicinal plants is still in its early stages and also has several bottlenecks such as a lack of genome information and genetic transformation technology. This chapter highlights the applications of CRISPR/Cas9 technology in improving secondary metabolites in various medicinal plants such as *Atropa belladonna*, *Cannabis sativa*, *Cichorium intybus* L., *Dendrobium officinale*, *Dioscorea zingiberensis*, *Salvia miltiorrhiza*, *Rehmannia glutinosa*, *Symphytum officinale*, *Papaver somniferum* L., and *Monochasma savatieri*. It also discuss the future directions of using this approach in other medicinal plants for developing ideal germplasm, creating biotic, and abiotic stress-tolerant plants.

Keywords CRISPR/Cas9 · Secondary metabolite · Medicinal plant · Genetic improvement

3.1 Introduction

Genome editing is a genetic engineering technique in which DNA is inserted, altered, or substituted in the genome of an organism. Genome editing comprises of various techniques such as zinc finger nuclease (ZFNs), transcriptional activator-like

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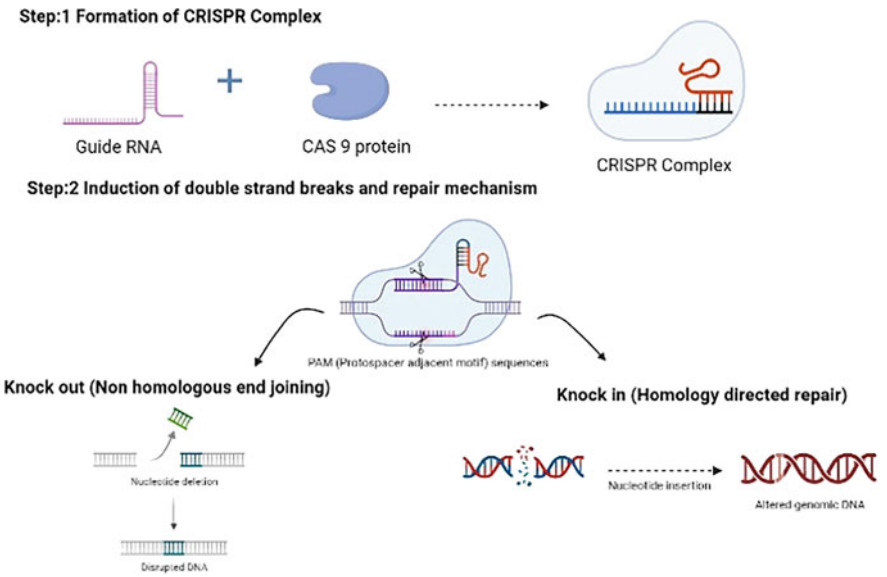

Fig. 3.1 Mechanism involved in CRISPR/Cas9 technique

effector nuclease (TALENs), and the most recently developed clustered regulatory interspaced short palindromic repeat (CRISPR)/CRISPR associated nuclease 9 system. This technique was developed from the natural bacterial immune system which is distinct and effective in nature. Due to its low cost and easy adaptation, this method is employed for a variety of directed genome editing applications (Chandel 2023). The CRISPR-Cas system is divided into two functional classes based on the structure of the effector nuclease genes. The class I CRISPR system includes the type I, III, and IV systems which are identified by the multisubunit effector nuclease complexes. The class II CRISPR systems are categorized into type II, V, and VI14, according to the factors necessary for pre-crRNA processing and the diversity of the effector protein's domains. The class II CRISPR system has only one effector protein, called Cas9 (Devi 2022; Yildirim and Ekinci 2022). It also enables the scientific community to correlate the parallel relationship between the genetic makeup of plants and their respective biological attributes (Vidya and Arun 2023). CRISPR/Cas9 has two components called Cas9 and sgRNA. The sgRNA along with Cas9 complex cleaves the double-stranded DNA causing double-stranded breaks (DSB). When this break occurs, nonhomologous end-joining (NHEJ) or homology-directed repair (HDR) DNA repair mechanisms are initiated. Most of the time, the NHEJ repairs the double-stranded breaks. It is a straightforward method to produce mismatches and gene deletions (indel), which results in gene knockout. In the presence of an oligo template, homology-directed repair (HDR) causes selective gene substitution (insertion) called knock-in (Fig. 3.1) (Liu et al. 2017; Halka et al. 2022). Although homologous recombination is the cornerstone of genome

engineering, the effectiveness of editing is limited by its low frequency (Afzal et al. 2020). On top of that, the CRISPR/Cas system recently developed a few advancements such as CRISPR with no PAM (protospacer adjacent motif) limitation, CRISPRi gene knockdown, CRISPR with epigenetic modification, and small-sized new CRISPR systems (Manghwar et al. 2019). Recently, CRISPR/Cas9-based imaging methods were equipped with several new features to improve the fluorescence for effective visualization of chromatin and gene loci. It includes supernova tagging systems, RNA aptamers, halo tags, molecular beacons, bimolecular fluorescence complementation, and RNA-guided endonuclease in situ labeling (Singh and Jain 2022). Moreover, this effective gene editing also established the groundwork in medicinal plants for investigating the molecular activities of genes, producing top-notch germplasm, hastening domestication, and boosting the secondary metabolite's productivity and quality. In addition to that, it advances plant molecular breeding by producing the most desirable features in medicinal plants (Li et al. 2021a). Only a few studies are available investigating the gene function in metabolic pathways of medicinal plants due to inadequate genome data, higher heterozygosity, and lack of genetic transformation technology. Besides, the lack of functional genomics research has impeded the development of restrictions in functional gene mining and genetic enhancement of medicinal plants. Yet, through the CRISPR/Cas9 technology, researchers have recently solved the hidden riddles with self-incompatible genes of *Rehmannia glutinosa* (Scrophulariaceae family) which prevent the production of homozygous lines through self-pollination by knocking out the *RgPDS1* gene (Li et al. 2021b). Hence, this chapter intends to summarize the potential applications of CRISPR/Cas9 in improving clinically significant secondary metabolites in different medicinal plants.

3.2 Applications of CRISPR in Medicinal Plants

CRISPR/Cas is a precise and efficient genome editing technique that improves the quality, infers pathways, and even upgrades valuable secondary metabolites. This technique is helpful in modulating the phytochemical profile of medicinal plants and even increases the production of plant-derived metabolites suitable for commercial purposes (Li et al. 2017). Genetic or metabolic engineering methods are used to manipulate the production of secondary metabolites obtained from plants because these methods are capable of changing a number of the genes involved in the biosynthetic pathway. Any uncertainty in the biosynthetic pathway is likely due to the alterations observed in the transcription level of the whole system. These alterations may change the plant's regulatory system which in turn is found to control secondary metabolite productions in medicinal plants. Further, when the rate-limiting enzyme is overexpressed, there is a drastic change in the transcriptional level for the closely related secondary metabolite genes (Rehman Summia et al. 2021). Further, the applications of CRISPR/Cas for the production of secondary metabolites in medicinal plants are summarized (Fig. 3.2 and Table 3.1).

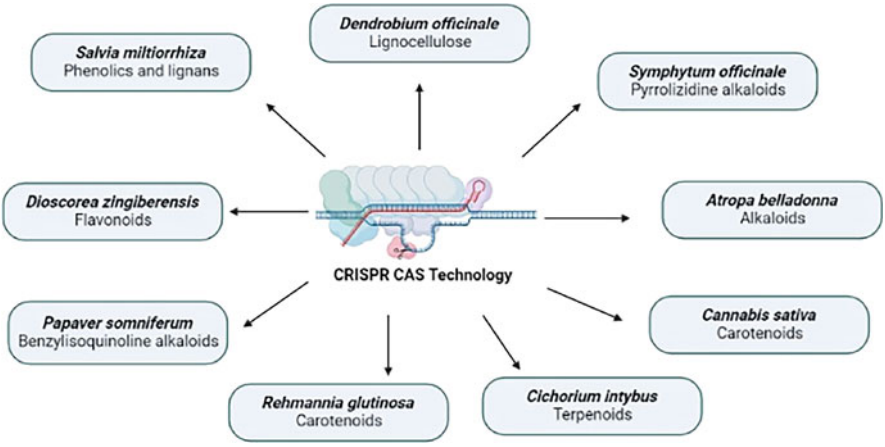

Fig. 3.2 Summary of CRISPR/Cas9 technology in medicinal plants for developing various secondary metabolites

3.2.1 Atropa belladonna

Atropa belladonna L. is one of the most important herbal plants with anisodamine, scopolamine, hyoscyamine, and atropine metabolites. Zeng et al. (2021) developed *A. belladonna* plants for the first time devoid of anisodamine and scopolamine utilizing the CRISPR/Cas9 technology to disrupt *hyoscyamine 6-hydroxylase* (*AbH6H*). It was observed that out of 11 transgenic plants, only 4 plants exhibited mutation and the mutation rate was approximately 63.6%. Thus, hyoscyamine synthesis was considerably increased in the *A. belladonna* plants, but neither anisodamine nor scopolamine was produced. Hasebe et al. (2021) claimed that the disruption of *pyrrolidine ketide synthase* (*PYKs*) reduces the accumulation of tropane alkaloids in *A. belladonna* using CRISPR/Cas 9 technology.

3.2.2 Cannabis sativa

Cannabis sativa is an annual and dioecious crop. Since it is enriched with phytocannabinoids, the demand for this plant is substantially increasing. Zhang et al. (2021) edited the *phytoene desaturase* (*CsPDS1*), a well-linked marker gene, in *C. sativus* using the CRISPR/Cas9 technology. Further, they developed four transgenic plants with albino phenotype by using *Agrobacterium*-mediated transformation system.

Table 3.1 List of medicinal plants improved by CRISPR/Cas9 technology

Species	Target gene	Secondary metabolite	Vector	Promoter	Mutation frequency	References
Salvia miltiorrhiza	*Committed diterpene synthase* (*SmCPS1*)	Tanshinone biosynthesis	pCAMBIA 1300	CaMV35S/ AtU6–26	11.5%	Li et al. (2017)
Salvia miltiorrhiza	*Rosmarinic acid synthase* (*SmRAS*)	Phenolic biosynthetic pathway	pCAMBIA 1300	CaMV35S/ AtU6–26,OsU3	50%	Zhou et al. (2018)
Salvia miltiorrhiza	*Laccase genes* (*SmLACs*)	Phenolic acid and lignin biosynthetic pathway	pCAMBIA 1300	AtUBQ/AtU6	90.6%	Zhou et al. (2021)
Salvia miltiorrhiza	*Basic leucine transcription factor* (*SmbZIP2*)	Phenolic acid biosynthetic pathway	pCAMBIA 2300	CaMV35S/ AtU6–26	12%	Shi et al. (2021)
Dendrobium officinale	*Coumarate 3-hydroxylase (DoC3H), Cinnamate 4-hydroxylase (DoC4H), 4-Coumarate coenzyme A ligase (Do4CL), and Irregular xylem5 (DoIRX)*	Lignocellulose biosynthetic pathway	pCAMBIA 1301-35SN	MtHP, CVMV, MMV, PCISV, CaMV 35S/OsU3	16.7%, 20%, 33.3%, 33.3%, and 6.7% for *C3H*, *C4H*, *4CL*, *CCR* and *IRX*	Kui et al. (2016)
Cannabis sativa	*Phytoene desaturase* (*CsPDS1*)	Carotenoid biosynthesis	pKSE401	AtU6	2.5% and 51.6% for the homozygous and chimeric mutants	Zhang et al. (2021)
Papaver somniferum	*3s-Hydroxyl-N-methylcoclaurine 4′-o-methyltranferase* (*Ps4'OMT2*)	Biosynthesis of benzylisoquinoline alkaloids	pK7WGF2	CaMV35S/AtU6	85%	Alagoz et al. (2016)
Dioscorea zingiberensis	*Farnesyl pyrophosphate synthase* (*Dzfps*)	Biosynthesis of squalene	pCAMBIA 1300	CaMV35S/OsU3	60%	Feng et al. (2018)
Rehmannia glutinosa	*Phytoene desaturase* (*RgPDS*)	Carotenoid biosynthesis	PKSE401	AtU6–26	Produced 45.5% albino phenotype with reduced	Li et al. (2021a, b)
Cichorium intybus	*Germacrene A synthase* (*CiGAS*)	Biosynthesis of sesquiterpene lactones	PEG mediated transfection	–	–	Cankar et al. (2021)

(continued)

Table 3.1 (continued)

Species	Target gene	Secondary metabolite	Vector	Promoter	Mutation frequency	References
Symphytum officinale	*Homospermidine synthase* (*SoHSS*)	PA biosynthetic pathway	pDE	AtU6–26	–	Zakaria et al. (2021)
Atropa belladonna	Disruption of *hyoscyamine 6 β-hydroxylase* (*AbH6H*)	Alkaloid biosynthesis	pCAMBIA 1300	CaMV35S/AtU6	63.6%	Zeng et al. (2021)
Atropa belladonna	*Pyrrolidine ketide synthase (AbPYKS)*	Tropane alkaloids	pMGP237-T1-T4	–	–	Hasebe et al. (2021)
Monochasa savatieri	*Cellulose synthase like D* (*MsCSLD3*)	Phenolic biosynthetic pathway	pRGEB31	CaMV35S/AtU6	6.49%	Bai et al. (2023)

3.2.3 Cichorium intybus *L.*

Cichorium intybus var. sativum is a type of industrial crop grown for extracting prebiotic and low-calorie sweeteners called inulin. The chicory taproot is accumulated with squalene and more phenolic chemicals. These compounds have a punitive taste and could not be eliminated during inulin extraction. Thus, CRISPR/Cas9 technique was able to inactivate the genes responsible for encoding the *germacrene synthase* (*CiGAS*). As a result of blocking the STL biosynthesis pathway, there is a reduced STL level which helps in facilitating inulin extraction without bitter-tasting compounds, and there is a substantial increase in the availability of farnesyl pyrophosphate (FPP) which increases the phenolic content in the chicory roots (Cankar et al. 2021).

3.2.4 Dendrobium officinale

Dendrobium officinale is a valuable medicinal herb, used in medical treatment for more than 2000 years. It possesses a broad spectrum of medicinal qualities, including hepatoprotective (Liang et al. 2018), antitumor (Liang et al. 2019), hypoglycemic (Chen et al. 2020), gastro-protective (Zhang et al. 2019), and anti-inflammatory (Yang et al. 2020) properties. Four genes such as *coumarate 3-hydroxylase* (*C3H*), *cinnamate 4-hydroxylase* (*C4H*), *4-coumarate coenzyme A ligase* (*4CL*), and *irregular xylem5* (*IRX*) in the lignocellulose biosynthesis pathway were successfully altered. Additionally, they measured the mutation rates of several target sites between 10% and 100% using PCR amplification and sequencing techniques (Kui et al. 2016).

3.2.5 Dioscorea zingiberensis

Dioscorea sp. is well recognized for producing diosgenin, which is steroidal hormone with anti-inflammatory, anti-allergic, cardiovascular, antitumor, and neuroprotective actions. Its rhizomes are extremely useful for isolating diosgenin and for making Dun-Ye-Guan-Xin-Ning tablets. Feng et al. (2018) reported that CRISPR/Cas9 editing tool used in this plant was carried out using the *Agrobacterium*-mediated transformation method. High mutant frequency for the secondary metabolite squalene was made possible by excluding the *farnesyl pyrophosphate synthase* (*Dzfps*), which led to decreased levels of squalene.

3.2.6 Monochasma savatieri

Monochasma savatieri is a perennial medicinal plant that is extensively used for treating various diseases. In this study, Bai et al. (2023) successfully transformed *M. savatieri* hairy root mutants through the targeted knockout of the *CSLD2/3* in a phenolic biosynthetic pathway using CRISPR/Cas 9 technique.

3.2.7 Salvia miltiorrhiza

Salvia miltiorrhiza is a Chinese medicinal herb that belongs to the family Labiatae and is widely used for treating cardiovascular and cerebrovascular diseases and diabetes (Ren et al. 2019). Due to the presence of lipid-soluble compounds such as tanshinones and water-soluble phenolic acids such as rosmarinic acid, salvianolic acid, and lithospermic acid, this plant is in great demand (Luo et al. 2014). Another study reported that CRISPR/Cas technique is helpful in knocking out the *SmCPS1*, which is an effective *diterpene synthase* involved in tanshinone biosynthesis. Further, it is reported that three homozygous and eight chimeric transgenic hairy root mutants were produced through *Agrobacterium*-mediated transformation in *Salvia.* Three major predominant tanshinones such as tanshinone I, tanshinone IIA, and cryptotanshinone are completely absent in homozygous mutants. These results demonstrated that this gene is crucial for the formation of tanshinones and laid the groundwork for further research into the production of secondary metabolites in *Salvia miltiorrhiza* (Li et al. 2017). Zhou et al. (2018) successfully generated *S. miltiorrhiza* hairy root mutants by employing CRISPR/Cas9 technology to knock down the *rosmarinic acid synthase SmRAS* in the phenolic acid synthesis pathway. From 16 distinct transgenic hairy root lines, a total of 5 biallelic, 1 homozygous, and 2 heterozygous mutants were produced. The mutants had higher concentrations of the RA precursor 3,4-dihydroxyphe nyllactic acid, whereas phenolic acids like RA, salvianolic acid B (SAB), and salvianolic acid had much lower concentrations.

Recent studies reported that CRISPR/Cas9 dual-locus editing technique was able to eliminate more than 20 genes from the laccase family in *S. miltiorrhiza*. The expression levels of the target laccase genes and critical genes for phenolic acid production were dramatically increased in the editing lines. The formation of hairy roots was also greatly slowed down in the CRISPR lines. These results revealed the function of *SmLACs*, which are crucial for phenolic acid synthesis as well as root growth and lignin formation in *S. miltiorrhiza* (Zhou et al. 2021). Shi et al. (2021) targeted *SmbZIP2*, a new basic leucine zipper transcription factor identified from *S. miltiorrhiza*, using overexpression (OE) and the CRISPR/Cas9 technique. Further, analyzing the transgenic lines showed that the phenolic acid content was increased in the CRISPR/Cas9 lines but decreased in the OE lines. The study showed

that *SmbZIP2* acts as a negative regulator in phenolic acid biosynthesis offering a unique strategy for the production of phenolic acid.

3.2.8 Rehmannia glutinosa

Rehmannia glutinosa is a vital component of traditional Chinese medicine that has special pharmacological and economic importance. In this study, it is successfully modified with the CRISPR/Cas9 cassette by precise editing of the *RgPDS1*. More intriguingly, a few partially albino shoots were able to develop in MS medium in a similar way to wild-type plants, indicating that the chlorophyll and carotenoid content in the leaves of these *PDS* mutants were still present with special emphasis in molecular breeding to generate desired traits into the plant (Li et al. 2021a, b).

3.2.9 Symphytum officinale

Comfrey, also known as *Symphytum officinale* L. Boraginaceae, is a plant with anti-inflammatory, analgesic, and proliferative properties. Its potential role in pharmaceuticals is constrained by the large concentrations of toxic pyrrolizidine alkaloid (PA) throughout the entire plant. Zakaria et al. (2021) claimed that CRISPR/Cas9 technique helps in eliminating *homospermidine synthase* (*HSS*), the first specialized enzyme in the PA biosynthesis pathway. The homospermidine and PA concentrations in the hairy roots (HRs) appeared to have decreased, as evidenced by the successfully acquired HSS-deficient HRs. This work showed the effectiveness of using gene editing as well as the ability to create nontoxic transgenic comfrey varieties.

3.2.10 Papaver somniferum *L.*

Benzylisoquinoline alkaloids (BIAs) are used in the biosynthesis of the opium poppy to create the therapeutically relevant narcotic morphine. Morphine heightens the brain's reward response because it has stronger effects on the central nervous system. CRISPR/Cas9-based gene knockout systems were able to specifically target the gene *4′ OMT2*, which is involved in the manufacture of BIAs (morphine, codeine, s-reticuline, noscapine, thebaine, laudanosine, and papaverine) (Alagoz et al. 2016).

3.3 Limitations of CRISPR/Cas9 Technique

This genome editing technique also possesses a few disadvantages such as off-targets of the desired gene, the identification of the genetic makeup related to particular plant traits, the competent delivery of the CRISPR/Cas9 construct to plant cells, and a reliable plant transformation system. In recent years, these shortcomings have been effectively overcome by the CRISPR/Cas variants and Cas9 orthologs with high precision and specificity (Fig. 3.3) (Vidya and Arun 2023).

3.4 Future Perspectives

This CRISPR/Cas9 technique has emerged as a promising tool in altering the medicinal plant genome, which subsequently escalates their metabolic profile. This can result in user-designed medicinal plants that aid in the large-scale production of commercially important secondary metabolites (Niazian 2019). It revolutionizes the agricultural field by enhancing the nutritional content as well as yield while making them more resilient to biotic and abiotic stresses. These upgraded plant attributes are much more significant and most required to satisfy the demands of a growing global population worldwide (El-Mounadi et al. 2020). On the other hand, a quick method for creating germplasm with disease and herbicide-resistance traits in medicinal plants is made possible by CRISPR/Cas9 technology. Also, researchers can create these systems that can remove harmful genetic elements or induce gain-of-function mutations by carefully altering the genome of therapeutic medicinal plants. CRISPR/Cas9 technique is the most efficient, safest, and cost-effective strategy to control diseases which encourages the sustainable cultivation of medicinal plants (Guo et al. 2022). Moreover, this technique also helps us to unravel the most complex

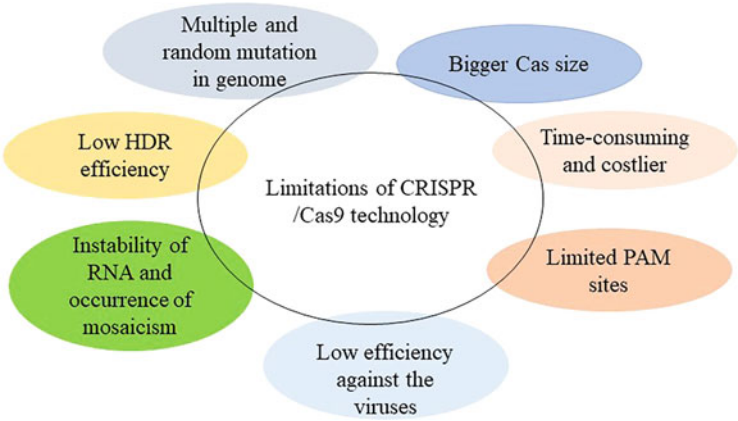

Fig. 3.3 Limitations of CRISPR/Cas9 system

biosynthetic network and multiple regulatory functions of secondary metabolites in medicinal plants (Li et al. 2021a, b). In order to use this technique efficiently, the scientific community must solve the different biosafety and societal problems around this technology in the long term. However, there is a strong need for the re-evaluation of the laws governing genome-edited medicinal plants and the creation of biosafety awareness among the people (El-Mounadi et al. 2020).

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Chapter 4
Enhancement of Plant Secondary Metabolites by Genetic Manipulation

Harsharan Singh

Abstract Secondary metabolites comprise the large repository of biomolecules, which are biosynthesized by bacteria, plants, and microorganisms. The metabolites necessary to perform day-to-day routine activities are termed primary metabolites and are the outcome of primary metabolism. Secondary metabolism forms more diverse and complex biomolecules termed as secondary metabolites; these are the end product of secondary metabolism. Depending upon the diverse functional group or basic carbon skeleton, secondary metabolites are categorized as terpenes, phenolics, and alkaloids. All the secondary metabolites are biosynthesized in either one of the shikimic acid, malonic acid, mevalonic acid, and methylerythritol phosphate pathways. These secondary metabolites enable the plant's survival in different habitats and fluctuating environment conditions. The secondary metabolites are more economical and have lesser side effects as compared to chemical drugs, therefore an indispensable part of the traditional healthcare system. They are also useful in food, aroma, spices, and perfume industry. Owing to their diverse and multiple uses, there exists a huge gap between their production and demand. Due to the uniqueness and complexity in the chemical structures of secondary metabolites, often complete plants/organisms are used for harvesting secondary metabolites. The production of secondary metabolite in their native systems has the problems, such as low yield, tissue- and organ-specific compartmentalization, and accumulation in response to specific growth or environmental and geographical conditions. Moreover, harvesting secondary metabolites from the wild or native stage is often not a sustainable way, as this might result in the overharvesting of concerned plant as well as to deterioration of biodiversity. Apart from this, the pharmaceutical industry demands homogeneous samples having uniform compositions of the bioactive principles that is difficult to be achieved when harvesting, or collection is done randomly from the wild. The practices, such as cultivation, culturing, and domestication of the source organism, might be a valuable alternative, providing more

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uniform conditions and delivering homogenous composition of desired valuable secondary metabolites. But in most of the cases, the feasibility of this approach is limited because of various reasons. To overcome these hurdles concerning to the low synthesis, heterogeneity in composition, and accumulation in response to specific cues or specific stage of secondary metabolites, genetic manipulation of host organism seems to be a viable option. The research related to secondary metabolism through genetic manipulation is expanding at a fast pace and is challenging in molecular biology and biotechnology, holding unlimited opportunities. New advents in molecular biology, functional genomics, metabolomics, and proteomics are expanding our understanding of the pathways, networks, genes, and enzymes involved in the synthesis of secondary metabolites. These inputs from different dimensions of genetic manipulations are contributing determinant role in developing efficient strategies for targeted biosynthesis of valuable secondary metabolites. With the ever-increasing demand for novel drugs related to recently identified molecular targets, genetic manipulation will likely become more and more relevant. The lucrative economic aspects of commercial and industrial production of secondary metabolite related to pharmaceuticals, food, nutraceutical, aromatic, and perfume industries could magnetize investments and interest and build up new opportunities in this promising research field. This chapter discusses the various approaches and strategies used for the genetic manipulation of secondary metabolites and manipulation of the biosynthetic pathway of secondary metabolite products, leading to an improved quantity of secondary metabolites or more valuable and desired biomolecules. The various examples concerned with each approach have been also mentioned.

Keywords Secondary metabolites · Genetic manipulation · Medicinal plants · Elicitor · Biosynthetic pathways

4.1 Introduction

Plants and microorganisms act as biosynthetic hubs for the production of primary as well as secondary metabolites (SMs). Primary metabolites include biomolecules like sugar, nucleic acid, lipids, and amino acid; these molecules directly play an indispensable role in the growth and developments of an organism. SMs include the biomolecules like terpene, alkaloids, glycosides, flavonoids, and volatile oil. The SMs include diverse types of low-molecular-weight compounds with little to no direct role in the plant's routine activities. However, SMs had been proved to have determinable contributions to plant survival from the external environment, defense against pathogen attack, and coping up with unfavorable physical factors, like high and low light intensity, fluctuation in temperature, UV irradiation, and harsh drought conditions. They play a critical role in pollination, oviposition, seed dispersal, pharmacophagy, etc. The SMs also act as a competitive weapon against other living forms. They act as agents for symbiotic association with other organisms, metal transportation, communication, reproduction, and effectors for differentiation. They also play a critical role in the germination and sporulation of plants. The SMs, like

phytoproteins and phytolexins, confer insect-pest and herbivore resistance to plants. SMs are also meant for human consumption and impart flavor, color, and aroma to diet. The SMs are used to produce drugs, colors, insecticides, flavors, and perfumes, for which different plant parts and flowers are used. SMs of plant origin are of utmost importance commercially in the pharmaceutical industry, as major portion of used pharmaceutical come directly or indirectly from plants.

Based upon the biosynthetic pathway, composition, structure, and function SMs can be classified as terpenes, steroids, phenolics, alkaloids, fatty acid-derived compounds, N-containing compounds, S-containing compounds, etc. Terpenoids and steroids are groups of compounds originating from mevalonate (MVA) and non-mevalonate (MEP) pathways. Terpenes, which consist of carbon and hydrogen and are formed by head-to-tail condensation of basic isoprenoid units (C_5H_8), show considerable diversity in their structures. Steroids have tetracyclic carbon ring in their structure. Phenolics consist of simple sugar and benzene ring in their basic structures. Alkaloids have basic amine group in their structures and biosynthesized from amino acids. Fatty acid-derived SMs are originated from fatty acyl derivatives like acetyl-CoA, propionyl-CoA, and methylmalonyl-CoA. The N-containing compounds contain a heterocyclic ring of aromatic amino. The S-containing compounds are unusual metabolites of plant origin, although their number is small as compared to other classes of SMs, but their role in host-pest interaction is critical (Burow et al. 2008).

The biosynthesis of all the known SMs is carried out via basic metabolic precursors, such as isoprenoid unit, phenylalanine lyase, acetyl-CoA, chalcone synthase, acetoacetate, malonyl-CoA, etc. Phenolics are derived from phenylpropanoid pathway and originate from basic precursors, like phenylalanine or chalcone, and are responsible for providing color to flower and fruits. Alkaloids and N-containing and S-containing compounds are the metabolic product of shikimic acid pathway.

The SMs are indispensable in the life of living biota as they form bioactive principle of ayurvedic preparation used to treat different diseases (Hussain et al. 2012; Parsaeimehr et al. 2011). They act as a nutraceutical and play their role in disease prevention. They are utilized in preparation of dyes, polymer, fibers, glue, oil, wax, paint, perfumes, and drugs (Grindberg et al. 2011). The SMs fulfill the requirement of basic raw materials required for the synthesis of herbal formulations, food colors, pesticides, perfumes, and aromas. The major share of active principle compounds of any pharmaceutical formulation is directly or indirectly derived from plants; therefore, secondary metabolism and secondary metabolites are attractive target for plant breeding.

The remote availability, overexploitation, deforestation, and difficulty in the cultivation of source plant pose major hurdles to the commercial use of these secondary metabolites. The other limiting factors include low yield, seasonal variation in composition, unique structure-specific compartmentalization, difficulty in extraction, and purification of these active principles from plants. The other important bottleneck factors for industrial or commercial production of SMs are the economic cost involved in screening and bioassay of these phytochemicals from

cultivated plants. Furthermore, the highly complex structure and stereospecificity of these phytochemicals rule out the possibilities of chemical synthesis of this active principle for commercial use. Moreover, homogeneity in the composition, which is strictly desired by industry, is often absent in native collection or harvest of source organism.

The array of biotechnological approaches, such as cell culture, suspension culture, callus culture, and micro propagation of whole plants, have been employed for the production of these SMs from cultivated plants in past time. However, to date, these brought low or remote commercial success because of decrease productivity and high cost involved in these cell cultural processes.

Molecular breeding, via genetic manipulation, is an attractive and feasible approach. Various genetic manipulation approaches, like overexpression or downregulation of important gene, preregulation of transcription factor, and controlled expression of regulatory steps of concerned biosynthesis pathway, resulted in enhanced yield and deposition of SMs. The last few decades had been dedicated to research in this direction; lot of progress has been done in this field (Tiwari and Rana 2015; Yeoman and Yeoman 1996), and a major hurdle has been the lesser understanding of biosynthesis pathways at the level of intermediates and enzymes involved in secondary metabolism. Genetic manipulation attempt to overexpress or suppress the quantity of a single or multiple phytochemical of valuable biomolecules (Verpoorte et al. 2000; Collin 2001; Demain 1999; Demain and Fang 2000; McMurry 2015). To downregulate the targeted metabolite(s), an array of genetic manipulation elements is available. The enzyme controlling the rate-limiting step in the pathway can be manipulated, via downregulation of the expression of concerned gene or by immunomodulation of target the enzyme (Verpoorte et al. 2000). Other strategies involve redirection of the metabolites into a divergent pathway or increasing the catabolism rate of the concerned metabolite. The ultimate and primary aim of genetic manipulation is to increase the quantity of targeted SM (s) in the native organism or to express the biosynthesis pathway in other microbial organisms (heterologous expression). To accomplish the elevated synthesis of a (group of) metabolites(s), either the expression of single or multiple regulatory transcripts is altered, hence manipulating unique regulatory steps in the biosynthesis, restricting the futile metabolic pathways, and suppressing degradation of the targeted biomolecule. The other approaches involve the alternation of regulatory genes, controlling multiple steps in the biosynthesis of the concerned biomolecule.

Genetic manipulation is more beneficial than traditional available technologies, such as plant breeding and backcrossing to harness crops for valuable traits or varietal development. For example, traditional approaches are only applicable to almost similar species, and different species with large genetic gap cannot be crossbred. Therefore, the feasibility and success of the classical method of harnessing desired traits are solely dependent on the availability of a natural pool of resistance, whereas genetic manipulation enabled one to transfer traits between organisms from a wide range of distant species for manipulation of different traits related to immunity from insects/pests, low water content, heat, and survival from herbicides and high salt, improving photosynthetic efficiency. Plants from hundreds

of different species have been transformed, which have conferred us with commercial and ecological benefits (Ahmad et al. 2012). The direct DNA sequence manipulation to regulate gene expression in nutraceutical plant research is a favorable approach adopted for the production of bioactive compounds. For instance, terpenes biosynthetic pathway of *Mentha* spp. were manipulated to alter the essential oils in trichomes (organs dedicated to production and accumulation of SMs) and improved resistance to fungal and abiotic stress (Lucchesini et al. 2006; Wang et al. 2011). The developments with interesting examples pertaining to genetic manipulation are discussed here, covering important secondary metabolite groups.

4.2 Genetic Manipulation for SM

Secondary metabolites have a large share of active principles in healthcare drug formulation, veterinary medicines, and crop management chemicals (Hussain et al. 2012; Parsaeimehr et al. 2011; Facchini 2001). Availability of sequencing of the entire genome at an economical cost by utilizing next-generation sequencing (NGS), pyrosequencing, and sequencing by hybridization techniques has been accelerating the discovery of the latest and unique phytochemicals biosynthesized by plant and microbes. The genetic manipulation strategies generally adopted for enhancements of SMs are as follows:

4.2.1 Mutagenesis and Recombination

Tryptophan found to have a critical nutritional role in mankind and animal and is well documented as a critical component in cereals (Bravo et al. 2013; Corpas et al. 2021). Thus, tryptophan is a crucial breeding aim in cereals. The level of tryptophan accumulation is determined by the regulatory enzyme anthranilate synthase (AS) in plants via feedback inhibition. This AS enzyme has dual α and β subunits. The β subunits' transfer of amido group from glutamine to the α subunits hence act as a glutamine amidotransferase. The binding site for tryptophan is found to be present on α subunits involved in the amination of chorismate and enolpyruvyl via feedback inhibition. The α subunit AS has two isoforms in rice, written as OASA1 and OASA2. Studies on recombinant AS showed greater sensitivity of the OASA2 as compared to OASA1 for tryptophan accumulation (Kanno et al. 2004). These findings highlighted that for a better accumulation of tryptophan in rice, the manipulation of OASA2 α-subunit is an appropriate target. The manipulation of OASA2 protein via S126F and L530D mutations resulted in increased catalytic activity of AS insensitivity to tryptophan, respectively (Kanno et al. 2005). The rice callus culture of transformed cells, having manipulated OASA2 gene harboring S126F/L530D mutations, resulted in a higher accumulation of free tryptophan (Kanno et al. 2005). The mature seeds and leaves of transformed rice plants showed higher levels of

GABA (γ-aminobutyric acid) and vitamin B. The higher level of new indole alkaloid, 2-[2-hydroxy-3-β-D-glucopyranosyloxy-1-(1*H*-indol-3-yl) propyl]-tryptophan, was found in leaves, hull, and seeds of transgenic plants (Saika et al. 2012).

4.2.2 Chemical and Physical Mutagenesis

The mutagens are chemical and factors that induce changes in DNA structures. The widely used chemical mutagens for enhancement of SMs are sodium azide (SA; NaN3), *N*-methyl-*N*′-nitro-*N*-nitrosoguanidine (MNNG), ethyl methanesulfonate (EMS), or nitrous acid (NA). The radiation, such as UV and X-rays (physical mutagens), had been used in the development of strains for SM production efficiency for many years (Baltz, Company). For example, the chemicals, such as NaN3, EMS, and the X-ray, had been successfully employed in *Catharanthus roseus*. NaN3 is a well-known bactericide and pesticide, employed to induce mutation in a wide range of organism. This azide is an organic metabolite that generates point mutation in the genome after entering the cell nucleus and generally replaces G with A and C with T. Similarly, EMS is known to alter the nucleotides via changing guanine to O6-ethylguanine and results in pairing with thymine in place of cytosine and hence a transition from G/C pairing to A/T (Baltz 2014a, b, c). EMS is also capable of creating mutations, such as G/C to C/G or G/C to T/A and A/T to G/C (Baltz 2012, 2014a, b, c). The level monoterpenoid indole alkaloids (MIA), namely, vinblastine, vindoline, catharanthine, and ajmalicine, were found to increase many folds after mutagenesis was carried out in *Catharanthus roseus*. This finding is important for pharmaceutical applications as this alkaloid possesses anticancer properties.

4.2.3 Manipulation of Biosynthesis Genes

The biosynthesis pathway for flavonoid and anthocyanin biosynthesis was the primary target for genomic manipulation, because their biosynthesis is well elucidated and any alternation could be evaluated by a phenotypic marker, such as variation in the color of petal and sepal (Dixon and Steele 1999; Forkmann and Martens 2001). The multiple strategies have been followed, such as enhancing the efficiency of one or more genes of concerned pathway, aiming to harness the pigmentation of floral parts by feeding different metabolites in the plant. The enhancement of the antioxidant activity of anthocyanin and flavonoid in food is the second attractive objective. The flavonoid pathway enzyme chalcone isomerase (CHI) was found to be the rate-controlling enzyme governing the level of flavonol biosynthesis (Muir et al. 2001). The higher induction of this gene from *Petunia* resulted in an enhancement of flavonoid levels in the tomato peel by as much as

78 times. This high valve of flavonoid was maintained even after processing, and tomato paste has 21 times higher flavonols in comparison to non-transgenic. Isoflavones were found to possess phytoalexin action in response to microbial infection in legumes. The higher level of isoflavone synthase, a cytochrome P450 enzyme, was found to confer phytoalexin activity to *Arabidopsis*, *tabacum,* and *Zea mays* plants, wherein this type of activity was absent (Yu et al. 2000; Jung et al. 2000). The precursors required for biosynthesis of the isoflavones are provided by the phenylpropanoid pathway. The manipulation of phenylpropanoid pathway is of utmost necessity to achieve a higher level of isoflavone in heterologous systems.

4.2.4 Elicitation with Biotic and Abiotic Elicitor

Elicitation using various biotic and abiotic elicitors is a favorable biotechnological approach for the induction, accumulation, and enhancement of an array of SMs. Elicitors induce their effect via signaling molecule; application of elicitors activates multiple signaling pathways of intracellular defense in plants (Bent and Mackey 2007). Elicitors are quite stable at the intracellular level and activate plant's immune system. Elicitor requires a receptor to be recognized by plant immune system, which induces the expression of defense genes. Elicitor can be biogenic and abiogenic (Holopainen et al. 2009; Ferrari 2010; Spoel and Dong 2012). Biogenic elicitors can be exogenous (isolated from pathogens or culture medium) and endogenous (isolated from some plants) (Mejía-Teniente et al. 2010). The abiogenic elicitors consist of heavy metal ions, inhibitors of certain metabolic stages, UV radiation, and some kinds of antibiotics and fungicides. The biogenic elicitors are preferred as they are active at very low doses; they cause no symptoms of demand and stress accumulation of toxic compounds upon application that activate the plant tissue and modulate their resistance to subsequent infections (Boller and Felix 2009; Mejía-Teniente et al. 2010; Spoel and Dong 2012). The cell suspension cultures of French bean (*Phaseolus vulgaris*) treated with polysaccharide elicitor molecules from the cell walls of the anthracnose fungus, *Colletotrichum lindemuthianum*, resulted in the rapid accumulation of isoflavonoid phytoalexins, phenolic compounds, and synthesis of hydroxyproline-rich glycoproteins. The selective induction of gene products, enzymes L-phenylalanine ammonia-lyase, cytochrome P450-dependent cinnamic acid 4-hydroxylase, chalcone synthase, chalcone isomerase, prolyl hydroxylase, and protein: arabinosyl transferase, was also observed in response to the application of this elicitation (Dixon et al. 1986).

The role of SA as a signaling molecule in modulating plant responses to various external biotic and abiotic stimuli has been documented (Ashraf et al. 2010). SA has been found to elicit the production of secondary metabolites (Ding and Ding 2020) in plants. The role of SA has been documented to enhance the production of stilbenes (Xu et al. 2015), alkaloids (Figueroa-Pérez et al. 2015), anthraquinones (Lee et al. 2013), terpenoids (Xu et al. 2012), etc. Salicylic acid (SA) treatment significantly altered the levels of the secondary metabolites in soybean roots. The levels of

coumestrol were enhanced 16-fold, and daidzein (both are phenolic metabolites) was increased sevenfold on a dry weight basis in the roots of soybean (Kim et al. 2022). Coumestrol is a phytoalexin in the soybean plant and is associated with insect attacks and senescence (Morandi 1996; Mun et al. 2021). Moreover, it has high commercial value because of its multiple biological activities, such as its anti-low density lipoprotein (anti-LDL) oxidation (Jin-Hee et al. 2006), anti-inflammation (Yuk et al. 2016), anticancer (Singh et al. 2017), anti-obesity, and skin protection (Park et al. 2015) benefits. Daidzein is another important phytochemical that is highly valuable in the nutraceutical field because of its antioxidant properties (Dwiecki et al. 2009) and its use as a plant-based alternative to estrogen (Vitale et al. 2013).

Jasmonates (JAs) play a role in diverse cellular activities, such as plant growth and development and plant responses to biotic and abiotic stresses (Afrin et al. 2015). JAs have been specially used as conserved elicitors for the production of secondary metabolites in both gymnosperms and angiosperms (Zhao et al. 2005; Pauwels et al. 2009). Apart from their role in plant growth and development, JAs act as a major elicitor for the enhancement of secondary metabolites. Methyl jasmonate is a widely used elicitor to enhance a wide range of secondary metabolites. It has been reported to influence the production of phytochemicals in different in vitro culture systems, such as adventitious root culture, callus culture, multiple shoot culture, cell suspension culture, and hairy root culture (Nabi et al. 2021). MJ is the routinely used elicitor, and it has been found to have a profound effect on secondary metabolite accumulation in plant cells and organs (Baenas et al. 2014; Giri and Zaheer 2016). MJ application was found to increase the concentration of phenols (Ahn et al. 2014), alkaloids (Zhou et al. 2015), terpenoids (Onrubia et al. 2013), coumarin (Dučaiová et al. 2016), anthocyanin (Ram et al. 2013), and polyamines (Cao et al. 2014), not only in plant cell cultures but also in whole plants (Ahn et al. 2014; Dučaiová et al. 2016; Ho et al. 2020). MJ was also proved as effective elicitor in the root suspension of *Ajuga bracteosa*, wherein its application enhanced phenolic and flavonoid content (Saeed et al. 2017). In addition, increased expression of genes and transcription factors related to secondary metabolite biosynthesis has also been reported.

4.2.5 Transcription Factors

Transcription factors can regulate the induction of one or more genes related to a specific pathway dedicated to SM biosynthesis. The manipulation of transcription factors is an attractive approach for enhancing SMs. For example, anthocyanin biosynthesis in maize is under the control of dual transcription factors, R and C1. The R factor is similar to proto-oncogene *c-MYC* of vertebrate, and the C1 factor is similar to the proto-oncogene *c-MYB*; these genes encode the basic helix loop helix, which is a transcription factor. The higher-level expression of these transcription factors in callus culture of maize has been found to activate the flavonoid pathway (Grotewold et al. 1998). The higher-level expression of these transcription factors

C1 and R, along with the CHI gene, was found to induce anthocyanin biosynthesis that confers better resistance to fungi (Gandikota et al. 2001). Similarly, higher expression of PAP1, MYB-type transcription factor in *Arabidopsis*, is responsible for dark purple pigmentation during entire development (Borevitz et al. 2000). These studies proved the high level of intense regulation of SM biosynthesis in the course of plant growth and in heterologous systems can be achieved by regulation of single or multiple transcription factors.

The negative effect and suppression in the accumulation of SMs could also be induced by transcription factors. The knockout for the MYB-type transcription factor of MYB4 gene resulted in better tolerance to UV-B irradiation and higher sinapate esters in the leaves in *Arabidopsis* (Jin et al. 2000). In another example, overexpression of FaMYB1, a MYB-type protein from strawberry when expressed in tobacco, resulted in decreased flower pigmentation, anthocyanin, and flavonol compounds, suggesting a repressive role of FaMYB1 in strawberry fruit in the flavonoid pathway (Aharoni et al. 2001). These findings highlight the necessities of fine knowledge of transcription factors and their regulatory circuits to achieve efficient pathway modulation.

4.2.6 Manipulation of Biosynthesis Gene Cluster or Cryptic Gene

The biosynthesis gene clusters (BGCs) are group of genes having complete genetic information necessary for regulation, modification, and biosynthesis of SMs (Cimermancic et al. 2014). Some microbial species have inactive genes in their nucleus governing the production of SMs. These cryptic BGCs have been shown to be crucial in the bio-prospection of new SMs (Skinnider et al. 2020). The modern genetic engineering techniques are useful in the elucidation of the latest and unique BGCs. An array of genetic manipulation techniques has been developed for the induction, activation, and expression of target BGC in homologous as well as heterologous system. These include ZFNs (zinc finger nucleases) (Urnov et al. 2010), TALENs (transcription activator-like effector nucleases) (Miller et al. 2011; Mussolino et al. 2014), and CRISPR (clustered regularly interspaced short palindromic repeat) or CRISPR/Cas9 (CRISPR-associated protein 9 (Cas9) system (Chylinski et al. 2014; Ran et al. 2013; Zhang et al. 2017). These systems make use of nucleases for cutting of both strands of nuclear DNA known as double-strand breaks (DSBs). These breaks are subsequently joined by the innate mechanism of DNA repair, utilizing homologous recombination (HR) or nonhomologous end-joining (NHEJ) system. At this stage, the desired manipulation is achieved via the addition or removal of targeted nucleotides. All the abovementioned approaches have their specific merits and demerits; however, CRISPR-Cas9 has been proved to be the most favorable, efficient, and reliable method adopted by a large group of researchers for research, innovation, discovery, and elevated accumulation of

desired SMs (Puchta 2017; Zaidi et al. 2017; Zhang et al. 2017; Svitashev et al. 2015).

The regulatory role of the *4′-O-methyltransferase* (*4′OMT2*) enzyme in the biosynthesis of benzylisoquinoline alkaloids (BIAs) from *Papaver somniferum* L. had been studied using the CRISPR/SpCas9 system via gene knockout of 4'OMT2. The elevated rate of induction and suppression of (*R, S*)-*reticuline 7-O-methyltransferase* (*7OMT*) and *3′-hydroxyl-N-methylcoclaurine 4′-O-methyltransferase* (*4′OMT2*) genes was found to influence the BIA accumulation in various tissues (Duda et al. 2014). The knockout *4′OMT2* significantly reduces the level of thebaine, codeine, noscapine, and papaverine in the stem of *Papaver somniferum* (Ran et al. 2013). The other products, namely, *S*-reticuline and laudanosine content, were also found to decrease in *4′OMT2* knockout plants (Alagoz et al. 2016). The CRISPR/Cas9-mediated targeted mutagenesis in the starting exon of the *HOS1* gene from *Arabidopsis* was performed. This gene acts as a signal regulator in response to cold stress. The mutation caused frameshift and resulted in premature stop codons and ORF disruption. The resultant mutant plants were compared with the control for survival from abiotic stresses, amount of secondary metabolites, and gene expressions of concerned genes. The resultant mutant showed the altered level of phytoalexins, glucosinolates content was decreased by 1.5 times, and flavonol glycosides increased by 1.2 to 4.2 times in mutated plants. The RNA coding for MYB and bHLH transcription factors was found to be modified in mutated *Arabidopsis* as compared to control. These observations establish the coordinated role exerted by HOS1 signaling on phytoalexin accumulation (Alagoz et al. 2016).

4.3 Cell Cultures as Biofactories

The plant cell culture is frequently used for genetic manipulation as they facilitate addition, deletion of desired gene, as well as expression of gene in response-specific cues. The suspension culture system plays indispensable roles in the commercial production of natural metabolites that have minute natural accumulation (Hellwig et al. 2004; Espinosa-Leal et al. 2018). The suspension cultures have many advantages, like being economical to known cultivation methods (Kieran et al. 1997; Roberts 2007) and freedom from the collection of wild plants (Efferth 2019), and moreover, all the recombinant organism can be expressed in suspension culture. The suspension culture enabled the researcher to perform manipulation, like posttranslational modifications that otherwise are absent in simple and early cell types like prokaryotes, and with the latest DNA-manipulating system, the desired gene can be expressed in heterologous system. The production of metabolites (Shih 2018; Arya et al. 2020) as well as proteins (Hellwig et al. 2004) could be possible by designing cell culture utilizing synthetic biotechnology approaches in plants (Kowalczyk et al. 2020). The gene editing technologies, which can be coupled with *Agrobacterium tumefaciens* infection followed by cell culture, provide reliable means for high-

precision gene modification related to target biomolecules of agricultural, industrial, and pharmaceutical interest.

Various studies had documented the role of plant cell culture, as general cultures developed from the plant organs or tissues in which the desired SMs compartmentalized. For example, ginsenosides are produced using root culture (Rahimi et al. 2016), diosgenin is produced from seeds culture of *Trigonella* spp. (Chaudhary et al. 2015), and vinblastine in *Catharanthus roseus* are produced utilizing leaves culture (Antonio et al. 2013; Parthasarathy et al. 2020). Lipid production is achieved by utilizing the cell culture of *Jatropha curcas* (Correa et al. 2020). Similarly, plant cell culture is suitable for the production of phenolics, alkaloids, terpenes, and steroids (Smetanska 2008). The important herbal compound, namely, paclitaxel, shikonin, and podophyllotoxin (Wilson and Roberts 2012); perfumery compounds (sesquiterpenoids patchoulol and a/b-santalene); antioxidants (Buttner-Mainik et al. 2011); and pigments (Wolf et al. 2010) are also biosynthesized utilizing cell culture approach. Phenolics are known to have anticancer, anti-inflammatory, antimicrobial, and antioxidant activities. The synthesis of the aforementioned commercially relevant metabolites can be revolutionized by using plant cell culture (Ratnadewi 2017).

4.3.1 *Metagenomics as a Source of Genetic Parts for SM Production in Bacteria*

Metagenomics enabled the researcher to identify novel gene clusters related to SMs of interest by directly sequencing and cloning the nucleic acids extracted from the microbes that inhabit a specific habitat. Metagenomics is an excellent way to enhance and broaden SMs' innovation from ecological hotspots, harboring unique DNA sequences (structural genes and regulatory sequences) and acting as an important benefactor to the progress of the synthetic biology. The DNA sequences concerned with SMs are generally found in well-ordered groups or operons for the biosynthesis of SMs via multiple ordered-sequential steps through a group of functionally interlinked enzymes (Cimermancic et al. 2014; Smanski et al. 2014). The appropriate arrangement and reorganization of these functional enzymes dedicated to specific biochemical reactions, coupled with appropriate intonation of enzymatic activity, would lead to the design and biosynthesis of novel SMs. For example, *Klebsiella oxytoca* nitrogen fixation gene cluster was rearranged by combinatorial design, and biological parts were assembled to achieve the functional optimization of the operon and alter the modularity of nitrogen fixation (Smanski et al. 2014). Ren and coworker have credit for the development of the rearrangement working plan applicable in *E. coli* and *S. cerevisiae* for the construction of metabolic pathway delivering higher output of metabolites by assembling expression cassettes from different genetic parts (Ren et al. 2017). Following the same approach, conjunctional pathways meant for carotenoid biosynthesis were developed

successfully (Ren et al. 2017). A new phosphonoacetic acid was discovered by rearranging a group of genes of *Streptomyces* sp. strain NRRL F-525 and expressed in *S. lividans* for phosphonoacetic acid biosynthesis (Freestone et al. 2017). Geller and coworker used metagenomics and metatranscriptomics to discover bacterial gene clusters (BGCs) in free-living and particle-associated bacteria from the water column of the Cariaco Basin, Venezuela. He successfully identified 1154 diverse BGCs from 565 bacterial and archaeal metagenome-assembled genomes (MAGs). This study established that the variable water redox potential and lifestyle of particle-associated as well as free-living bacteria were due to the different composition and biosynthesis of SMs. This finding suggests that bacterial communities, like *Planctomycetota*, potentially biosynthesize variable types of SMs in these anoxic/euxinic waters (Geller-Mcgrath et al. 2023a, b).

4.3.2 Heterologous Systems for SM Production

Elucidation of the biosynthetic pathways leading to desired SMs begins with genetic manipulation of plant secondary metabolism as well as specific regulation of individual enzymes. Identification of regulatory factors and their effect on changes in metabolites and physiological networks is of utmost importance. A robust balance of enzymes, cofactors, ATP, and other metabolites is necessary for heterologous expression in the bacterial system. The simple pathways with fewer enzymes are easy to express in heterologous system; however, complex pathways also have been expressed successfully into the microbial systems. Many studies have documented the successful production of several classes of plant natural products in microbial systems. Many SMs related to this class, flavonoid, alkaloid, betalain, and glucosinolate, have been produced successfully in the bacterial system. *E. coli* is the most extensively used organism for genetic manipulation, and a number of strategies have been developed for engineering this organism. For example, cocultures of *E. coli* for the production of flavan-3-ols (flavonoid) resulted in a 970-time increase in amount and provided freedom for the optimization of factors, such as carbon source, induction temperature, induction point, inoculation ratio, and strain choice (Jones et al. 2016). Similarly, the heterologous expression of carotenoid genes from *Pantoea ananatis* in *E. coli* enhanced the level of zeaxanthin (Li et al. 2015), which is a carotenoid synthesized by some plants, bacteria, and fungi (Sajilata et al. 2008), used against age-related macular degeneration and also in the food industry (Hadden et al. 1999; Snodderly 1995). The tunable intergenic regions' approach was used to coordinate the expression of the *crtY* and *crtZ* genes (Li et al. 2015). The genes concerned with the biosynthesis of myrcene, an acyclic monoterpene, are also being customized in *E. coli* strains (Kim et al. 2015). Myrcene acts as a precursor for the biosynthesis of flavors, fragrances, cosmetics, vitamins, and pharmaceuticals (Behr and Johnen 2009). The expression of mevalonate (MVA) pathway in heterologus system resulted in 34 fold increase in mycrene level (58.19 ± 12.13 mg/L) (Kim et al. 2015). 2-Pyrrolidone is an important C4 "Top Value-Added Chemical from Biomass" (Werpy and Petersen 2004) due to its great

commercial significance. This is used in ring-opening polymerization to produce nylon-4, which possesses more thermal tolerance and water retention than its precursors. To accomplish, this recombinant *E. coli* strain has been manipulated for the synthesis of 2-pyrrolidone using glutamate as a substrate (Park et al. 2013). Two complete cDNAs belonging to the type I PK gene clusters, which were AMP-dependent synthetases, were identified using in silico tools. The glutamate decarboxylase and AMP-dependent synthetases, when expressed in recombinant *E. coli,* resulted 25% more efficiency in the 2-pyrrolidone biosynthesis (Zhang et al. 2016).

Pseudomonas putida, which is a Gram-negative bacterium, can metabolize different natural and synthetic organic compounds. By virtue of this competence, *P. putida* is used as a bio-catalyze in the various processes related to industry and environment (Martınez-Garcıa et al. 2015; Martins Dos Santos et al. 2004). Two genes, which are related to c-di-GMP production and degradation, were inserted into *P. putida* (Simm et al. 2004). The p. putida harboring two transferred genes from *E. coli* found to form biofilm as per the requirement of specific catalyze for example, was able to degrade the environmental pollutant 1-chlorobutane. The *P. putida* biofilm-forming cells were found to form upon addition of cyclohexanone to the culture medium, SMs (Benedetti et al. 2016).

4.3.3 Plastid Manipulations

The plant has specialized organelles covered by a double membrane termed plastids. Chloroplasts are plastids, which are present in the leaves of green plants. Chloroplasts have an independent gene pool in addition to the nuclear genome called plastome, which is present in higher copy numbers up to 10,000 copies of ptDNA per cell in certain tissues (with reductions over time in senescent leaves). This property of the plastid genome enabled chloroplasts (plastids of plant origin) from a single plant to accept thousands of copies of genes belonging to diverse species, leading to elevated levels of expressed proteins of interest (Ahmad et al. 2012a, b, c; Ruhlman et al. 2010; Oey et al. 2009). In angiosperms and gymnosperms, chloroplast transformations are governed by maternal inheritance, which means genes belonging to female cytoplasm dominate in progeny irrespective of the genotype of male (Birky 1995). It is advantageous, as inserted foreign genes using chloroplast transformations will have no chance to spread to other varieties or plants through pollens. The second advantage offered by the plastid of higher plants is that phenomena like gene silencing, site-specific recombination, and mendelian inheritance are absent. The new-generation plant after chloroplast transformation displays uniform expression of the foreign gene (Ahmad et al. 2012a, b, c). Moreover, the expressed protein remains compartmentalized to plastid only, which permits the toxic proteins to be expressed in plants, whereas nuclear expression of these gene is toxic to host plant. For instance, *Nicotiana tabacum* transformed by the insertion of the cholera toxin β subunit (CTB) in the cytoplasm even at low concentration (0.3%

TSP) resulted in stunted plant growth (Arakawa et al. 1997), whereas the chloroplast expression of said gene had not shown any growth retardation, despite a 14-fold increase in expression (Daniell et al. 2001). The genetic manipulation of plastid genome had been carried out in different plants with the aim of modifying the metabolic pathways leading to biosynthesis of polyhydroxybutyrate (PHB) (Lössl et al. 2003, 2005), β-carotene (Wurbs et al. 2007; Apel and Bock 2009; Harada et al. 2014), insertion of the mevalonate pathway (MEV) (Kumar et al. 2012), artemisinin production (Saxena et al. 2014; Fuentes et al. 2016), and higher content of vitamin E in tobacco (Lu et al. 2013) and lettuce (Yabuta et al. 2013); the cyanogenic glucoside dhurrin pathway from *Sorghum bicolor* had been transferred to *tobacco* (Gnanasekaran et al. 2016) and the biosynthesis of squalene, which is a terpene (Pasoreck et al. 2016). Endeavors have been in progress to increase photosynthesis efficiency (Longoni et al. 2015; Ort et al. 2015; Sharwood et al. 2016), by using the improved versions of Rubisco enzyme (Lin et al. 2014; Occhialini et al. 2016), insertion of a C4-type photosynthetic pathway into plants with C3-type leaf morphology (Leegood 2013; Kellogg 2013; Hibberd and Furbank 2016), or insertion of carbon concentration mechanisms (CCMs) from cyanobacteria into higher plants (Price et al. 2011; Whitney et al. 2015; Rolland et al. 2016).

4.3.4 Glycosylation of Secondary Metabolites

Glycosylation (attachment of a sugar moiety) is an important modification that affects the stability, solubility, and availability of biomolecule (Gachon et al. 2005). Moreover, it also plays a determinal role in the compartmentalization of secondary metabolites. For example, glycosylated forms of monolignols and anthocyanins are compartmentalized in the vacuole (Le Roy et al. 2016; Cheng et al. 2014). Glycosyltransferases (GTs) are a group of enzymes that catalyze the glycosylation of plant secondary metabolites, involving the transfer of sugar moiety to the respective metabolite. These GTs have been grouped as family 1 in the Carbohydrate-Active EnZyme (CAZy) database (Lombard et al. 2014) and are named as uridine diphosphate (UDP) GTs, or simply written as UGTs. UDP-glucose is the preferred donor, although other activated sugars acting as donors are UDP-galactose, UDP-rhamnose, UDP-xylose, and UDP-glucuronic acid (Bowles et al. 2006). The UGTs consist of a GT-B fold and an inverting mechanism, that is, the anomeric configuration of the product is inverted with respect to the nucleotide sugar donor (Lairson et al. 2008). The C-terminus of glycosyltransferase consists of around conserved 40-amino-acid motif known as PSPG (plant secondary product glycosyltransferases) box (Hughes and Hughes 1994). The PSPG box of UGTs is of extreme importance for expression in heterologous hosts (Osmani et al. 2009). The nucleotide sugar donor is recognized by the PSPG box, whereas the N-terminus recognizes and accepts a substrate of the concerned secondary metabolite (Vogt and Jones 2000). The less conserved residues in the PSPG box of UGTs have a role in the catalytic activity. For example, the exchange of the PSPG box of a

Catharanthus roseus curcumin UGT with that of a *tobacco* UGT resulted in the total absence of catalytic activity in the hybrid, and this activity was regained by replacing the non-conserved arginine residue with cysteine (the original amino acid present in the curcumin UGT (Masada et al. 2007). Enzymatic modifications via replacement of these domains have been attempted with the aim of developing hybrid UGTs with enhanced catalytic efficiencies and a wider, more flexible substrate range. For example, the UGT domains of *Arabidopsis* flavonol 3-O-glucosyltransferase AtUGT78D2 and flavonol 3-O-arabinosyltransferase AtUGT78D3 replaced the result enzyme found to utilize both UDP-glucose and UDP-arabinose as substrates (Kim et al. 2013).

The C-GTs are a special class of UGTs performing C-glycosylation, which is a process of C-C bond formation between the sugar donor and acceptor metabolites, thus resulting in the synthesis of relatively stable C-glycosides as compared to hydrolysis-sensitive O-glycosides (Brazier-Hicks et al. 2009). These C-GTs are more valuable and demanding as the resultant secondary metabolites, after acquiring this modification, are found to possess higher activities, such as antioxidant and anti-inflammatory activities, obesity, and diabetes management (Falcone Ferreyra et al. 2013; Xiao et al. 2016).

The UGTs with bifunctional activity, capable of displaying both C- and O-glucosyltransferase activities, simultaneously have been identified in maize (a bifunctional UGT, UGT708A6), have the ability to speed up the formation of C-glycosides using 2-hydroxyflavanones as substrate, and can catalyze the formation of O-glycosides from flavanones (Falcone Ferreyra et al. 2013). These bifunctional enzymes can be important in studies related to elucidating the mechanism governing the formation of C-glycosidic bonds that are more stable and more resistant to hydrolysis when compared to O-glycosidic bonds (Xiao et al. 2016).

The active site motifs swapping (Ile-Asp and Asp-Ile) between a rice C-GT and a pear O-GT showed that I117D and D118I substitutions in the pear O-GT double mutant resulted in the 100% formation of the C-glucoside nothofagin (Gutmann and Nidetzky 2013). These types of enzyme engineering approaches can play a crucial role in the creation of secondary metabolites with enhanced stability and bioavailability with potential interest and application in the pharmaceutical/nutraceutical industries. The genome editing with CRISPR/Cas9 system enables precise site-directed mutagenesis for UGT modification in plants (Soda et al. 2017). Glycoengineering of bioactive molecules by modified plant UGTs is more attractive than organic synthesis, due to drawbacks like unwanted anomers' production during the synthesis steps, the use of heavy metal catalysts, and low yields (Kren and Thiem 1997).

4.3.5 Epigenetic Modifications

Epigenetic approach creates transferable changes in the expression of functional protein of interest without altering the concerned genome. The SM biosynthesis via

epigenetic modification has been emerged as an influential and powerful approach. Secondary metabolism in fungi and bacteria is under the control of complex and interdependent controlling circuit, which is affected by the diverse array of transcription factors and chemical modifiers that generate epigenetic modifications (Cichewicz 2010). Enzyme-specific inhibitor compounds or gene knockout can be used to alter the function or level of specific protein related to metabolic biosynthetic pathway, and novel transformed biomolecules can be synthesized utilizing epigenetic modifications. Epigenetic modification may be achieved through the following: (1) methylation of target gene, known as DNA methylation; (2) winding and unwinding or chromatin, known as chromatin remodeling; and (3) single-stranded RNA transcripts which are used to regulate the expression of specific gene, called RNA interference. The basic mechanism of DNA methylation involves the retardation or inhibition of transcription machinery at 5′ regulatory region of the gene. By using epigenetic modification reagent related to DNA methylation, different derivatives of tryptophan like cytosporone, indigotide, and tenuipyrone had been reported in *Torrubiella luteorostrata* (Asai et al. 2011). Biosynthesis of a new polyketide glycoside, indigotide B, has been reported from *Cordyceps indigotica* (Asai et al. 2012). The inhibitor of methyl transferase, namely, 5-azactytidine (5-AZA), is used to produce penicillin in *Penicillium citreonigrum*. The biosynthesis of diverse types of phytochemical, namely, sclerotiorin, sclerotiorimine, ochrephilone, dechloroisochromophilone III, dechloroisochromophilone IV, atlantinone A, and atlantinone B had been reported in response to addition of AZA in different type of suspension culture of corn, oat, rice, and vermiculite, resprctively (Wang et al. 2010). Similarly, when suberohydroxamic acid (SBHA) and RG-108 (epigenetic modifiers) are added in the culture of *Isaria tenuipes* cells, they initiate the synthesis of tenuipyrone (Asai et al. 2012). The decrease in aflatoxin content had been reported in response to the addition inhibitor of DNA methylation in different species belonging to aspergillus, namely, *Aspergillus flavus,* and *Aspergillus parasiticus* (Yang et al. 2014a, b). The addition of nicotinamide or sodium butyrate (NaBut) in the culture medium of *Penicillium brevicompactum* leads to a ten times more phenolic compound synthesis (Hawary et al. 2018).

Chromatin is comprised of genomic DNA and histone proteins localized in the cell nucleus of higher organisms. In the chromatin, DNA is circled by the octamer of histone protein in a highly condensed manner, forming a bead-like appearance termed a nucleosome. A total 146 nucleotides and eight different histone proteins make up a single nucleosome. The histone packaging and accessibility of DNA are varied in response to external stimuli such as changes in physiological factors or developmental stages (Cedar and Bergman 2009). Different chemicals have been tried in different species of aspergillus and zygomycete by modifying chromatin packaging with the aim of enhancing SM production (Gacek and Strauss 2012). Histone deacetylase and methyltransferase have been found to play crucial roles in the identification and activation of the silent gene cluster related to the biosynthetic pathway (Asai et al. 2012). Chromatin packaging has been modified by either the induction of an artificial histone modification gene or by enzyme inhibitors specific to the histone deacetylase enzyme (HDAC) to activate SM biosynthesis. HDAC was

found to affect the DNA replication, transcription, and repair processes via acetyl group removal from lysine amino acids present in histone proteins (Robyr et al. 2002). Many studies have documented the effects of deacetylation of histone on heterochromatic regions and inhibition of gene activity (Bulger 2005). The HDAC inhibitors, namely, trichostatin A (TSA), SAHA, and NaBut, are widely employed in filamentous fungi. These HDAC inhibitors alter the level of gene product and stimulate the modification of protein after translation (Kim and Bae 2011). The deletion in the hdaA gene encoding the HDAC enzyme activates the gene cluster related to the biosynthetic pathways of sterigmatocystin, penicillin, and terrequinone A from *A. nidulans* strain A89 at the transcriptional level (Shwab et al. 2007). Deletion of the same gene (hdaA) in *A. fumigatus* enhanced the content of fumitremorgin B and pseurotin and decreased the gliotoxin content. The transcriptional activation of the NRPS gene cluster increased following the deletion of hdaA, as reported in *A. fumigatus* strain AF293 (Lee et al. 2009). A similar type of deletion in hdaA was found to enhance the SM biosynthesis gene up to 75%, along with the synthesis of novel metabolites in *Calcarisporium arbuscula* (Mao et al. 2015). A reduction in acetyl content of H4 histone resulted in the inhibition of toxin production in species of filamentous fungi (Roze et al. 2011). The hdaA deletion gene related to the histone deacetylase enzyme had been found to affect the SM pathway leading to the formation of the indole alkaloid meleagrin in *P. chrysogenum* (Ding et al. 2020). Methylation of lysine amino acid is another alternation that acts at the transcription level and controls the induction and suppression of gene (Rolando et al. 2013). Methylation at lysine 9 leads to the activation of the genes of the sterigmatocystin cluster. The methylation of H3K9 associated with heterochromatin protein A (HepA) was found to transform chromatin into heterochromatin. The removal of the HepA gene was found to activate the stc gene cluster (Brakhage 2013). The SM regulator LaeA indirectly influences H3K9 methylation and hence is involved in histone methylation. LaeA has been employed in SM biosynthesis in different species of fungi, like *Aspergillus terreus* (Palonen et al. 2017), *Penicillium expansum* (Kumar et al. 2018), *A. ochraceus* (Wang et al. 2019), *A. flavus* (Zhi et al. 2019), and *P. dipodomys* (Yu et al. 2019). The SM gene cluster was expressed, and biosynthesis of emodin, monodictyphenone, and its derivatives was achieved by silencing the cclA gene, linked with histone H3 lysine 4 methylation (Bok et al. 2009). The deletion of Set1 gene, encoding for histone methyltransferase in *F. graminearum*, resulted in the biosynthesis of aurofusarin and deoxynivalenol (Liu et al. 2015).

The coding RNA synthesized by the cell but not processed into protein is termed as non-coding RNA (ncRNA). These ncRNA had a regulatory effect on the gene function at the transcriptional and posttranscriptional levels. Depending upon the number of nucleotides, ncRNAs are named as short ncRNAs and long ncRNAs. Short ncRNAs have further divided in to microRNA (miRNA), short interfering RNAs (siRNA), and Piwi-interacting RNAs (piRNA). The miRNAs and siRNAs can bind to target mRNA, inhibit the translation ultimately degrading them. The long ncRNAs are found in the cells of all the higher organisms as well as fungi (Donaldson and Saville 2012). Some ribosomal components, such 5.8S, 18S, and

26S rRNA, are included in long ncRNAs and act as natural antisense transcripts (NATs).

The cis and trans-NATs forms are highly active species and exert their effects on double stranded RNA, chromatin transformation, and transcriptional interference. These RNA subtypes are used to suppress specific genetic element and to regulate the biosynthesis of different biomolecules. The gene suppression resulted in the production of methyltransferase involved in the conversion of meleagrin into glandicolin B in *P. chrysogenum*. When two additional genes belonging to same gene cluster were suppressed, there was no biosynthesis of roquefortine C and meleagrin (García-Estrada et al. 2011). The inhibition of oxalate biosynthesis gene resulted in increased biosynthesis of the cephalosporin precursor, adipolyl-6 aminopenicillin acid, in another strain of *P. chrysogenum*. In *P. expansum*, the inhibition of the gene related to the toxin patulin, a contaminant of orange juice, resulted in two new strains, wherein patulin content was low (Sanzani et al. 2012).

4.4 cDNA-AFLP as a Functional Genomics Tools

The cDNA-amplified fragment length polymorphism (cDNA-AFLP) has been widely used to identify genes and transcriptomic profiling of genes involved in SM biosynthesis in non-model plants. It offers advantages like the fact that no pioneer genomic data is required at the start of experimentation (Breyne and Zabeau 2001; Breyne et al. 2003). It makes cDNA-AFLP most suitable for SMs discovery as neither genomic data nor cDNA libraries are available for most medicinal plants. The cDNA-AFLP involves mRNA extraction from the source and its subsequent conversion to cDNA. The resultant transcripts are treated with two different nucleotide digesting enzymes, followed by adapters annealing at ends, and these fragments are selectively amplified. These transcripts are resolved on acrylamide gels and can be identified with the help of software dedicated to AFLP analysis, such as AFLP-QuantarPro (Keygene, Wageningen, The Netherlands). This approach has been successfully employed in suspension cultures of tobacco BY-2 and *Catharanthus roseus* after jasmonate elicitation for monitoring transcript profiling and change in alkaloid content (Goossens et al. 2003). After critical functional validation, full-length cDNAs were isolated, and their functional validation was performed in transgenic plant. The cDNA-AFLP combined with a microarray was used to analyze and compare the benzylisoquinoline biosynthesis in morphine-containing and morphine-free *Papaver* species. This led to the identification of o-methyltransferase as a critical gene (Ziegler et al. 2005). The high-valued phenolics and tanshinones from *Salvia miltiorrhiza* are effective in the treatment of coronary heart disease, myocardial infraction, atherosclerosis, chronic renal failure, and cirrhosis (Zhou and Memelink 2016). The cDNA-AFLP analysis coupled with metabolic profiling was successfully employed to identify novel genes concerning phenolics and tanshinone biosynthesis from the genus *Salvia* (Yang et al. 2013). The

cDNA-AFLP was employed in lavander for the identification of genes related to signal transduction, defense, and translation under extreme salt condition.

4.4.1 Modification of Translation Apparatus

It is a well-known fact that ribosomes are the workbench for protein synthesis and are Orchester of translation. Therefore, ribosomes and translation machinery might be attractive targets for enhanced SM biosynthesis. Studies had shown that mutations in the rpsL gene related to streptomycin resistance (StrR) encoding ribosomal protein S12 enhanced SM biosynthesis in *actinomycetes* (Baltz 2011a, b; Ochi and Hosaka 2013; Tanaka et al. 2009). Moreover, higher expression of the frr gene related to ribosome recycling factor resulted in enhanced production of actinorhodin in *Streptomyces coelicolor* and avermectin in *Streptomyces avermitilis* (Baltz 2011a, b; Li et al. 2010; Hosaka et al. 2009). The disruption of rimP, encoding for the 30S ribosome subunit assembly cofactor, caused higher synthesis of actinorhodin and calcium-dependent antibiotics in *S. coelicolor* and improved jadomycin level in *Streptomyces venezuelae* (Pan et al. 2013). Moreover, *S. coelicolor* strain having disruption in rimP showed higher synthesis of heterologously expressed polyoxin and nikkomycin (Nik) biosynthetic genes (Pan et al. 2013).

4.4.2 Synthetic Biology

The synthetic biology approach utilizes an array of methods to join PCR-amplified and synthetic DNA fragments in vivo in one host for cryptic expression of gene cluster or strain improvement (Cobb et al. 2014; Smanski et al. 2014; Shao et al. 2013; Weber et al. 2015). For example, the spectinabilin biosynthetic gene cluster from *Streptomyces orinoci* was unable to express in *S. lividans* in its native configuration (Shao et al. 2013). To express this gene cluster in *S. lividans*, via synthetic biology approach, there are three types of modules: (1) for different types of promoters, (2) for eight biosynthesis genes, and (3) helper modules for replication and selection in *S. cerevisiae* and *E. coli,* and selection and site-specific integration in *Streptomyces* was assembled. The DNA assembler was used to assemble these modules in *S. cerevisiae* and subsequently transferred to *E. coli* and then finally to *S. lividans*. The transformed *S. lividans* with these recombinant modules were able to biosynthesize 100 μg/l of spectinabilin. In another study conducted in *Streptomyces griseus,* a cryptic polycyclic tetramate macrolactam (PTM) was refactored using six orthogonal promoters hyperactive in *S. lividans*. They used *S. cerevisiae,* as the DNA assembler, and ultimately transformed the refactored PTM gene cluster into the chromosome of *S. lividans*. The resultant recombinant synthesized three novel PTMs. By a similar method, single and multiple gene deletions were generated in

order to achieve a better understanding of the biosynthetic pathway for PTM assembly (Luo et al. 2015). These studies had validated the role of synthetic biology refactoring approach related to heterologous expression of cryptic secondary metabolite gene cluster.

The construction of improved host strain for expression, having a minimized genome or having deletion for multiple cryptic secondary metabolite gene cluster for genome mining applications or carrying deletion of targeted steps of specific pathways for combinatorial biosynthesis applications, is another area where synthetic biology approach might be game changer. The traditional methods using homologous recombination for strain improvement take a long time and are tedious. For example, the construction of *S. coelicolor* strain carrying a deletion for 900 kb of subtelomeric DNA along with 10 polyketide synthase and non-ribosomal peptide synthetase gene clusters by sequential deletions took 6 years (Zhou et al. 2012). The other methods based on site-specific recombination like Cre/loxP or Dre/rox may be employed in some cases (Herrmann et al. 2012; Komatsu et al. 2010), but there are technical limitations with their use (Myronovskyi et al. 2014). The CRISPR-Cas9 system can be employed in different types of eucaryotic cells (Mali et al. 2013) and single-cell eubacteria (Jiang et al. 2013) for the simultaneous creation of desired multiple variations in the genome. The CRISPR-Cas9 system is used for genome manipulation in *Streptomyces* species (Cobb et al. 2015) in plants and bacteria. The efficient deletion of small segments of SM genes as single or double deletions in *S. lividans, S. albus J1074,* and *Streptomyces* chromogenes had been reported using CRISPR-Cas9 (63). It has also been documented that a codon-optimized Cas9 under the control of the inducible tipA promoter is capable of creating deletions of the following: (1) single or double unlinked genes, (2) single or double SM gene clusters, and (3) the gene cluster meant for the biosynthesis of calcium-dependent antibiotic (Huang et al. 2015). The CRISPR-Cas9 system is an efficient, faster method for genome manipulation in *Streptomyces* species. The CRISPR-Cas9 approach can be employed to create deletion of different length in *Streptomyces coelicolor,* and reversible modulation of gene expression can also be achieved (Tong et al. 2015).

4.5 Conclusion and Future Perspective

In the near future, our knowledge about secondary metabolites and their biosynthesis pathways will be deeper and finer. The technology of cell culture on a large scale opens an attractive alternative for screening new biologically active compounds and for using this technology during the earlier development of new drugs from rare species. Cell cultures will prove to be an important alternative. The futuristic growth of various strategies for improving the productivity of secondary metabolites will be rewarding. The advent of the latest tools, like ZFNs, TALENs, and CRISPR/Cas9, creates precise and efficient desired variation in the target genome; these are supposed to improve SM discovery. Moreover, technologies like chloroplast

manipulation allow the engineering of difficult parameters related to photosynthesis and metabolism of plants, which was impossible in older times using traditional techniques. Plastid transformation of the plants becomes a more valuable strategy for SM manipulation as it avoids pollen-mediated mixing and spread of transgenes with wild cultivar. The metabolic engineering approach is especially promising, as it explores the opportunity of enhancing levels of known compounds, producing transformed compounds or even producing novel compounds. The outcomes of studies on secondary metabolite biosynthesis and modification in metabolic pathway can be applied on the cell cultures, which means they might be helpful to enhance the quantity of targeted valuable biomolecule. We are hopeful that in the future a multiomic-based approach, including genomics coupled with proteomics and subsequently metabolomics, will be followed to enable rapid exploration of the links between the metabolite level and the enzyme and gene levels, including posttranslational modification. The latest development related to synthetic biology, bioinformatics computational tools and strategies that have emerged, will make possible the discovery of new regulatory controls and biosynthesis pathways for valuable metabolites. In future time, utilization of strong computational tools might be used more frequently, resulting in noticeable progress in the concerned field by permitting efficient design or new pathways, accelerating the biomolecule research. Moreover, the discovery of the latest genomic techniques and their utilization in microbial communities devoid of appropriate genetic machinery will permit the employment of new microorganisms and their efficient utilization. Similarly, metagenomic approaches have the capability to act as a source for the identification of unique, latest genetic parts for the redesigning of microbial metabolism for biosynthesis of novel biomolecules and as a host for the expression of the artificially designed transcripts. All the approaches mentioned herein, together with mass screening protocols and recombinant biotechnology, will contribute to astonishing progress in the rate of metabolite research. These will all explore new possibilities related to the secondary metabolite research and innovation. These strategies will also actively contribute to improving the properties of crop plants, like higher resistance, better taste, smell, other colors, increased of nutraceutical, decreased of toxic level, or other unwanted compounds.

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Chapter 5
Role of Mutation and Stresses in the Production of Secondary Metabolite in Plants

Swapnil Singh, Harleen Kaur, Ravneet Kaur, Neetu Jagota, and Ashish Sharma

Abstract Plants face various challenges in their environment, both from living organisms and nonliving factors, which prompt them to develop a defense system using a wide range of chemical compounds. These compounds, known as secondary metabolites, allow plants to interact with their surroundings and protect themselves. The production of secondary metabolites in plants involves physiological and biochemical processes, with primary metabolites serving as the building blocks. When plants are exposed to nonliving stressors, such as extreme temperatures or drought, they produce secondary metabolites that play vital roles in their defense mechanisms, helping them survive and defend against living stressors, such as pests and pathogens. Secondary metabolites have significant applications in various industries, including pharmaceuticals, medicine, pigments, flavorings, and other products. To facilitate the study and production of plant secondary metabolites, several plant cell culture methods have been developed. These methods include elicitation, hairy root culture, suspension culture systems, and other in vitro techniques, all of which have proven effective in enhancing the production of secondary metabolites. While suspension culture and elicitation are the primary methods for increasing secondary metabolite production, organ cultures like hairy roots have also shown promise in meeting the demand for these compounds. Advancements in understanding the mechanisms underlying the production of plant secondary metabolites have made it easier to control and manipulate their production. In this chapter, we aim to provide an overview of the impact of different environmental stresses on plants and the diverse methods employed for the production of secondary metabolites.

Keywords Alkaloids · Flavonoids · Phenolics · Secondary metabolites · Stress · Mutation

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5.1 Introduction

Plants produce certain chemicals that are vital for their survival but are not directly involved in plants metabolism are called as secondary metabolites. Kossel (1891) is the one to be credited to distinguish it from primary metabolism. Secondary metabolites refer to an assortment of plant defense chemicals produced naturally by various pathways. Plants have been known to produce substances, such as alkaloids, flavonoids, polyphenols, amides, and flavones, along with the fundamental nutrients that define how the plant would interact with its environment, including both biotic and abiotic conditions especially stresses (Yang et al. 2018). Secondary metabolites have a crucial role defensive role in protecting plants against pathogens, herbivores, and even against other competing plants. Furthermore, these compounds can also be used to attract agents for pollination and seed dispersal, as well as to communicate with symbiotic microbes. The chemical nature of the compound forms the basis for the classification of plant secondary metabolites, with terpenoids, alkaloids, phenolic acids, and flavonoids being notable classes linked to the activation and enhancement of plant defense mechanisms (Bourgaud et al. 2001). These secondary metabolites have been traditionally used as significant sources of active traditional medicine, perfumes, and industrial raw materials due to their remarkable biological activity. They have also found wide-ranging applications in pharmaceutics, cosmetics, fine chemicals, and nutraceuticals, underscoring their economic value and utility (El-Khattouti et al. 2014).

The production and accumulation of phytochemical components in plants are significantly influenced by environmental factors. Factors such as light, temperature, soil moisture, soil fertility, and salinity can have a profound impact on plant growth, development, and the synthesis of secondary metabolites. These environmental stimuli can lead to changes in the overall phytochemical profiles necessary for the production of bioactive substances. Plant secondary metabolism can be understood as the plant's ability to adapt and survive in response to environmental cues throughout its life, establishing ecological relationships with other organisms (Musilova et al. 2016). Environmental stresses, both abiotic and biotic, can affect the synthesis of secondary metabolites by altering plant metabolism. These stresses often result in reduced morphological characteristics in plants, including height, leaf number, leaf area, number of branches, root volume, and so on, ultimately leading to decreased biomass production (Pradhan et al. 2017).

Abiotic stress takes various forms, including drought, extreme temperatures, salinity, alkalinity, UV radiation, ozone exposure, metal ions, and more, all of which influence plant functioning. Under standard metabolic conditions, in the absence of stress, plant's defense mechanisms do not operate, resulting in reduced production of secondary plant products (Indrajeet and Rajesh 2018). Metabolite synthesis is a common trait among microorganisms (both eukaryotes and prokaryotes) in their natural environments. Bacteria regulate biotic stress mediated by the production of a variety of metabolites with diverse functions (Salazar et al. 2022). Secondary metabolites, comprising of different classes including terpenes and

phenolics play a defensive role for plants for protection against herbivory and pathogenic microbes like fungi, bacteria, and other parasites (Ruparelia et al. 2022). Plants are a valuable and abundant source of novel medicinal compounds that can be utilized for the development of drugs. The secondary metabolites produced by plants offer distinctive opportunities for the development of pharmaceuticals, food additives, flavors, and various industrial applications. As a result, there has been an increasing focus on exploring tissue culture technology to enhance the production of secondary metabolites. In recent years, there has been a growing interest in studying and utilizing tissue culture techniques to improve the production of these valuable compounds (Jain et al. 2019).

In vitro cultivation of plant cells and tissues through plant tissue culture provides a well-established technological foundation for generating plant natural products under controlled conditions. Micropropagation of plants in vitro, as well as the culture of plant organs (usually roots) or callus in vitro, often yields plant material capable of producing secondary metabolites (Espinosa-Leal et al. 2018). Alongside plant tissue culture, there is an increasing demand for novel technologies, including immobilization techniques for enhanced metabolite production, as well as the utilization of metabolic engineering and biotechnological tools. The combination of increased demand for enhanced and novel secondary metabolites, along with advancements in genetic manipulation, holds significant potential for future economic benefits. This chapter examines secondary metabolite production under various environmental conditions and explores emerging trends in in vitro cell/tissue culture and plant transformation that can be employed to produce secondary metabolites.

5.2 Plant Metabolites

5.2.1 Primary Metabolites

Primary metabolites are a necessity for normal cellular development. They assist fundamental metabolic functions, including respiration and photosynthesis, and are produced during the many stages of cell growth. Amino acids, tricarboxylic acids, carbohydrates, building blocks, and energy sources are among the most well-known metabolites in the majority of organisms. Proteins, nucleic acids, and polysaccharides are additionally regarded as primary metabolites in addition to the molecules indicated above (Saddique et al. 2018).

5.2.2 Secondary Metabolites

To produce secondary metabolites, primary metabolites must be synthesized initially (Kumar et al. 2014). These secondary metabolites typically do not participate

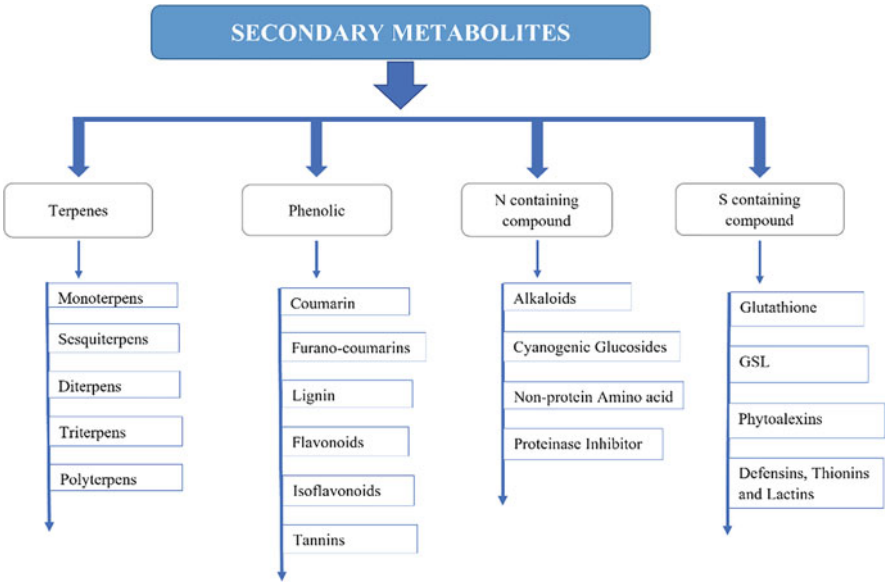

Fig. 5.1 Major classes and subclasses of various secondary metabolites found among plant kingdom

directly in an organism's essential growth and developmental processes. Instead, their absence jeopardizes the organism's defense mechanisms rather than causing immediate death. Bacteria, algae, fungi, animals as well as plants naturally produce a diverse array of secondary metabolites. Saddique et al. (2018) classified these metabolites mainly into terpenes, alkaloids, and phenolics, based on their biosynthetic origin (Fig. 5.1). Since secondary metabolites are not ubiquitous in plants, making their essentiality for plant growth is questionable, thereby allowing the plants to thrive even in their absence.

Secondary metabolism plays a critical role in providing the genetic expression necessary for mutations to trigger natural selection, thereby facilitating the fixation of advantageous traits through evolution. Moreover, secondary metabolism is recognized as a vital component of overall metabolism. Fundamental metabolism, as explained by Roze et al. (2011), encompasses the cellular machinery, essential enzymes, and energy production required for the long-term survival of plants.

Of all the available secondary metabolites, most of them can be principally categorized into three major groups, viz.: terpenes, nitrogen- and sulfur-containing compounds, and phenolic compounds.

5.2.2.1 Terpenes

A variety of compounds have been reported from plants called as secondary metabolites, which have been harnessed for various beneficial biological activities in

humans. The term "terpene," coined by Dumas in 1866, originates from the Latin word "turpentine" (*Balsamum terebinthinae*), which refers to a liquid extract obtained from pine trees. Being the most abundant naturally occurring plant products, terpenes exhibit a wide range of structural variations, including straight hydrocarbons or carbocyclic skeletons. According to a study by Reynolds and Enriquez (2017), there are approximately 55,000 different members in this class of compounds. Numerous terpenoids fulfill crucial roles in plant defense mechanisms against both biotic and abiotic stressors, as well as serving as signaling molecules to attract pollinating insects. Many of the terpenoids that have been extensively researched exhibit significant pharmacological and biological properties, making them valuable in medicine and biotechnology. The initial step in the biosynthesis of terpenoids involves the production of C5 units, such as isopentenyl diphosphate (IPP) or dimethylallyl diphosphate (DMAPP), as explained in a study by Singh and Sharma (2015).

Naturally occurring terpenes can be classified into the following major categories:

5.2.2.1.1 Monoterpenes (Having 10 Carbon Atoms)

Numerous chemical compounds produced by plants play a crucial role as insecticides. For instance, pyrethroids (monoterpene esters) are synthesized by *Chrysanthemum* species in their leaves and flowers. These compounds exhibit potent pesticide activity against pests like moths and are commonly used in commercial pesticides due to their low environmental persistence and low toxicity to mammals (Taft et al. 2015). Monoterpenes, which consist of two isoprenoid units, are present in angiosperms (both monocots and dicots), fungi, bacteria, and gymnosperms. They are volatile chemicals responsible for the fragrances of many flowers and fruits. In rice, around 18 monoterpenoids have been identified, serving various roles, including defense against diseases and pests (Wang et al. 2018). Acyclic, monocyclic, and bicyclic monoterpenes are examples of different types of monoterpenes. They are major constituents of essential oil compounds that contribute to the scent and flavor of plants and are essential for numerous active chemicals used in agriculture, medicine, cosmetics, and food applications. Active compounds, such as pinenes, carveol, camphor, menthol, and limonene, find utility in various industrial applications (Ninkuu et al. 2021).

5.2.2.1.2 Sesquiterpenes (Having 15 Carbon Atoms)

Sesquiterpenes are well-known for their role in plant defense as antiherbivore agents. These compounds consist of a five-membered lactone ring and act as potent feeding repellents for various herbivorous insects (Jiang et al. 2016). The structural arrangement of the 15-carbon skeletons, the functional group stacking, and the substituents on the backbone contribute to the diversity of terpenes. Within this group, hydrocarbons (such as humulene and farnesene), aldehydes (like farnesal and lepidozenal),

oxygenated hydroxyl or carbonyl derivatives, and esters (including torilin and ejaponines) are commonly found constituents. Additionally, some sesquiterpenes, such as -elemanol and -germacrenol, are alcohols. Sesquiterpenes exhibit antibacterial, antifungal, anticancer, and anti-inflammatory properties (Lorigooini et al. 2020). One specific sesquiterpene, abscisic acid, plays a crucial role in regulating seed and bud dormancy and modulating plant responses to water stress by modifying membrane characteristics (Jiang et al. 2016).

5.2.2.1.3 Diterpenes (Having 20 Carbon Atoms)

Diterpenes are diverse hydrocarbons with a C20 structure that are derived from four isoprene units (Abdallah and Quax 2017). They come in various forms, such as linear, bicyclic, tetracyclic, pentacyclic, and macrocyclic. What sets diterpenes apart is the presence of multiple oxygenated keto and hydroxyl groups (Ashour et al. 2018). These compounds have demonstrated inhibitory effects on pathogenic microorganisms, herbivore pests, and weeds, making them valuable secondary metabolites in agriculture for potential biopesticide production (De Sousa et al. 2018). Rice plants naturally produce diterpenes, including phytoalexins and allelochemicals, to protect themselves against infections, pests, and weeds. However, their accumulation is limited and can be enhanced through genetic modification or the introduction of external elicitors (Ninkuu et al. 2021). Legumes produce a diterpene called abietic acid, which is found in resin canals within the tree trunk. When these canals are breached by pests, the release of resin acts as a potent deterrent to feeding. Another diterpene, phorbol, is produced by plants in the *Euphorbiaceae* family and functions as an irritant to epithelial tissues and an internal toxin for pest insects and mammals (Saddique et al. 2018).

5.2.2.1.4 Triterpenes (Containing 30 Carbon Atoms)

Triterpenes, which have over 20,000 known members, are derivatives of the C30 precursor, squalene. The majority are plant kingdom members (Thimmappa et al. 2014). However, microorganisms and sea cucumbers create triterpene glycosides that aid in defense. Triterpenes are formed by connecting two sesquiterpene molecules head-to-head. The most important constituents are cyclic triterpenes (1–5 rings). Alcohols, aldehydes, and carboxylic acids are the most common. Sterols and phytosterols are defined as triterpenes by a cyclopentane perhydrophenanthrene ring structure (Ludwiczuk et al. 2017). Saponins and other glycosylated triterpenes have a protective role in plants against pathogens and insects. Therefore, their presence has been found in many components of food, health and biotechnology industries (Ninkuu et al. 2021). Certain triterpenes play crucial roles in plant cell membranes by regulating protein channels and facilitating the uptake of particles through the reduction of lipid droplets (Fatope et al. 1990).

5.2.2.1.5 Polyterpenes (Having At Least 35 Carbon Atoms)

Plants produce a variety of high-molecular-weight terpenes, including tetra- and polyterpenes (Silvestre and Gandini 2008). Polyterpenes, such as tetraterpenes, have large molecular sizes due to the presence of numerous isoprene units. One well-known polyterpene is rubber, which is a polymer composed of multiple repeating isoprene units. Rubber is commonly found in laticifers and serves as a defense mechanism against herbivores while aiding in wound healing. Polyterpenes, similar to waxes, function as viscosity diluents and co-tackifiers (Jan et al. 2021).

5.2.2.2 Phenolic Compound

An essential component of a plant's defense mechanism against herbivorous pests involves the synthesis of secondary metabolites containing phenol groups, characterized by an OH functional group attached to a benzene ring, among other components (Wuyts et al. 2006). According to Malcovska et al. (2014), plants contain a crucial class of aromatic secondary metabolites termed as phenolics. Phenolics encompass a diverse family of molecules, soluble in both water and organic solvents (Taiz and Zeiger 2006). Shikimic and malonic acid pathways are the major biosynthetic pathways for the formation of phenolic compounds (Świeca et al. 2014). Phenolics consist of various chemical groups, including arylpyrones, styrylpyrones, stilbenes, tannins, coumarins, flavonoids, lignins, and lignans (Fang et al. 2011). The phenylpropanoids are a comparatively simple class containing chemicals such as p-coumaric acid, cinnamic acid, and their derivatives (Marienhagen and Bott 2013). Conversely, the phenylpropanoid groups can also be found in a highly complex class of branched phenolics. Lignin, a compound abundant in plants, represents a prime example of a complex phenolic molecule, second only to cellulose (Taiz and Zeiger 2006). Phenolic compounds play crucial roles in plant growth, reproduction, and the ability to withstand different abiotic and biotic stresses (Giménez et al. 2014). Plants that produce allelopathic phenolics can inhibit the growth of neighboring plants (Ashraf et al. 2018). These compounds possess antioxidant properties due to their redox capabilities, allowing them to neutralize singlet oxygen and serve as detoxifying agents (Vallverdú-Queralt et al. 2014). Additionally, phenolics have been found to have significant cancer-preventive effects in humans (Verma and Shukla 2015). Moreover, phenolic compounds are involved in various plant processes, including food digestion, enzyme activity, photosynthesis, and protein synthesis (Fayez and Bazaid 2014). Furthermore, the production of phenols in plants is highest when exposed to diverse stresses and toxic substances, making them useful indicators of stress (Verma and Shukla 2015).

5.2.2.2.1 Coumarin

Vascular plants produce simple phenolic chemicals through the shikimic acid pathway as a defense mechanism against insect herbivores and fungi, exhibiting varying capacities (Ghasemzadeh et al. 2011). These chemicals are highly active and possess antimicrobial properties against bacteria and fungi. Within plants, these cyclic compounds have been found to serve as natural pesticides, effectively combating a range of fungal infections. Specifically, coumarin, derived from halogenated compounds, demonstrates potent inhibition of fungal growth in vitro. For instance, the formation of 7-hydroxylated simple coumarins inhibits parasitism by *Orobanche cernua*, facilitating successful germination, disruption, and establishment of a connection to the host plant's transport system (Saddique et al. 2018).

5.2.2.2.2 Furanocoumarins

Furanocoumarins are frequently present in plants of the *Umbelliferae* family, and they are noteworthy due to their phytotoxic properties. These compounds can become toxic when exposed to ultraviolet radiation, causing disruptions in transcription and DNA repair and ultimately resulting in cell death (Sajjadi et al. 2013). Psoralen, a significant furanocoumarin, is commonly utilized by plants as a defense mechanism against fungal toxicity (Ali and Sharma 2003).

5.2.2.2.3 Lignin

The secondary cell wall of vascular plants contains a phenolic polymer, viz., lignin (Lee et al. 2019). Lignin gives cell walls strength and imperviousness, to support high-pressure water transport in vascular tissues (Barros et al. 2015). Plant lignin is mainly produced from the polymerization of three different alcohols, i.e., coniferyl, sinapyl, and coumaryl alcohols (Hatfield and Vermerris 2001; Saddique et al. 2018). The chemical nature of lignin also prevents herbivory in many species (Walters 2011). In addition to its responsibility in growth and development, it also acts as a physical barrier (Cesarino 2019). It has been shown that lignin level increases under pathogen attack (Miedes et al. 2014).

5.2.2.2.4 Flavonoids

Flavonoids are a group of polyphenolic compounds that are abundant in photosynthesizing cells and can be found in various sources, such as fruits, vegetables, nuts, seeds, stems, flowers, tea, wine, propolis, and honey. These compounds have a long history of therapeutic use dating back to ancient times and are still widely utilized today. Flavonoids exhibit potent antioxidant properties, acting as water-soluble antioxidants and scavengers of free radicals. This ability helps protect

cells from oxidative damage and shows promising potential in combating cancer. Flavonoids have also been utilized in the development of medications for improving aquaresis, as well as for their anti-inflammatory, antispasmodic, anti-allergic, and antibacterial properties (Mills and Bone 2000). Furthermore, flavonoids have been observed to enhance blood circulation and reduce blood pressure (Jain et al. 2019). In plants, flavonoids serve as a defense mechanism against various stressors, including insect pests. They enhance plant resistance to herbivory, regulate the levels of reactive oxygen species (ROS), and reduce ROS production through metal chelation (Treutter 2006). Flavonoids can be classified into different groups, including anthocyanins, chalcones, aurones, flavonols, flavanones, dihydroflavonols, flavones, and proanthocyanidins. Some flavones have been extensively studied for their role as deterrents against insect feeding (Saddique et al. 2018).

5.2.2.2.5 Isoflavonoids

Isoflavonoids are derivatives of flavonones that are predominantly found in plants. They serve important roles in plant growth and are synthesized by leguminous plants, particularly in supporting the formation of root nodules through symbiotic interactions with bacteria (Saddique et al. 2018). Plants produce isoflavonoids as an active defense mechanism against reactive oxygen species (ROS), protecting themselves from oxidative damage (Posmyk et al. 2009).

5.2.2.2.6 Tannins

Tannins are the most common type of polyphenolic secondary metabolites found in plants, constituting approximately 5 to 10% of the dry weight of vascular plant materials. They are predominantly present in various plant parts, such as bark, stems, seeds, roots, buds, and leaves (Giovando et al. 2019). Tannins can also be found in a variety of foods, including grapes, blackberries, strawberries, walnuts, cashew nuts, hazelnuts, mangoes, and tea (Das et al. 2020). In plants, tannins serve as defensive agents, offering protection against fungi, diseases, insects, and herbivorous animals (Sharma 2019). Tannins can be categorized into two main groups based on their structures and biosynthesis: condensed tannins (CTs) and hydrolyzable tannins (HTs) (Dai et al. 2020). CTs, also known as proanthocyanidins (PAs), are oligomeric or polymeric flavan-3-ols. Flavan-3-ols can be esterified by adding gallic acid (GA) to the 3-hydroxyl group, leading to the formation of galloylated flavan-3-ols such as epigallocatechin-3-gallate (EGCG) and epicatechin-3-gallate (ECG). CTs, flavan-3-ol gallates, and flavan-3-ols are abundant in seeds, fruits, and tissues of various crop plants, including grape seeds, pecans, sorghum, cacao beans, and green tea, among others (Smeriglio et al. 2017). Hydrolyzable tannins encompass gallotannins (GTs) and ellagitannins (ETs), which are derived from penta-O-galloyl-b-D-glucopyranose (PGG). GTs and ETs differ in their structures, with GTs containing a meta-digalloyl group and ETs featuring hexahydroxydiphenoyl

(HHDP) residues. PGG serves as the fundamental GT, while strictinin is a representative ET (Dai et al. 2020).

5.2.2.3 Sulfur-Based Compounds

Protection against most of the microbial diseases in plants is provided by a group of S-containing metabolites that include phytoalexins, glycosphingolipids, thionins, allinins, and defensins (Halkier and Gershenzon 2006).

5.2.2.3.1 Glutathione

GSH, a naturally occurring chemical substance, contains sulfur (S) and is found in various amino acids, such as cysteine and methionine. It serves as a mobile pool of sulfur during plant development and growth, and under stress conditions, it acts as an antioxidant by enhancing the plant's defense system (Kang et al. 2007). Previously, it was considered an indicator of excessive sulfur in plants, which could hinder the regulation of sulfur uptake and utilization by roots. Certain specialized cells, such as trichomes, produce additional enzymes for GSH synthesis and other compounds required for defense against heavy metal stress in plants (Choi et al. 2001).

5.2.2.3.2 GSL

GSLs, which are S- and N-containing glucosides with a small molecular weight, are produced by higher plants as a defense mechanism against parasites and predators. These compounds can release volatile by-products with toxic or repellent properties. Examples of such compounds include glucosides found in mustard plant oils and S-allyl cysteine sulfoxides present in *Allium* species (Van-de-Mortel et al. 2008). The volatile compounds with distinctive odors belong to the GSL group, and they are formed through the action of the enzyme myrosinase. This enzyme cleaves the glucose-S-C bond, leading to rearrangement of the aglycone and the absence of sulfate, resulting in the production of chemically reactive and pungent compounds like nitriles and isothiocyanates. These compounds play a role in defending against herbivores by acting as poisons (Saddique et al. 2018).

5.2.2.3.3 Phytoalexins

According to Jeandet et al. (2013), plants respond to biotic stress by producing phytoalexins, which help prevent the spread of pathogens within the plant by accumulating around the site of infection. This defense mechanism against insect pests is common in many plant species. Various plant families produce organic phytoalexins, with isoflavonoids being associated with *Leguminosae* and

sesquiterpenoids with *Solanaceae*. However, the *Crucifereae* family stands out as the sole producer of distinct secondary metabolites, separate from other GSL compounds. Cruciferous crops are of great importance, as they contain specific phytoalexins known as cruciferous phytoalexins (Monde et al. 2003).

5.2.2.3.4 Defensins, Thionins, and Lactins

Plants respond to biotic stress by synthesizing sulfur-rich proteins, the majority of which provide resistance against fungal infections. In turn, pathogens induce the expression of defensin genes (Gu et al. 2002). Lectins are proteins that bind to carbohydrates and play a protective role against damage caused by various pests (Fondevilla et al. 2011). Lectins, as natural insecticides produced by plants, exhibit resistance to high pH ranges and inflict damage to the epithelium, leading to digestive and nutrient absorption problems. This disruption in lipid, carbohydrate, and protein metabolism results in tissue injury while altering hormonal and defensive responses, ultimately reducing the proliferation of insect pests. Agglutinin was the first identified plant lectin with anti-insect activity (Li et al. 2014). The effectiveness of agglutinin found in wheat germ against various insect pests has been studied (Saha et al. 2006). Numerous lectins are produced in leaf tissues of monocots, such as barley, maize, rye, rice, and wheat (Jiang et al. 2006).

5.2.2.4 Nitrogen-Based Compounds

Apart from their importance for plants, some secondary metabolite groups hold immense importance as medicinal compounds for humans also. Some such compounds are alkaloids, cyanogenic glucosides, and nonprotein amino acids (Wink and Mohamed 2003).

5.2.2.4.1 Alkaloids

Alkaloids are naturally occurring chemical substances that are widely distributed, with approximately 5500 known alkaloids, making them the largest class of secondary plant metabolites. They possess pharmacological properties and have been utilized in medicinal applications, recreational substances, and entheogenic rituals. Alkaloids, as secondary metabolites, are believed to provide protection to plants against herbivores and diseases. Due to their potent biological activities, nearly 12,000 identified alkaloids have been used as medicines, stimulants, narcotics, and poisons. The use of plants containing alkaloids for purposes such as dyes, spices, medications, or poisons can be traced back to the earliest civilizations (Jain et al. 2019).

5.2.2.4.2 Cyanogenic Glucosides

Plants produce a variety of N-containing compounds as a defense mechanism against pathogens. These compounds can generate the toxin hydrogen cyanide (HCN) and are commonly found in plants belonging to the *Rosaceae* and *Gramineae* families. Although these compounds are not inherently dangerous, when a plant is injured, slugs and snails tend to avoid it due to the presence of these plant components. Similarly, the presence of glucosides in cassava extends its storage life by protecting it against insect attacks (Adamolekun 2010).

5.2.2.4.3 Nonprotein Amino Acid

Crop plants commonly contain nonprotein amino acids, which are uncommon amino acids that can be present either in their free form or in conjunction with proteins. These nonprotein amino acids serve as protective compounds for plants (Glover et al. 2014). When plants are injured, they produce a compound called canavanine, as observed by Cornara et al. (2016). Canavanine is recognized by an enzyme in herbivores, which typically binds arginine to the arginine tRNA molecule. This disrupts the precise positioning required for protein synthesis. In *Arabidopsis*, arginine is utilized as an active nitrogen source, while proline functions as a component during periods of water scarcity, as reported by Boller and He (2009). The resistance of individual plants to pests can significantly vary due to the identification of specific pathogenic elements that are crucial in triggering defense responses in different plant species. Numerous genes have been identified in plants, which play a role in defense against biological stress. These genes encode receptors that can detect and bind to specific molecules from pests, enabling the plant to prepare for potential pest attacks.

5.2.2.4.4 Proteinase Inhibitor

Proteinase inhibitors (PIs) are a crucial group of protective proteins that are synthesized in higher concentrations in storage organs. They play a vital role in defense by inhibiting various enzymatic processes. PIs bind to enzymes in the insect gut, leading to a reduction in protein digestion. This results in an amino acid shortage and disrupts insect growth. When plant parts are damaged by lepidopteran and hemipteran insects, many plants produce PIs against these insect pests to provide a defensive function. However, certain insects have developed mechanisms to produce proteases that are insensitive to PIs. This enables them to evade the inhibitory effects of PIs, potentially causing more damage to the affected plants (Saddique et al. 2018).

5.3 Biosynthesis

The production and accumulation of secondary metabolites in plants are highly regulated processes that can be affected by a range of abiotic and biotic factors (Fig. 5.2). Throughout their growth and development, plants interact with their environment, including elements such as water, light, temperature, soil composition, and chemicals. Adverse abiotic conditions, such as drought, flooding, extreme temperatures, excessive light exposure, or the presence of harmful substances in the soil, can trigger secondary stresses that influence the synthesis of secondary metabolites. As a result, the production and diversity of these metabolites are significantly influenced by environmental factors (Li et al. 2020).

Plants exhibit stress responses to detect and cope with various biotic and abiotic stressors, leading to the activation of molecular, cellular, and signaling processes. These responses are triggered by the recognition of specific stress events and can result in the production of secondary metabolites. Plants have evolved mechanisms to detect stress, including the recognition of evolving pathogen or microbial patterns, the production of specific proteins, and the synthesis of secondary metabolites to adapt to stressful conditions. These adaptations allow plants to thrive in challenging environments (Dawid and Hille 2018).

The stress response in plants involves the modulation of physiological processes through signal transduction mechanisms. These processes enable plants to adapt to the stimulus by regulating primary and secondary metabolites. These metabolites play roles in regulating cell osmotic pressure, protecting against oxidative damage, inhibiting microbial growth and infection, and deterring herbivores through various biochemical and physiological pathways (Isah 2019).

Secondary metabolites in plants are synthesized in specific organs and can be distributed throughout the plant through vascular tissues or via symplastic and apoplastic transport, depending on their polarity. Hydrophilic compounds like alkaloids, glucosinolates, and tannins are stored in vacuoles or specialized cells called idioblasts. On the other hand, lipophilic compounds, such as essential oils derived from terpenes, are found in various plant structures, including thylakoid membranes, cuticles, resin ducts, and trichomes (Rai et al. 2017).

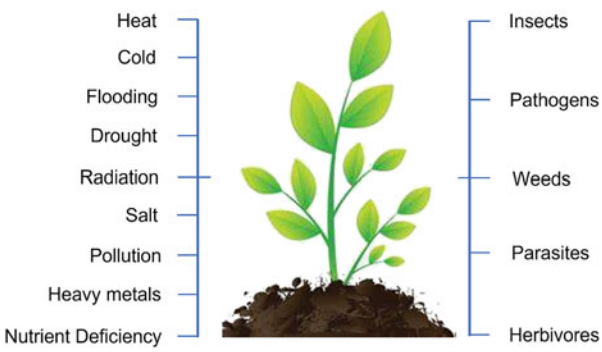

Fig. 5.2 Schematic representation of different factors affecting plants by induction of either abiotic or biotic stress

5.3.1 Abiotic Stress

Throughout different stages of development, plants are often exposed to various abiotic stresses, including factors such as salt, chemical fertilizers, soil composition and type, temperature stress, light intensity, and temperature. The availability and appropriate levels of abiotic components play a significant role in the biosynthesis of secondary metabolites, affecting plant growth and productivity (Verma and Shukla 2015) (Table 5.1). Abiotic stress can negatively impact plants by disrupting cellular metabolic activity and causing an accumulation of reactive oxygen species (ROS) within the plant. Reactive oxygen species serve both harmful and regulatory functions in plants, influencing various biological processes, such as growth, programmed cell death, cell cycle regulation, hormone signaling, and cellular responses and development. Under abiotic stress conditions, certain aromatic and medicinal plants respond by increasing the production of secondary metabolites, leading to enhanced synthesis of phytomedicines and essential oils in aromatic plants (Pradhan et al. 2017).

5.3.1.1 Light Irradiation

Light is one of the most important abiotic factors for plants as it powers photosynthesis, growth, and the accumulation of secondary metabolites, which consists of photons with varying wavelengths and intensities (Li et al. 2018). However, excessive exposure to light can lead to the inactivation or damage of photosynthetic reaction centers in chloroplasts, resulting in photoinhibition and impeding plant growth (Szymańska et al. 2017). The optimal amount of light is crucial for efficient photosynthesis and can significantly impact the quality of metabolites and their accumulation, including total alkaloids, hexadecanoic acid, total flavonoids, phenolic acids, and spermine (Li et al. 2018).

While higher light levels can have a positive impact on plant growth and the production of secondary metabolites in certain cases (Zhang et al. 2015), the response may differ, depending on the plant species and the specific metabolites involved. For instance, *Erigeron breviscapus* leaves grown in sunlight were found to have higher levels of the flavone glycoside scutellarin compared to those grown in shade. Similarly, increased light intensities have been linked to enhanced essential oil production (Kong et al. 2016; Li et al. 2018). However, contrasting results have also been reported. *Flourensia cernua* plants grown in partial shade displayed higher levels of camphene, sabinene, b-pinene, borneol, bornyl acetate, and Z-jasmone compared to fully irradiated control plants (Estell et al. 2016). These findings highlight the variability in the influence of light intensity and photoperiod on secondary metabolite synthesis among different plant species. Therefore, optimizing light conditions, both in terms of quality and quantity, can be adjusted to maximize plant yield and promote the desired therapeutic characteristics of secondary metabolites. It is important to recognize that each plant species may have unique light

Table 5.1 The effect of different abiotic stresses on production of secondary metabolite

Abiotic stress	Plant	Synthesis of secondary metabolites	Role of secondary metabolites	References
Light	*Vanilla planifolia*	Vanillin	Promote photosynthesis	Indrajeet and Rajesh (2018)
Light	*Withania somnifera*	Phenol	Antioxidant activity	Li et al. (2020)
Light	*Mahonia bodinieri*	Alkaloids	Effect on photoperiod	Kong et al. (2016)
Light	*Hordeum vulgare*	Gingerol and zingiberene	Promote photosynthesis	Anasori and Asghari (2008)
Temperature	*Medicago sativa L.*	Quercetin and kaempferol	Synthesis of cryoprotectant compounds	Molmann et al. (2015)
Temperature	*Camellia japonica*	Fatty acid	Gene regulation	Li et al. (2016a, b)
Temperature	*Sorghum bicolor L.*	Polyphenols	Increase concentration of apigeninidin and luteolinidin	Wu et al. (2016a, b)
Temperature	*Capsicum annuum L.*	Proline and phenolic compound	Decrease in chlorophyll content	Esra et al. (2010)
Heavy metal	*Brassica juncea*	Oil content (35%)	Increase antioxidative activity	Indrajeet and Rajesh (2018)
Heavy metal	*Abelmoschus esculentus L.*	Thiol, proline, total phenolics,	Comprises endogenous antioxidant	Sharma et al. (2010)
Heavy metal	*Vaccinium corymbosum L.*	Phenolic compound	Antioxidant activity	Manquián-Cerda et al. (2016)
Heavy metal	*Matricaria chamomilla L.*	Phenolics	Antioxidant activity	Kováčik et al. (2009)
Salinity	*Carthamus tinctorius L.*	Total flavonoids, proline	Increase antioxidative activity	Golkar and Taghizadeh (2018)
Salinity	*Ricinus communis*	Alkaloids	Induce oxidative stress	Ali et al. (2008)
Salinity	*Plantago ovate*	Flavonoids	Induce oxidative stress	Haghighi et al. (2012)
Salinity	*Coriandrum sativum*	Essential oils	Increase the number of oil gland	Neffati and Marzouk (2008)
Drought	*Scutellaria baicalensis*	Phenols	Production of antioxidant enzyme	Cheng et al. (2018)
Drought	*Artemisia*	Terpene	Increase in PSMs production	Verma and Shukla (2015)
Drought	*Chrysanthemum* sp.	Polyphenols, flavonoids	Promoting antioxidant capacity	Hodaeia et al. (2018)
Drought	*Labisia pumila*	Total phenols	Promoting antioxidant capacity	Jaafar et al. (2012)

requirements to achieve optimal production of secondary metabolites (Li et al. 2020).

5.3.1.2 Temperature Stress

According to Yadav (2010), appropriate temperature ranges are directly correlated with plant growth and development. Extreme temperatures can have negative effects on plant development and productivity. Heat stress, which affects plants growing in hot climates, can lead to reduced stomatal conductance and net CO_2 fixation, resulting in decreased plant growth and productivity. Heat stress is also linked to changes in secondary metabolite production (Verma and Shukla 2015). While some studies in the literature report a decrease in secondary metabolites under heat stress, a literature review by Ashraf et al. (2018) suggests that high-temperature stress generally increases the synthesis of secondary metabolites. The photochemical efficiency of photosystem II decreases in plants experiencing heat stress. However, the response of secondary metabolites to high temperatures can vary, depending on the species and other factors. High temperatures can either increase or decrease the levels of secondary metabolites. High temperatures can up- or downregulate specific genes, which in turn influence plant growth and development (Li et al. 2016a, b).

Conversely, exposure to low temperatures can have detrimental effects on plant growth, the biosynthesis of secondary metabolites, and their storage (Verma and Shukla 2015). Plants undergo cold acclimation to adapt to low-temperature stress, which involves significant changes in physiological, chemical, and molecular processes such as cellular dehydration, water absorption, and metabolic reactions (Ashraf et al. 2018). In the case of *Medicago sativa* L., Molmann et al. discovered that higher temperatures resulted in increased levels of quercetin and kaempferol, while putrescine content was higher at lower temperatures. Hummel et al. (2004) found that *Pringlea antiscorbutica* seedlings exhibited elevated levels of polyamines (agmatine and putrescine) under low temperatures, indicating a response to chilling stress. In the *Brassicaceae* family, Indrajeet and Rajesh (2018) observed a modest reduction in carotenoid levels, particularly β-carotene, at high temperatures. To summarize, temperature is a critical factor influencing plant growth and the production of secondary metabolites. Heat stress and low-temperature stress can yield divergent effects on secondary metabolite synthesis, with some metabolites increasing while others decreasing, depending on the specific conditions and plant species involved.

5.3.1.3 Salinity Stress

Nutrient imbalance is one of the foremost effects of the presence of salt in soil; the presence of salt also causes a decline in photosynthetic rate and plant growth (Banerjee and Roychoudhury 2017). Osmotic stress caused by salinity or specific ion toxicity can alter the concentration of secondary metabolites in plants (Akula and

Ravishankar 2011). *Plantago ovata* plants growing under salinity stress, for example, have shown increased concentrations of alkaloids, tannins, phenolics, saponins, flavonoids, and proline. Recent studies have also indicated a decline in oil production under salt stress (Li et al. 2020). For instance, species such as *Mentha suaveolens, M. pulegium, Salvia officinalis, Matricaria chamomilla, Majorana hortensis, Origanum vulgare, Thymus maroccanus, Mentha piperita*, and *Trachyspermum ammi* have experienced a decrease in essential oil production due to saline stress. On the other hand, *Satureja hortensis, Salvia officinalis*, and *Matricaria recutita* have shown an increase in essential oil production under salt stress (Said-Al Ahl and Omer 2011). Hence, high salt content in soils can cause various stresses on plants, leading to changes in the concentration of secondary metabolites. While some plants may show an increase in certain secondary metabolites under salinity stress, others may experience a decrease. The response to salt stress and its impact on secondary metabolite production can vary among different plant species.

5.3.1.4 Drought Stress

In addition to its involvement in cellular processes, water plays a critical role in nutrient and metabolite transportation within plants. Drought stress occurs when plants experience insufficient water availability or heightened transpiration rates, which can impact the production of secondary metabolites. Several medicinal plants, such as *Catharanthus roseus, Hypericum perforatum*, and *Artemisia annua*, have exhibited increased levels of endogenous secondary metabolites in response to drought stress (Jogawat et al. 2021). For example, *Trachyspermum ammi* subjected to drought stress showed elevated levels of phenolics and photosynthetic pigments, albeit with reduced fresh and dry biomass. Another study highlighted the positive impact of drought stress on the quality of essential secondary metabolites like rutin, quercetin, and betulinic acid in *Hypericum brasiliense*, as well as artemisinin in *Artemisia* species (Ashraf et al. 2018). Similarly, Verma and Shukla (2015) observed that drought stress enhanced the quality of significant secondary metabolites, including rutin, quercetin, and betulinic acid, in *Hypericum brasiliense*. St. John's wort plants exposed to water-limited conditions exhibited a notable decrease in photosynthesis but an increase in concentrations of secondary metabolites, like pseudohypericin, hypericin, and hyperforin. Additionally, *Glechoma longituba* plants grown under drought-like conditions displayed an increase in total flavonoid content. Water scarcity significantly affected the concentrations of various macronutrients, proline, carbohydrates, and essential oils in *Ocimum americanum* and *Ocimum basilicum* (Ashraf et al. 2018).

5.3.1.5 Heavy Metal Stress

Heavy metals play a vital role in plant biology as they are essential components of numerous enzymes. For example, Zn is necessary for carbonic anhydrase, Mo and Fe for nitrogenase, and Cu for superoxide dismutase. However, certain metals such as Cr, Cu, Mn, and Fe can also have detrimental effects on plants by participating in Haber-Weiss and Fenton reactions. These reactions generate reactive oxygen species (ROS) or oxygen free radicals within plants, leading to disturbances in cellular homeostasis, DNA damage, protein degradation, cell membrane impairment, and cell death. To counteract the effects of heavy metals, plants employ various strategies and produce a wide array of secondary compounds. Ni stress, for instance, has been shown to decrease the anthocyanin content in lettuce leaves (*Lactuca sativa* L.) (Indrajeet and Rajesh 2018). In *Brassica juncea* L. plants, the accumulation of Cr, Fe, Zn, and Mn has been found to increase oil production by up to 35% (Singh and Sinha 2005). Cu^{2+} stimulates the production of betalains in *Beta vulgaris* L. plants. Furthermore, Sharma et al. (2010) observed that increasing concentrations of Cd in the soil led to elevated levels of thiol, proline, total phenolics, ascorbic acid content, and peroxidase activity in lady's finger (*Abelmoschus esculentus* L.) plants. Plants possess mechanisms to mitigate oxidative stress by scavenging free radicals from their surroundings. This is accomplished through the action of various endogenous enzymes such as SOD, CAT, APX, GPX, and GR, as well as nonenzymatic antioxidants, including ascorbate (AsA), glutathione (GSH), carotenoids, alkaloids, tocopherols, proline, phenolic compounds, flavonoids, tannins, and lignin. Together, these components form the antioxidant defense system within plant cells (Indrajeet and Rajesh 2018).

5.3.2 Biotic Stress

All living organisms must contend with a variety of biotic and environmental constraints throughout their entire existence (Anjali et al. 2023). Many organisms can defend themselves against the negative impacts of biotic and abiotic stressors, enabling them to grow, spread, and survive (Herrmann et al. 2021). Numerous living things, such as worms, bacteria, viruses, and fungi, produce biotic stressors on plants. However, plants are unable to move to another location to escape the demanding environment. Plant's tolerance to the attack by pathogens can be demonstrated by the rise in the production of secondary metabolites (Ashraf et al. 2018) (Table 5.2). There exist approximately 8100 species of fungi that have been directly or indirectly related to the development of plant diseases (Tarkowski and Vereecke 2014). However, viruses can cause comparably more damage than fungi and are therefore considered hazardous plant pathogens. These microbes can induce various symptoms in plants, including wilting, leaf spots, root rot, and seed damage. They can infect any plant part and can also serve as a source of bacterial and viral

Table 5.2 Production of secondary metabolites in response to the presence of biotic stressors in different plant species

Biotic stresses	Species	Plant	Secondary metabolites	References
Pathogen	*Tobacco mosaic virus*	*Citrus reticulate*	Reticine A	Wang et al. (2021)
Pathogen	*Alternaria solan*	*Allium sativum*	Terpenoids, flavonoids,	Kumar et al. (2021)
Pathogen	*Aspergillus* spp.	*Carthamus tinctorius*	Alkaloids	Hussain et al. (2021)
Pathogen	*A. solani*	*Azadirachta indica*	Saponins, steroids,	Chohan et al. (2019)
Pathogen	*F. graminearum*	*Zea mays*	Flavonoid and Terpenoid	Bai et al. (2021)
Pathogen	*B. cinerea*	*Vitis vinifera*	Resveratrol	Wang and Wang (2019)
Pathogen	*Verticillium dahlia*	*Gossypium* spp.	Lignin	Zhu et al. (2022)
Pathogen	*Magnaporthe oryzae*	*Cassia alata*	Methyl 2,4,6-trihydroxybenzoate	Pham et al. (2021)
Pathogen	*Rhizoctonia solani*	*Datura metel*	Pentadecanoic acid	Hanif et al. (2022)
Pathogen	*Burkholderia glumae*	*Zingiber officinale*	Geranial	Gunasena et al. (2022)
Pathogen	*Cladosporium cladosporioides*	*Aloe succotrina*	Flavonoids	Mohamed et al. (2022)
Pathogen	*P. infestans*	*Solanum tuberosum*	Alkaloids	Yogendra et al. (2017)
Pathogen	*Fusarium graminearum*	*Hordeum vulgare*	Hydroxycinnamic acid amide	Karre et al. (2019)
Pathogen	*F. graminearum*	*Triticum aestivum L.*	Lignin	Soni et al. (2021)
Herbivory	*Honeybee and bumblebee*	*Nicotiana* sp.	Pyridine alkaloid	Stevenson et al. (2017)
Herbivory	*E. coli, S. aureus*	*Xanthium cavanillesii*	Diterpene kaurene	Chen et al. (2015)
Herbivory	*S. sulfureana, Lymantria dispar L.*	*Vaccinium myrtillus*	Phenol	Hernandez-Cumplido et al. (2018)
Herbivory	*Bark beetle*	*Pinus and Abies*	Terpenoids	Zaynab et al. (2018)
Herbivory	*Insect*	*Pteris*	Steroids	Canals et al. (2005)
Herbivory	*Spodoptera litura*	*Nicotiana tabacum*	Terpenoids	Zaynab et al. (2018)
Herbivory	*Operophtera brumata*	*Salix glauca L.*	Terpenoids	Ruuhola et al. (2001)
Herbivory	*Galerucella lineola*	*Willow plant*	Phenolics	Zaynab et al. (2018)
Herbivory	*Tetranychus urticae*	*Fragaria ananassa*	Phenolics	Luczynski et al. (1990)

transmission to uninfected plant parts and other plants also (Saddique et al. 2018). Along with these disease-causing organisms, weeds are another source of damage to the plants as they compete with plants of economic importance and can restrict or delay the growth of these plants (Dass et al. 2016).

5.3.2.1 In Response to Pathogen

Pathogens invade host cells, replicate, and exploit the host plant's biological processes, posing a risk to plant growth. Plants naturally produce a wide range of secondary metabolites, which primarily serve as defense mechanisms against predators, bacteria, and other harmful organisms. These secondary metabolites have been extensively studied in the context of plant-pathogen interactions, as highlighted in a notable research by Zaynab et al. (2018). They encompass various substances like amino acids, chemical compounds, and nucleosides, which regulate specific metabolic pathways and enzymes involved in primary metabolism. The evolutionary role of these metabolites is closely tied to their functionality, such as attracting pollinators, fending off insects and pathogens, or aiding plant cells' survival in biotic environments, as explained by Jan et al. (2021) and Katyal (2022). Plant defense mechanisms activate multiple secondary metabolites through the recognition of defense proteins known as MAMPs (microbe-associated molecular patterns) by pattern recognition receptors. The classification of plant immunity and secondary metabolites takes into account factors like compound production, defense-associated phytochemicals, phytoalexin and phytoanticipin formation, common precursors, structures, and mechanisms of action, as discussed in the research by Piasecka et al. (2015).

5.3.2.2 In Response to Fungi

Plants are capable of producing significant quantities of secondary chemicals with antibacterial properties. Fruit skin and leaves are rich in phenolic compounds, such as flavonoids and associated phenolics, which make up a large category of phytochemicals (Anjali et al. 2023). These metabolites discovered crucial protection against disease, UV resistance, and pigmentation (Tuladhar et al. 2021). Additionally, it is known that these phenolic metabolites change cellular permeability and cause casing proteins to disintegrate structurally and practically. This disrupts pH balance and causes ATP production, substrate consumption, and membrane-bound enzymes (Anjali et al. 2023). Antimicrobial substances also prevent pathogen proliferation in plant apoplast. As demonstrated by a high-antifungal-activity saponin found in tomatoes, called α-tomatine, it activates G-protein and phosphotyrosine kinase pathways before binding to plant cell membranes and inducing a sudden increase in ROS and Ca^{2+} concentration in response to *Fusarium oxysporum* attack (Zaynab et al. 2018). According to Ludwiczuk et al. (2017), turpentine and camphor are terpenes composed primarily of unsaturated isoprene units. Induced defenses in

crop plants, such as cotton and solanaceous vegetables, heavily rely on sesquiterpenoid phytoalexins like rishitin, gossypol, and capsidiol, as emphasized by Shukla et al. (2019). Rice plants, as highlighted by Azizi et al. (2019), produce several diterpenoid phytoalexins that aid in combating fungal diseases like rice blast caused by *Magnaporthe oryzae*. Kauralexins, a type of diterpenoid phytoalexin produced by maize plants, concentrate at the interface between the plant and pathogens and exhibit effectiveness against *Rhizopus microspores* and *Colletotrichum graminicola*. The response of maize to *Fusarium graminearum* infection led to the identification of sesquiterpenoid phytoalexins, including zealexin, which confer resistance against *F. graminearum*, *Aspergillus flavus*, and *R. microspores*, as discovered by Anjali et al. (2023). Flavonoids are present in the liquid extracts of *Zapoteca portoricensis*, a leguminous crop known for its antioxidant, antitumor, and anti-inflammatory properties. Chahal's 2018 research investigated the antifungal effects of dill seed oil on fungi, like *Ustilago segetum* var. tritici, *Alternaria triticina*, and *Bipolaris sorokiniana*. The study revealed that the presence of key components, such as carvone, camphor, and their polar fractions, contributed to these antifungal effects.

5.3.2.3 In Response to Bacteria

According to Zaynab et al. (2018), the root exudates have a protective role against the bacterial presence in the soil as was demonstrated with *Arabidopsis*, in which the root exudates protected the plants in the presence of *Pseudomonas syringae*. The efficacy of methanol extracts prepared from *Polyalthia longifolia*, *Terminalia chebula*, and *Zingiber officinale*, has also been demonstrated against *Xanthomonas campestris pv. campestris* (Yumlembam et al. 2016). The defensive enzymes peroxidase and polyphenol oxidase have also been reported to boost plant immune response. According to Ezzat and Moussa (2016), systemic resistance is produced by *Bacillus subtilis, B. polymyxa*, and *P. fluorescens* via boosting phenolic chemicals and molecules associated with defense. Caffeine, a secondary metabolite in plants, has been identified as an effective antibacterial agent against various harmful plant pathogenic bacteria, including *Ralstonia solanacearum, Clavibacter michiganensis* subsp. *sepedonicus, Dickeya solani, Pectobacterium carotovorum subsp. carotovorum, P. atrosepticum,* and *X. campestris* subsp. *campestris* (Uche-Okereafor et al. 2019).

5.3.2.4 Mode of Action

The plant defense system involves various chemicals with diverse modes of action. Secondary metabolites, such as phenolics, carotenoids, terpenes, terpenoids, and alkaloids (e.g., nicotine, morphine, caffeine, atropine, and quinine), play a crucial role in developing complex defense mechanisms against invading pathogens, enhancing plant survival, and building disease resistance, as highlighted by Abegaz

and Kinfe (2019). Flavonoids, phenols, and phenyl propanoids are prominent secondary metabolites found in plant systems, as noted by Ramírez-Gómez et al. (2019). These chemicals primarily affect cell signaling pathways or interact with pathogen components through mechanisms like enzyme inhibition, DNA alkylation, and disruption of reproductive systems. Secondary metabolites accumulate in plants in response to various forms of stress or signaling molecules. Biotic or abiotic elicitors, known as secondary metabolite modulators, stimulate plants to increase the production or accumulation of secondary metabolites, as discussed by Narayani and Srivastava (2017) and Thakur et al. (2019). Signaling factors in plants can be generated by compounds like oligogalacturonic acid from plant cell walls, chitin, and phytoalexin biosynthesis in fungal cell walls. For instance, *Hypericum perforatum* increases the production of phenolic compounds as a defensive response against pathogens when exposed to stress, as indicated by Gadzovska et al. (2015) and Ghorbanpour et al. (2016). Rhizobacteria serve as valuable models for secondary metabolites with therapeutic potential. They colonize the plant's rhizosphere, promote plant growth, and act as signal transducers and enzyme inducers in bioactive metabolic pathways, facilitated by the production of jasmonic acid, as discussed by Vafadar et al. (2014) and Kumar et al. (2019).

5.4 Mutation and Secondary Metabolites Production

Throughout history, plants have been valued as a bio-cultural resource and recognized as the primary source of natural products derived from various plant components, such as barks, stems, leaves, and roots. Plants have the capacity to produce both primary and secondary metabolites, and various techniques have been used to increase the production of secondary metabolites. Secondary metabolites, including alkaloids, phenolics, and essential oils, play crucial roles in plant metabolism, such as regulating growth, development, and defense against pathogens, insects, and herbivores. They also contribute to pigmentation and have potential as valuable compounds for the development of drugs and medicines.

Traditional plant-based manufacturing techniques, such as callus or suspension culture, have long been utilized to obtain secondary metabolites from plants. However, recent advancements have introduced novel techniques that have demonstrated promising results in enhancing the production of these metabolites. Modern techniques, including elicitation therapy, antecedent feeding, plant tissue culture, as well as technologies like Crispr Cas, nanotechnology, and genome editing, are being employed to improve the yield of secondary metabolites. These techniques offer effective and environmentally friendly alternatives when chemical synthesis is not feasible or when the natural supply of secondary metabolites is limited (Sharma et al. 2020).

5.4.1 *Plant Tissue Culture and Secondary Metabolites Production*

The concept of using plant cell, tissue, and organ culture as a method for producing secondary metabolites was first proposed in the late 1960s. Over the years, various methods have been employed in cell culture systems to achieve high yields of secondary metabolites. Plant cell cultures have the ability to produce secondary metabolites in different quantities and qualities compared to intact plants, and these properties can vary over time. The process of plant cell culture involves isolating cells from the plant, culturing them under optimized conditions, and then harvesting the desired secondary metabolite from the cultured cells. Advances in plant tissue culture techniques have significantly contributed to the improved production of secondary metabolites, allowing for their commercial production and providing valuable tools for research in cell biology, genetics, and biochemistry, as discussed by Fazili et al. (2022).

In vitro synthesis of secondary metabolites is a two-step process that involves biomass aggregation and subsequent secondary metabolite production. Various organized structures, such as shoots, roots, calli, and cell suspensions, have been utilized for the production of secondary metabolites, as highlighted by Chandran et al. (2020). Callus formation is commonly induced in plant cell cultures by employing high concentrations of auxin or a combination of auxin and cytokinin. Callus cultures hold great potential for the commercial production of secondary metabolites with medicinal applications and provide a reliable alternative to sourcing plant materials from the wild for obtaining medicinal metabolites, as noted by Wu et al. (2016a, b). Callus and suspension cultures not only serve as platforms for the production of desired secondary metabolites but also allow for the manipulation of the biosynthesis pathways of these metabolites. Several secondary metabolites, such as tropane alkaloids, α-tocopherol, ajmaline, serpentine, reserpine, flavonoids, scopolamine, paclitaxel, stilbene, resveratrol, and anthocyanins, have been successfully produced using callus cultures, as discussed by Efferth (2019). These cultures can also be utilized for micropropagation to generate numerous plant clones and for single-cell suspension cultures through batch or continuous fermentation to produce the desired secondary metabolites.

5.4.1.1 Suspension Culture

Systems for cell suspension culture offer a faster, more efficient way for production of secondary metabolites at an industrial scale. This process is the best and most trustworthy way to produce natural products (Vanisree et al. 2004). Calli are initially produced from chosen mother plants in the best-suited growing medium. For dedifferentiation and differentiation mechanisms, this suitable media is beneficial. Despite the fact that this task requires a lot of experience and is crucial, it can also be completed using surface response methods or incomplete factorial trials (Fazili et al.

2022). These calli can then be propagated or subjected to organogenesis and embryogenesis in suspension cultures as such (Barrales et al. 2019).

5.4.1.2 Elicitation

An elicitor is a chemical that initiates or enhances the production of a specific compound. For increasing the production of secondary metabolites, the use of elicitation has proven to be highly effective. In living cell systems, the presence of even negligible concentrations of elicitor molecules has shown promising results for improving the production of secondary metabolites (Radman et al. 2003). In plant tissue culture, the introduction of chemical or physical stimuli can be employed for elicitation purposes. Stresses caused by elicitation lead to the production of secondary metabolites that are not typically synthesized by plants. Biotic elicitors, such as chitosan, various protein extracts, and sterilized mycelium of pathogenic fungi, as well as abiotic factors including heavy metal salts, extreme temperature, pH variations, and UV radiation, are commonly used for elicitation (Fazili et al. 2022). Multiple studies have provided evidence for the efficacy of elicitors in augmenting the production of secondary metabolites in plant tissue culture. Scientists worldwide have utilized diverse elicitors to enhance the production of secondary metabolites in in vitro systems. Elicitors function as signals, initiating the elicitation process through the recognition of signals by specific receptors on the plant cell membrane. This recognition triggers a series of signal transduction events that ultimately result in alterations in the expression levels of regulatory transcription factors/genes and key genes involved in secondary metabolic pathways. As a consequence, there is an upsurge in the synthesis and accumulation of secondary metabolites (Halder et al. 2019).

5.4.1.3 Organ Culture

Differentiated organ cultures, such as shoots or roots, have been found to produce metabolites and exhibit a metabolite profile similar to that of natural plants. Among the alternative methods for producing plant-derived chemicals, plant hairy root cultures have shown great promise. The controlled transformation of *Agrobacterium rhizogenes* has been utilized to induce hairy roots in plants, enabling the in vitro production of secondary metabolites typically synthesized in plant roots (Chandran et al. 2020). This technique has provided a valuable biological tool for studying the production of various bioactive substances, including nicotine and tropane alkaloids, as well as ginsenosides, anthraquinones, and artemisinin. The advantage of utilizing hairy roots for compound production lies in their high productivity, consistency, and efficiency (Cardoso et al. 2019).

5.4.1.4 Hairy Root Culture and Shoot Culture

The term "hairy root" was first mentioned in scientific literature by Stewart et al. (1900). Hairy root cultures have emerged as an advanced technique in plant tissue and organ culture, aimed at enhancing the production of secondary metabolites. This is accomplished by transforming the targeted plant species using *Agrobacterium rhizogenes*, a natural vector system. *Agrobacterium rhizogenes* induces hairy root disease in dicotyledonous plants (Giri and Narasu 2000; Bourgaud et al. 2001). Studies have demonstrated that the roots of *Hordeum vulgare*, when infected with the vesicular arbuscular mycorrhizal fungi *Glomus intraradices*, can synthesize a terpenoid glycoside. Additionally, the combination of hairy roots from *Catharanthus roseus* with *Acaulospora scrobiculata* in a culture system has been found to regulate and enhance the production of indole alkaloids (Rao and Ravishankar 2002; Sharma et al. 2020). Apart from hairy root cultures, shoot culture is another significant technique utilized for the production of secondary metabolites. This can be achieved by either infecting the aerial parts of plants with *Agrobacterium tumefaciens*, leading to the formation of transgenic shoots (shooty teratomas) (Massot et al. 2000), or by applying appropriate hormone doses to induce the development of non-transgenic shoots.

5.4.1.5 Biotransformation

Plant cell suspension cultures are well-established as an effective method for producing significant amounts of secondary metabolites. However, these cell cultures also possess the ability to utilize biotransformation mechanisms, which involve the conversion of external substances into new substances with different properties. Biotransformation is a process in which primary substrates are transformed into different substrates with new properties, often facilitated by appropriate enzymes or microorganisms (Zhang et al. 2007; Zhao et al. 2007). Plant enzymes play a vital role in the production of specific metabolites or compounds with altered properties, making the enzymatic capacity of plant cells advantageous in biotransformation processes (Ishihara et al. 2003). Biotransformation has been observed in various plant species. For example, in *Eucalyptus perriniana*, thymol, carvacrol, and eugenol can be converted into glycosides through biotransformation. In tobacco, hyoscyamine can be transformed into scopolamine through this process. In Catharanthus roseus, the glycosylation biotransformation of capsaicin and 8-nordihydrocapsaicin occurs in cell cultures (Fazili et al. 2022).

5.4.2 Secondary Metabolite Production and the Role of Genetic Engineering

Secondary metabolite genetic engineering is a technique used to manipulate the production of specific chemicals or groups of target compounds. Various methods

can be employed to decrease the production of undesirable combinations. The initial application of genetic engineering focused on flavonoid and anthocyanin production due to the well-understood biosynthesis pathways and easily observable differences in flower color (Rastegari et al. 2019). In addition to traditional methods, modern approaches are now being utilized to produce secondary metabolites. Excessive expression of gene pathways has been investigated to develop new flower colors and plant chemicals. Flavones, which have antioxidant properties and are important in the diet, have structures similar to those found in food additives and plant pigments. In the fermentation industry, the insertion of microbial strains for increased production and mutagenesis are popular methods. For example, the induction of the p-fluorophenylalanine strain and various tobacco species resulted in elevated phenolics production (Sharma et al. 2020). Metabolic engineering using *E. coli* has been employed to produce 1-valine, an important pharmacological precursor.

Bioreactors and immobilization techniques are two methods that can enhance the production and accumulation of secondary metabolites. Bioreactor systems such as feed batch, batch, and continuous cultures can be used for large-scale production. Immobilization of *Plumbago rosea* cell cultures in calcium alginate and cultivation on Murashige and Skoog media have shown increased production of secondary metabolites (Gaspar et al. 1996). The terpenoid indole alkaloid pathway is another target of genetic engineering efforts, as it includes economically important alkaloids, such as vinblastine, vincristine, and camptothecin, which have anticancer properties. Isoquinoline alkaloids, including pharmaceuticals like morphine and codeine, are essential secondary plant metabolites. Terpenoids constitute a significant portion of secondary chemicals. Recent discoveries in the 2-C-methyl-D-erythritol-4-phosphate (MEP) pathway have led to the duplication of multiple genes involved in plastidial terpenoid biosynthesis, including red fat-soluble pigments, monoterpenes, and diterpenes (Rastegari et al. 2019).

5.4.3 Micropropagation in Secondary Metabolites Production

Plants possess pharmacological characteristics that rely on their phytochemical constituents, especially secondary metabolites, which serve as valuable sources of bioactive compounds. Secondary metabolites are synthesized in response to various types of stress and exhibit complex chemical compositions to fulfill diverse physiological functions within plants. These metabolites find applications in industries such as food and beverage, cosmetics, and pharmaceuticals. To enhance production and optimize large-scale cultivation, research efforts have focused on utilizing plant tissue culture (PTC) techniques and bioreactors. By employing PTC methods, secondary metabolites can be continuously, sustainably, economically, and efficiently produced, irrespective of climate or location (Chandran et al. 2020). The in vitro micropropagation approach enables the production of different

phytoconstituents, including pterocarpans, flavonoids, and alkaloids, from therapeutic plants (Sharma et al. 2021).

5.4.4 Secondary Metabolite Production and the Role of Plant Growth Regulators

Plant growth regulators (PGRs) are simple compounds that exhibit specific effects on plant development, even at low concentrations. They have demonstrated their efficacy as elicitors for stimulating the production of secondary metabolites in plants. PGRs encompass both naturally occurring phytohormones and their synthetic counterparts. Besides their role in regulating antioxidant potential and fundamental growth and developmental processes, PGRs have been found to influence the biosynthesis of plant secondary metabolites in plant tissue culture (Jamwal et al. 2018). The response of plants to PGRs can vary depending on various factors, including species, plant age, variety, environmental conditions, developmental stage, physiological and nutritional status, and endogenous hormonal balance (Aftab et al. 2010).

Azeez and Ibrahim (2014) discovered that the optimization of cultural conditions and the introduction of PGRs to the medium during the initiation of callus in *Hypericum triquetrifolium* resulted in higher levels of active chemicals in cultured cells, leading to increased production of secondary metabolites. The stimulation of cell proliferation and division by PGRs contributes to the enhancement of secondary metabolite production. PGRs have proven useful in stimulating secondary metabolite production in *Saintpaulia ionantha* and *Hypericum mysorense* due to their significant impact on secondary metabolite metabolism (Shilpashree and Rai 2009).

According to the findings of Jamwal et al. (2018), the application of naphthalene acetic acid (NAA) and benzyl adenine (BA) in callus cultures of *Phyllanthus acidus* led to an increase in the accumulation of secondary metabolites. Similarly, the use of indole acetic acid (IAA) and naphthalene acetic acid resulted in enhanced secondary metabolite production in *Coscinium fenestratum*, while 2,4-dichlorophenoxyacetic acid (2,4-D) showed effectiveness in *Nicotiana tabacum*. Baque et al. (2010) observed that treating adventitious roots with a combination of kinetin (KIN), indole 3 butyric acid (IBA), and thidiazuron (TDZ) caused a decrease in fresh weight (FW) and dry weight (DW) but an increase in secondary metabolite content. Amoo et al. (2012) found that altering cytokinin concentrations, particularly in conjunction with equimolar amounts of NAA, significantly influenced secondary metabolite production in *Aloe arborescens*. Rawat et al. (2013) reported that 6-benzylaminopurine (BAP) exhibited superior efficacy to thidiazuron in enhancing shoot regeneration and secondary metabolite production in *Aconitum violaceum*. Liu et al. (2007) demonstrated that cytokinins promoted secondary metabolite production in *Hypericum sampsonii* and *Hypericum perforatum* plantlets. Treatment with abscisic acid (ABA) was shown to induce oxidative stress and stimulate secondary

metabolite formation in *Orthosiphon stamineus* (Kalt et al. 2001). Additionally, gibberellic acid (GA3) was found to promote hairy root growth and influence secondary metabolite production to varying degrees across different species, including Cichorium intybus (Bais et al. 2001).

5.4.5 Other Method to Enhance the Production of Secondary Metabolites

Additional factors used to enhance the production and quantity of secondary metabolites include precursors and biotic or abiotic elicitors. Biotic elicitors mainly consist of low-molecular-weight organic acids, glucans, fungal cell wall components, and glycoproteins. Abiotic elicitors, on the other hand, include chemical salts, heavy metals, and exposure to ultraviolet or infrared radiation (Hoopen et al. 2002). Elicitors, which encompass physical, molecular, and chemical stimuli, trigger specific responses in plants and their cultures. Elicitation is a strategy employed to enhance plant lifespan by increasing the production and synthesis of secondary metabolites. For instance, the effect of elicitor treatment on the generation of glycyrrhizin from suspension cultures of *Abrus precatorius* was demonstrated. High-yielding cell lines are established on MS media using plantlets and genetically modified *Agrobacterium tumefaciens* and *Agrobacterium rhizogenes* (Sharma et al. 2020).

It has been observed that the generation of secondary metabolites is also influenced by the growth medium, the carbon source utilized, and biotic and abiotic stresses. *Catharanthus roseus* cultures exhibited an increase in alkaloid production when provided with higher amounts of sucrose (ranging from 4% to 10%). In tobacco cells, supplying lower quantities of sucrose resulted in increased production of ubiquinone-10 (Komaraiah et al. 2002). Somaclonal changes commonly observed in plant cultures can modify the genetic characteristics of the cultured plant, leading to improved biomass yield and secondary metabolite production. Synthetic biological technologies such as Crispr/Cas9 have been used to produce secondary metabolites from *Streptomyces*. Ion-mobility-mass spectrometry (IM-MS) has been widely employed for structural elucidation and characterization, and matrix-assisted laser desorption assays have also been utilized, enhancing the discovery of secondary metabolites. Crispr/Cas9 has been employed to introduce gene modifications in the genomic contents and can contribute to the synthesis of secondary metabolites, representing a promising area of research for future applications that can benefit mankind (Chinnusamy et al. 2004).

Despite significant advancements in the biotechnology of cultured plant cells for secondary metabolite production (Khojasteh et al. 2014; Georgiev and Weber 2014), the development of new bioengineering techniques remains a crucial process. MiRNAs (microRNAs) may be particularly suitable for controlling secondary metabolism in cultured plant cells due to their ability to regulate entire gene families.

MiRNAs exert various effects on plants, including posttranscriptional mRNA cleavage, translation suppression, RdRP-mediated second-strand synthesis, and generation of transacting siRNAs (ta-siRNAs) triggered by miRNA activity.

5.5 Conclusion

Plants respond to different stressors by producing secondary metabolites, including phenolics, flavonoids, terpenoids, and anthocyanins, which are crucial for their overall physiological well-being. To maximize the production of these compounds, plants can be exposed to specific stress conditions. Recent advancements in cell culture technology have revolutionized the production of bioactive secondary metabolites. In vitro cultivation provides a controlled environment for plant cells to grow, independent of soil and climate, ensuring consistent and reliable production of natural compounds unaffected by environmental variations. Biotechnological tools, such as genetic engineering, have played a significant role in enhancing the synthesis of secondary metabolites. By manipulating plant cells, their capacity to produce these valuable compounds can be improved. Furthermore, a better understanding of the structure and regulation of pathways involved in secondary metabolite formation has facilitated the achievement of commercially viable levels of these compounds. Cell culture-based production of bioactive secondary metabolites offers several advantages. It is a sustainable and eco-friendly approach to obtaining natural compounds and allows for scalability to meet the increasing demand in industries like pharmaceuticals, cosmetics, and food. In summary, the combination of stress induction in plants and advanced cell culture techniques has opened up new opportunities to maximize the production of secondary metabolites. With a deeper understanding of metabolic pathways, the sustainable and consistent production of diverse bioactive compounds has become a reality.

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Chapter 6
Genetic Manipulation in Medicinal Plants for Enhancement of Secondary Metabolites

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Abstract Medicinal plant is hike in demands for the production of secondary metabolites, important for the pharmaceutical industry. However, their yield is limited under de novo synthesis. The amount of production is often limited by the availability of source plant, presence of suitable elicitor, environmental conditions, and method of extractions. Genetic engineering provides the tools for engineering elicitor pathway in the plant so that a quick and prolonged response can be generated for the higher accumulation of target secondary metabolites. Tissue culture provides the mass multiplication of plantlets of endangered and rare medicinal plants and facilitates the production of secondary metabolites throughout the year using plant cell as a bioreactor. Plant with higher biomass, short duration, and minimal maintenance are ideal bioreactor for secondary metabolite production. Gene responsible for secondary metabolite production can be transformed in the plant-based bioreactor to enhance their accumulation. Thus, biotechnological tools, tissue culture, genetic transformation, gene editing, and RNAi approaches provide the avenue for development of medicinal plant with higher production of target secondary metabolites.

Keywords Biotechnology · Genetic engineering · Tissue culture · Secondary metabolites · Medicinal plants

6.1 Introduction

Medicinal plants are being used since centuries for treating a variety of ailments. These plants are important source of compounds that play important role in improvement of immune power and health (Constabel 1990). Often these organic

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compounds are produced in low quantity and play an important role in growth and development of the plant (Kim et al. 2002). These compounds are essential for the improvement of physiological and biochemical activities to ensure the fitness and survival of plant under biotic and abiotic stress are called as secondary metabolites (Hussain et al. 2012; Yeshi et al. 2022). Secondary metabolites are small organic molecules with various chemical structures having biological functions. However, primary metabolites are essential for various function that work as life support system such as cell formation, development, and proliferation, but secondary metabolites are also necessary for better performance of the plant under competitive environment. Secondary metabolites produced by the plants are used as therapeutic purpose, agrochemicals, nutritive value, fragrance, food coloring agent, insecticides, and pesticides (Balandrin and Klocke 1988). It is being speculated that around one lakh, types of secondary metabolites are known and found in 50,000 different plant species. Each year, more than 4000 unique metabolic compounds are being identified and characterized from several plant species. Since prehistoric times, humans are using such valuable plant extract for treating various life-threatening disease. Secondary metabolites have been used for centuries for their therapeutic potential due to their anti-inflammatory, antimicrobial, and antioxidant properties. Some examples of secondary metabolites and their medicinal value include:

1. Alkaloids: Many alkaloids have been used as painkillers, e.g., morphine and codeine, which are derived from opium poppy. Alkaloids have also been used as stimulants, such as caffeine, and as anticancer agents, such as vinblastine and vincristine.
2. Terpenoids: Terpenoids have been used as anti-inflammatory agents, such as curcumin from turmeric, and as anticancer agents, such as taxol from the Pacific Yew tree.
3. Phenolics: Phenolics are the antioxidants that have potential in prevention of life-threatening diseases, cancer, and cardiovascular disease. Resveratrol, a phenolic found in grapes, has been found to have anti-inflammatory and anticancer properties.
4. Flavonoids: Flavonoids have been used as antioxidants and have been found to have potential to reduce and treat diseases such as cancer and cardiovascular disease. Quercetin, a flavonoid of various fruits and vegetables, has been found with anti-inflammatory and anticancer properties.
5. Glycosides: Glycosides have been used as medicines for heart and malaria such as digitalis from the foxglove plant and artemisinin from *Artemisia annua* respectively.

Due to these medicinal properties of secondary metabolites, around 70–80% of the global population predominantly depends on traditional, largely herbal medicines to meet their primary healthcare needs (Farnsworth and Soejarto 1991). An estimated 50,000–70,000 plant species are used in traditional and modern medicine throughout the world. Global demand of the herbal medicine is high and increasing with time. More than 25% of the pharmaceutical drugs used globally are derived from plant natural products (Newman and Cragg 2016). Some of the medicinal

Table 6.1 Secondary metabolites with their source plant and medicinal properties

Secondary metabolites	Source	Properties
Ajmalicine	*Rauvolfia serpentine*	Antiarrhythmic, antihypertensive
Caffeine	*Coffea Arabica*	Stimulant
Camptothecin	*Camptotheca acuminate*	Antineoplastic
Cocaine	*Erythroxylum coca*	Analgesic, narcotic, local anesthetic
Codeine	*Papaver somniferum*	Antitussive, analgesic
Emetine	*Carapichea ipecacuanha*	Antiamoebic, emetic, expectorant
Hyoscyamine	*Atropa belladonna*	Anticholinergic
Morphine	*Papaver somniferum*	Analgesic, narcotic
Nicotine	*Nicotiana tabacum*	Stimulant
Pilocarpine	*Pilocarpus jaborandi*	Cholinergic
Quinidine	*Cinchona* spp.	Antiarrhythmic
Quinine	*Cinchona* spp.	Antimalarial
Reserpine	*Rauwolfia serpentine*	Tranquilizer
Scopolamine	*Hyoscyamus niger*	Sedative, anticholinergic
Strychnine	*Strychnos nux-vomica*	Stimulant, poison
Taxol	*Taxus brevifolia*	Antineoplastic
Vinblastine and vincristine	*Catharanthus roseus*	Antineoplastic

secondary metabolites with their source and medicinal properties are listed in Table 6.1.

6.2 Enhancement of Secondary Metabolites in Medicinal Plants

The production of the secondary metabolites is influenced by several factors such as genotype of the plant, environmental conditions, and other biotic as well as abiotic stresses (Wink 2018). As many of these compounds have medicinal and therapeutic properties, enhancing the production of secondary metabolites in medicinal plants is important that can be achieved through various methods, including:

1. Breeding: Traditional plant breeding techniques can be used to select and propagate plants with desirable traits such as increased production of specific secondary metabolites. These methods may involve hybridization of different varieties/species or selection of plants with desirable traits from a population.
2. Biotechnology: Genetic engineering can be used to modify the genetic makeup of plants to enhance production of specific secondary metabolites, introducing genes from other plants or organisms that are known for their involvement in the biosynthesis of the desired compound.
3. Stress induction: Certain stress conditions such as drought or nutrient deprivation may induce the production of secondary metabolites in plants. Hence, this

approach may be helpful in increasing the production of specific secondary metabolites.
4. Plant growth regulators: Plant growth regulators such as hormones and elicitors may be used to enhance the production of secondary metabolites in plants. These substances may stimulate the production of secondary metabolites by activating specific metabolic pathways.
5. Environmental factors: Environmental factors such as light and temperature affect the production of secondary metabolites in plants. By optimizing these factors, it may be possible to enhance the production of specific secondary metabolites.
6. Elicitors: Elicitors are substances that can stimulate the production of secondary metabolites in plants. These can be natural compounds such as plant hormones or synthetic compounds such as methyl jasmonate. These elicitors can be applied to plants either by spraying or by root treatment.

Out of these approaches, only plant breeding and biotechnological techniques are the methods, which involves manipulation of the genetic constitution/change in the genetic makeup of plants, which are heritable and maintained generation after generation (Kuiper et al. 2001; Narula et al. 2005). Other methods may enhance the production of secondary metabolites by providing favorable environmental condition for secondary metabolism. In this chapter, we will deal with genetic manipulation methods for enhancement of secondary metabolite concentration in medicinal plants.

6.3 Genetic Manipulation in Medicinal Plants

Genetic manipulation refers to the range of methods deliberately used to modify the organism's genetic makeup including transfer of genes within and across species/genera/kingdom boundaries to produce improved or novel organisms. Genetic manipulation in medicinal plants may be used to improve their medicinal properties or to increase their yield. The use of genetic manipulation in medicinal plants has been an area of intense research and development for several decades. One of the earliest examples of genetic manipulation in medicinal plants was the production of recombinant human insulin using genetically modified bacteria in the 1980s. Since then, advances in genetic engineering techniques have enabled scientists to manipulate the DNA of medicinal plants more precisely and efficiently (Tzfira and Citovsky 2016). Genetic manipulation has been used to enhance the production of specific medicinal compounds in plants, such as artemisinin in *Artemisia annua*, a plant used to treat malaria (Zhang et al. 2011). In 1999, a gene encoding a key enzyme involved in the biosynthesis of ginsenosides, bioactive compounds in ginseng, was introduced into *Panax ginseng*. In recent years, genome-editing techniques such as CRISPR/Cas9 have been developed, which allow precise modification of specific genes in the plant genome. This technique has been used to modify genes involved in the biosynthesis of secondary metabolites in several medicinal

plants, including *Hypericum perforatum*, *Catharanthus roseus*, and *Salvia miltiorrhiza* (Zárate and Verpoorte et al. 2007). There are many reasons to manipulate and alter the levels and profile of secondary metabolites in plants. Sometimes it is not possible to use the biochemical synthesis pathway of secondary metabolites at industrial level due to the complexity of the metabolic pathways and the complexity exhibited by these compounds (Giri and Narasu 2000). The production of these secondary metabolites is regulated by the transcriptional and posttranscriptional regulatory mechanism of the gene encoding for the enzyme responsible for the synthesis of the desired products. Naturally, the expression level of these genes or enzymes is kept very low under normal condition, and thus the gene active status can be triggered by several environmental or external factors called elicitors. Several approaches are being implied to enhance the levels of a biochemicals in a target plant (Yuan et al. 2020). Among these approaches, genetic manipulation/modification has emerged as promising tool and one of the important techniques to enhance the production of secondary metabolites in medicinal plants for its medicinal use for human welfare (Pandey et al. 2013; Pandey and Tripathy 2017; Ramawat and Arora 2021).

6.3.1 Strategies for Genetic Manipulation in Medicinal Plants

There are several strategies for genetic manipulation including traditional breeding, crossing, or hybridization, mutation breeding, and modern biotechnological techniques like genetic engineering and genome editing (Pauwels et al. 2017). Traditional methods involve crossing plants with desirable traits to produce offspring with improved traits. It has been used for centuries to improve the production of secondary metabolites in medicinal plants. However, these methods are time-consuming and can be limited by the natural genetic variation in the plant population. The advance biotechnological tools are included for the genetic engineering of target plant to promote the target-based insertion and modification of gene of interest into the genome of desired plants (Lusser et al. 2012). The production of efficient and reliable genetically modified plant needs tissue culture condition for mass propagation (Fazili et al. 2022). The identification of restriction enzyme, vector, and ligation as tools for recombinant DNA technology (RDT) paved the path for the amelioration of plant to produce biochemicals of high value for the pharma industry, food, and nutritional security through its immense potential in the field of plant biotechnology (Siahsar et al. 2011; Sharma et al. 2019). RDT has already proved itself for the providing food and nutritional security with nutritional quality in the field of crop improvement programs (Datta 2013; Qaim and Kouser 2013).

6.3.2 Methods of Genetic Manipulation

In the context of medicinal plants, genetic manipulation can be used to create plants with improved medicinal properties or to produce pharmaceutical compounds more efficiently. This can be done using various methods as described below.

6.3.2.1 Genetic Engineering

Genetic engineering has emerged as a powerful tool for the improvement of medicinal plants. This method involves the introduction of foreign genes into the plant genome to enhance the production of secondary metabolites. The genes encoding the enzymes involved in the biosynthesis of secondary metabolites are often isolated from other plant species or microorganisms and introduced into the plant genome using various transformation methods (Niazian 2019; Nielsen et al. 2019). The overexpression of these genes in the plant can result in the enhanced production of secondary metabolites. Genetic engineering involves targeted change in gene sequence using rDNA technology (Sinha et al. 2019). This targeted change in genetic constitution can be done by direct method of gene transfer like particle bombardment, electroporation, chloroplast transformation, and indirect method of gene transfer through microbial vector like agrobacterium-mediated gene transfer.

6.3.2.1.1 *Agrobacterium*-Mediated Gene Transfer

The most popular technique of transformation for introducing desired genes into agricultural plants is *Agrobacterium*-mediated gene transfer, either in differentiated plant cells or into undifferentiated callus cells. This technique makes use of *Agrobacterium*'s natural ability to infect and transfer genes into plant cells. Discovery and development of efficient regeneration and suitable *Agrobacterium*-mediated genetic transformation protocols laid the foundation for advancement in crop biotechnology (Chilton et al. 1977; De La Riva et al. 1998). *Bacopa monnieri* was genetically altered using the *Agrobacterium tumefaciens* strain EHA105 that carried the binary vector pBE2113 encoding the genes for glucuronidase (GUS) and neomycin phosphotransferase. *Agrobacterium tumefaciens* transformation system is well developed for *Taxus* (Han et al. 1994), *Echinacea* (Wang and To 2004), *Scrophularia* (Park et al. 2003), foxglove (Sales et al. 2003), *Thalictrum* (Samanani et al. 2002), and *Artemisia* (Chen et al. 2000). In transgenic *Artemisia annua* developed through agrobacterium transformation, analysis of artemisinin revealed that about 8–10 mg per liter distilled water of artemisinin was detected in transgenic plants regenerated from fine shoot lines, which was almost two to three times higher as compared to control. Therefore, regular genetic modification of many therapeutic plants was done through agrobacterium transformation. *Agrobacterium rhizogenes* have similar potential for gene transfer like *A. tumefaciens*, mainly in the roots of

Table 6.2 Genetic modification in the medicinal plants through *Agrobacterium*-mediated transformation methods

Plant species	Gene manipulated	Product of interest	References
Catharanthus roseus	Geraniol 10-hydroxylase gene	Monoterpenoid indole alkaloid	Pan et al. (2012)
Panax ginseng	PgSS1 gene	Triterpene	Seo et al. (2005)
Panax notoginseng	3-hydroxy-3-methylglutaryl CoA reductase (PnHMGR) and squalene synthase (PnSS)	Triterpene saponins	Ding et al. 2015)

medicinal plants. A. *rhizogenes* cause hairy root disease in dicot plants (Chilton et al. 1982; Giri and Narasu 2000; Bourgaud et al. 2001). A similar approach to the *A. tumefaciens* was performed to infect the root of higher plant using *A. rhizogenes* in order to produce secondary metabolites (Ackermann 1977). Transgenic hairy root cultures and plantlets have been developed using *A. rhizogenes*-mediated transformation. An example of *A. rhizogenes* transformation in medicinal plants is the introduction of 6-hydroxylase gene from *Hyoscyamus muticus* to hyoscyamine-rich *Atropa belladonna*. *A. rhizogenes*-engineered roots showed increased enzyme activity and five times higher concentration of scopolamine (Table 6.2).

6.3.2.1.2 Particle Bombardment

Klein et al. (1987) discovered that naked DNA could be transferred to the cells by blasting plant cells with small pellets (diameter: 1–4 μm) to which DNA was adhered. This is an effective physical method of DNA delivery in species where *Agrobacterium* does not naturally transform (Dai et al. 2001). A variety of plant components, including callus, cell suspension culture, and organized tissues like embryos and meristems, have been employed as transformation targets. Cell suspension culture is created from gentian (*Gentiana triflora* * *G. scabra*) leaf explants for the purpose of creating transgenic plants by particle bombardment (Leo et al. 2000). The transfer, expression, and stable integration of a DNA fragment into chromosomal DNA can be achieved by an effective and stable transformation as achieved in garlic plants (Sawahel 2002). This technique should definitely be used in the future to create genetically modified creatures due to its relative simplicity.

6.3.2.1.3 Electroporation

In electroporation process, an electrical impulse helps plant protoplast to absorb macromolecules from their surrounding fluid. Protoplast is the product of the removal of the protective cell walls from cells developing in a culture medium. Known DNA is added to the protoplast culture medium, which enters the cell. After

that, transformed cells sprout new cell walls and develop into whole, fertile transgenic plants. The development of organ from the protoplast of woody medicinal plant is tricky. It is accelerated by exposing them to the three sequential pulses of voltage of 250–1250 V per centimeter for 10–50 μs (Chand et al. 1988). Compared to tissues from protoplasts that were not treated, these tissues showed greater morphogenesis in a shorter time in culture. Additionally, regenerated shoots had more robust root systems than untreated protoplast shoots.

6.3.2.1.4 Chloroplast Transformation

The aim of high production of valuable compound under containment with high stability can be achieved by the chloroplast transformation. It was first used for transferring the foreign genes into the chloroplast genome of single cell green algae *Chlamydomonas reinhardtii* in 1988 (Boynton et al. 1988) and then quickly followed for the production of various compounds of medicinal value in the tobacco plant and sometimes in *Arabidopsis thaliana*. Around 40 genes for the vaccine, antigen, antibody, and pharmaceutical compound have been transformed in chloroplast genome with stable integration and expression (Daniell et al. 2005). In medicinal plants, chloroplast transformation has the potential to improve the production of bioactive compounds and to develop plant-based systems for the production of pharmaceuticals. For instance, chloroplast transformation has been used to increase the yield of artemisinin, a key antimalarial drug in *Artemisia annua.* Similarly, the production of ginsenosides, the active compounds in ginseng, has been improved through chloroplast transformation in *Panax ginseng*. Chloroplast transformation has also been used to produce vaccines and other therapeutic proteins in plants including medicinal plants. For instance, chloroplast transformation has been used to produce a malaria vaccine in tobacco plants and to produce human growth hormone in lettuce.

This technique has several advantages over nuclear transformation, including high levels of transgene expression, ability to express multiple genes from a single transformation event, and the absence of gene silencing.

6.3.2.2 Genome Editing

Conventional genetic engineering has few shortfalls such as complex insertion, public acceptance, and various apprehensions associated with the reporter genes (Naqvi et al. 2010). However, conventional approach of genetic transformation is still demanding to insert the construct of genome editing tools. Genome editing tools provide the target insertion based on customized construct against the genomic region. Target-based insertion provides the opportunity for PCR-based transgenic detection in contrary to the conventional marker-based detection (Liu et al. 2013).

Genome editing involves the precise modification of specific genes in the plant genome using techniques such as zinc finger nucleases (ZFNs), transcription

activator-like effector nucleases (TALENs), clustered regularly interspaced short palindromic repeats, and CRISPR-associated protein (CRISPR/Cas9). This technique can be used to delete, insert, or modify specific genes involved in the biosynthesis of secondary metabolites. Genome editing has the advantage of precise modification of the plant genome, which reduces the pleiotropic effect of target gene modification and insertion. ZFNs, TALENs, and CRISPR/Cas are specific nucleases that can cleave the double strands of DNA using customized construct against the target. These are fusion proteins made up of a nonspecific DNA-cleavage domain with a programmable and sequence-specific DNA-binding domain (Gaj et al. 2013). The DNA-cleavage domain is derived from the FokI restriction enzyme in both ZFNs and TALENs. ZFNs is a member of zinc finger protein family having DNA binding motifs, whereas TALENs has *a* TALE protein as a DNA binding motif. For the CRISR/Cas9 system, a 20-nucleotide-long sequence guide RNA (sgRNA) is the DNA binding motif, while Cas9 nucleases function for the target cleavage. All three CRISPR/Cas, TALEN, and ZFN DNA-binding domains are programmable and have customizable DNA-binding domains that can recognize and attach to any sequence of interest based on interactions (Gaj et al. 2013). The 20-nucleotide structure of sgRNA recognizes the target based on sequence complementarity and induces cleavage at protospacer adjacent motif (PAM) sequence of the target region; as a result, gene mutation, deletion, insertion, as well as transcriptional activation and repression may induce (Xu et al. 2014). The type II CRISPR system (CRISPR/Cas9) is the most widely used gene editing and transformation techniques for the metabolic engineering of medicinal plants. It can target multiple genes of same metabolic pathway using customized sgRNA against the various target genes. Rosmarinic acid synthase gene (SmRAS) in *Salvia miltiorrhiza* has been suppressed using the CRISPR/Cas9 system (Zhou et al. 2018). The expression of CRISPR/Cas system is mainly driven with U3 promoters of rice origin and *Arabidopsis*-derived U6 promoter. The HPLC-MS/MS study revealed that the amount of salvianic acid A sodium (SAAS) and sodium salt form of DHPL is raised in the hairy root extract of CRISPR/Cas9-edited *Dioscorea zingiberensis* mutants (Feng et al. 2018). The biotechnological approaches provide the best and sustainable approaches for the enhancement of diosgenin production from Dioscorea sp (Nazir et al. 2021). The SmCPS1 gene, a negative regulator of tanshinone production, has been knocked out using the CRISPR/Cas9 system in the root of *Salvia miltiorrhiza* utilizing *Agrobacterium rhizogenes* to enhance the accumulation of tanshinones (Li et al. 2017) (Table 6.3). These findings highlight the importance of the CRISPR/Cas9 system for the targeted genetic manipulation in medicinal plants. The development of minimal plant cell, as a bioreactor for the production of target secondary metabolites, can be achieved through CRISPR/Cas9 (Noman et al. 2016).

6.3.2.3 RNA Interference (RNAi)

The RNAi strategy is one of the alternative methods of gene modification and allows enhanced accumulation of the positively regulated enzyme for the secondary

Table 6.3 Genetic modification in the medicinal plant through CRISPR/Cas mediated gene editing

Plant species	Gene manipulated	Product of interest	References
Nicotiana benthamiana	Glycosyltransferase gene	Pharmaceutical protein	Jansing et al. (2019)
Papaver somniferum	(R, S)-reticuline 7-O-methyltransferase (7OMT) and 3′-hydroxy-N-methylcoclaurine 4′-O-methyltransferase (4′ OMT2)	Benzylisoquinoline alkaloid	Alagoz et al. (2016)
Salvia miltiorrhiza	Knock out the committed diterpene synthase gene (SmCPS1)	Tanshinone	Li et al. (2017)
Salvia miltiorrhiza	Rosmarinic acid synthase gene (SmRAS)	Phenolic acid	Zhou et al. (2018)

metabolite production. RNAi involves the use of small RNA molecules, called short-interfering RNAs (siRNAs), designed against the target gene. Binding of small RNA to the target gene induces the degradation of target RNA using transcriptional and posttranscriptional gene silencing approaches (Jain and Khurana 2009). This method has proven to be a breakthrough in the study of molecular biology and has prompted the creation of RNAi-focused biotechnology firms. Gene silencing is straightforward and effective for conducting extensive functional analyses in a variety of species (Agrawal et al. 2003; Eamens et al. 2008). RNA interference is catalyzed by a number of enzymes: Drosha, DICER, and RNA-induced silencing complex (RISC). Reaction initiated with the formation of long dsRNA and hairpin molecules like structure between transcript and antisense RNA. The long double-stranded RNAs are recognized by DROSHA and DICER that cleave them into small RNA of size 21–25 nucleotides called small siRNAs (small interfering RNA) and miRNAs (microRNAs). SiRNA and miRNA bind with the multimeric protein with nuclease complex called RISC (RNA-induced silencing complex) (Wilson and Doudna 2013). The antisense strand of siRNA/miRNA is integrated into RISC complex that is termed as guide RNA and the target complementary RNA strand called as passenger RNA. SiRNA and miRNA bind with RISC complex recognized the target based on base complementarity and cleave them with the utilization of ATP. The catalytic component of RISC complex is present in the Argonaute (AGO) protein that helps to cleave the target transcript (Leuschner et al. 2006). The 5′ end of guide RNA acts as driver for the recognition and cleavage of passenger RNA. Although RNA interference is a potent tool in gene silencing, still it is not being exploited much for manipulation of plant secondary metabolite production (Ossowski et al. 2008). This technique has been used to produce secondary metabolites in plants such as *Papaver somniferum*, *P. ginseng*, *A. annua*, and *W. somnifera* that are useful in the pharmaceutical industry (Hussain et al. 2012). Similarly, reticuline a non-narcotic alkaloid found in opium poppies and more powerful medicine than morphine, has been produced by researchers through suppression of codeinone reductase (COR), a critical enzyme in the biosynthesis of morphine (Wijekoon and Facchini 2012) (Table 6.4).

Table 6.4 List of medicinal plants transformed through RNAi method

Plant species	Gene manipulated	Product of interest	Reference
Artemisia annua L.	Squalene synthase	Artemisinin	Zhang et al. (2009)
Catharanthus roseus	Tryptophan decarboxylase	Monoterpene indole alkaloids	Runguphan and O'Connor (2009)
Coffea canephora	CaMXMT1	Caffeine	Ogita et al. (2003)
Nicotiana tabacum	Cytochrome P450, CYP82E2 family	Conversion of nicotine to nornicotine	Siminszky et al. (2005)
Panax ginseng	Squalene epoxidase gene	Ginsenoside and phytosterol	Han et al. (2009)
Panax ginseng	Protopanaxadiol 6-hydroxylase gene	Triterpenoid saponins	Park et al. (2016)
Papaver somniferum	Salutaridinol 7-O-acetyltransferase (SalAT	Alkaloid salutaridine	Allen et al. (2008)
Papaver somniferum	COR 1, COR2	Non-narcotic alkaloid reticuline	Allen et al. (2004)
Withania somnifera	Cycloartenol synthase (CAS)	Withanolides	Mishra et al. (2016)

6.3.2.4 Ploidy Engineering

Ploidy engineering is the manipulation of the number of chromosomes in a cell or organism, and it has been used in medicinal plants to improve the quantity and quality of secondary metabolites form the target plant species having high vigor and wider adaptability. The multiplication of the genomic component of any organism is achieved for adding their significant effect over the quantity and quality of types of products. The high vigor and quality performance are the specialties of polyploids compared to their diploid forms and species (Noori et al. 2017). A number of studies reported that the secondary metabolite accumulation is enhanced in polyploid genotype of a medicinal plant than their diploids (Pradhan et al. 2018). Thus, the artificial induction of polyploids in various medicinal plants is being implied for improvement of breeding performance and adding quality to their chemical composition such that overall quantitative and qualitative performance can be enhanced (Salma et al. 2017). Induced polyploidy in Brazilian ginseng (*Pfaffia glomerata*) increased the level of 20-hydroxyecdysone by 31% as compared to diploid plants. The polyploidization is induced with the application of antimitotic agents. The colchicine is the most frequently used antimitotic agents for the induction of artificial polyploids compared to trifluralin and oryzalin (Salma et al. 2017). However, the types of antimitotic agent, their concentration, and exposure period need to be standardized as per the species to get the polyploidization benefits for secondary metabolite productions. Colchicine is the most applied antimitotic agent for the medicinal plants. Different colchicine concentrations, namely, 0.05, 0.1, and 0.2% (w/v), are being used for the induction of tetraploid in *Bletilla striata* for different exposure times, viz., 12, 24, 36, 48, and 60 h. The application of 0.2% colchicine for

36 h is the most effective treatment for the autotetraploidy induction in *Bletilla striata* (Pan-pan et al. 2018). Similarly, haploids are another aspect of polyploidy engineering in medicinal plants for enhancement of secondary metabolites (Germanà 2011). The production of haploids in various plants is being implied through tissue culture-based techniques such as gynogenesis (Piosik et al. 2016), androgenesis (Kasha 2005), and extensive hybridization-chromosome aberration (Forster et al. 2007). The most common technique for haploid induction in many medicinal plants is through the androgenic route, which includes anther and isolated microspore culture (Sharma et al. 2018). The first isolated microspore culture was performed for the medicinal plants of Asteraceae family in 2006. However, due to small capitula of the targeted plants, there were a number of difficulties for haploid production (Bal and Touraev 2009). The induction of haploids may be achieved through the various types of culture media, antimitotic chemicals (colchicine and n-butanol), and external environment or imposed physical factors (centrifugation and electroporation), which were tested in a borage (*Borago officinalis* L.) anther culture. The highest percentage of doubled haploid plants (65%) may be achieved in borage (*Borago officinalis* L.) using the pretreatment of anthers with 0.2% n-butanol for 5 h and B5 salt medium supplemented with 200 mg/L colchicine for 4 days (Hoveida et al. 2017). The haploid production in black cumin (*Nigella sativa*) may be achieved through treatment with various concentrations of plant hormones, BA, kinetin, NAA, and 2, 4-D PGRs, to ovule culture in MS medium (El-Mahrouk et al. 2018). Another technique for inducing haploid cells is CENH3, which is not dependent on tissue culture and is applicable to any desired plant species. This technique results in haploid inducer lines by knocking off or downregulating the native CENH3 gene, which connects chromosomal centromere regions to spindle microtubules (Ravi and Chan 2010; Britt and Kuppu 2016). However, it is outside the purview of the current study.

The inclination of public interest towards natural drug has been rejuvenated because the medicines developed with chemical formulations are often associated with side effect and various health consequences in the long term. Natural drug is high in demand by the pharmaceutical industry that urges them in pressure to discover and identify novel secondary metabolites and make them available as an effective medicine with potentiality to treat various diseases. The onset of advanced genetic engineering tools and techniques provides the avenue to understand the traditional and natural drug with the new perspectives. The various applications of secondary metabolites of plants draw the attention of researchers for the investigation of their potential role as agrochemicals, medicinal value, and nutritive value. The conventional method of genetic manipulation of medicinal plant using breeding approaches often compromised with the availability of donor, linkage drag, time-consuming characterization, and enhancement of secondary metabolite production. However, the biotechnological interventions have now emerged as king pin techniques with the amelioration power of immense capabilities. One such technique is the genome editing construct (CRISPR/Cas9, TALLENs, ZFNs)-based technology that can edit and modify specific region of the target genome of medicinal plants. CRISPR/Cas9-based genome editing techniques are the most powerful methodology

to achieve the target-specific insertion and modification of genome (Feng et al. 2018; Li et al. 2017; Zhou et al. 2018). The artificial polyploidy induction and haploid generation are also techniques for achieving high yield of secondary metabolites in few species.

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Chapter 7
Elicitors: Role in Secondary Metabolite Production in Medicinal Plants

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Abstract Elicitors are the stimuli from the living, nonliving, and environmental sources that expedite the synthesis of secondary metabolites in the plants. Secondary metabolites are the byproduct and end-product of various signal transduction pathway that inculcate the response of signal by biotic and abiotic stimuli. Secondary metabolites are generated in response to the various elicitors having key role in stress tolerance mechanism for the plants. It plays a role in defense mechanism against biotic factor and enhances tolerance to the abiotic stresses. The medicinal property of secondary metabolites makes it eye catching for the researchers. The yield of secondary metabolites is often limited in plant; thus, role of various elicitors for secondary metabolites is important to study. Elicitors are key regulators for few secondary metabolites and enhance the yield, but doses and duration of exposure to the elicitor need to be standardized.

Keywords Elicitors · Secondary metabolites · Medicinal plant · Elicitation · Biotic elicitor · Abiotic elicitor

7.1 Introduction

The backbone of traditional medicines is the medicinal plants. It contains the rich source of essential elements required for medicine preparation or blending. Approximately 8000 species have been identified as medicinal plants by rural as well as by tribal communities of India. The medicinal plants are mainly seen in core of the ecosystem, which are being used in Ayurveda. Natural extracts from the medicinal plants are being used since ancient time for human health benefits. The use of herbal

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remedies has accentuated the globe in recent years (Mosihuzzaman 2012). A growing population results in large impact over the effectiveness and safety of herbal medications as a result of this rising demand. Although there is a never-ending potential for therapeutic plants, there are some significant barriers that prevent widespread adoption by the modern medicine system. Low rate of reproducibility (up to 40%) is the major point of consideration with respect to the doses that exhibit by many plant extracts during testing and that piqued the interest in development of plant-based medicines (Cordell 2000). Plant-based medicines are mainly a secondary metabolite from plants that make the plant self-reliant against a number of external biotic and abiotic factors. According to Poulev et al. (2003), genetic polymorphism among different genotypes of plant can be a significant source of various qualitative and quantitative secondary metabolites generated by plants. Secondary metabolites are the organic compound of low molecular weight produced as byproduct or/and end-product of primary metabolites that make plant self-reliant in the competitive environment (Wang and Wu 2013). There are four major categories of plant secondary metabolites based on their chemical properties: phenolics, alkaloids, terpenoids, and sulfur-containing compounds. Different types of secondary metabolites are being used in pharmaceutical industries for various purposes: pesticides, weedicides, agrochemicals, food additives, food flavoring agents, food coloring agents, essential oils, cosmetics, and medicine. Various metabolites of plant origins, namely, quinine, vincristine, papaverine, ephedrine, and caffeine, are in high value compounds, and thus their demand in the global market is high (Verpoorte et al. 2002). The demand of secondary metabolites expedites the role of scientist toward medicinal plants.

The current production of phytochemicals is insufficient to meet the global demand based on their natural rate of production from the intact plant system. Sometimes, use of the whole plant for the extraction of secondary metabolites leads to the shortfall of future source plant for their production. Biotechnological techniques such as plant tissue culture techniques can be utilized to counteract the issue related to the environmental and seasonal availability of source plant and accumulation of level of secondary metabolites in the plant (Yukimune et al. 1996; Karuppusamy 2009). Various plant tissue culture techniques are being used to improvised the accumulation of plant-based chemicals: single-cell culture, cell suspension culture, culture of root and shoot, and callus cultures (Biswas et al. 2016, 2018; Awad et al. 2014; Buitelaar et al. 1992; Murthy et al. 2014). However, the production of some valuable secondary metabolites such as morphine, vinblastine, taxol, capsaicin, vincristine, and berberine using tissue culture is limited (Vanisree et al. 2004; Varma 2010). These barriers are being tackled by various biotechnological strategies to engineer the metabolic pathway, such as biotransformation methods and use of plant cell system as a bioreactor with several media compositions (Naik and Al-Khayri 2016).

7.2 Factors Affecting Secondary Metabolite Productions

The secondary metabolites (SMs) and/or phytochemicals are involved in the development of drugs that are being used by the pharmaceutical industry to act against various diseases (Kliebenstein 2013). Plants generate a wide range of these organic substances to help them engage with their biotic and abiotic environments and develop defense mechanisms (Wang and Wu 2013). More than 50,000 secondary metabolites are known in plant kingdom (Teoh 2015). The interaction between the plant and its surroundings can stimulate the accumulations of the phytochemicals. Normally, the yield of secondary metabolites from the plants is less than one percentage dry weight. The accumulations of secondary metabolites depend on various endogenous and exogenous factors to the plant (Dixon 2001; Oksman-Caldentey and Inzé 2004). The synthesis of secondary metabolites is highly regulated by the elicitors. Elicitor is the stimulus that initiates the secondary metabolite production in the plant. Sometimes, growth and developmental stage is also regulating the production of SMs.

7.3 Growth and Development Factors

The synthesis, distribution, and accumulation of secondary metabolites classify the medicinal plant in to four medicinal parts: (1) roots and shoots, (2) leaves, (3) flowers, and (4) fruits and seeds (Belkheir et al. 2016). The synthesis and accumulation of different secondary metabolites may occur through unique regulatory pathways and transport routes in the specific cell, tissues, and organs. Therefore, understanding the biosynthesis and accumulation of secondary metabolites is important. However, the accumulation of SMs in the organs and reproductive parts of the plant is regulated with the developmental growth stage, season, and circadian rhythm.

7.3.1 Root and Stem

The root and stem are the primary organs of plants that accumulate most of secondary metabolites with significant medicinal properties. The production of SMs in root and stem of herbaceous species is primarily influenced by the developmental stage, planting seasons, and age of plants. The roots of *Echinacea purpurea* produce more cichoric acid during the fruiting stage (Xu et al. 2014). Similarly, *Scutellaria baicalensis* accumulate more flavonoids in the roots before full-bloom stage (Xu et al. 2018).

7.3.2 Leaf

Leaves are major sites for the photosynthesis and photochemical reaction, and thus, it is the major site to produce the number of byproduct that may be involved in secondary metabolite (SM) synthesis and storage in the plants. The SM content in the leaves of medicinal plant is influenced by the age (Vázquez-León et al. 2017), developmental stage (Li et al. 2016), and harvesting season (Gomes et al. 2019) of the leaves. The synthesis of certain monoterpenes and sesquiterpenoids occurs at early stage of first cotyledons in *Melaleuca alternifolia* (Southwell and Russell 2002). Similarly, the accumulation of essential oil in *Cinnamomum verum* is observed in young leave stage of less than 1 year (Li et al. 2016).

7.3.3 Flower

The aroma in most flowering plants is mainly made up of terpenes and aromatic compounds, which are regulated by developmental stages, biological clock, and environmental factors (Figueiredo et al. 2008). The accumulation of volatile oil in the flower buds of *Magnolia zenii* is high during early bud stages, and highest accumulations are observed in October (Hu et al. 2015).

7.3.4 Fruits and Seed

The developmental phase of fruits and seeds of various plant species has a prominent role on the composition and content of medicinal components. The secretory cavity development of citrus fruits affects the content of volatile oils, which is the main active ingredient (Liang et al. 2006). Typically, the highest volatile oil content can be found when the fruit turns light yellow, which can serve as a morphological indicator for harvesting. Similarly, a significant increase in essential oil, α-thujone, β-pinene, carene, and γ-terpinene during the mature phase of *Citrus medica* L. var. *sarcodactylis* can be observed (Wu et al. 2013). The capsules of *Papaver somniferum* L. are the opulent for morphine, codeine, and thebaine, but the maximum morphine content is estimated at maturity (Shukla and Singh 2001).

7.4 Elicitors and Its Classification

Elicitors are the stimuli of living and nonliving origin that enhance the biochemical reaction to stimulate the biosynthesis of the targeted bioactive compound (Cabrera-De la Fuente et al. 2018). The production of bioactive compound, organic

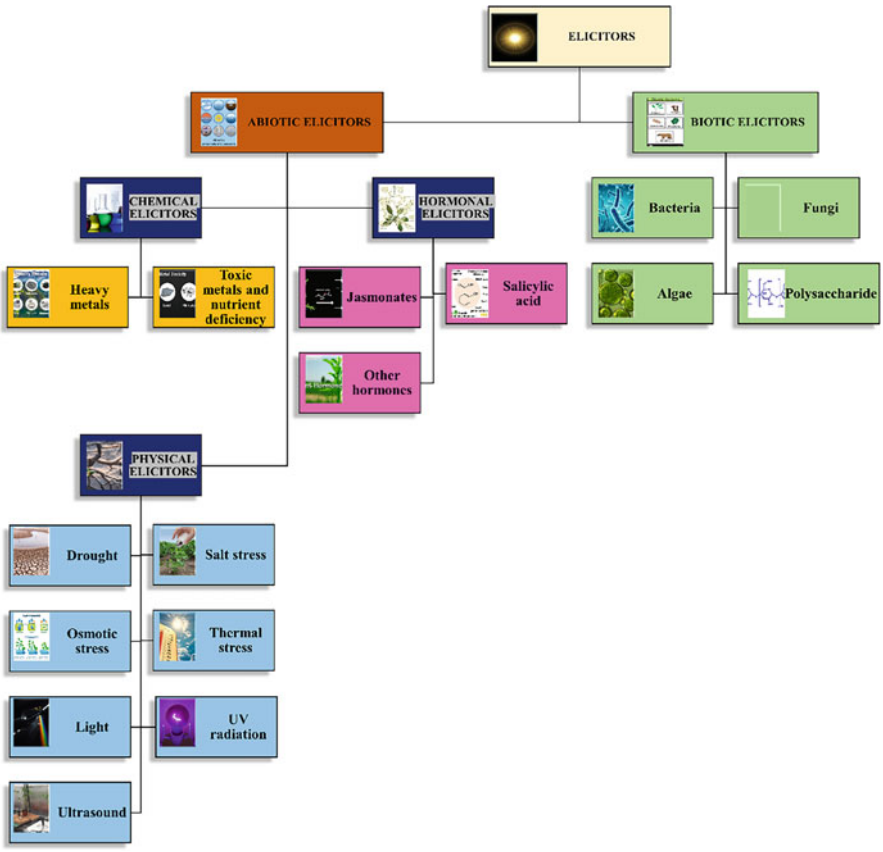

Fig. 7.1 Classification of elicitors

substances, and metabolites using various types of elicitors is known as elicitation. A number of biotechnological tools and techniques are being proposed and applied for enhancement of the metabolite production. The method of elicitation is the most widely used techniques for enhancing SMs from cells, organs, and plants (Namdeo 2007). Based on the source of origin, elicitors can be categorized into two groups: (1) abiotic elicitors and (2) biotic elicitors (Thakur et al. 2019). The biotic elicitors comprise the substances of biological origin. Biotic elicitors of plant origin may be regarded as endogenous and pathogen origin as exogenous elicitors. Polysaccharides from plant, fungi, and bacterial origin are the major example of biotic elicitors. On the contrary, the environmental factors that are nonbiological origin are abiotic elicitors that further grouped into physical, chemical, and hormonal origins (Fig. 7.1).

7.4.1 Biotic Elicitors

The term "biotic elicitors" refers to compounds with biological origins. They can be further subdivided as exogenous and endogenous elicitors. Endogenous elicitors are plant-derived component such as polysaccharides, while exogenous elicitors are pathogen/microbe-derived elicitors such as chitin, lignin, pectin, cellulose, etc. Cell extraction of yeast, bacteria, and fungus is included in microorganism-based elicitors (Namdeo 2007). However, all elicitors initially interact with the receptors that may be present on the plasma membrane or inside the cell. The binding of elicitors to the receptors initiates a cascade of different pathways that lead to biochemical and physiological functions, producing a range of secondary metabolites of medicinal importance (Ferrari 2010; Shasmita et al. 2018). The signal transduction pathway for SM production is initiated with the attachment of elicitors to the receptor and is the first step in the process. The binding of elicitors to receptor is perceived by the alteration ionic balance across the cell membrane that intricates the production of signaling molecules such as ions, potassium ions (K^+), calcium ions (Ca^{2+}), chloride ions (Cl^-), hormones, ABA, ethylene, etc. and amplifies the response of elicitors (Jabs et al. 1997; Shabala and Pottosin 2014). The elicitors of biotic and abiotic sources activate the calcium channels immediately within a minute and cause change in the flux of ion across membranes that in turn change in pH and depolarization of the cytoplasmic membrane (Mathieu et al. 1996; Sakano 2001; Zhao et al. 2005). Some elicitors cause enhancement pH in the apoplast region, which results in a proton flux, and some elicitors cause apoplast to become acidic, which results in a proton efflux of the vacuole (Bolwell et al. 2002; Angelova et al. 2006), therefore increasing phospholipase activity and protein phosphorylation in the plant (Zhao 2015). As a result, secondary messengers like inositol triphosphate (InsP3), diacylglycerol (DAG), and Ca^{2+} are produced that activate a number of kinases to activate the signal transduction pathway components (Gillaspy 2011; Aldon et al. 2018). One such pathway activates the mitogenic-activated protein kinase that translocated to the nucleus via nuclear pore and activates a specific transcriptional factor by phosphorylation (Pitzschke et al. 2009; Colcombet et al. 2016). Signal of some elicitor pathway is also perceived by membrane protein NADPH oxidase, which produces active oxygen species (AOS) that G protein-coupled receptor (GPCR) interaction is activated (Mishra et al. 2012). This acidifies the cytoplasm and activates the glycoproteins, chitinases, hydroxyproline-rich glycoproteins, glucanases, and protease inhibitors which in turn activates transcriptional factors that cause the expression of jasmonates, ethylene, salicylates, and other defense-related genes produced by the plant as secondary metabolites called phytoalexins as a defense reaction (Zhao et al. 2005; Angelova et al. 2006) (Fig. 7.2).

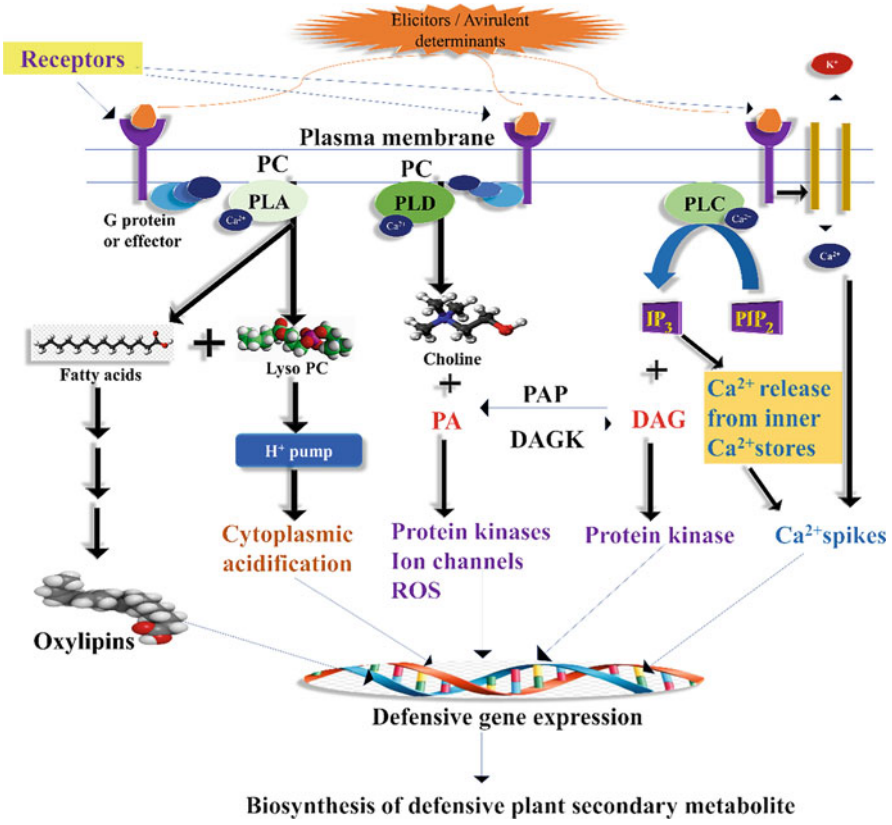

Fig. 7.2 Elicitor response pathway generated

7.4.1.1 Bacterial Elicitation

Single-celled microbes called bacteria are omnipresent in nature (Dzhavakhiya and Shcherbakova 2007). Bacterial elicitation is the process of inducing a reaction in plants using their surface tentacles, cellular contents, and bacterial cell formulations. Different SMs can be accumulated by different bacterial cellular preparations. Different types of bacterial components are used for the induction of SMs, including live cells (Park et al. 2006; Awad et al. 2014), cellular extracts (Gandi et al. 2012; Chodisetti et al. 2013), cell homogenates (Buitelaar et al. 1992; Jung et al. 2003), and culture filtrates (Biswas et al. 2016). The membrane receptor binds with the elicitor to induce G protein-coupled signaling that in turns the cytosolic acidification and the production of reactive oxygen species (Zhao et al. 2005; Biswas et al. 2016).

Bacterial cultures of *Bacillus*, *Agrobacterium*, and *Rhizobium* are used to produce glycyrrhizic acid from the root cultures of *Taverniera cuneifolia* (Roth) (Awad et al. 2014). The formation of isoflavone (daidzein and genistein) in Albizia kalkora is induced by the bacterial colonies of coculture strain of *Rhizobium radiobacter* and

Rhizobium rhizogenes (Park et al. 2006). The coculture bacterial colonies of *B. cereus* and *D. metel* induce the production of Tanshinone (Wu et al. 2007). The production of rosmarinic acid in the shoot culture of *Rosmarinus officinalis* L. is enhanced by *Pseudomonas* sp. (Yang et al. 1997).

In order to elicit the production of secondary metabolites, some plants need formulation of different concentrations of various bacterial strains applied with media formulations called bacterial cell formulations. The culture filtrates of *Bacillus circulans* and *Pseudomonas monteilii* are used for ginsenoside synthesis from *Panax quinquefolius* L. cell suspension culture (Biswas et al. 2016). Similarly, the cell suspension cultures of *Panax sikkimensis* and *Serratia marcescens* are elicited with the 2.5% *B. subtilis* to enhance ginsenoside synthesis (Biswas et al. 2018). The production of ginsenosides in root cultures of *Panax ginseng* is elicited by various gram-negative bacteria such as *Bradyrhizobium ganzhouense*, *Mesorhizobium huakuii*, *Mesorhizobium amorphae*, and *Azotobacter beijerinckii* and gram-positive bacteria such as *Leuconostoc* sp., *Lactobacillus plantarum*, and *Bacillus* sp. However, the gram-negative bacteria is the better elicitor than gram-positive bacteria (Le et al. 2018). However, the diosgenin synthesis in *Helicteres isora* L. cell suspension culture is elicited with B. subtilis and E. coli culture filtrates (Shaikh et al. 2020). The anthocyanin production in *Daucus carota* L is elicited with the culture filtrate of *S. aureus*, *B. cereus*, *E. coli*, and *P. aeruginosa*. The elicitor is playing a significant role in the synthesis of hypericin through shoot culture of the *Hypericum perforatum* L. and *Stenotrophomonas maltophilia* (Mañero et al. 2012). The enhancement of gymnemic acid content in *Gymnema sylvestre* is achieved by *A. rhizogenes* under tissue cultures (Chodisetti et al. 2013). *E. coli* is the most efficient elicitor for the andrographolide production from *Andrographis paniculata* cell suspension culture (Gandi et al. 2012). The alkaloid production is enhanced by the cellular extracts of *Pseudomonas* sp. and *Enterobacter species* in *Pinellia ternata* (Liu et al. 2010b). Similarly, the cellular extract of *Cronobacter sakazakii* is used to induce the accumulation of antioxidants and phenolic substances in medicinal plant cells of *Dionaea muscipula* (Makowski et al. 2020). The callus culture of *R. officinalis* accumulates caffeic acid, rosmanol, rosmarinus acid, and carnosic acid using the cellular lysate of *P. aeruginosa* as an elicitor (Rashid et al. 2011). *Enterobacter sakazakii* cellular extract increased scopoletin in cell suspension culture, while umbelliferone and bergapten production is enhanced in the hairy root culture of *Ammi majus* (Staniszewska et al. 2003). The synthesis of xanthone was significantly enhanced by the cellular extracts of *A. tumefaciens* and *A. rhizogenes*, by treating the cell suspension cultures of *H. perforatum* L. (Tusevski et al. 2013). The production of acteoside, baicalin, wogonin, scutellarin, and wogonoside increased in *Scutellaria lateriflora* L., by *A. rhizogenes*, *Pectobacterium carotovorum*, *E. sakazakii*, *Klebsiella pneumonia* (Wilczańska-Barska et al. 2012).

7.4.1.2 Fungal Elicitation

Fungal elicitors are the most significant and frequently used biotic elicitors for the production of various commercial SMs. The interactions between the fungus and the plant induce hypersensitive reactions that trigger the plant's defense mechanisms, resulting in an increase in phytoalexins (Baldi et al. 2009) and more effectively induced SM synthesis (Zhai et al. 2017). Usually, hyphal tips are used to create pure fungal colonies (Salehi et al. 2019).

Aqueous extracts of *Aspergillus favus*, *Aspergillus niger*, *Penicillium notatum*, and *Fusarium oxysporum* were used to stimulate the production of anthocyanins in *D. carota* L. Mycelial extracts of *A. favus* are one of the best elicitors for the anthocyanin production in *D. carota* L. (Rajendran et al. 1994), thiophene in *T. patula* L. (Buitelaar et al. 1992), menthol in *Mentha piperita* L. (Chakraborty and Chattopadhyay 2008), and gymnemic acid in *Gymnema sylvestre* (Devi 2011). The *A. niger* and *Rhizopus stolonifera* stimulate the production of glycyrrhizin from *Abrus precatorius* L. (Karwasara et al. 2011). It is observed that biotic elicitor is more efficient in secondary metabolite production than abiotic elicitors. Dye is a secondary metabolite produced from the essential shrub *Oldenlandia umbellata* L. which is enhanced with the biotic elicitors: *Mucor prayagensis*, *A. niger*, and *Trichoderma viride* (Saranya 2019). *Trichoderma atroviride* and *T. harzianum* culture filtrates are used to produce the most ginsenoside and anthocyanin in *P. sikkimensis* cell suspension cultures (Biswas et al. 2018). The production of SMs by few fungal elicitors may inhibits the plant cell biomass and their growth such as inhibitory action of *F. oxysporum* on shoot growth of *Centella asiatica* L. (Prasad et al. 2013). Similarly, the extracts of *Phoma exigua*, *F. oxysporum*, and *Botrytis cinerea* increase the phenylpropanoid and napthodianthrone, while biomass of *H. perforatum* L. cell culture is decreased. Sometimes, the production of SMs is increased initially and then decreased drastically. The production of hypericin and pseudohypericin levels is increased rapidly and then decreased progressively (Gadzovska Simic et al. 2015). The culture extracts of *Micromucoris abellina* enhance the production of indole alkaloid, catharanthine, and ajmalicine in *Catharanthus roseus* (Dicosmo et al. 1987). The sanguinarine is synthesized only with the elicitation of the cultures of *Papaver somniferum* using *Botrytis* sp., *Rhodotorula rubra*, *Helminthosporium gramineum*, and *Sclerotinia sclerotiorum* (Eilert et al. 1985). *Paraconiothyrium brasiliense* and *Chaetomium globosum* are used to elicit the suspension cultures of *Corylus avellane* L. for the synthesis of paclitaxel (Salehi et al. 2019).

7.4.1.3 Algal Elicitation

Algae and cyanobacteria are the majority of phytoplankton. It is the true representative of thallophytes. The use of algae in elicitation research is one of their most promising uses. Generally, marine phytoplankton is underutilized, and only a few of

it is used for food medicine and other biological purposes (Vinoth et al. 2012). The effective elicitor is made up of the cell wall and their parts (oligosaccharides and polysaccharides) of seaweed, offering profuse benefits like plant protection, enhanced biochemical output, and increased tolerance to various stresses (Arman and Qader 2012; Sbaihat et al. 2015). When the algal polysaccharides are introduced in the plant, a number of defense cascades are activated that produce various byproducts as secondary metabolites (Stadnik and Freitas 2014). In addition to the elicitor, the algal sources are macro- and micronutrients and growth factors (Satish et al. 2015). Hence, the complete algal cells or its constituents are useful for secondary metabolite production under in vitro conditions.

The major finding is the use of cyanobacterium strains for the plantlet formation (Wake et al. 1991). The major role of algal elicitor is to increase biomass production. The elicitor treatment to the neem cell suspension cultures with *Anabaena* sp. and *Nostoc carneum* led to rise in callus biomass. The protein concentration is also raised by *N. carneum* and *Anabaena* sp. (Devi et al. 2008). Various microalgae, *Anabaena cylindrica*, *Nostoc linckia*, and *A. variabilis*, were used to induce the production of red pigment using *Carthamus tinctorius* L. cell culture (Hanagata et al. 1994). Phycocyanin, a biliprotein extracted from the blue-green algae *Spirulina platensis*, is used to boost the production of capsaicin from the cell cultures of *Capsicum frutescens* L. and *D. carota* L. (Rao et al. 1996). Similarly, acetone extracted from Botryococcus braunii, a colonial Chlorophyceae microalga, is used to enhance the total chlorophyll content, seed viability, and leaf area in *C. frutescens* L. (Sharma et al. 2010). Aqueous extracts of *Haematococcus pluvialis* and *Spirulina platensis* are used to elicit the production of food coloring agent and betalain production from *Beta vulgaris* L. while thiophene from *T. patula* L. (Rao et al. 2001). The production of picroside-I from the *Picrorhiza kurroa* plant is initiated with the treatment of the red seaweed *Kappaphycus alvarezii*. The treatment enhances the production of the biomass, plant length, number of roots, and shoot length. Thus, this quantity continues to be ideal for promoting root induction and plant growth (Sharma et al. 2015a).

7.4.1.4 Elicitation Through Polysaccharides

Polysaccharides are biopolymers synthesized by polymerizing the monosaccharides and disaccharides (Fukui et al. 1990). The structural orientation of polysaccharides determines their physical and chemical characteristics (Nartop 2018). Based on biochemical functions within organisms, polysaccharides are divided into two groups: storage polysaccharides and structural polysaccharides (Fukui et al. 1990). It plays crucial role in cell signaling, protecting plants from adverse environmental conditions, etc. It is derived from the biotic source, thus regarded as biotic elicitors, which is present in all living system: plants, animals, and microorganisms. There are two types of polysaccharide elicitors: (1) endogenous polysaccharide (cellulose, pectin, etc.) and (2) exogenous polysaccharide (chitin, chitosan, etc.) (Nartop 2018). It is very efficient to elicit the SMs of antimicrobial activities (Paulert et al.

2009; Lu et al. 2019). It acts as one of the most prominent elicitors for the production of SMs and induction of plant defense reactions (Putalun et al. 2007).

The polysaccharide elicitor from yeast is mostly used for the production of various metabolites (Funk et al. 1987; Jeong et al. 2005). The yeast extract is used to elicit the synthesis of isoflavonoid from the *Pueraria candollei* (Udomsuk et al. 2011). Similarly, tanshinone is produced from *Salvia miltiorrhiza* Bunge cell cultures using yeast extract (Zhao et al. 2012). It is often used to elicit the vinblastine, vincristine, and other alkaloids from *C. roseus* (Maqsood and Abdul 2017). Yeast extracts elicit both the biomass and metabolites (Funk et al. 1987; Putalun et al. 2007). Various elicitors, namely, chitosan, chitin, and mannan, originated from the yeast used to induce the production of SMs in vitro (Baque et al. 2012). Chitosan is used to elicit the production of Withaferin-A in the *Withania somnifera* L. (Thilip et al. 2019), antimalarial compound artemisinin in *Artemisia annua* L. (Putalun et al. 2007), and plumbagin in *Plumbago rosea* L. (Komaraiah et al. 2003). Chitosan in combination with hormones like jasmonates and salicylic acid is used to elicit *Azadirachta indica* for the production of azadirachtin (Prakash and Srivastava 2008). Additionally, chitosan and chitin have been found to induce the production of various phytoalexins, phenylpropanoids, and naphthodianthrones in plants (Orlita et al. 2008; Gadzovska Simic et al. 2015).

Sometimes, polysaccharides derived from endophytic fungus are also involved in the elicitation process (Cheng et al. 2006; Wiktorowska et al. 2010). Mannan, which is a highly active polysaccharide produced by these endogenous organisms, is primarily extracted from the yeast cell wall and stimulates the production of pseudohypericin, hypericin, and other SMs (Fukui et al. 1990; Yamaner et al. 2013). An endogenous fungus, *F. oxysporum*, enhances the production of diosgenin from *Dioscorea zingiberensis* (Li et al. 2011a). Plant-derived pectin is commonly utilized as an elicitor in studies (Veerashree et al. 2012). The production of metabolite naphthoquinone shikonin in cell suspension culture of *Lithospermum erythrorhizon* is induced by agro-pectin (Fukui et al. 1983). The highest accumulation of anthocyanin is observed with pectin elicitors (Cai et al. 2012). However, the saponin production is induced by yeast cell wall from the hairy root cultures of *P. ginseng* (Jeong et al. 2005). Dextran, a bacterial-derived polysaccharide synthesized from sucrose with the help of dextran sucrase, has been identified as an effective elicitor (Nagella and Murthy 2010; Rahpeyma et al. 2015). Treatment of *B. cinerea*-infected wounds on *Solanum lycopersicon* L. with dextran and laminarin resulted in a high production of phenylpropanoids and flavonoids (Lu et al. 2019).

Alginate is a seaweed-derived polysaccharide that is frequently used as an elicitor in SM production (Paulert et al. 2009). In addition to alginate, ulvan, carrageenan, and laminarin have also been found to be effective in activating secondary metabolite pathways (Ben Salah et al. 2018). Some novel seaweeds are also being investigated for their potential in enhancing SM production (Thilip et al. 2019). Sodium alginate and ulvan are used to induce the phenolics in *V. vinifera* L. under tissue culture (Cai et al. 2012). Phenolic compounds are used to remove wilt disease in shoot cultures of *Olea europaea* L. (Ben Salah et al. 2018). Laminarin is used to

accumulate the isoflavonoids in *Pueraria candollei* cell culture (Korsangruang et al. 2010).

7.4.2 Abiotic Elicitors

Elicitors derived from the nonliving origin are termed as the abiotic elicitors that may be categorized into three main domains: physical, chemical, and hormonal elicitors. Abiotic elicitors stimulate the number of pathways that affect the gene expression network which inculcate the synthesis of various phytochemicals. Numerous genes, molecular network, proteins, and metabolites are involved in plant reactions to heavy metals, light, dehydration, salt, thermal, endocrine, and other abiotic elicitors which have been recently described in studies (Rodziewicz et al. 2014).

7.4.2.1 Physical Elicitors

Physical elicitors include all the environmental conditions that induce various stresses over the plant system. The physical agents, namely, drought, salinity, osmotic, thermal, light, ultraviolet rays, and ultrasound, are the factors that affect the metabolite productions.

7.4.2.1.1 Drought

Plants under drought stress exhibits a number of signal transduction pathway in response to primary and secondary messenger to synthesize and accumulate secondary metabolites (Al-Gabbiesh et al. 2015). The short duration of water deficiency enhances the accumulation of glycyrrhizic acid in *Glycyrrhiza uralensis* roots (Li et al. 2011b). Various phenols and betulinic acid significantly increase in *Hypericum brasiliense* in response to the drought (de Abreu and Mazzafera 2005). The production of oleanolic acids, rosmarinic, and ursolic in *P. vulgaris* is increased with SMs (Chen et al. 2011) and salvianolic acid, tanshinone, and content of other bioactives in roots of *Salvia miltiorrhiza* (Liu et al. 2011). Water deficiency enhanced the levels of flavonoids in *Crataegus* (Kirakosyan et al. 2004).

7.4.2.1.2 Salt Stress

Salt stress stimulates the production of phenols, terpenes, and alkaloids in plants (Selmar 2008; Haghighi et al. 2012). Stress caused by combination of KCl and $CaCl_2$ on in vitro cells of *Bacopa monnieri* increased the production of the medicinally active compound bacoside A (Ahire et al. 2014). The production of vinblastine and vincristine in *C. roseus* is induced by NaCl (Fatima et al. 2015). For elicitors the

secondary metabolite concentration and type of salt need to be standardized. 250 mM of NaCl treatment enhances the sitosterol concentration in *Nitraria tangutorum* cell suspension (Ni et al. 2015). The application of saline water enhances the anthocyanin production in *Grevillea* (Kennedy and De Filippis 1999) and alkaloids in young *Datura innoxia*. The findings showed that tropane alkaloid accumulation is linked to the plant at the organ level (Brachet and Cosson 1986). Similarly, polyamines, diamines, polyphenol, and sugar alcohol are induced in various species: *Trifolium repens* (Varshney and Gangwar 1988), *Triticum aestivum* (Krishnamurthy and Bhagwat 1989), and *Oryza sativa* (Krishnamurthy and Bhagwat 1989), in response to salinity.

7.4.2.1.3 Osmotic Stress

Osmotic stress is a secondary stress crop up due to the onset of drought, salt stress, chilling and freezing, etc. Thus, it may act as a significant abiotic elicitor for the production of SMs (Liu and Cheng 2008). Sucrose is used as a standard osmotic stress agent to produce SMs. Osmotic stress increased the capsaicin in *Capsicum chinensis* cell suspension cultures (Kehie et al. 2012). The production of hypericin and hyperforin in *Hypericum perforatum* plants is stimulated with water deficiency and osmotic disequilibrium (Pavlik et al. 2007). For some osmolytes, proline is synthesized in response to osmotic stress and plays a vital role as protective agent for cytoplasmic enzymes, a regulator for protein synthesis, pH control, and generation of reactive oxygen species (Verbruggen and Hermans 2008). The prime role of proline under stress is to maintain the integrity of plasma membrane (Xu et al. 2009). Artificially, water stress is induced by osmotic substance polyethylene glycol (PEG), also known as nonpenetrating osmoticum (Van den Berg and Zeng 2006). PEG and proline increased steviol glycoside production in *Stevia rebaudiana* solution and callus cultures (Gupta et al. 2015). PEG also enhances hypericin and pseudohypericin, which are pharmacologically active substances present in *Hypericum adenotrichum* (Yamaner and Erdag 2013).

7.4.2.1.4 Thermal Stress

Thermal stress is perceived as heat and cold stress. Both the heat and cold stress stimulate the production of SMs. High temperature strongly influences the metabolic activity of the plant and ultimately results in premature leaf senescence (Morison and Lawlor 1999). Extreme temperatures are a harmful climatic factor that restricts plant development and expedites the senescence. Plant growth is often intervened by a variety of metabolic processes that are often associated with the creation and breakdown of primary metabolites (Xu et al. 2013). In the medicinal plant *Panax quinquefolius*, elevated temperature raised the concentrations of both root secondary metabolites and leaf senescence. The raise of temperature causes increase in the amount of ginsenoside in *P. quinquefolius* stems (Jochum et al. 2007). The hairy root

cultivation of *Panax ginseng*, temperature, and light quality affect the synthesis of ginsenoside (Yu et al. 2005).

Temperature changes the metabolism, permeability, and pace of intracellular responses (Morison and Lawlor 1999). The range of temperature is important for the development of callus tissues and proliferation of cultured cells under in vitro conditions (Rao and Ravishankar 2002). The variation in temperature under in vitro condition affects the anthocyanin production from *Melastoma malabathricum* cell cultures (Chan et al. 2010). The duration of applied temperature is also the point of consideration. The production of hypericin and hyperforin in *Hypericum perforatum* stems considerably increased after 15 days at 35 °C (Zobayed et al. 2005).

7.4.2.1.5 Light

The light is a physical factor which drives major growth, developmental, physiological, and reproductive phase of plant. It drives most of photochemical reaction: photo synthesis and photo respiration that affect the metabolite production in plants. It promotes the synthesis of secondary metabolites under in vitro conditions too. Light induces the synthesis of gingerol and zingiberene in callus cultures of *Zingiber officinale* (Anasori and Asghari 2009). The hairy roots and light affect both development and the synthesis of secondary metabolites. In addition, light drives both the development of hairy roots and alkaloid production in *Hyoscyamus albus* (Sauerwein et al. 1992). The synthesis of secondary metabolites is also affected by the quantity and quality of light. The importance light spectra for the secondary metabolite production in *P. ginseng* are achieved in terms of alkaloid synthesis (Yu et al. 2005). Similarly, the synthesis of indole alkaloids is triggered by light in *Catharanthus roseus* hairy root cultures (Bhadra et al. 1998) and the production of artemisinin from *Artemisia annua* (Liu et al. 2002).

7.4.2.1.6 Ultraviolet (UV) Radiation

The ultraviolet (UV) is also an environmental factor that seized attention because of climate change. UV light perceived as a light component but artificially can be used to induce the plant system for secondary metabolite synthesis. UV radiation spectrum is distinguished into three regions: UV-C (wavelengths below 280 nm), UV-B (280–315 nm), and UV-A (315–400 nm). Although UV-C is the most harmful, it is mostly absorbed by the atmosphere. The stratospheric ozone layer only partly absorbs UV-B energy and completely rejects UV-A radiation. The accumulation of phenolic compounds like flavonoids and glycosylates in plants is stimulated in response of UV-B radiation (Schreiner et al. 2016). Similarly, the synthesis of nitric oxide (NO) and flavonoids is activated as per the enhanced activities of nitric oxide synthase and phenylalanine ammonia lyase in *Ginkgo biloba* callus as a result of UV-B exposure (Hao et al. 2009). UV-B radiation was found to increase catharanthine and vindoline synthesis in suspension cultures of *C. roseus* (Ramani

and Jayabaskaran 2008). The total terpenoid indole alkaloid (TIAs) amount in the pubescent roots of *C. roseus* increased in response to UV-B (Binder et al. 2009). The high radiation of UV-B rays enhances the vitamin C content in *Turnera diffusa* plants cell under in vitro conditions (Soriano-Melgar et al. 2014). Similarly, UV-C rays are used to enhance the production of resveratrol and piceatannol in peanut callus cultures (Ku et al. 2005). It encourages the synthesis of stilbene in grape calli of various varieties (Liu et al. 2010a). UV-C is also used in conjunction with methyl jasmonate (MeJA) or salicylic acid (SA) to increase stilbene synthesis (Xu et al. 2015).

7.4.2.1.7 Ultrasound

The low-energy ultrasound (US) has been reported as an abiotic elicitor to promote the synthesis of secondary metabolites in plants (Yu et al. 2016). Additionally, US increases the permeabilization of the cell membrane that enhances the release of secondary metabolites. The cell-permeabilizing effect is applicable for two phase-based secondary metabolite production under in vitro conditions. US stimulation and in situ solvent extraction in a *Lithospermum erythrorhizon* cell culture led to increased yield of shikonin (Lin and Wu 2002). Taxol production is enhanced with US treatment per week in *Taxus chinensis* (Wu and Lin 2003). Similar result is found with *Taxus baccata* cell culture for taxol production (Rezaei et al. 2011) and ginsenoside and saponin production in Panax ginseng (Lin et al. 2001).

7.4.2.2 Chemical Elicitors

Chemical elicitors include heavy metals, mineral salts, toxic organic complex as insecticides, herbicides, and gaseous toxins. Plants exposed to chemical elicitors initiate several biochemical pathways. The byproduct of which is accumulated as secondary metabolites.

7.4.2.2.1 Heavy Metals

Heavy metal mostly intercalates with structural and functional component of concentrations which adversely affect the growth and development of plants. Growth effects are caused by changes in physiological factors such as photosynthesis, respiration, enzyme activity, lipid composition, and nutrient distribution (Rout and Das 2009; Shanker et al. 2005). Changes in the metabolic activity of plants caused by heavy metals can affect the production of photosynthetic pigments, sugars, proteins, and nonprotein thiols. Inhibition of enzymes involved in the production of natural products may cause these effects, most likely due to impaired substrate utilization (Nasim and Dhir 2010). The presence of metals such as Ni, Ag, Fe, and

Co has been demonstrated to stimulate the production of secondary metabolites in a various species of plants (Zhao et al. 2001).

The most common species that is being used to elicit by heavy metals is *Brassica juncea* in which oil content is stimulated with heavy metals Cr, Fe, Zn, and Mn (Singh and Sinha 2005). Copper (Cu^{2+}) and cadmium (Cd^{2+}) ions are used for accumulations of secondary metabolites shikonin (Mizukami et al. 1977) and digitalin (Ohlsson and Berglund 1989). The betalains and other secondary metabolites in *Beta vulgaris* are stimulated with Cu^{2+} and Co^{2+} (Trejo-Tapia et al. 2001; Rudrappa et al. 2004). The betacyanins in callus cultures of *Amaranthus caudatus* are stimulated with Cu^{2+} (Obrenović 1990). Various heavy metals, including Co^{2+}, Ag^{+}, Cd^{2+}, Cu^{2+}, Ce^{2+}, La, Mn^{2+}, and Zn^{2+}, are used as elicitors to accumulate bioactive chemicals in *Salvia miltiorrhiza*. Silver metal (Ag^{+}) is a useful elicitor for phenolic compound and tanshinone synthesis in S. miltiorrhiza bushy roots and enhances the production of rosmarinic acid, salvianolic acid B, and tanshinones (Yan et al. 2006; Zhao et al. 2010; Zhou et al. 2012; Shi et al. 2014). Lepidine content is enhanced with Zn^{2+} and Cu^{2+}in *Lepidium sativum* (Pande et al. 2000). Silver nitrate ($AgNO_3$) or cadmium chloride ($CdCl_2$) induced the overproduction of two tropane alkaloids, scopolamine and hyoscyamine, in hairy root cultures of *Brugmansia candida* (Angelova et al. 2006). The rare-earth element lanthanum acts as a stimulator for the taxol synthesis in *Taxus* sp. cell culture (Pitta-Alvarez et al. 2000). The amount of umbelliferone in the leaves of *Matricaria chamomilla* adult tetraploid plants is enhanced with $CuCl_2$ application in the field (Repčák et al. 2001).

Various studies emphasize cobalt as an elicitor for secondary metabolites. It was reported to increase the levels of endogenous hormones (auxins, gibberellins, and abscisic acid) in olive fruit along with the oil content (Gad et al. 2006). It enhances the flavone and anthocyanin content in roselle leaves (Aziz et al. 2007) and total phenol and oil content in canola (Gad 2010).

7.4.2.2.2 Toxic Metals and Nutrient Deficiency

Secondary metabolite production is induced by availability and mineral deficiency in the soil. The biochemical changes occur in response to the availability of metals in soil solution and are quick and reversible that improved the metal absorption in and eliminate the toxic metals from the soil (Dinkelaker et al. 1989; Jin et al. 2007; Lambers et al. 2002; Marschner et al. 1987; Wang et al. 2007). Many plant species produce biochemicals from their roots after attaching with metal called as chelation (Wang et al. 2007). Phosphorus deficiency in the soil induces citrate, malate, and oxalate in *Glycine max*, while aluminum deficiency caused malate and citrate accumulations (Liao et al. 2006). Phenolic compounds secreted from roots that are iron deficient (Jin et al. 2007) and aluminum deficient release various secondary metabolites, e.g., roots of *Lupinus albus* (Wang et al. 2007). Allelopathic interactions under iron deficiency accumulate chemical 8-hydroxyquinoline from the roots of *Centaurea diffusa* (Tharayil et al. 2009).

7.4.2.3 Hormonal Elicitors

Plant hormones are signaling molecules often synthesized in response to biotic and abiotic elicitors. Application of hormone to the plant enhances the secondary metabolites without compromising the plant health in response to various stresses. Jasmonic acid (JA), salicylic acid (SA), abscisic acid (ABA), brassinosteroid (BR), and gibberellins (GA) are the important hormones and growth factors that play role in secondary metabolite production.

7.4.2.3.1 Jasmonic Acid (JA)

Jasmonic acid is one of the signaling components synthesized in response to defense mechanism in the plant that triggers the production of secondary metabolites (Jeandet et al. 2002). Flavonoids, terpenoids, and alkaloids are produced in response of jasmonic acid. Methyl jasmonates boosted the alkaloid raucaffricine production from *R. canescens* (Parchmann et al. 1997). JA elicitation elicited the *Mentha piperita* to produce rosmarinic acid (Krzyzanowska et al. 2012), *V. vinifera* to produce anthocyanin (Curtin et al. 2003), and bushy stems of *Plumbago indica* to produce plumbagin (Gangopadhyay et al. 2011). Methyl jasmonate (MeJA) elicitation enhances the phytoecdysteroids and phenolic and flavonoid content in the root solution of *A. bracteosa* (Saeed et al. 2017). MeJA and cyclodextrin (CD) together increased the synthesis of bioactive alkaloids in cambial meristematic cells (CMCs) of C. roseus (Zhou et al. 2015). Cyclodextrin is used to trigger the biosynthesis of ajmalicine (Almagro et al. 2011). MeJA in hairy root culture with transgenic technology greatly increased the tanshinones in *S. miltiorrhiza* (Hao et al. 2015).

7.4.2.3.2 Salicylic Acid (SA)

Salicylic acid plays a substantial role in eliciting of medicinal plant species and producing different forms of secondary metabolites: terpenes or terpenoids, phenolics, and N-containing compounds (Taiz and Zeiger 2006). External application of salicylic acid (SA) increases the overall triterpenoid concentration in *Centella asiatica* (Buraphaka and Putalun 2020). SA are elicitors for the synthesis of triterpene glycosides in *Actaea racemose* (black cohosh) (De Capite et al. 2016). The SA treatment enhances the glycyrrhizin in the stems of in vitro-cultivated licorice (Glycyrrhiza glabra) (Shabani et al. 2009). The oxygenated monoterpene and sesquiterpenes are produced in response to the SA in lemon balm (*Melissa officinalis*) plants (Pirbalouti et al. 2019). The addition of SA to the *Panax ginseng* adventitious root cultures promotes the formation of farnesol, isochiapin B sesquiterpenoids, camphor, and cineole monoterpenoids (Rahimi et al. 2014). The stimulation of *Crocus sativus* (saffron) with salicylic acid increases the accumulation of phenolics and flavonoids (Tajik et al. 2019), capsaicinoids (Gutiérrez-Carbajal

et al. 2010), and vanillin (Rodas-Junco et al. 2013) in *Capsicum chinense*. The phytochemicals such as chlorogenic acid, apigenin, luteolin, quercetin, harpagide, aucubin, harpagoside, catalpol, and 8-o-acetyl harpagoside increase in *Ajuga integrifolia*, when treated with SA (Abbasi et al. 2020). Increase in flavonoids concentration was reported in *Ginkgo biloba* species when treated with SA (Ni et al. 2018).

Essential oil is produced from various species of *Thymus* with different concentration; however, when elicited with SA, it enhanced the accumulation of essential oils (Ghasemi Pirbalouti et al. 2014). Treatment of SA in combination with drought stress produces the optimum level of essential oil in *Thymus vulgaris* (Khalil et al. 2018) and in *T. kotschyanus* (Mohammadi et al. 2019). *Ruta graveolens* when exposed to mild drought stress along with SA treatment increased the essential oil in it by long-chain methyl ketones (94.2%), followed by 2-undecanone and 2-nonanone, both in leaves and flowers (Attia et al. 2018). The accumulation of volatile compounds such as trans-pinocarveol, cis-isopinocarveyl acetate, trans-carveol, and trans-pinocarvyl acetate in *Egletes viscosa* is accumulated with SA (Batista et al. 2019). The concentrations of monoterpene in the essential oil and the total phenolic content in *Mentha piperita* (peppermint) plants are enhanced in response to the exogenous SA treatment (Cappellari et al. 2019).

7.4.2.3.3 Other Hormones

Abscisic Acid

Abscisic acid (ABA) acts as a stress hormone accumulated in response to the various abiotic stresses and seed development and maturation phase (Bari and Jones 2009). It regulates the number of pathways for initiating tolerance pathway in the plants. Osmolytes, signaling components, enzyme activations, and compatible solutes are accumulated in response of ABA signaling in the plant. Most of them are secondary metabolites of medicinal importance which includes anthocyanins in *Arabidopsis thaliana* (Loreti et al. 2008) and terpenoid indole alkaloids in *C. roseus* (El-Sayed and Verpoorte 2004).

Brassinosteroids

Brassinosteroids are new-generation hormone that plays a key role in the control developmental process in the plant that imparts resilience against a variety of biotic and abiotic stressors (Müssig 2005; Sasse 2003). According to several studies, brassinosteroids are the important hormonal elicitor that boost the synthesis of various secondary metabolites like phenolics, shikonin, and forskolin by the plant (Choudhary et al. 2011; Swamy and Rao 2011; Yang et al. 1999). Artemisinin is one of important secondary metabolites synthesized in response to brassinosteroid homobrassinolide to the culture media of *Artemisia annua* hairy roots (Wang et al. 2002).

Gibberellic Acid

The phytohormone, Gibberellic acid (GA) is playing role in the induction of seed germination, stem elongation, flowering in long day plant and etc. Apart from this, it is also playing a role in secondary metabolite production in few plant species. The treatment of GA to the root hairs of *Salvia miltiorrhiza* yields tanshinones (Yuan et al. 2008); similarly, caffeic acid is produced in *Echinacea purpurea* (Abbasi et al. 2012).

7.5 Factors Affecting Elicitations

Elicitation is a well-established method for inducing de novo synthesis or increasing the production of secondary metabolites in the plant system or under in vitro plant cell cultures (DiCosmo and Misawa 1985). Elicitors provide a new avenue for the pharmaceutical industry to enhance the secondary metabolite production that could have significant economic benefits. However, the enhanced production of secondary metabolites is influenced by quantity, quality, and duration of elicitor exposure with respect to the source of cell, age of the plant cell culture, cell line, growth regulators, nutrient composition of media, and quality of cell wall materials (Ganapathi and Kargi 1990). These parameters play a crucial role in the elicitation of some medicinal plants for the production of secondary metabolites. The concentration of elicitors used in the elicitation process is also a crucial factor that affects the production of secondary metabolites. An excessive amount of elicitor can cause hypersensitive responses and cell death; thus, an optimal level is required for inducing production (Collinge and Slusarenko 1987; Mukundan and Hjortso 1990; Roewer et al. 1992). For instance, the minimal concentration of sodium chloride (0.1% w/v) leads to optimal accumulation of ginseng saponin content (Jeong and Park 2006). The accumulation of salvianolic acid B and caffeic acid in *S. miltiorrhiza* cell cultures is regulated with minimum concentration of salicylic acid (SA) (Dong et al. 2010). The accumulation of gymnemic acid is regulated with various concentrations of MeJA and SA in cell suspension cultures of Gymnema sylvestre (Chodisetti et al. 2013, 2015). Different biotic elicitors, including *A. rhizogenes*, *Bacillus subtilis*, *Escherichia coli*, *Aspergillus niger*, and *Saccharomyces cerevisiae*, required varying time durations for induction of secondary metabolite accumulation (Sharma et al. 2015b). The production of secondary metabolites is also affected by the type and age of cell culture. The accumulation of withanolide A, withanone, and withaferin A needs an organized cell culture such as hairy root culture of *W. somnifera* (Sivanandhan et al. 2013). The production of ajmalicine from *C. roseus* cell cultures needs a 20-day-old cell culture elicited with *A. niger* and *F. moniliforme* (Namdeo et al. 2002; Namdeo 2004). The selection of culture medium is an important factor in the elicitation process, affecting the production of bioactive compounds. The production of cocaine, cinnamoylcocaine, chlorogenic acid (CGA), and 4-coumaroyl quinate (CQA) from *Erythroxylum coca* callus culture was significantly influenced

by the culture medium (Docimo et al. 2015). Cocaine production is significantly higher on Anderson rhododendron medium (ARM), Gamborg B5 (GB5), and Murashige-Tucker medium (MMT) compared to other media (Anderson 1978; Gamborg et al. 1976; Murashige 1969). The media compositions are highly influenced with the amount of nitrogenous compound, growth factor, and total ion concentrations (Docimo et al. 2015).

Secondary metabolites are the major output of medicinal plant for the pharmaceutical industry. The yield of secondary metabolites is enhanced with elicitor. Elicitor may be of biotic and abiotic origin that may be derived endogenously and exogenously. The process of elicitation with the help of elicitor depends upon the types of elicitors with their concentration and duration of exposure that need to be standardized. For the production of secondary metabolites from rare species, yield can be enhanced with tissue culture approaches. However, the production of secondary metabolites is also depending upon the source, age, and duration of plant cell culture with media compositions. Thus, to maintain their yield, each component needs to be standardized with the duration of elicitations. The inherent capacity of SM production through plants can also be enhanced through biotechnological tools.

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Chapter 8
Regulation of Phytochemical Properties of Hawthorn: A *Crataegus* Species

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Abstract Hawthorn (*Crataegus*) is a shrub or small tree that originates from Asia, North America, and Europe within the Crataegus genus. This genus comprises over 1000 species and belongs to the *Rosaceae*'s subfamily, Maloideae. Throughout centuries, hawthorn has been utilized for its medicinal properties, which can be attributed to its rich concentration of phytochemicals. A global examination of hawthorn has uncovered the existence of diverse advantageous components like flavonoids, titerpenoids, procyanidins, and phenolic acids. These compounds are responsible for the pharmacological activities associated with hawthorn. The fruits and leaves of hawthorn have been recognized for their medicinal properties, primarily as cardiotonic, antispasmodic, hypotensive, diuretic, and atherosclerotic agents.

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Crataegus fruits are abundant in phenolic compounds, known for their various biological activities, and have been traditionally utilized for medicinal purposes. Renowned antioxidant compounds such as hyperoside, isoquercetin, epicatechin, cholorogenic acid, quercetin, rutin, and protocatechuic aids are abundant in these fruits and leaves, which are rich in phenolic components. Consequently, *Crataegus* is recognized as a valuable natural source of antioxidant. To obtain a comprehensive comprehension of the medicinal uses of hawthorn for addressing diverse health-related concerns, further investigation is necessary. Research findings have indicated that hawthorn possesses antioxidant, anti-inflammatory, antihypertensive, anti-arrhythmic, and lipid-lowering characteristics. These findings highlight the potential therapeutic benefits of hawthorn in combating different health complications. Additionally, it improves myocardial contractility and enhances blood circulation. Clinical studies have demonstrated that hawthorn extracts can alleviate symptoms of heart failure and angina, reduce blood pressure, and enhance lipid profiles. As a result, hawthorn shows promise as a natural remedy for preventing and treating cardiovascular diseases. Nevertheless, additional exploration is required to unveil the exact mechanisms of operation, ascertain the ideal dosage, and create appropriate formulations for the utilization of hawthorn.

Keywords Hawthorn · *Crataegus* · *Rosaceae* · Antioxidant activity · Medicinal benefits

8.1 Introduction

For a long time, hawthorn and other edible wild plants have held a considerable importance in human existence. These plants have served various personal and social functions, including food, medicine, and environmental beautification (Hricova et al. 2016). The genus *Crataegus*, a part of the *Rosaceae*'s subfamily Maloideae, encompasses more than 1000 species distributed widely across Asia, North America, and Europe (Alirezalu et al. 2018). Hawthorn goes by different names in different regions, such as Ring kul in the Kashmir valley and Pingyat in Lahaul, Himachal Pradesh. It has been utilized for medicinal purposes for centuries, notably in traditional Chinese medicine and Native American healing practices. Its leaves, flowers, and berries are all utilized for medicinal purposes, with the berries being the most commonly employed component (Anwar et al. 1979; Shah and Hussain 2012; Kumar et al. 2009; Haq 2012; Rawat et al. 2010; Rafeeq et al. 2022).

Crataegus has a broad geographic range encompassing Afghanistan, Iran, northern India, northern Pakistan, Tadzhikistan, Kyrgyzstan, Uzbekistan, Kazakhstan, and Sinkiang. It thrives at elevations between 800 and 2700 m. This species is predominantly present in the mountainous areas of India, particularly in the temperate Himalayas of Kashmir and Himachal Pradesh. Its growth is most favorable in river valleys and on slopes of ravines, thriving at altitudes ranging from 1800 to 3000 m (Nadkarni 1976). Furthermore, hawthorn can also be observed in Afghanistan and Uttar Pradesh, where it occurs at elevations ranging from 1500 to 2700 m.

Fig. 8.1 Hawthorn, *Crataegus songarica*, leaves

Within Jammu and Kashmir, *Crataegus* is distributed across various areas such as Lolab, Sind valley, Gulmarg, Pahalgam, and Pir Panjal Range. In the Kashmir Valley specifically, the species is commonly encountered at altitudes of 1700 to 1900 m above sea level (Stewart 1972).

Most *Crataegus* species exhibit two leafy bracts on their leaves, with the leaf stalks connecting to the twig. The leaves of *Crataegus* species typically range from 15 mm to 5 cm in length and have a smooth texture. Their shape is either broad-ovate or obovate, featuring margins with toothed edges and three to seven lobes (Fig. 8.1). The flowers gather in clusters of 5–12 and showcase a range of colors, spanning from white to pink or transitioning from pink to red (Fig. 8.2). These flowers possess both male and female reproductive components and rely on insect pollination due to their appealing scent. In certain regions of England, the local names for the red fruits include pixie pears, cuckoo's beads, or chucky cheese. These fruits, known as Hawthorn berries, are ovoid pseudo-fruits that initially appear greenish-red (Fig. 8.3). As they mature, they transition to bright red and eventually deep red. The berries contain fleshy white pulp that encloses one or two hard stone-like seeds (Huang et al. 2009).

Hawthorn is renowned for its diverse medicinal properties, with its edible parts, viz., fruits, leaves, and flowers, being utilized as hypotensive, cardiotonic, antispasmodic, antheroscelerotic, and diuretic agents (Nabavi et al. 2015). The berries of hawthorn are abundant in phenolic compounds, which exhibit various biological activities and are commonly employed in medicinal remedies, as stated by Li and Wang (2011). Additionally, studies conducted by Zugic et al. (2014) and Barros et al. (2010) have highlighted the abundant presence of antioxidants in hawthorn

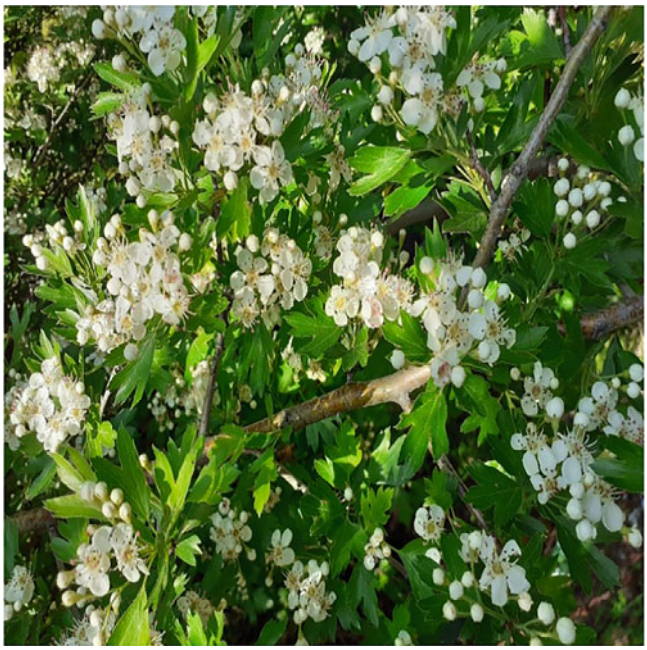

Fig. 8.2 Hawthorn, *Crataegus songarica*, flowers

Fig. 8.3 Hawthorn, *Crataegus songarica*, fruits

fruits and leaves. These antioxidants include hyperoside, isoquercetin, epicatechin, chlorogenic acid, quercetin, rutin, and protocatechuic acids.

This chapter aims to present a summary of the phytochemical composition and medicinal benefits of Hawthorn, specifically *Crataegus* species.

8.2 Phytochemical Composition of Hawthorn

Extensive global research has been dedicated to exploring the phytochemical composition of hawthorn, which encompasses a diverse array of biologically active compounds such as triterpenes, flavonoids, procyanidins, catecholamines, polysaccharides, and oligomerics (Kumar et al. 2009). Hawthorn possesses numerous medicinal properties, and its various components, including leaves, berries, and flowers, are commonly employed as cardiotonic, antispasmodic, diuretic, antherosclerotic, and hypotensive agents (Nabavi et al. 2015). Phenolic compounds, found abundantly in hawthorn fruits, exhibit a wide range of biological activities and are frequently utilized in medicinal remedies (Li and Wang 2011). Due to their abundant phenolic compositions, hawthorn leaves and fruits are acknowledged as remarkable sources of antioxidants. These compositions consist of a variety of well-known antioxidant compounds, such as hyperoside, isoquercetin, epicatechin, chlorogenic acid, quercetin, rutin, and protocatechuic acids (Zugic et al. 2014; Barros et al. 2010).

Furthermore, the flowers, leaves, and fruits of hawthorn harbor diverse bioflavonoid complexes, with prominent biflavonoids like vitexin, oligomeric procyanidins (OPC), hyperoside, and quercetin commonly found in *Crataegus* species (hawthorn). Alongside these biflavonoids, hawthorn encompasses additional chemical constituents such as vitamin C, saponins, derivatives of purine (adenosine, adenine, guanine, caffeic acid, amygdalin), tripene acids, and cardiotonic amine ursolic acid.

The chemical composition of different *Crataegus* species was examined by analyzing specific compounds, including quercetin, hyperoside, rutin, and vitexin. This analysis was conducted using HPLC-UV and UV-vis spectrophotometry techniques (Sagaradze et al. 2019). The concentration of the hyperoside flavonoid in hawthorn fruit was analyzed using spectrophotometry at a wavelength of 285 ± 2 nm, and it ranged from 0.112% to 0.183% (w/w). Using HPLC-DAD and LC-MS/MS techniques, the presence of polyphenol compounds including epicatechin, epicatechin gallate (ECG), rutin, caffeic acid, and caftaric acid was detected in extracts of *Crataegus oxyacantha* (Benabderrahmane et al. 2018). Rutin and quercetin were also detected in extracts of *Crataegus oxyacantha* fruit through HPLC extraction (Cuevas-Durán et al. 2017).

Numerous compounds with significant biological activity have been discovered in hawthorn, totaling over 150. Phenolic acids such as ferulic acids, gallic acid, p-coumaric acid, syringic acid, chlorogenic acid, and caffeic acid are among the identified compounds. Additionally, other compounds including quercetin, pyrocatechin, phloridzin, terpenoids, lignans, steroids, organic acids (such as

fumaric acid, tartaric acid, succinic acid, citric acid, citric acid, malic acid), and sugars (such as maltose, sucrose, glucose, fructose) have been detected (Cuevas-Durán et al. 2017). Flavanols and flavanol polymers have been detected in *Crataegus pinnatifida*, which include (+)-catechin, (−)-epicatechin, leucocyanidin, proanthocyanidin A2, and procyanidin B2, B4, and B5. Additionally, trimers such as procyanidin C1, procyanidin D1, and epicatechin-(4β-6)-epicatechin-(4β-8) have also been identified (Wu et al. 2014). In a study conducted by Alirezalu et al. (2018), the phenolic content of *Crataegus* leaves was found to range from 7.21 to 87.73 mg GAE/g of dry weight. The total flavonoid content, on the other hand, varied across different species and plant organs, ranging from 2.27 to 17.40 mg/g of dry weight. The main phenolic compounds detected in *Crataegus* leaf extracts were vitexin, chlorogenic acid, and vitexin 2”-O-rhamnoside. Furthermore, Alirezalu et al. (2020) found that the total phenols, total flavonoid content, and antioxidant activity in *Crataegus* species ranged from 21.19 to 69.12 mg GAE/g of dry weight, 2.44 to 6.08 mg QUE/g dry weight, and 0.32 to 1.84 mmol Fe++/g dry weight, respectively. The most prevalent phenolic compounds in Crataegus fruit extracts were hyperoside, chlorogenic acid, and isoquercetin (Alirezalu et al. 2020).

Polyphenolic compounds have been identified as the primary cause of the antioxidant and pharmacological effects in *Crataegus* species, according to Keser et al. (2012). Liu et al. (2011) utilized HPLC-UV/ESI-MS to detect various phenolic constituents, including hyperoside, procyanidins B2/C1, epicatechin, and C-glycosyl flavones in hawthorn. Using nuclear magnetic resonance spectrometry, Lund et al. (2020) identified chlorogenic acid, rutin, hyperoside, vitexin-2”-O-rhamnoside, and naringenin as the major flavonoids in *Crataegus* species. Moreover, Sydora et al. (2018) reported the extraction of glucose, mannose, and fructose from hawthorn fruits using 2 M trifluoroacetic acid through acid hydrolysis, and these sugars were identified through gas chromatography/mass spectrometry.

A summary of primary compounds found in Hawthorn is shown in Table 8.1.

8.3 Medicinal Properties of Hawthorn

Hawthorn fruits contain abundant polyphenols, which are potent antioxidant compounds present in plants, as noted by Zao et al. (2017). These polyphenols are linked to numerous health advantages, such as a decreased likelihood of developing cancer, diabetes, heart issues, asthma, and premature skin aging (Pandey and Rizvi 2009; Wu et al. 2017). Hawthorn fruits are widely utilized in Europe and China to prepare food items like jam, jelly, beverages, and wine (Chang et al. 2002). Additionally, hawthorn has a long history of use in traditional medicine for addressing various human ailments, as noted by Barros et al. (2010) and Ozcan et al. (2005). The medicinal attributes of hawthorn were initially recorded by Dioscorides in De Materia Medica during the first century A. D, establishing the foundation for the European premodern pharmacopoeia. Hawthorn has a rich history in traditional Chinese medicine and was mentioned in the Tang Ben Cao, the first state-approved

Table 8.1 Main compounds found in hawthorn, *Crataegus*, species

Species	Compounds identified	References
Crataegus oxyacantha	Naringenin, epicatechin, quercetin-3-O-β-glucoside, and quercetin	Benabderrahmane et al. (2018)
Crataegus oxyacantha	Rutin and quercetin	Cuevas-Durán et al. (2017)
Crataegus songarica	Quercetin 3-O-galactoside and kaempferol-3-O-glucoside	Mraihi et al. (2015)
Crataegus azarolus	Quercetin 3-O-methyl ether, and 3-β-O acetylursolic acid	Abu-Gharbieh and Shehab (2017)
Crataegus pubescens	(+)-catechin and (−)-epicatechin	Guo et al. (2019)
Crataegus oxyacantha	Epicatechin, epicatechin gallate (ECG), rutin, caffeic and caftaric acids	Benabderrahmane et al. (2018)
Crataegus oxyacantha	Rutin and quercetin	Cuevas-Durán et al. (2017)
Crataegus oxyacantha	Chlorogenic acid, vitexin 2”-O-rhamnoside, vitexin, rutin, hyperoside, quercetin, and isoquercetin	Alirezalu et al. (2018)
Crataegus oxyacantha	Vitexin, rutin, hyperoside, quercetin, and isoquercetin	Alirezalu et al. (2020)
Crataegus monogyna	b-carotene, ascorbic acid, quercetin, and epicatechin	Barros et al. (2012)

pharmacopoeia, in 659 A.D. Various species of hawthorn are used for medicinal purposes in different regions. In Europe, *Crataegus monogyna* and *Crataegus laevigata* are commonly employed, while in China, *Crataegus cuneata* Siebold & Zucc. and *Crataegus pinnatifida* Bunge are more well-known and utilized (Kirakosyan et al. 2003; Jalali et al. 2012; Chang et al. 2005). Hawthorn has been traditionally employed in folk medicine for treating cardiac diseases, hypertension, and hyperlipidemia and as an anti-atherosclerotic agent. It has demonstrated particular efficacy in addressing cardiovascular issues such as heart failure, hypertension with myocardial injuries, angina pectoris, arrhythmia, and atherosclerosis. Furthermore, it has been used to improve blood circulation and alleviate blood stasis (Schmidt et al. 1994; Thompson et al. 1974). Hawthorn has also been utilized for the treatment of gastrointestinal disorders, promoting digestion and enhancing stomach functions. Additionally, it has been employed to relieve indigestion, epigastric distension, abdominal pain, and diarrhea. In the European tradition, hawthorn is known for its anti-spasmodic, cardiotonic, astringent, and diuretic properties (Edwards et al. 2012; Bullitta et al. 2007; Fakir et al. 2009).

Hawthorn berries contain bioactive compounds, including flavonoids, oligomeric procyanidins (OPCs), triterpenoids, and phenolic acids, which contribute to many of the plant’s therapeutic properties.

A summary of important medicinal benefits of Hawthorn is shown in Fig. 8.4 and Table 8.2.

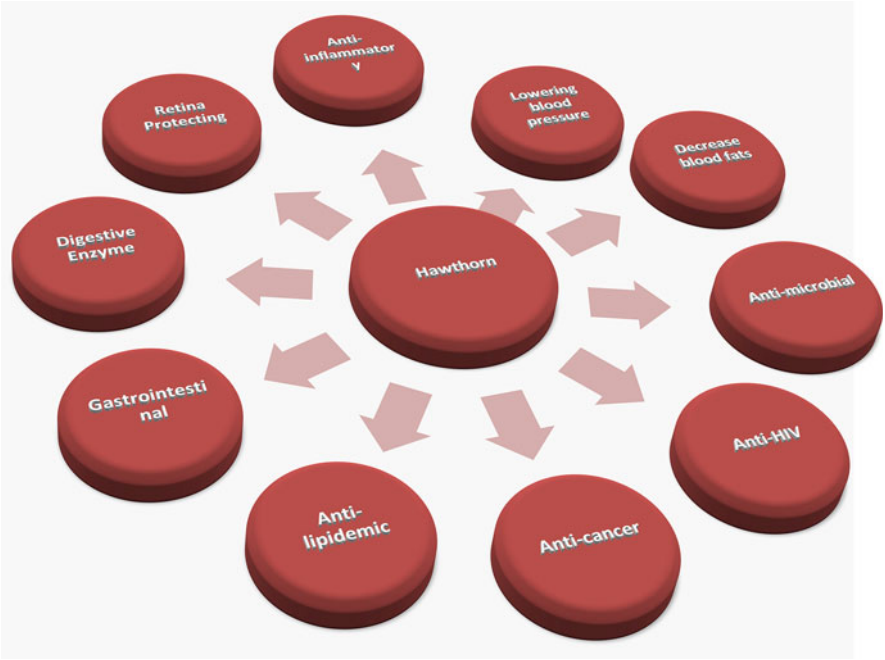

Fig. 8.4 Medicinal properties of hawthorn

8.3.1 Anti-Inflammatory Properties

Hawthorn contains a variety of anti-inflammatory compounds that have been shown to effectively reduce inflammation in the body. These properties make it beneficial for individuals with conditions such as arthritis, asthma, and inflammatory bowel disease. Numerous researchers have reported the anti-inflammatory properties of hawthorn, including a study by Pandey and Rizvi (2009). It is important to highlight that chronic inflammation has been associated with various diseases, including type 2 diabetes, asthma, and certain cancers, as discussed by Hunter (2012). Furthermore, a study by Han et al. (2016) indicated that extracts of hawthorn significantly reduced inflammatory compounds in mice with liver disease. Similarly, hawthorn fruit extracts have been found to decrease inflammation and significantly alleviate asthma symptoms in mice, as shown in a study by Shin et al. (2012).

8.3.2 Lowering Blood Pressure

According to Zou (2016), hawthorn fruit is utilized in traditional Chinese medicine for reducing blood pressure. Multiple animal studies, such as Kim et al. (2000), have

Table 8.2 Medicinal benefits of hawthorn, *Crataegus*, species

Species	Part used	Medicinal benefits	References
Crataegus monogyna	Flowers, fruits	Antioxidant, negative chronotropic effect	Kirakosyan et al. (2003), Degenring et al. (2003)
Crataegus aronia	Flowers, leaves, fruits	Antioxidant; antidiabetic	Ljubuncic et al. (2006)
Crataegus oxyacantha	Leaves, stem, fruits	Reducing heart rate, strengthening the heart, treating congestive heart failure, scavenging free radicals, reducing inflammation, protecting the stomach lining, fighting against microbes, treating myocardial infarction, acting as antioxidants, and inhibiting the production of thromboxane A2	Long et al. (2006), Degenring et al. (2003), Tadic et al. (2008), Jayalakshmi et al. (2006a, b), Anna et al. (2007)
Crataegus pinnatifida	Berries, fruits, leaves (dichloromethane, ethyl acetate, acetone, ethanol, heptane, hot water)	Lower lipids, reduce inflammation, combat oxidative stress, potentially prevent cataracts, and exhibit immunobiological effects	Lin et al. (2009), Kao et al. (2005)
Crataegus pentaegyna	Fruits (methanolic, aqueous)	Antioxidant	Ebrahimzadeh and Bahramian (2009)
Crataegus azarolus var. eu-azarolus	Flower (ethyl acetate)	Antioxidant, treatment of ischemic heart failure	Bahri-Sahloul et al. (2009)

provided evidence that hawthorn possesses the ability to decrease blood pressure. In a 10-week study conducted by Walker et al. (2002) involving 36 individuals with high blood pressure, the administration of 500 mg of hawthorn on a daily basis did not lead to a significant decrease in blood pressure. The study also indicated a noticeable trend toward lower diastolic blood pressure among the patients. In a separate study conducted by Walker et al. (2006a, b), the daily intake of 1200 mg of hawthorn extract showed promising result in reducing blood pressure and addressing diabetes-related concerns in individuals diagnosed with type 2 diabetes and hypertension.

8.3.3 Decrease Blood Fats

Hawthorn possesses the capability to decrease blood lipids. As per the research conducted by Yoo et al. in 2016, the administration of two doses of hawthorn extract to mice resulted in a reduction in cholesterol and triglyceride levels when compared

to the mice that did not receive the extract. Similarly, another study by Xu et al. in 2009 found that hawthorn extract exhibited the ability to lower total cholesterol and triglyceride levels in mice. Furthermore, a study involving 264 individuals with anxiety discovered that a combination of hawthorn, magnesium, and California poppy flower significantly alleviated anxiety levels in comparison to a placebo (Hanus et al. 2004).

8.3.4 Antimicrobial Properties

Hawthorn has been the subject of several studies that have demonstrated its antimicrobial effects. According to Barros et al. (2012), hawthorn fruit extract exhibited moderate bactericidal activity against gram-positive bacteria.

Belkhir et al. (2013) conducted a study on *Crataegus monogyna* and found that both leaf and fruit extracts exhibited antiradical and antibacterial properties against gram-positive bacteria, including *Streptococcus faecalis* and *Staphylococcus faecalis*. Another research conducted by Bisignano et al. (2016) demonstrated that Apigenin-7-O-glucoside and luteolin-3,7-diglucoside, derived from hawthorn, effectively eliminated *Ureaplasma urealyticum*. The minimum inhibitory concentration values for these compounds were found to range from 0.48 to 3.9 μg/mL and 0.48 to 1.95 μg/mL, respectively.

8.3.5 Anti-HIV Properties

The human immunodeficiency virus (HIV) utilizes serine protease to release itself from infected cells and attack other cells. However, *Crataegus pinnatifida* has demonstrated the ability to hinder the activity of serine protease, thereby reducing the diffusion rate of HIV in the body (Wang et al. 2009).

Furthermore, Xu et al. (1996) discovered that maslinic acid, derived from *Crataegus pinnatifida*, demonstrated a significant ability to inhibit HIV-1 protease activity. At a concentration of 17.9 mg/mL, it achieved a remarkable 100% inhibition rate. These findings suggest that maslinic acid holds potential as a prospective candidate for the development of novel anti-HIV therapeutics.

Additionally, Shahat et al. (1998) made a noteworthy discovery that flavonoids and trimeric procyanidin derived from *Crataegus sinaica* possess antiviral properties against HIV. They identified hyperoside, vitexin, 2”-O-rhamnosylvitexin, (4”-O-acetyl)-2”-O-rhamnosylvitexin, epicatechin, (+)-taxifolin, and 3-O- β-xylopyranosyl-(+)taxifolin as potential compounds with antiviral effects. These compounds are believed to function by either binding to the protein coat of the virus or inhibiting reverse transcriptase in retroviruses like HIV.

8.3.6 Anticancer

There is evidence suggesting that hawthorn may possess anticancer properties. Research indicates that hawthorn extracts can impede the growth of cancer cells in laboratory settings and hinder cancer metastasis.

In a study conducted by Numata et al. in 1989, maslinic acid obtained from *Crataegus pinnatifida* was shown to exhibit cytotoxic effects on P-38 cancer cells. The ED50 value, indicating the concentration at which 50% of the cells were affected, was found to be 13.0 μg/mL.

Flavonoids isolated from *Crataegus pinnatifida* did not have an impact on normal cells but were able to increase the calcium levels in tumor cells. This increase in calcium concentration led to the inhibition and destruction of Hep-2 tumor cells by overwhelming them with calcium and inhibiting DNA (Zhang et al. 2004). The aqueous extracts of *Crataegus pinnatifida* were discovered to reduce sperm distortion caused by cyclophosphamide in mice. This beneficial effect was attributed to the presence of linoleic acid and vitamin C (Cui et al. 2002).

In 2020 study conducted by Ma et al., HPS (hydroxypropyl starch) derived from hawthorn exhibited anticancer activity against human colon cancer cells (HCT116) in the concentration range of 125–1000 μg/mL. The anticancer effect was achieved by halting the cell cycle and inducing cell apoptosis through both extrinsic and intrinsic mechanisms. These mechanisms involved P38 mitogen-activated protein kinase and the phophatidylinositol-3-kinase/AKT/mammalian target of rapamycin signaling pathway.

Furthermore, Mraihi et al. in 2015 found that quercetin 3-O-galactoside and kaempferol-3-O-glucoside inhibited the growth of MCF-7 human breast cancer cells.

In a separate study conducted by Huang et al. in 2013, pinnatifidanin BVI, extracted from hawthorn, demonstrated a preventive effect against Mrc% human lung cells.

8.3.7 Antilipidemic

Guo et al. (2019) conducted a study to examine the impact of fermented hawthorn juiced treated with *L. plantarum* grade A pasteurized milk ordinance on rats that were fed a high-fat diet for 28 days. The findings revealed a hypolipidemic effect of the juice, which led to various positive outcomes. These included the regulation of adipose tissues and liver morphology, restoration of liver tissue, and decrease in levels of low-density lipoprotein cholesterol, serum total cholesterol, lipid vacuolization, and lipid metabolism. The study emphasized the favorable effects of fermented hawthorn juice on the lipid profiles of rats.

Research has identified hyperin and ursolic acid as the primary compounds responsible for reducing high cholesterol levels in *Crataegus pinnatifida*. Two different animal models of hyperlipidemia were created in mice, and these models were treated with hyperoside or ursolic acid extracted from *Crataegus pinnatifida* at two different doses. Compared to the control groups, the treatment resulted in significant reductions in total cholesterol (TCH) levels, while high-density lipoprotein (HDL) and superoxide dismutase (SOD) activity increased. The treatment also reduced the ratio of total cholesterol to high-density lipoprotein (TC/HDL), which could potentially prevent atherosclerosis by reducing damage to the vascular endothelium caused by oxygen-free radicals (OFR) in hyperlipidemia. This effect may be attributed to elevated levels of nitric oxide (NO) in the serum and decreased synthesis of endothelin (ET), both of which are associated with improved vascular function in hyperlipidemia model rats. The studies conducted by Li et al. (2002) and Yang et al. (2008) provided insights into the mechanisms and benefits of hyperoside and ursolic acid in managing hyperlipidemia.

8.3.8 Gastrointestinal Function Regulating Effect

The effects of *Crataegus pinnatifida* on gastrointestinal function can vary depending on the method of extraction, whether using alcohol (60% alcohol) or water. The alcohol extract obtained from charred fruits of Crataegus pinnatifida demonstrated a dose-dependent reduction in the contractility of rat gastric and intestine smooth muscle strips in a concentration range of 2–8 mg/mL (crude drugs). Similarly, the alcohol extract, in a concentration range of 5–20 mg/mL (crude drugs), dose-dependently reduced the contractility of rat gastric and intestine smooth muscle strips, and at a concentration of 20 mg/mL (crude drugs), it inhibited acetylcholine-induced stimulation. In contrast, the aqueous extract of *Crataegus pinnatifida* significantly increased the contractility of rat gastric and intestine smooth muscle strips in a dose-dependent manner within a concentration range of 5–20 mg/mL (crude drugs). Furthermore, at a concentration of 20 mg/mL (crude drugs), the aqueous extract enhanced the intensive contraction induced by acetylcholine and counteracted the relaxation of intestinal smooth muscle induced by atropine (Huang et al. 2009; Deng et al. 2009).

Moreover, a study demonstrated that children who consumed hawthorn slices orally while receiving intravenous injections of azithromycin experienced a lower incidence of gastrointestinal side effects compared to the control group ($p < 0.05$). This research suggested that *Crataegus pinnatifida* can help reduce the gastrointestinal side effects caused by azithromycin without any additional reported side effects (Wen et al. 2010; Yang et al. 2010).

8.3.9 Digestive Enzyme Promotion Effects

Traditionally, hawthorn has been renowned for its impact on digestive well-being. Recent research suggests that it can provide multiple advantages to the digestive system. For instance, it has been demonstrated to enhance the production of digestive enzymes, improve the absorption of nutrients, and reduce inflammation in the digestive tract. Additionally, it may help alleviate symptoms of indigestion such as bloating and gas.

Crataegus pinnatifida, a plant rich in nutrients such as organic acids, carotene, vitamin C, and vitamin B2, exhibits the potential to augment the secretion and activity of digestive enzymes in the stomach. Specifically, the presence of amylase can enhance lipase activity, directly facilitating the digestion of fatty foods. Additionally, the protease agonists derived from *Crataegus pinnatifida* have shown the ability to increase protease activity.

Furthermore, the organic acids present in *Crataegus pinnatifida* have been observed to enhance gastrointestinal motility in mice and counteract the relaxation of intestinal smooth muscle caused by atropine. However, these organic acids did not affect the stimulation of intestinal smooth muscles induced by neostigmine. These findings from the study conducted by Wu and Sun in 2009 suggest a unidirectional regulation of intestinal motility.

8.3.10 Retina Protecting Effects

During the experiment, rabbits were subjected to continuous inhalation of CS2 for a duration of 3 h daily, 6 days a week, over a period of 3 weeks. Prior to the contamination, the rabbits in the experimental group received a water decoction called "haw drink compound," prepared using *Crataegus pinnatifida*, *Lycium barbarum*, and *Fructus jujubae*. Upon completion of the 3-week study, the findings revealed that the retinal tissues of the control group exhibited greater abnormalities compared to the treatment and normal groups. Specifically, the cells in all layers of the control group's retinal tissue displayed degenerative changes, whereas those in the treatment group remained normal. This investigation effectively demonstrated the ability of the haw drink compound to enhance the rabbits' resilience against CS2 toxicity and protect against retinal damage (Tian et al. 1999).

8.3.11 Application of Hawthorn in Humans

Numerous clinical trials have documented the various health benefits of hawthorn (Song et al. 2019; Kadas et al. 2014; Asher et al. 2012; Al-Gareeb 2012; Asgary et al. 2004; Degenring et al. 2003; Zapfe 2001). One particular study investigated the

effects of hawthorn extract (900 mg/day) over a 620-day period on 2681 patients with congestive heart failure. The results demonstrated a reduced risk of sudden cardiac death among individuals with lower left ventricular function (Holubarsch et al. 2008). A different study, which included 120 mobile patients experiencing symptomatic chronic heart failure, discovered that usage of hawthorn (450 mg, twice daily) over a period of 6 months did not have a substantial impact on inflammation, oxidative stress, neurohormones, functional capacity, or quality of life. Nonetheless, there was a slight enhancement observed in the left ventricular ejection fraction (Zick et al. 2009).

Moeini et al. (2016) discovered that the consumption of 5 mL of hawthorn fruit extract after each meal for a duration of 4 weeks effectively managed the primary symptoms of gastroesophageal reflux disease in male and female patients. This led to a significant alleviation of 94.2% for regurgitation and 93.5% for heartburn. Trexler et al. (2018) reported that the intake of 160 mg of hawthorn supplementation for 1 week in adult subjects did not affect electrocardiographic indices. Similarly, Erfurt et al. (2014) conducted a study indicating that sphygmomanometric blood pressure measurements taken before and after intervention did not show any significant effects on hypertension.

However, in a recent clinical trial conducted by Walker et al. (2006a, b), a high reduction in diastolic blood pressure was seen in patients with type 2 diabetes over a span of 16 weeks, following daily consumption of 1200 mg of hawthorn extract. Another study by Walker et al. (2002) revealed that patients with mildly hypertensive who took hawthorn extract (500–600 mg/day) for 10 weeks experienced a decrease in both diastolic and systolic blood pressure. Furthermore, Werner et al. (2009) found that short-term usage of camphor from *Crataegus* berry extract in women resulted in improved mental performance and blood pressure.

Table 8.3 presents a summary of the application of Hawthorn in humans.

8.3.12 Hawthorn Studies in Animals

Numerous research studies have provided evidence of the positive effects of hawthorn through experiments conducted on living organisms. These studies have observed various beneficial properties of hawthorn extract. For instance, it has been found to reduce atherosclerosis by inhibiting apoptosis and inflammation signaling pathways, preventing calcium accumulation in vascular smooth muscle cells, lipidosis, and proliferation, as well as regulating lipid levels. Additionally, it decreases levels of interleukin-1β, hypersensitive C-reactive protein, and monocyte chemoattractant protein-1 while enhancing adiponectin levels in the bloodstream and Bcl-2 levels in the aorta (Wang et al. 2019).

Similarly, administration of hawthorn leaf flavonoids to apo-lipoprotein E knockout mice resulted in improved atherosclerosis by promoting reverse cholesterol transport, inhibiting foam cell synthesis, and inducing the expression of antioxidant-related genes (Dong et al. 2017). Hawthorn fruit extract also

Table 8.3 Application of hawthorn, *Crataegus*, species in humans

Activity/disease	Dosage	Effect on patients	Reference
Antihypertensive effect	Over the course of 3 months, 60 hypertensive patients were given a hawthorn extract dosage of 450 mg twice daily	The intervention resulted in an increase in high-density lipoprotein levels and a decrease in low-density lipoprotein, total cholesterol, diastolic blood pressure, and systolic blood pressure levels	Al-Gareeb (2012)
Antihypertensive effect	For a period of 4 days, 21 patients were administered hawthorn extract two times a day at doses of 1000 mg, 1500 mg, and 2500 mg	The treatment lowered blood pressure	Asher et al. (2012)
Treatment of patient with New York heart association class II heart failure	Patients with NYHA class II heart failure were given *Crataegus* berry extracts three times a day, with a dosage of 30 drops per administration	After a period of 8 weeks, there was a confirmed improvement in tolerability and an increase in exercise tolerance	Degenring et al. (2003)
Treatment of patient with New York heart association class II heart failure	*Crataegus* extract was administered to subjects with congestive heart failure (NYHA class II)	After a period of 12 weeks, the in vitro parameters confirmed the safety and well-tolerated nature of *Crataegus* extract in the treatment of congestive heart failure (NYHA class II)	Zapfe (2001)
Anti-inflammatory effect	Over a period of 30 days, 37 diabetic patients were given 20 mL of diluted hawthorn vinegar (mixed with 40 mL of water) after their meals	The intervention resulted in a decrease in serum levels of triglyceride, LDL, cholesterol, and glucose, as well as a reduction in glycated hemoglobin, blood pressure, and body weight	Kadas et al. (2014)

demonstrated vascular protective activities in hypocholesterolemic rats by reducing reactive oxygen species and cholesterol levels and promoting bile acid production. Furthermore, the combination of resveratrol with hawthorn flavonoids after coronary artery bypass graft surgery led to a reduction in thrombotic restenosis and endothelial cell injury (Kwok et al. 2013).

Studies have shown that hawthorn leaf extract exhibited cardioprotective functions in rats by enhancing the antioxidant defense system, improving heart antioxidant biomarkers, increasing inflammatory cytokine biomarkers, and enhancing serum parameters related to heart function (Zhu et al. 2018). In rats with diabetes-induced cardiomyopathy, hawthorn leaf flavonoids demonstrated anti-inflammatory

and antioxidant effects by suppressing the activation of PKC-α (Turkistani 2019). Moreover, hawthorn displayed antiarrhythmic activity in rats through the administration of *Crataegus oxyacantha* alcoholic extract and reduced nutritive stress, lipid peroxidation, and apoptotic processes in rats with isoproterenol-induced myocardial infarction using the alcoholic extract of *Crataegus oxyacantha* berries (Vijayan et al. 2012; Min et al. 2017).

A study revealed that hawthorn extract possesses several properties, including antimelanogenesis, antioxidant, and antitumor effects. Administration of total oligomer flavonoids from hawthorn extract to tumor-implanted mice at a dosage of 150 mg/kg body weight for 21 days resulted in decreased tumor weight and volume, reduced melanin production, and inhibition of tyrosinase in melanoma cells. Additionally, it exhibited intracellular free radical scavenging activity, effectively preventing oxidative damage (Mustapha et al. 2016). Yonekubo et al. observed genotoxic effects when different concentrations of *Crataegus oxyacantha* fruit extracts were used in mice for 1 week (Yonekubo et al. 2018).

Furthermore, when hawthorn extract was combined with resistance training at a dosage of 100 mg/kg per day for 10 consecutive weeks, it significantly improved memory and learning in rats with type I diabetes. This improvement was attributed to the reduction of lipid peroxidation and the increase in total antioxidant capacity (Zarrinkalam et al. 2018). Another study demonstrated that administration of *Crataegus oxyacantha* leaves at dosages of 200 mg/kg and 400 mg/kg enhanced memory and learning in rats with scopolamine-induced amnesia. This effect was achieved by inhibiting dementia and oxidative damage (Paul et al. 2017).

Lee et al. discovered that an ethanol extract derived from the fruits of *Crataegus pinnatifida* showed potential in the treatment of Alzheimer's disease by inhibiting the buildup of amyloid β (Lee et al. 2019). Gan et al. observed that treating rats fed a high-fat diet with L. plantarum grade A pasteurized milk ordinance-fermented hawthorn juice for 28 days resulted in hypolipidemic activity. This activity was achieved by regulating adipose tissues and liver morphology, restoring liver tissue, and reducing levels of low-density lipoprotein cholesterol, serum total cholesterol, lipid vacuolization, and lipid metabolism (Gan 2019).

Additionally, Kim et al. demonstrated that administering *Crataegus pinnatifida* to mice with high-fat diet-induced obesity influenced gut microbiota activity, leading to decreased serum triglyceride levels, reduced fat and body weight, inhibition of adipogenesis and inflammation, and alterations in gut microbial abundance and diversity (Kim et al. 2019).

In a recent study, Lee et al. investigated the effects of different concentrations of HT048 (derived from *Citrus unshiu* peel and *Crataegus pinnatifida* leaves) in rats. After 12 weeks, they found that HT048 exhibited an anti-obesity effect by suppressing adipocyte differentiation and stimulating glycerol release. Moreover, it reduced the expression of peroxisome proliferator-activated receptor gamma and CCAAT/enhancer binding protein-alpha mRNA, resulting in decreased body weight, lower serum lipid content, reduced expression of hepatic lipogenesis-related genes, and increased expression of β-oxidation-related genes. These results indicate

that HT048 has beneficial effects in preventing obesity by inhibiting adipogenesis and lipogenesis (Lee et al. 2015).

The effects of hawthorn leaf flavonoids on rats with diabetic nephropathy were investigated, leading to improved renal function and reduced renal damage. This improvement was attributed to the reduction of oxidative stress injury and regulation of the p38/MAPK signaling pathway (Qin et al. 2019). Rats with hyperglycemia and dyslipidemia were treated with a methanolic extract of *Crataegus oxyacantha* at a dosage of 100 mg/kg body weight for 12 weeks (Kanyonga et al. 2011). Hawthorn exhibited hepatoprotective effects in rats with alcoholic liver damage by decreasing LDL and total cholesterol levels, regulating serum lipids such as triglycerides, and reducing various liver abnormalities, including sinusoidal distension, congestion, necrosis, steatosis, fibrosis, and cell damage markers (acid phosphatase, γ-glutamyl transpeptidase, alanine aminotransferase, and aspartate aminotransferase) (Aierken et al. 2017). Moreover, hawthorn demonstrated antioxidant activity by eliminating bilirubin, regulating glycogen levels in liver tissue, increasing serum total antioxidant capacity levels, and reducing lipid peroxidation (Martínez-Rodríguez et al. 2016). In mice fed with a high-fat diet, the administration of hawthorn pectin pentaglaracturonide at dosages of 150 mg/kg/day and 300 mg/kg/day for 10 weeks inhibited hepatic lipid accumulation and prevented fatty acid synthesis in the liver by reducing the expression of high-fat diet-induced sterol regulatory element binding factor-1c, pyruvate kinase, acetyl-CoA carboxylase, and fatty acid synthase (Li et al. 2017).

Mustapha et al. (2016) discovered that hyperoside and ethyl acetate extracts from *Crataegus azarolus* leaves exhibited antioxidant and immunomodulatory effects on macrophages, cytotoxic T lymphocytes, and natural killer cells. Hatipoglu et al. (2015) observed that daily administration of hawthorn extract (100 mg/kg/day) for 11 days prevented alveolar bone loss in rats with periodontal disease by regulating oxidative stress and serum total antioxidant levels. Wang et al. (2018) noted that the methanol extract of *Crataegus dahurica* fruit accelerated gastrointestinal tract movement and activated the antioxidant system. Liu et al. (2019) found that hawthorn polyphenol extract controlled skin damage induced by UVB radiation by suppressing p53, reducing DNA damage, eliminating excess ROS, downregulating pro-apoptotic BAX, and upregulating anti-apoptotic BCL-2, thus preventing apoptosis and suppressing caspase-3/9 activation. Shin et al. (2013) observed that the consumption of *Crataegus pinnatifida* extract promoted hair growth in mice by inducing the anagen phase, activating cellular signaling resulting in high proliferation and survival rates of human dermal papilla cells, and increasing the Bcl-2/Bax ratio, thereby protecting against cell death. Furthermore, Shi et al. (2019) reported that hawthorn leaf flavonoids exhibited protective effects on rats with dehydroepiandrosterone-induced polycystic ovary syndrome. Table 8.4 provides a summary of the various applications of hawthorn in humans, as demonstrated by several studies.

Table 8.4 Application of hawthorn, *Crataegus*, species in animals

Activity/disease	Effect on patients	Reference
Anti-atherosclerosis effect	In Wistar rats, the oligomeric proanthocyanidins extracted from *Crataegus oxyacantha* caused a decrease in the differentiation of monocytes to macrophages by reducing inflammation and downregulating the levels of monocyte chemoattractant protein-1 and vascular cell adhesion molecule-1	Mohana et al. (2015)
Radioprotective effect	Treating mouse bone marrow cells with phenolic compounds extracted from hawthorn (at a dosage of 200 mg/kg) resulted in a decrease in the stress and genotoxicity induced by 2-Gy γ-radiation	Hosseinimehr et al. (2006)
Dyslipidemia therapy effect	*Crataegus pinnatifida* fruit extract (250 mg/kg) for 7 days in high-fat diet-fed mice with hyperlipidemia reduced blood lipid and lipid degradation by enhancing the hepatic expression of peroxisome proliferator-activated receptor α	Niu et al. (2011)
Antibacterial effect	The extract from hawthorn fruit, containing monomers of (+)-catechin, (−)-epicatechin gallate, and (−)-epigallocatechin, exhibited the ability to regulate methicillin-resistant *Staphylococcus aureus* (MRSA) in septic mice by increasing the accumulation of daunomycin inside MRSA cells and downregulating the expression of the norA, norC, and abcA mRNAs which are the main efflux pumps of MRSA	Qin et al. (2013)
Analgesic and central nervous system activities	Administering hawthorn seed and pulp extracts to mice at a dosage of 1000 mg/kg resulted in a decrease in pain, sleep disorders, nervousness, and stress, with minimal toxicity	Can et al. (2010)
Cardioprotective effect	Administering an alcoholic extract of *C. oxycantha* to rats at a dosage of 0.5 mL/100 g body weight per day for 1 month prevented isoproterenol-induced myocardial infarction by decreasing the activity of enzymes involved in the Krebs cycle. This extract also prevented the peroxidative injury of mitochondrial lipids and preserved the mitochondrial antioxidant balance	Jayalakshmi et al. (2006a, b)
Cardioprotective effect	Administering an aqueous extract of *Crataegus tanacetifolia* leaf to rats at a dosage of 100 mg/kg for 4 weeks prevented the development of hypertension	Koçyoldoz et al. (2006)
Anti-atherosclerosis effect	Administering sugar-free *Crataegus pinnatifida* aqueous extract to rats with atherosclerosis regulated endothelial function, reduced inflammatory responses, and lowered serum lipid levels	Zhang et al. (2013)

8.4 Conclusion

The medicinal properties of *Crataegus* species, which include antispasmodic, cardiotonic, hypotensive, diuretic, and atherosclerotic effects, are present in various parts such as fruits, leaves, and flowers. The beneficial properties mentioned above are attributed to the diverse phytochemicals present in *Crataegus* species. However, additional research is needed to establish a definitive connection between the chemical composition of these plants and their efficacy in treating different medical conditions.

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Chapter 9
Hairy Root Cultures for Secondary Metabolite Production

Ravi S. Singh, Prakash Singh, Ruchi Kumari, and Sanjeev Kumar

Abstract Hairy root culture (HRC) is a promising biotechnological tool for the production of plant secondary metabolites under in vitro condition. This has been harnessed for the production of several molecules of medicinal and commercial importance. The hairy root phenotype appears at the site of infection of plants by *Rhizobium rhizogenes* which possess characteristics like fast growth even without hormone in the media, high genetic stability, geotropism, profuse lateral branching, differentiated tissue type, and high productivity. The content of secondary metabolites in these hairy roots is usually comparable to that of the fully grown plants. But, the major bottleneck for commercial exploitation of HRC is the developing protocol for scaling up production in bioreactors, as easy handling of interconnected hairy roots that are unevenly distributed in the vessel, is difficult. This chapter is focused on HRC with latest information available on basic method of induction of hairy roots to its uses in production of low volume and up-scaling in bioreactors, for commercial viability of HRC-based technology.

Keywords Secondary metabolites · Hairy roots · *Rhizobium rhizogenes* · *Agrobacterium rhizogenes*

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9.1 Introduction

Hairy root formation is induced by *Rhizobium rhizogenes* (earlier known as *Agrobacterium rhizogenes*) bacteria (Gutierrez-Valdes et al. 2020), at the site of infection by integration of T-DNA of the root-inducing (Ri) plasmid into the host genome. The hairy roots usually grow faster than plant cell cultures and yield more biomass. These hairy roots show rapid growth in hormone-free medium with extensive lateral branching and ageotropic nature, making these roots to be used as an attractive system for producing secondary metabolites. Further, their high genetic stability ensures the uniform productivity for longer period, which also makes these hairy roots as preferred material for HRC-based in vitro production. Several phytochemicals of medicinal and commercial importance, i.e., pharmaceuticals, cosmetics, dye, pesticides, food colorant, and food additives, have been reported. These hairy roots have potential to be utilized for in vitro production of secondary metabolites. Though several in vitro culture methods such as callus culture, cell suspension culture, adventitious root culture, and organ culture are available for secondary metabolites (Singh et al. 2010; Silja and Satheeshkumar 2015; Purwianingsih et al. 2016; Shoja and Shishavani 2021), the genetically transformed hairy roots are very attractive and promising keeping in view their differentiated nature and genetic stability and high production and productivity of secondary metabolites. The hairy root culture (HRC)-based production systems for secondary metabolites were reported for shikonins, camptothecins, azadirachtin, paclitaxel, hyoscyamine, harpagide, ginsenosides, and scopolamine (Sim and Chang 1993; Satdive et al. 2007; Almagro et al. 2015; Zhang et al. 2017; Balasubramanian et al. 2018; Singh et al. 2020; Barba-Espin et al. 2020; Wawrosch and Zotchev 2021; Table 9.1). As the plant-based phytoceuticals are in great demand worldwide, as per the estimate of World Health Organization (WHO), up to 80% of people take traditional herbs as medicines (Khan et al. 2009).

HRCs are usually capable of producing the structurally identical compounds similar to the ones found in roots of the naturally occurring intact parent plant, in contrast to as frequently observed in callus or cell suspension cultures. In comparison to undifferentiated cell culture, higher and stable yield of secondary metabolites are obtained with differentiation hairy roots. HRCs usually surpass the spatial barrier of production or accumulation of metabolites and could be employed also in cases where secondary metabolites accumulate or produced in other aerial tissues (stem, leaf, fruit, flower, seeds). HRC is considered to be more biosynthetically efficient than their mother plants. The hairy roots are also used for the production of those metabolites, which are synthesized and accumulate in aerial part of plants only, for example, lawsone and artemisinin, so irrespective of their origin or site of production, the metabolites could be obtained in hairy roots (Patra and Srivastava 2016; Bakkali et al. 1997). High genetic stability, differentiated tissue type, and high productivity are the features of HRC leading it to be used as valuable alternative method for the production of plant secondary metabolites. High branching, high growth rate, and genetic stability of these roots also suit for commercial up-scaling

Table 9.1 HRCs for secondary metabolite production

Secondary metabolite	Plant	Biological activities	Reference
Shikonin	*Lithospermum erythrorhizon, Arnebia euchroma*	Wound healing	Sim and Chang (1993), Singh et al. (2010)
Flavonoid/ biflavonoid	*Selaginella bryopteris*	Antimicrobial	Singh et al. (2018a, b, c, 2020)
Ginsenosides	*Panax quinquefolium*	Antimicrobial	Kochan et al. (2013)
Terpenoid indole alkaloids	*Catharanthus roseus*	Anticancer drugs (vinblastine, vincristine)	Almagro et al. (2015)
Curcumin	*Atropa belladonna*	Antimicrobial, wound healing	Singh et al. (2021)
Quercetin	*Raphanus sativus*	Antioxidant and anti-inflammatory effects	Balasubramanian et al. (2018)
Tanshinones	*Salvia miltiorrhiza*	Antioxidant, anti-inflammatory, and antitumor activities	Zhang et al. (2017)
Anthocyanin	Black carrots (*Daucus carota*)	Antioxidants	Barba-Espin et al. (2020)
Flavone	*Scutellaria baicalensis*	Antioxidants	Park et al. (2021)
Phenolic compounds	*Momordica dioica*	Antioxidants	Thiruvengadam et al. (2016)

even in bioreactors; hence, in recent years, focus has been on designing appropriate bioreactors suitable to culture the delicate and sensitive hairy roots (Rekha and Thiruvengadam 2017). But, the major bottlenecks for commercial exploitation of HRC are the developing protocol for scaling up production in bioreactors, as easy handling of interconnected hairy roots that are unevenly distributed in the vessel is difficult. In this chapter, we have discussed the HRC with latest information available on basic method of induction of hairy roots to its uses in production low volume and up-scaling in bioreactors for commercial viability of technology.

9.2 Molecular Mechanism of Hairy Root Development

Root loci (*rol*) genes present on T-DNA of the root-inducing (Ri) plasmid of *R. rhizogenes* are integrated into the host genome causing hairy root formation. The most studied agropine-type strains, *R. rhizogenes* strains, have two T-DNA regions designated as the TL-DNA and TR-DNA on their Ri plasmid (Nemoto et al. 2009). These regions get independently transferred to the nuclear genome of infected plant cells. The TL-DNA contains about 18 potential genes, of which 4 genes, *rol* A, B, C, and D, are implicated to induce the formation of hairy root in plants (Nemoto et al. 2009). The *rol*A gene suggested as an activator of growth and secondary

metabolism while *rol*B gene as stimulator or growth-suppressor (Bulgakov 2008). The *rol*C has self-activation property and reported to play a significant role in hairy root growth (17-fold increase); however, *rol* A, B, and C together had 75-fold increase in *Atropa belladonna* (Bonhomme et al. 2000), while the TR-DNA possesses the genes for opine synthesis and the genes involved in the auxin biosynthesis, i.e., *aux1* and *aux2*. The synergistic function of *rol*B, *rol*C, *ORF13*, and *ORF14* of TL-DNA of *A. rhizogenes* in hairy root induction in *Nicotiana tabacum* and the effect of these genes on the rolB-mediated rooting were in the order ORF13 > rolC≤ORF14 (Aoki and Syono 1999). The Ri plasmid of *R. rhizogenes* strain has *rolC* and *aux1* genes present on T-DNA designated as TL-DNA and TR-DNA, respectively. The detection of these genes in the hairy roots of host is indicative of T-DNA integration into the host genome. The virulence (*vir*) loci lying outside the T-DNA encode trans-acting products involved in early events in the plant-pathogen interaction. The *vir* loci are composed of six tightly regulated transcriptional units, *vir*A, *vir*B, *vir*C, *vir*D, *vir*E, and *vir*G, in different *Agrobacteria*. The *virD2* gene is localized outside the T-DNA of Ri-plasmid, and this feature serves as diagnostics for the presence of any leftover *Agrobacteria* in the root tissue (Thiruvengadam et al. 2016). These genes play a role in inducing the expression of defense genes, thereby eliciting the production of secondary metabolite in and development of transformed roots.

9.3 Establishment of Hairy Root Cultures

9.3.1 Induction of Hairy Roots

The hairy root induction is affected by the type of strains of *A. rhizogenes*, co-cultivation period, type of explants, media composition, and PGRs. We have investigated these factors in our earlier study (Singh et al. 2020) and discussed here along with updated information available in literature, in brief.

9.3.1.1 *A. rhizogenes* Strains

The virulence of the *A. rhizogenes* strains has been observed to vary strain-wise, and it has effect on the frequency of the hairy root formation. In our experiment (Singh et al. 2020), in *S. bryopteris* the strain LBA 1334 was found to induce hairy root formation, while other two MTCC series strains (MTCC 532 and MTCC 2364) could not. Perhaps, it was believed that the recalcitrance in *S. bryopteris* to genetic transformation and the virulence of these strain affected the hairy root formation. Kim et al. (2015) reported HRC of *Silene vulgaris* by infecting leaf explants with *A. rhizogenes* strains, LBA9402, R1000, A4, 13333, and 15834, and the strain LBA9402 had induced the most hairy roots per plant. In *Withania somnifera*, the hairy root induction by above strains of *A. rhizogenes* (MTCC 2364, MTCC

532, R1000) showed the strain R1000 to be highly virulent in inducing hairy roots (50.6%), in comparison to MTCC 2364 (29.3%) and MTCC 532 (18.6%) (Chandrasekaran et al. 2015). Explants infected by MTCC 2364 and MTCC 532 showed maximum 77.6% and 67.6% of hairy root induction, respectively (Balasubramanian et al. 2018). Among different strains (R1000, 15834, and A4), R1000 was the most promising for hairy root stimulation as it was found to induce the highest growth rate, root number, root length, and transformation efficiency in *Fagopyrum tataricum* (Thwe et al. 2016). Joseph Sahayarayan et al. (2020) investigated effects of *A. rhizogenes* strains such as 15834, 13333, A4, R1200, R1000, LBA9402, R1301, and R1601 hairy root induction in *Cucumis anguria*. Their finding also supported the R1000 strain of *A. rhizogenes* as the most virulent and the best strain for hairy root initiation of *C. anguria* from cotyledon explants. Yousefian et al. (2020) reported hairy root induction in *Mentha spicata* by direct injecting of explants with *A. rhizogenes* strains (A13, R318, ATCC15834 A4, and GMI 9534). They found that of these four strains, the strain A13 exhibited the highest transformation efficiency (~75%).

9.3.1.2 Co-Cultivation Period

The co-cultivation period during transformation of explants with the *R. rhizogenes* strain has also shown to impact the hairy root formation. In *S. bryopteris*, the effect of co-cultivation period was examined for hairy root induction with the strain LBA1334 grown along with explants for 24 and 48 h (Singh et al. 2020). It was observed that the hairy root appeared after 6 days of infection with this strain in case of 48 h co-cultivation only. In *Raphanus sativus*, co-cultivation of explants with *A. rhizogenes* strain in half-MS medium containing acetosyringone (100 μM) for 2 days had the maximum effect on hairy root induction (Balasubramanian et al. 2018).

9.3.1.3 Type of Explants

Hairy roots usually induced in explants such as leaf, cotyledon, hypocotyl, node, stem, and root within 1–4 weeks of culture. Singh et al. (2020) reported hairy root induction after 6 days of infection using *A. rhizogenes* strain LBA1334 co-cultivated with root network as explants for 48 h after transformation. Yousefian et al. (2020) found that the middle and lower internodes of stem were highly susceptible to infection by *A. rhizogenes*, and these showed a higher rate of transformation. In *Cucumis melo,* cotyledon as explants was observed to give higher frequency of hairy root formation (Pak et al. 2009).

9.3.1.4 Media

The media compositions do affect the hairy root formation. Singh et al. (2020) tested different media like MS, SHFR (Stag Horn Fern Rooting), and Knops during transformation for hairy root induction by *A. rhizogenes* and found that SHFR plus TDZ (2 mg/L) and Bavistin (0.1%) showed good response in transformation and hairy root formation. The culture media incorporated with acetosyringone (an amino acid-derived phenolic compound) utilized by the invading *Agrobacterium* for food enhances the transformation rate by inducing the expression of *vir* gene (Veluthambi et al. 1989). Rana et al. (2016) reported the effect of medium supplements (30 g L^{-1} sucrose, 0.1 g L^{-1} l-glutamine and 5 g L^{-1} polyvinylpolypyrrolidone) on *A. rhizogenes*-mediated hairy root induction in *Camellia sinensis* var. sinensis. In HRC of *A. indica*, different media varying in ionic strength such as Ohyama and Nitsch (ON), Gamborg's B5, and MS basal were used that yielded maximum amounts of biomass and azadirachtin in correlation with their ionic strength (Satdive et al. 2007). As ON medium contained the higher ionic concentrations of inorganic salts than the MS and B5, it was found to favor the growth of hairy roots as well as azadirachtin production.

9.3.1.5 PGRs

Though hairy roots have inherent ability to grow even in the absence of PGRs, the addition of PGR elicits the secondary metabolite production. Singh et al. (2020) reported SHFR media modified with PGRs in combination or alone (Kinetin, TDZ and Bavistin). The SHFR media with TDZ (2 mg/L) and Bavistin (0.1%) was found to increase the transformation efficiency and propagation of hairy roots (65%), while with Kinetin (2 mg/L) alone the response was 40% only. The phytohormone treatment, 0.3 mg L^{-1} IBA and 100 μM MeJA, in *M. spicata* was reported to substantially increase in the hairy root growth and phenolic acid accumulation (Yousefian et al. 2020). Joseph Sahayarayan et al. (2020) found that MS medium supplemented with IBA + NAA (2.46 + 1.07) had the maximum accumulation of biomass (0.68 g/L dry wt. and 6.52 ± 0.49 g/L fresh wt.) in 21-day-old transgenic hairy roots of *Cucumis anguria*. Methyl jasmonate elicited the biosynthesis of triterpenoid saponins in the hairy roots of *S. vulgaris* leading to increased level of segetalic acid (fivefold) and gypsogenic acid (twofold) than control hairy root (Kim et al. 2015). In transgenic hairy root line of *Taxus x media* var. Hicksii carrying a *taxadiene synthase* gene, the paclitaxel production and phenyl ammonia lyase activity upon elicitation with nitric oxide and methyl jasmonate yielded the highest paclitaxel content (7.56 mg L^{-1}) (Sykłowska-Baranek et al. 2015).

9.3.2 Molecular Confirmation of Hairy Roots

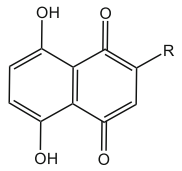
The integration of Ri T-DNA into the genome of host plant cells causes the induction of hairy roots. The Ri T-DNA contains *rol* and *aux* genes, so these genes also integrate into the host genome along with T-DNA. The transgenic nature of hairy roots could be confirmed by simply amplifying these genes by PCR using *rolC* and *aux1* gene primers. While the non-transgenic hairy root line can be detected by amplifying the *virD2* gene present in in Ri-plasmid but not on T-DNA, it could be used as a diagnostic marker for the presence of any remaining *Agrobacteria* in the root tissue (Thiruvengadam et al. 2016). In *S. bryopteris*, *rol*A and *vir*C genes were PCR-amplified from the hairy roots indicating that the hairy roots developed were due to T-DNA integration and not due to any *A. rhizogenes* present (Singh et al. 2020).

9.4 Productions of Secondary Metabolites in Using Hairy Root

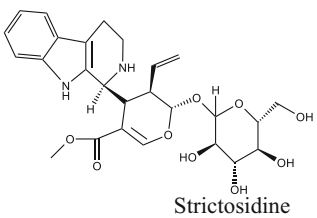
HRC-based production of several plant secondary metabolites with known bioactivities such as shikonins, stilbenes, lignans azadirachtin, camptothecins, paclitaxel, hyoscyamine, harpagide, ginsenosides, and scopolamine was reported (Sim and Chang 1993; Satdive et al. 2007; Almagro et al. 2015; Zhang et al. 2017; Balasubramanian et al. 2018; Singh et al. 2020; Barba-Espin et al. 2020; Wawrosch and Zotchev 2021; Table 9.1; Fig. 9.1).

9.4.1 Shikonin

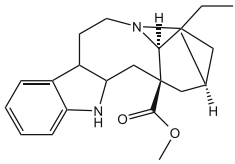
The HRC of *Lithospermum erythrorhizon* was reported long back in 1991 by Shimomura et al. (1991) using in vitro grown shoots for transformation with *A. rhizogenes* strain 15,834. They found that the hairy roots cultured on MS solid medium failed to produce any red pigments; however, in solid or liquid root culture media, a large amount of red pigments was produced. Further, they added adsorbents to the culture medium that stimulated shikonin production by approximately threefold. In *Echium plantagineum*, the hairy root lines developed using *A. rhizogenes* strain ATCC15834 (Fu et al. 2020) showed significant difference in the biomass and shikonin production in the 1/2B5 and M9 media. It was observed that the biomass in the 1/2B5 medium was fivefold than the M9 and the content of acetylshikonin was twofold in the 1/2B5 medium (36.25 mg/L on average) than the M9 medium.



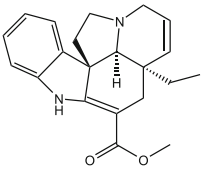
Shikonins



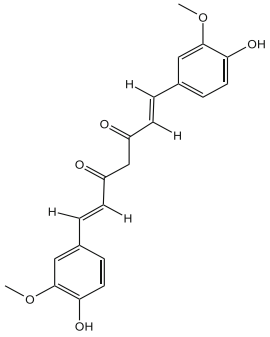
Strictosidine



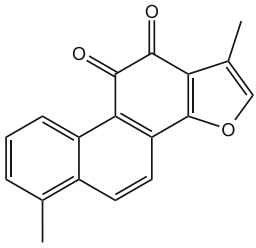
Catharanthine



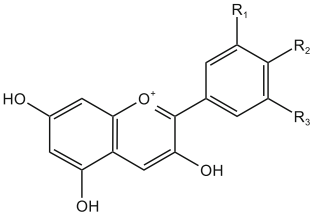
Tabersonine



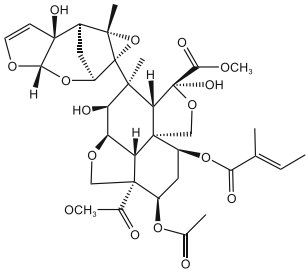
Curcumin



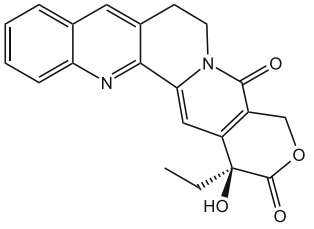
Tanshinones



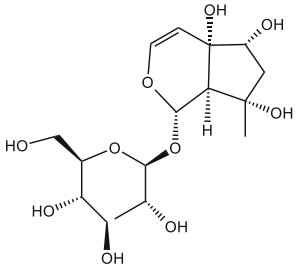
Anthocyanin



Azadirachtin

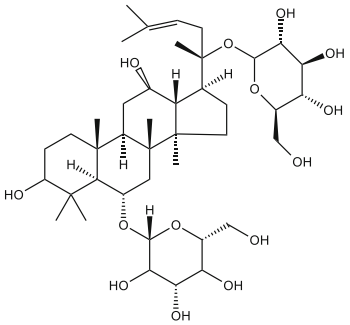


Camptothecins

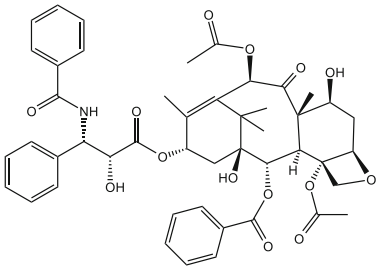


Harpagide

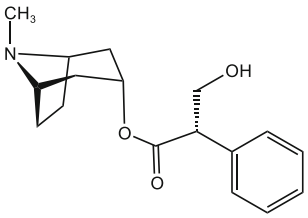
Fig. 9.1 Chemical structures of some of the well-known secondary metabolites produced in HRC



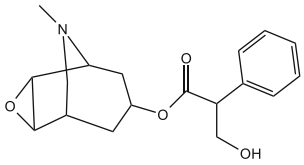
Ginsenosides



Paclitaxel



Hyoscyamine



Scopolamine

Fig. 9.1 (continued)

9.4.2 Phenolic Compounds of HRC

Several phenolic compounds such as rosmarinic acid, caffeic acid, chlorogenic acid, lithospermic acid B, and cinnamic acid were observed in the hairy roots of *M. spicata*, developed by infection with different *A. rhizogenes* strains (ATCC15834, A13, A4, 9534, and R318), and their content was compared (Yousefian et al. 2020). Ho et al. (2018) reported HRCs of *Polygonum multiflorum* from leaf explants infected by *A. rhizogenes* strain KCCM 11879 with more than 60% transformation efficiency after 21 days of co-cultivation. It was found that the line HR-01 yielded the highest biomass (9.7 g L^{-1} of DW) and total phenolic content (26.64 mg g^{-1} DW).

9.4.3 Triterpenoid Saponins

Phytochemical studies of *Gynostemma pentaphyllum* revealed nearly 90 dammarane-type saponin glycosides, known as gypenosides, that show pharmacological activities (Yin et al. 2004). HRC of *G. pentaphyllum* was established by infecting leaf discs with *A. rhizogenes* (Chang et al. 2005); it yielded 7.3 g L(−1) the dry wt. of biomass in MS medium for a period of 49 days with a gypenoside content

of 38 mg g(−1) dry wt. Kim et al. (2015) also reported the in vitro production of triterpenoid sapogenins in HRC of *S. vulgaris* by infecting leaf explants with five strains of *A. rhizogenes*.

9.4.4 Artemisinin

Mass cultivation of hairy roots in *Artemisia annua* was reported in a modified 3-L stirred tank bioreactor using optimized culture conditions (Patra and Srivastava 2014). In this bioreactor, it was possible to produce biomass, 18 g L(−1) (dry wt.), and artemisinin content, 4.63 mg L(−1), in a period of 28 days, which further increased to 10.33 mg L(−1) in response to methyl jasmonate. Elicitation of artemisinin production with 150 mg chitosan l(−1) in hairy roots of *Artemisia annua* was reported to be increased sixfold to 1.8 microg mg(−1) dry weight in 6 days (Putalun et al. 2007). Wang et al. (2006) reported use of oligosaccharide elicitor (MW < 2500) from an endophytic fungus, *Colletotrichum gloeosporioides*, for the stimulation of artemisinin production in hairy roots of *A. annua*. They observed that the 23-day-old hairy roots on exposure to the elicitor at 0.4 mg/mL for 4 days yielded the maximum artemisinin content, 13.51 mg/L (51.63% increase over the control). Further, it was also reported that the nitric oxide generated by an oligosaccharide elicitor from *Fusarium oxysporum* mycelium potentiates its role in induction of artemisinin production in *A. annua* hairy roots (Zheng et al. 2008). The combination of sodium nitroprusside (NO donor) with OE increased artemisinin content from 1.2 to 2.2 mg/g dry wt., whereas the content of artemisinin in HRC was 28.5 mg/L, a twofold increase over the OE treatment alone. Tetraploid clones of *Artemisia annua* hairy roots produce more artemisinin (six times) than diploid parent (De Jesus-Gonzalez and Weathers 2003). The physical and chemical factors such as light, pH value 5.4 of the medium, and 3% sucrose in the medium along with gibberellin (4.8 mg/L) had effect on the growth of the hairy roots and production of artemisinin (Cai et al. 1995).

9.4.5 Paclitaxel

Paclitaxel (trade name: Taxol®) is a potent anticancer agent extracted from the yew plants (*Taxus* spp.). Due to its low accumulation levels in the plants of *Taxus* spp., the yield of paclitaxel is limited and could not meet the demand for the pharmaceutical use. Therefore, alternative methods including HRC have been attempted for in vitro production of paclitaxel from *Taxus* spp. (Kim et al. 2009; Sykłowska-Baranek et al. 2015; He et al. 2022). HRCs have been reported in different *Taxus* spp. such as of *T. cuspidate* (Korean yew) (Kim et al. 2009), *Taxus x media* var. Hicksii (Sykłowska-Baranek et al. 2015); and *T. baccata* (He et al. 2022) using *A. rhizogenes*. Kim et al. (2009) reported that methyl jasmonate treatment of the

hairy root line (RC11106) led to accumulation of 52.5 mg/L of taxol over 2 weeks of incubation. Paclitaxel production in HRC of *Taxus x media* var. Hicksii with a *taxadiene synthase* transgene upon elicitation by nitric oxide and methyl jasmonate enhanced the paclitaxel content to 7.56 mg L^{-1} after 2 weeks of treatment (Sykłowska-Baranek et al. 2015).

9.4.6 Azadirachtin

Azadirachtin is a limonoid ($C_{35}H_{44}O_{16}$, tetranortriterpenoid) and the major component of widely used neem (*Azadirachta indica)*-based biopesticides. In HRC of *A. indica*, the effect of ON, Gamborg's B5, and MS basal media was investigated on yield of biomass and azadirachtin content (Satdive et al. 2007). Out of the three media, ON medium, that contained higher ionic concentrations of inorganic salts than the MS and B5 media, favored the growth and azadirachtin production (0.0166% dry weight, DW). Further, addition of biotic elicitor increased the production of azadirachtin by nearly fivefold (0.074% DW), while jasmonic acid and salicylic acid showed an approximately six- (0.095% DW) and ninefold (0.14% DW) increase, respectively, on ON medium (Satdive et al. 2007). The mass culture of hairy roots of *A. indica* in gas-phase reactors (nutrient spray and nutrient mist bioreactor) yielded the biomass 9.8 g/L dry wt. and azadirachtin accumulation of 2.8 mg/g (Srivastava and Srivastava 2012).

9.4.7 Camptothecin

Camptothecin, an important anticancer drug, is believed to be a potent topoisomerase inhibitor that interferes with the topoisomerase in DNA replication HRC of *Ophiorrhiza alata* which was developed by infection of nodal explants of in vitro-grown plant with *A. rhizogenes* TISTR 1450 for camptothecin (CPT) production (Ya-ut et al. 2011). The content of CPT in various parts of *O. alata* was analyzed by HPLC and found to be double in transformed hairy roots (785 ± 52 μg/g dry wt) than in the soil-grown plants (388 ± 32 μg/g dry wt). Kamble et al. (2011) reported hairy root induction in *O. rugosa*, another source of CPT, using *A. rhizogenes* strain LBA9402. The CPT contents in the hairy roots and in vitro-grown transformed shoots were 0.009% d.w. and 0.012% d.w., respectively.

9.4.8 Ginsenoside

Ginseng (*Panax ginseng*) roots contain ginsenosides (saponins), utilized for its bioactive properties such as immunomodulatory, hepato- and cardioprotective,

stamina booster, antifatigue, and physiological and pharmacological effects. Yu et al. (2005) investigated the impact of temperature and light quality on biomass and ginsenoside content of HRC in the bioreactor. They observed that biomass of hairy roots was highest under dark or red light, while ginsenoside content was optimum under fluorescent light. Kochan et al. (2013) reported three independently generated hairy root (A, G, and B) of *P. quinquefolium* upon infection by *A. rhizogenes*. It was found that the line A had the highest increase of dry biomass (above eightfold) followed by line G (sevenfold) and line B (fivefold) for the period of 28 days culture. In the developed hairy roots of *P. quinquefolium*, the total ginsenoside level in hairy root lines A, G, and B were recorded to be about 10, 8, and 6 mg/g dw, respectively (Kochan et al. 2013).

9.4.9 Hyoscyamine

In *Datura stramonium*, HRC developed with hairy roots of different ploidy (diploid and tetraploid) levels was investigated by Pavlov et al. (2009) for hyoscyamine biosynthesis. The ploidy level difference was observed in content of hyoscyamine and the influence of nutrients growth of hairy root. The HRC of tetraploid plants had the maximum yield of hyoscyamine (177 mg/L) in MS medium with 6% sucrose.

9.4.10 Scopolamine

Scopolamine is a tropane alkaloid that shows anticholinergic property. The overexpression of genes of scopolamine biosynthesis *putrescine N-methyltransferase* (*PMT*) and *hyoscyamine 6 β-hydroxylase* (*H6H*) in *Hyoscyamus niger*'s transgenic HRC produced significantly higher ($P < 0.05$) levels of scopolamine in comparison to the wild-type and transgenic lines harboring *pmt* or *h6h* gene alone (Zhang et al. 2004). The line (T3) overexpressing the genes, *pmt* and *h6h*, was observed to produce the highest content of scopolamine (411 mg/L) followed by single-gene (*h6h*) transgenic line H11 (184 mg/L) and wild type (43 mg/L). Dehghan et al. (2017) found that the tetraploidy improved the overexpression of *h6h* and scopolamine production of *H. muticus*.

9.4.11 Harpagide

Harpagide is an *iridoid* glycosides that show anti-inflammatory properties. Only a few literature are available on the hairy root line development for harpagide production. Piątczak et al. (2019) reported HRC in *Rehmannia elata* (Orobanchaceae) from shoot tips and leaves by the infection of *A. rhizogenes* strain (A4). They

observed the different levels of iridoid glycoside content, in the hairy root line (S1 line) were aucubin (0.2 mg/g DW) and harpagide (1.57 mg/g DW), and in L14 line, harpagoside (0.09 mg/g DW) were the highest.

9.5 Upscaling the Productions of Secondary Metabolites in Bioreactors Using HRCs

For scaling up the production of secondary metabolites and biomass of hairy roots, a large vessel (bioreactor) is needed that can provide the best conditions for optimum growth and secondary metabolite production. But the delicate and interconnected form of the hairy roots makes the growth measurement and designing of a large-scale culture system a difficult task. Stiles and Liu (2013) have discussed the major factors related to large-scale bioreactor cultures such as process intensification technologies and the mathematical models and computer-aided methods of bioreactor design and development. Wyslouzil et al. (2000) suggested that the bioreactor design for HRC is a balancing act between the biological needs of the tissues without inducing an additional and undesirable biological response. The bioreactors used in HRC are usually (1) liquid-phase, (2) gas-phase, and (3) hybrid reactors.

Bioreactor designs for HRC are challenging and should meet the criteria such as least mechanical agitation (may cause wounding and shearing of delicate hairy roots leading to callus formation), uninterrupted nutrient flow (hampers due to branching and interconnected forms hairy roots), regular oxygen supply, nutrient uptake, etc. in the medium. An air-lift fermenter system along with a XAD-2 column was used for the shikonin production from HRC of *L. erythrorhizon* (Shimomura et al. 1991), which continuously produced ~5 mg/day of shikonin during a period of over 220 days. Srivastava and Srivastava (2012) reported azadirachtin production by hairy roots of *A. indica* using HRC in gas-phase reactors (nutrient spray and nutrient mist bioreactor). They found the biomass production of 9.8 g/L dry weight with azadirachtin accumulation of 2.8 mg/g biomass (27.4 mg/L) during 25 days of batch cultivation period.

The study by Zhang et al. (2004) provided an effective approach for large-scale commercial production of scopolamine by using transgenic hairy root culture systems as bioreactors. Homova et al. (2010) investigated the capacity of HRC of *Harpagophytum procumbens* to accumulate four phenylethanoids, viz., glycosides beta-OH-verbascoside, verbascoside, leucosceptoside A, and martynoside in shake flasks and a stirred tank reactor. They observed equally highest contents of verbascoside in both kinds of culture (1.12 mg/g dry weight), while leucosceptoside A content was 1.6 times higher in bioreactor than in shake flask. Several reviews are available discussing bioreactor-based scale-up using HRCs (Valdiani et al. 2019).

9.6 Elicitors Increase the Secondary Metabolite Production in HRCs

Various biotic and abiotic elicitors are used in HRCs to elicit the biosynthesis and the accumulation of secondary metabolites (Yousefian et al. 2020; Singh et al. 2020; Joseph Sahayarayan et al. 2020). These elicitors are known to activate different genes such as secondary metabolic pathway, defense-related or signaling pathway, and transcription factors. In general, biotic elicitors used in HRC include fungal cell extracts, fungal or plant polysaccharides, and heavy metals, while the abiotic elicitors include PGRs (auxins, TDZ, cytokinins), signaling molecules (nitric oxide, hydrogen peroxide, salicylic acid, methyl jasmonate), UV radiation, ultrasound, shaking, and temperature. For the best result on elicitation of in vitro production of metabolites in the medium, the selection of suitable elicitor, its level to be added, and treatment duration need to be standardized. The elicitor treatments like other tissue culture methods also stimulate the production and release of intracellular products in the hairy roots.

9.7 Prospect and Challenges

HRC-based in vitro culture is a promising approach for production of a wide range of secondary metabolites as compared to other in vitro methods such as cell culture and adventitious root culture (Table 9.2). Several factors critical to increase in yield of HRC have been standardized such as light, oxygen, temperature, phytohormones, heavy metals, elicitors, external stimuli, etc. The metabolic pathway engineering for modulation of expression of key genes either by overexpression of genes and transcription factors or by downregulation of competing pathway genes has also

Table 9.2 Comparison of hairy root culture with other tissue culture methods

Description	Hairy root culture	Cell culture	Adventitious root culture
Production of secondary metabolite	Comparable to roots without elicitation	Elicitation may require	Elicitation may require
Tissue type	Differentiated	Undifferentiated	Differentiated
Phytohormone required	No	Yes	Yes
Growth rate	Higher	Slower but high in liquid cell suspension	Higher
Genetic and biochemical stability	Stable for several subculture	Unstable	Stable
Genetic engineering possible	Yes	Yes	Yes
Scale-up	Yes	Yes	Yes
Cost	Expensive	Expensive	Expensive

led to increased yield of end-products. With advances in genomics and molecular biology, today we have a better understanding of the biosynthetic pathway and their regulation, thus realizing that the biosynthetic potential of hairy roots is more feasible. Commercial production of secondary metabolites that have phytoceutical uses, from field-grown plants, can't meet the market demand, for example, the extraction of catharanthine and vindoline from field plants and then chemical semi-synthesis of vinblastine and vincristine, two chemicals usually found in the FDA drug shortage list due to supply chain problems (Kaiser 2011). Due to complex chemical structures, most of secondary metabolite economic feasibility for the industrial mass production through chemical synthesis is not possible and thus not popular as method of choice.

Despite vast potential and prospect of HRCs, very few companies (public or private sector) have initiated the HRC-based commercial production phytochemicals from hairy roots or any in vitro methods unlike a large number of companies worldwide involved in the mass production of tissue culture raised plants. ROOTec bioactives Ltd. (www.rootec.com) is the only company founded in 2005 in Switzerland for the commercial production of plant-derived molecules using bioreactor technology to provide products for the pharmaceutical and cosmetic markets. Besides in vitro production of secondary metabolites, the hairy roots have been useful for the functional validation of genes, plant synthetic biology, genome editing, symbiotic root colonization, antimicrobial excretion, and phytoremediation (Morey and Peebles 2022). Deciphering the competing biosynthetic pathways or identifying feedback or feed-forward reactions in metabolite production and then devising strategies is important for increasing the yield desired product. The major hurdle in commercial exploitation of HRC is the development of suitable protocol for the sustainable growth of hairy roots and designing of appropriate bioreactors for scaling-up without damaging the delicate hairy roots. Metabolic engineering for manipulation of biosynthetic pathways for high yield of secondary metabolites is easier nowadays with the availability of vast genomic and transcriptomic datasets helping better understanding of regulation of pathways.

9.8 Conclusion

Among various tissue culture methods, HRC has the potential to be harnessed for in vitro production of secondary metabolites at commercial level. It could be an economically viable option for sustainable in vitro production of secondary metabolites, as the hairy roots are normally high yielding due to their fast growth rates, possible growth in hormone-free media, and genetic and biosynthetic stability. These are also amenable to genetic manipulation using genetic engineering including recent gene-editing approaches. In future, the HRC efficacy and commercial viability could be enhanced by adopting advanced biochemical processing methods for metabolite extractions, genetic manipulations, and elicitations for enhanced yield and bioreactor-based up-scaling.

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Chapter 10
Secondary Metabolite Basis of Elicitor- and Effector-Triggered Immunity in Pathogen Elicitation Amid Infections

Ashfaq Ahmad Shah and Amit Gupta

Abstract Biotic stress refers to stress triggered by other living creatures like fungi, bacteria, viruses, parasitic organisms, nematodes, parasitic mites, insects, animals, noxious weeds, and farmed or natural plants wrecking an organism. Because of the enormous financial damages imposed on fruit and lucrative crops, biotic stresses are the primary focus of agricultural studies. Plants and their disease-causative agents evolved alongside one another over hundreds of millions of years of age. Such a coevolutionary dynamic has culminated in the development of a diverse variety of plant defenses toward their parasites, all of which serve to reduce the incidence and extent of assault. These defenses comprise both anatomical and physiological modifications, which can be exhibited persistently or, in numerous instances, only after being attacked. Microbe-associated or pathogen-associated molecular patterns (MAMPs/PAMPs) deemed to be ubiquitous across microbial groups are principally instrumental in inducing a defense outcome following biotic or abiotic duress. The plant's core immune apparatus operates to identify and generate defenses against these patterns, but constantly shifting microorganisms articulate an assortment of effectors that are race or species-specific and help promote parasite infectiousness by dampening these adaptations. In response to this, plants express specialized receptors known as R proteins which sense pathogenic effectors and launch powerful defenses. Infectious agents can then modify or eliminate their recognized effectors to evade defense stimulation, at the risk of incurring an adaptation cost as a result of the depletion of their recognized effectors. Plant resistance may be activated by both eliciting agents and effectors, which are referred to as PAMP-triggered and effector-triggered immunity (PTI and ETI, correspondingly). Salicylic acid, jasmonic acid, and ethylene are important drivers of interrelated channels of signaling of plant defense systems that help plants resist adversity. Cellular redox shifts, ion channel gating, oxidative burst, or protein kinase signaling cascades that right away stimulate

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cellular alterations like cell wall stimulation, secretion of antimicrobials, boosting the amount of reactive oxygen species (ROS) within the cell, and elevating the level of calcium inside the cell in question, leading to apoptosis, promote shifts in the expression of genes that subsequently upgrade phytoanticipins and phytoalexin levels. Current studies are increasingly concentrating on the secondary metabolite foundation of retaliation to parasites, and investigators employ metabolomic techniques to explore the defense-related compounds synthesized as a reaction to elicitors, effectors, and other stressful situations.

Keywords Elicitors · Microbe-associated molecular patterns · Secondary metabolites · Effectors · Pattern recognition receptors · Phytoalexins

10.1 Introduction

A disease trinity is a three-way relationship between the infectious agent, the organism, and the surroundings that determine disease incidence. Higher life forms in biological networks experience a profusion of biotic and abiotic adversaries that put a strain on the regular physiological operations in their bodies. The link between biological stress, plant development, and yield influences both socioeconomic and functional factors. Biotic damage has an effect on population trends, plant-stressor mutual evolution, and the ecological cycling of nutrients (Abdul et al. 2020). There are essentially two types of invading parasites: those that assault the cells of their hosts with toxic secretions and collect resources from cells that have been destroyed before right after colonization (necrotrophs) and those that need surviving cells as a supply for nourishment (biotrophs) (Ali et al. 2018). In plant life, biological stressors primarily influence photosynthesis because gnawing insects diminish the area of leaves, while virus and fungal infestations decrease the efficiency of photosynthesis per area of foliage. Vascular-wilt fungi impair water movement and photosynthesis by restricting stomatal opening (Bernard et al. 2017). Plants use built-up resistance to defend themselves from an extensive variety of plant pathogens, insects, and abiotic stresses when proper signals are detected. This robust resilience that arises in response to duress is termed as systemic acquired resistance (SAR) or induced systemic resistance (ISR). Additional terms, including plant immunization, translocated retaliation, and provoked resistance, are being proposed to describe gained systemic resistance over the course of time (Bolouri Moghaddam and Van Den Ende 2012). Necrotrophic, biotrophic, and hemibiotrophic (largely biotrophic stage followed by a necrotrophic period) infectious agents such as fungi, viruses, bacteria, and phloem-feeding bugs prompt systemic induced resistance (Ádám et al. 2018). Whenever eliciting agents or inducements activate the defense genes in plants, it results in that type of resistance. Higher life forms possess a plethora of preformed or developed defense systems to deal with a variety of stress, both biotic and abiotic. These defenses comprise a combination of physical and chemical modifications. The primary line of defense against pathogens is a hypersensitive response (HR), a type of local defense

barricade that governs cell demise at the region of invasion (Alabadí and Blázquez 2009). Using abundant mineral ion quantities acquired from the rhizosphere, for instance, allows plants to mitigate the detrimental impacts of biological stressors while additionally avoiding the onset of catastrophic metal poisoning by maintaining metal ions from dispersion all over the plant with vigilant biochemical mechanisms. Notwithstanding being devoid of a complex immune and circulatory system as in animals, plants have the knack of sensing intruders via the detection of non-self-impulses. The initial line of defense, dubbed PAMP-triggered immunity (PTI), manages the most likely invaders (Zipfel 2014). Elicitors and receptor proteins are two essential components of defense signaling in plant defense. The early identification of microorganism-associated molecular patterns (MAMPs), which operate as broad eliciting agents of defense reaction, is frequently a plant's primary means of defense against pathogenic microbes. MAMPs may be nucleic acids shared by microbes or bacterial plasma membrane endotoxins (LPS) identified by specialized pattern recognition receptors (PRRs) occurring on plant cell plasma membranes. Additional compounds that function as triggers and are recognized by plant PRRs include DAMPs (damage-associated molecular patterns) and HAMPs (herbivory-associated molecular patterns) (Wang et al. 2019). Elicitors promote the transcription of genes pertaining to pathogenesis in the host cell in order to establish systemically induced resistance. Exogenous and endogenous elicitors are the two types of elicitors. Endogenous elicitors are chemicals generated by cells in the aftermath of an infection by a pathogen and are largely plant cell wall ingredients, whereas exogenous elicitors are proteins or other chemicals synthesized by microbes. Fungal elements that include oligo chitin, hepta-glucoside, and oligochitosan, as well as plant cell wall constituents like oligogalacturonide, are commonly used as discrete oligosaccharide elicitors for elicitation. Oligosaccharides are among the most prevalent and well-known elicitors that have been well-studied. All the eliciting agents are recognized by an attachment protein designated after the elicitor, such as oligochitosan elicitor coupling protein, hepta-glucoside elicitor coupling protein, oligo chitin elicitor coupling protein, and glycopeptides/glycoprotein interaction proteins (Thakur and Sohal 2013). The pathogen's pathogenic effector compounds cause effector-triggered defenses. Effectors complement the virulence of the pathogens and allow them to flee from the PTI following the inhibition of the host's recognition mechanism by PTI signaling and successfully commence colonization. Thus, pattern recognition immunity (PTI) and effector-triggered immunity (ETI) are activated in response to the capture of eliciting and effector molecules, correspondingly (Suzuki et al. 2014). The enactment of the initial and second lines of defense conduces to the emergence of resistance that is triggered in tissues located at far from the point of contamination as an outcome of the transfer of a single remote impulse among healthy plant tissues in order to trigger more effective defense responses, known as systemic acquired resistance (SAR). The overexpression of several defense mechanisms, including defensive chemical signaling chemicals, is the functions of both PTI and ETI. For the most part, phytohormones like salicylic acid (SA), jasmonic acid (JA), and ethylene (ET) control provoked defense mechanisms. Salicylic acid buildup initiates a defense mechanism, which activates

biochemical defense weaponry and assists in the decrease of ensuing infection in adjacent tissues (Ballaré and Austin 2019). The ligand-receptor engagement and afterward signaling and stimulation lead to several phenomena like membrane channel gating; cellular redox alterations; protein kinase signaling; transcription of some genes that immediately stimulate oxidative burst; enhancement and de novo production of secondary bioactive compounds; activation of defensins, prohibitins, and defense enzymes; enhancement in the levels of calcium inside the plant cell via hormonal signaling; and hypersensitivity reaction that is a local defense barrier and regulates cell death (apoptosis) at the injection site (Cheng et al. 2012). The enhanced synthesis of metabolites particularly secondary bioactive compounds that in this context are termed phytoanticipins and the bioactive compounds that are synthesized de nova is as dubbed phytoalexins. By acting straightaway as antimicrobial and antioxidant agents, these kinds of chemicals lessen cellular damage, boost the ability to resist infections and pests, and slow the dissemination of intruders (Tiku 2020).

Secondary metabolites, also dubbed as specialized metabolites, are organic byproducts of metabolism generated by any living organism, such as fungi, bacteria, mammals, or plants, but have no primary role in the regular development, growth, or reproduction of an organism. Nevertheless, they often operate as mediators between interactions in the environment, which might provide the organism with an edge in selection by boosting its capacity to survive or reproduce. Secondary metabolites frequently regulate collaborative relationships like the pollination cycle and sharing of resources as well as conflicting relationships like predation and rivalry. Typically, secondary metabolites are restricted to just one lineage or a single species; however, there is substantial proof that the horizontal exchange of complete processes between genera or species has played an integral part in the diversification of microbes (Ashfaq et al. 2021). Secondary metabolites from herbs, fungi, and algae are used by humans as pharmaceuticals, flavorings, colors, and medications. Plant secondary metabolites may be categorized into many types based on their chemical makeup, including terpenes, phenylpropanoids (phenolics), S- and N-possessing compounds, polyketides, etc. (Ashfaq et al. 2022). As secondary metabolites, plants have the potential to synthesize and produce a wide range of chemical molecules. The plant is shielded against diseases by these chemicals possessing antibiotic, antimycotic, and antiviral capabilities. The phytoalexin hypothesis pertaining to disease resistance, in addition to any other single theory, has likely sparked an increased investigation into the biology of immunity to disease in plants. It has surely contributed to one of the most significant advancements in botanical pathology over the past two decades. Secondary metabolites that suit the phytoalexin idea are being discovered. In many plants, chemicals that are raised or synthesized from scratch in the aftermath of pathogenic assault are being sought for (Fu and Dong 2013). Metabolomics is frequently used to examine how resilient plants are to biotic stressors. With the help of this technique, it is possible to assess pathogen-triggered local and systemic changes in the composition and distribution of plant metabolites without making any inferences first. The precise situation regarding the physiological state of the tissue of the plant under attention may be determined by comparing the amount of metabolites

before and after infection by a pathogen. Sophisticated hyphenated techniques such as GC-MS-, LC-MS-, HPLC-, and NMR-based metabolomics are typically utilized to help in the detection and quantification of such protective amino acids, natural acids, sugars, and a diverse range of phenolic and polyphenolic compounds, terpenoids, nitrogen- and sulfur-containing compounds, and specialized phytoalexins. Desorption electrospray ionization mass spectrometry (DESI-MS) now allows for fine-scale assessment of chemicals on natural plant surfaces. De novo phytoalexins are also being studied using laser desorption/ionization time-of-flight mass spectrometry (LDI-ToFMS) (Sandip et al. 2023). This chapter delves into the secondary metabolite basis of elicitor and effector-triggered immunity in pathogen elicitation amid pathogenesis.

10.2 The Role of Elicitors and Effectors in the Development of Pattern-Triggered Immunity and Effector-Triggered Immunity

Elicitors are miniature molecular mass chemicals that activate the signaling cascade to cause a plant immune system reaction. These compounds are exceedingly varied compounds with no molecular similarities other than the fact that they cause the hypersensitive reaction (HR) in plants. Eliciting agents are now widely utilized to investigate the biochemical mechanisms of defense reactions. Pathogen-derived eliciting agents (exogenous elicitors) and plant-derived eliciting agents (endogenous elicitors) are the two categories of elicitors. The majority of external eliciting agents of plant defense mechanisms are heterogeneous and vary greatly in their chemical makeup, such as glycoproteins, proteins/peptides, oligosaccharides, and lipids (Dodds and Rathjen 2010). Endogenous eliciting agents tend to be more particular in their activities and have more deadly consequences. Damage associated molecular patterns (DAMPs) are the most common elicitors of that kind. Numerous microbes upon invasion release lytic proteins like polygalacturonase, which produce cell wall shards and peptides that prevent or eliminate physical obstacles in tissues of plants like the anterior lamella. These DAMPs generally appear in the apoplast as warning signs to activate the defense system (Elmore et al. 2011). Certain endogenous eliciting agents generated from the disintegration of the host cell wall of plants include dodeca-4-d-galacturonide, trideca-1,4-d-galacturonide, and pectic polysaccharides (proteinase inhibitor-inducing agents). The eliciting molecules are generated in some circumstances when plant-based lytic enzymes break down the cell walls of fungal cells. A beta-linked heptaglucoside, for instance, has been demonstrated to be an extremely potent inducer of the hypersensitive reaction in soybean roots afflicted with the fungus *Phytophthora megasperma* that causes rotting in roots (Shah and Gupta 2022a, b). The use of genetic engineering to produce elicitors in plants is an appealing method for improving plant durability against stressful conditions, and initiatives have already been tried to apply it to potatoes. Elicitors

are capable of safeguarding plants from stress, and wreckage when administered to plants causes an accumulation of defense chemicals toward vulnerable attackers, and people are increasingly opting for better options. For instance, chitosan which is present in insects, fungus, and crab shells is utilized as an organic biocontrol tool in farming to boost plant longevity and crop harvests. Chitosan functionality is determined by the rate of polymerization and N-acetylation. It has been demonstrated to affect cells that are differentiated, such as shaggy (transformed) roots, which are often refractory to regularly employed elicitors (Iqbal et al. 2020). Jasmonates, which are botanical hormones with a multifunctional influence on developmental and growth processes, are other intriguing elicitors. Other robust generalized eliciting agents that induce stimulation, hypersensitivity response (HR), hormonal signaling, phytoanticipins amplification, and phytoalexin synthesis include chitin oligomers produced by higher-level fungi from fungal cell wall chitin; ergosterol, produced by many fungi and the primary sterol of higher fungi; glucans found in the cell walls of phytophthora, pythium, and oomycetes, such as phytophthora glycoproteins; pectin oligomers produced by fungus and bacteria from the destroyed cell walls; harpins, the type III secretion proteins produced by numerous bacterial species that are gram-negative, yeast glycopeptide fragments; flagellin, a gram-negative bacterium flagellum component; bacterial toxins, such as *P. syringae* coronatine; sphinganine, a fumonisin analog produced by *F. moniliforme*; and other necrotrophic toxins. Abiotic agents of elicitors like metallic ions, UV radiation, and various chemicals that inhibit metabolism can also induce biological reactions of defense and assist in resilience. The impact they have is often transient and nonspecific (Amiołkowska 2020). Gene-specific eliciting agents are elicitors generated by avirulence genes in the infectious agent. Here the identification of a disease-causing agent occurs when the byproduct of the parasite's avirulence (Avr) gene interacts with the products of the plant's resilience (R) gene, which has been designated as the gene for gene paradigm. This genotype-specific resilience is determined by an overlapping set of genes present in a specific pathogen species and a host variety (Abdul et al. 2020). Examples of race-specific elicitors such as elicitation, hypersensitive reaction (HR), and phytoalexin defense include avr gene products by fungi and bacteria, elicitins by phytophthora and pythium, enzymes like endoxylanase by *Trichoderma viride*, viral proteins like viral coat proteins of TMV, acyl glycosides by *P. syringae*, protein or peptide toxins like victorin by *Cochliobolus victoriae*, etc. Almost all eliciting agents cause sensitization in nanomolar quantities (Jain and Khurana 2018). Figure 10.1 summarizes the present understanding regarding elicitor signal interpretation and transduction.

The subsequent tier referred to as effector-triggered immunity (ETI) is predominantly controlled by R gene derivatives. The pathogen's pathogenic effector compounds leading to effector-triggered defenses allow the infectious agent to flee from the PTI following the inhibition of the host's recognition mechanism by PTI signaling and successfully commence colonization (Jones and Dangl 2006). To get over plant-induced defenses, microbes may deliver the effectors right into the cells of their hosts. This is known as effector-triggered susceptibility (ETS), which undermines the host plant's defense mechanism (Ashfaq et al. 2022). Depending on the

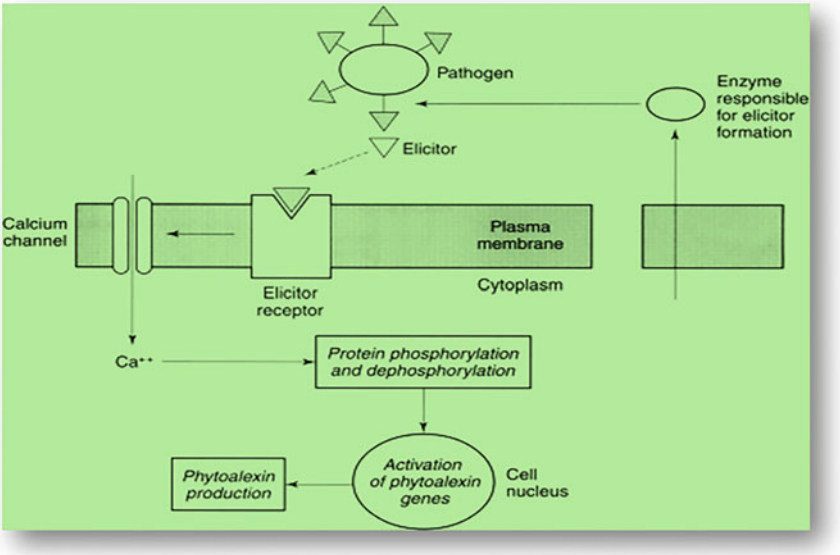

Fig. 10.1 A generalized transduction pathway of elicitor signals triggered in most elicitation cases

plant's capacity for sensing (the existence of the right surface proteins) and responsiveness (proper defense reaction), effectors may encourage resistance or damage it. Effector molecules that parasites developed to counteract plant defense mechanisms were subsequently shown to promote resilience through gene-for-gene interaction (Avr R), resulting in improved immunity, by coevolving R genes in plants. Eventually, effector-receptor interaction activates ETI, which consequently causes potent antimicrobial reactions. These interactions set off a series of signaling channels that, within minutes to hours, trigger the defensive mechanism. As a result of stimulation, the cell membrane's ion flow increases, ROS are produced, protein phosphorylation modifications are made, lipids are oxidized, cell wall is strengthened, defense enzymes are produced, and pathogenesis defending factors are synthesized. R gene (resistance gene) byproducts thus facilitate tolerance to microbe and pest strains of bacteria, nematode, oomycete, fungus, and insects that are wily (Kaloshian 2004). The apoptotic hypersensitivity reaction (HR), a type of programmable demise of plant cells at contamination locations, is frequently linked to ETI. Contrary to specialized immunity in animals, residual protection, known as basal defense, is usually inadequate to forestall disease. It can, however, restrict the dissemination of lethal infections in their hosts. Plant mechanisms crucial for both disease and stress tolerance can be found using effectors made by parasites. Numerous R genes produce NB-LRR protein molecules, also referred to as NLR proteins or STAND proteins. More than 600 distinct R gene counterparts are present in the majority of plant systems of immunity. A plant R gene possesses selectivity for an

infectious agent Avr gene, as originally recognized by Harold Flor in his articulation of the gene-for-gene link in the middle of the twentieth century. Some effectors have been discovered to be encoded by avirulence genes. For the microbe's Avr gene to provide immunity, it has to possess matching sensitivity with the R gene, implying a receptor/ligand association for Avr and R genes (Kaloshian 2004; Shah et al. 2023). On the other hand, an effector can change its host tissue targeting (or a biological decoy of that receptor), and when the R gene transcript (NLR protein) recognizes the transformed version of the host target or decoy, it stimulates defenses. A few hundred effectors are commonly transported inside the host via a type III secretory system in extensively researched bacterial pathogens of plants. A number of Gram-negative infections feature hypersensitive responses and pathogenic islands that encode the type III secretion apparatus. It is used by bacteria to deliver effector proteins inside the plant cell. It found that the *Pseudomonas syringae* effector proteins AvrRpt2 and AvrRpm1 reduced the PAMP-triggered defense reaction in *Arabidopsis* by decreasing callose buildup, and *Arabidopsis* genes encoding cell wall secretory and defense proteins were suppressed by *P. syringae* AvtPto effector (Zhang and Zhou 2010). Plant-associated pathogens such as fungi, oomycetes, and nematodes appear to encoder hundreds of effectors. The practical meaning of "critical" effectors is their generalization throughout a pathogen's community and their significant influence on disease pathogenicity. Genomics may be exploited to find core effectors and can subsequently be utilized to uncover novel R gene alleles imparting disease-resistant traits in the plant breeding process. Tremendous scientific work is exposing methods of MAMP sensing, host defense mechanisms, and particular host-specific proteins that infectious agent effectors target, ways to regulate R protein stimulation, and how pathogenic effector modules and R genes evolve, among others (Shah et al. 2022a, b, c). The two layers mentioned above are important in plant immunity, although they do not entirely characterize plant immune mechanisms. Furthermore, many specific cases of apparent PTI or ETI contradict standard PTI/ETI descriptions, implying a demand for greater abstractions and/or models.

10.3 Defense Against Stressors Via Pathways Complemented by Minerals and Phytohormones

Whenever parasites or other abiotic stressors strike a plant, it undergoes physiological stress, causing intrinsic biochemical transformations that may result in the upregulation or diminution of particular chemicals that might have an integral part in the plant's antipathy toward these foreign incursions. Hormones produced by plants are signaling chemicals that are generated by the plant (Lamers et al. 2020). Phytohormones influence biological activities in certain cells and can be transported into various areas of the plant (Fig. 10.2). Signaling chemical messengers such as salicylic acid, jasmonic acid, ethylene, and auxin modulate plant immune system

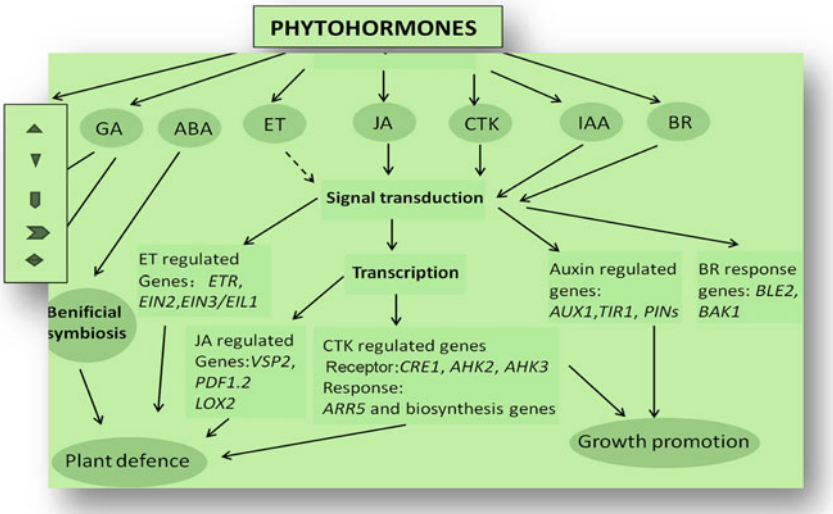

Fig. 10.2 Defense against stressors via pathways complemented by phytohormones

performance in part. For instance, in most plant stress defense systems, salicylic acid affects catalase isozyme operation, jasmonate controls phenylalanine ammonia-lyase action, and auxin binds to receptors, which subsequently engage and deactivate transcription activating regulators, therefore stimulating auxin-relying expression of genes (Lau and Deng 2010). The oxylipin jasmonic acid and its derivatives, dubbed jasmonates, influence biotic and abiotic stress responses via cell signaling. Jasmonic acid is akin to auxin in that it influences jasmonate-response signaling intermediates like JAZ proteins. These kinds of proteins relate jasmonate sensing to shifts in transcription (Cagnola et al. 2018). Mitogen-actuated protein kinases (MAPKs) are triggered once fatty acid amides are detected in the saliva of the insects. Phosphorylated proteins regulate the functioning of defense-related genes. Once the genes in question are active, they participate in the octadecanoid cascade. This network is critical for triggering the expression of plant defense genes. The route results in the formation of Jasmonic acid, an imperative phytohormone in defense. Jasmonic acid (JA) production spikes are additionally seen close to the foci of infection by pathogenic microbes. The ubiquitination of proteins with jasmonate ZIM (JAZ) motifs that hinder JA signaling is reportedly linked to this biological reaction to enhance JA synthesis. This, in turn, causes the proteins' disintegration and a surge in the number of defense genes that are activated by JA. Gibberellin alters the folding pattern of receptors, binds to, and degrades Della proteins, modulating the plant immune system by balancing JA/SA signaling, demonstrating the critical function of GA signaling in plant defense against parasite invasion. Additionally, research employing variant jasmonic acid production channels in *Arabidopsis* has revealed

that JA mutations are more vulnerable to infestation by soil-borne pathogens (Campos et al. 2016). Modulation of the cellular framework and vesicle trafficking aids in directing plant defense mechanisms in the direction of the pathogen attack. This is also under hormonal control in part. When protecting themselves from phloem suckers, plants have established a means of defense utilizing the salicylic acid route. Salicylic acid synthesis has been demonstrated to rise in response to an infectious agent. The transcription of pathogenesis-related genes is induced by a boost in SA, and this increases the resilience of plants to both biotrophic and necrotrophic diseases. Arabidopsis mutants installed with the bacterial NahG gene, which prevents the buildup and synthesis of SA, have been found to be significantly more vulnerable to infections than wild-type cultivars. The failure to develop vital safeguards, such as enhanced PR transcription, was assumed to be the cause of this. Salicylic acid (SA) production may have significance in limiting viral multiplication, according to other research that showed salicylic acid (SA) injections into tobacco plants, and *Arabidopsis* increased immunity to contamination by the lucerne and tobacco mosaic viruses (Lu 2009). Insect saliva contains eliciting agents that plants can identify via specialized pattern recognition receptors, and a signaling propagation cascade is triggered after detection. Ca^{2+} ions discharge into the cytosol in response to the existence of an elicitor. Protein targets like calmodulin are activated as a result of the rise in the cytosolic quantity of Ca^{2+} ions. Ca^{2+}-reliant protein kinases activate downstream pathways such as phosphorylation and transcriptional initiation of stimuli-specific reactions (Cheng et al. 2002). The calmodulin-linking transcription promoter IQD1 overexpresses itself in some plants, which inhibits herbivore exertion. The cell surface ion channels for H+, Ca++, K+, and Cl are impacted after the fungal oligopeptide-eliciting agent attaches to its sensor there. Calcium channels are implicated in elicitor-triggered cell signaling cascade because they reduce both the influx of calcium ions and phytoalexin synthesis when Ca++ is absent from the growth media or when inhibitors of calcium channels are added. Multiple proteins have been shown to undergo elicitor-peculiar, calcium-reliant phosphorylation in biological systems. Consequently, the imperative is the role of calcium ions in the cell signaling cascade pertaining to defense (ldon et al. 2018). Although pattern-triggered defense mechanism is assumed to have emerged separately in each, there are similarities among animal and plant defenses in terms of the usage of hormones, metal ions, and the development of specialized immune responses.

10.4 Elicitation by Elicitors Eventually Ending Up in the Enrichment of Defensive Secondary Metabolites and Specialized Phytoalexins

Plants manufacture a wide range of chemical compounds that seem to perform no apparent duties for their development or growth, i.e., no standardized contributions to the processes of photosynthesis, transportation, translocation, respiration, nutrient

assimilation, and proliferation. They have a far more restricted spectrum than primary metabolites across the plant realm, frequently being present only in a single species of plant or a botanically adjacent assortment of taxa. Secondary metabolites are thought to have a crucial part in the ecological, defense, and physiological processes and are not merely undesirable byproducts that result from primary metabolism. Most plants synthesize antimicrobial secondary metabolites during the course of typical plant growth, known as phytoanticipins, or synthesize them from scratch when confronted with bacteria, known as phytoalexins. In accordance with natural circumstances, phytoanticipins use a lot of carbon and energy and have a significant fitness cost, yet they are recognized as the initial line of biochemical defense that prospective parasites must conquer (Shah and Gupta 2022a, b). Conversely, phytoalexin synthesis can take up to 3 days since the system of enzymes and cell signaling cascades must first be processed. These plant antibiotic-like secondary metabolites are thought to defend plants from disease and other stressful conditions. Many phytopathogenic microbes, on the other hand, have catalysts that can neutralize the phytoanticipins or phytoalexins generated by their host. This might be a way for these organisms to avoid plant defense. As such, pathogens specific to the host that can dismantle phytoalexins tend to be more lethal than those that cannot (Ashfaq and Amit 2021; ldon et al. 2018). High quantities of secondary metabolites have been experimentally shown to provide greater plant defense against pathogenic intruders. Secondary metabolite formation involves several chemical processes and requires a significant quantity of ATP. Aside from their creation during parasite assault, their preservation in the vesicles demands vitality additionally. H+ − ATPase provides the necessary power for upward transportation and, in many cases, for retaining metabolites in the vacuoles. Furthermore, certain metabolites are carried into the vacuole by ATP-binding cassette carriers (ABC transporters), which are ATP-dependent (Shah and Gupta 2022a, b). Secondary metabolite synthesizing routes can be put into four types: shikimic acid, malonic acid, mevalonic acid, and MEP (methylerythritol-phosphate) pathways (Fig. 10.3). The isoprenoid, phenylpropanoid, alkaloid, and fatty acid/polyketide networks communicate the production of the majority of secondary metabolites. Plant protection mechanisms have been linked to a number of broad families of secondary metabolites, including phenolic and polyphenolic compounds, terpenoids, alkaloids, flavonoids, cardenolides, and cyanogenic and iridoid glycosides among others. Phytoalexins, which belong to various groups of secondary phytochemicals, may inhibit parasite growth by actively rupturing the cell wall, prolonging maturity, altering metabolic processes, or preventing pathogen replication. When phytoalexin production is blocked, plant tissue becomes more susceptible to infections, indicating its role in plant defense (Garcia-Guzman and Heil 2014). Mutants that are unable to produce phytoalexins demonstrate more parasite colonization than wild forms. The perception of the elicitor and effector signals by plant cells by PRRs signaling causes widespread alterations inside the plant, inducing the encoding of genes that guard against additional parasite entry, such as enzymes that participate in phytoalexin synthesis. When jasmonates or ethylene are produced from injured tissue, neighboring plants frequently generate phytoalexins in reaction. Elicitors served as an integral

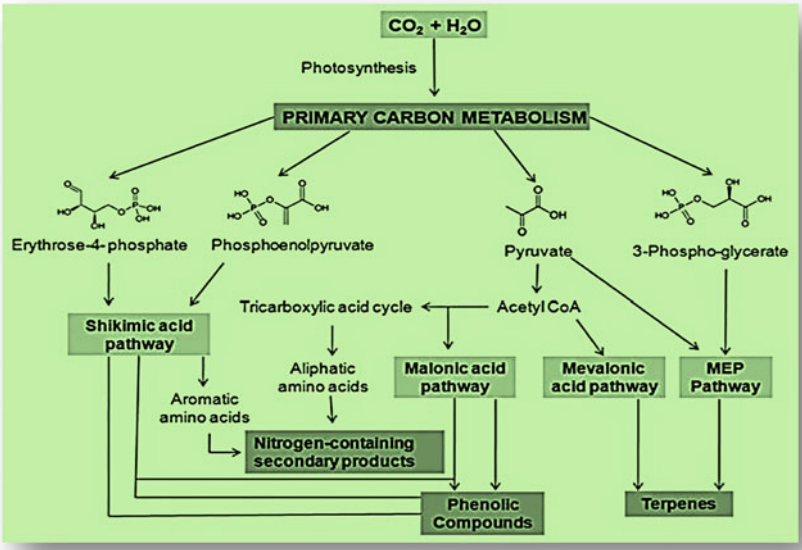

Fig. 10.3 Generalized pathways of secondary metabolite synthesis

part of secondary metabolite amassing as well as increasing their production. By modifying the biological machinery of the cell, both biotic and abiotic eliciting agents are utilized to increase the buildup of antibiotically potent secondary metabolites/phytoalexins in plants and in cultured plant cells (Islam et al. 2019). Since the middle of the 1990s, the usage of eliciting agents in vitro has significantly grown. Abiotic elicitor treatments of actinomycin D, psoralens, ultraviolet light, NaCl, $CaCl_2$, $AlCl_3$, $CdCl_2$, and biotic elicitors from yeast extract, *Aspergillus flavus*, *Fusarium oxysporum* like hepta-β-glucoside, glucan, polysaccharide containing Glc, Man, Ara, eicosapentaenoic acid and arachidonic acid, β-glucan, chitosan heptamer of β-1,4-glucosamine, high-molecular-weight glycan, glucomannan, glycoproteins, and galactoglucomannan to plant parts have been revealed to augment the secondary metabolites and synthesize phytoalexins like vincristine, waspisatin, vinblastine, ipomeamarone, ajmalicine, pisatin, reserpine, pterocarpans (e.g., phaseollin), orchinol, and rishitin among numerous others in various plants and crops (Barz et al. 2007). Multiple enzymes are stimulated by a variety of eliciting agents in the secondary metabolite's biosynthesis steps. For instance, the primary enzymes involved in the phenylpropanoid pathway, phenylalanine ammonia lyase (PAL) and p-coumarate 3-hydroxylase (C3H), are activated by harpin protein. Methyl jasmonate stimulates the functioning of γ-tocopherol methyltransferase (γ-TMT), lycopene β-cyclase (LCYB), UDP-glucuronosyltransferase, and luminescence duress actuates enzymes like C3H, F3H, DFR, LCYB, CHS, l-GalLDH, PAL,

SS, HPT, 4CL, and C4H (Ruiz-García and Gómez-Plaza 2013). In order to procure veggies and fruits with increased benign phytochemical levels and superior quality, eliciting agents seems to be a useful technique.

10.4.1 Categories and Instances of Secondary Metabolites as Players in Defense

Terpenes, phenolics, and compounds incorporating nitrogen and sulfur are the four fundamentally separate types of secondary metabolites found in plants (Fig. 10.4).

10.4.1.1 Terpenes/Terpenoids

Terpenes are the most abundant group of secondary metabolites, and they have a similar metabolic genesis from acetyl-CoA or glycolytic precursors. A large proportion of terpene complexes generated by plants as secondary metabolic products are thought to be engaged in defense. Several monoterpenes (C10) are major bug toxins. Pyrethroids (monoterpene esters) found in the foliage and petals of *Chrysanthemum* species, for instance, have powerful insect-killing (neurotoxin) effects (Ninkuu et al. 2021). Monoterpenes concentrate in resinous conduits present in the needles, twigs, and stems of conifers such as pine and fir, mostly as limonene, pinene, and β-myrcene, all of which are deadly to several pests notably peel beetles, a serious threat of conifer plants worldwide. Costunolide, a class of antiherbivore factors

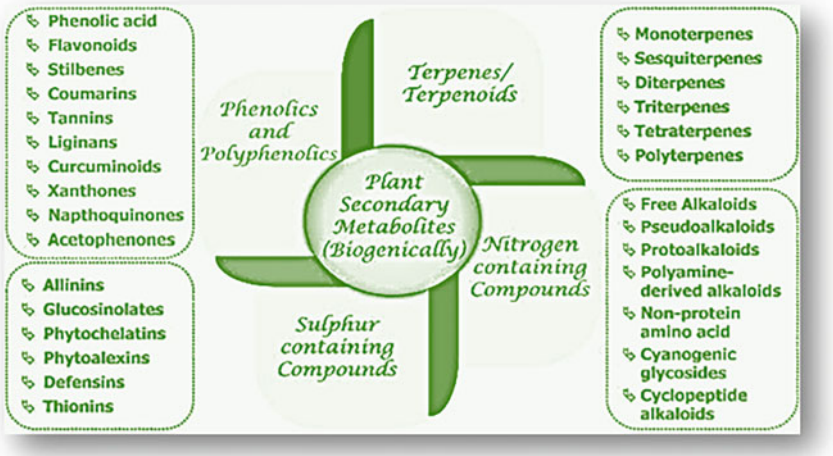

Fig. 10.4 Categories of plant secondary metabolites with typical examples

characterized by a five-membered lactone ring (a cyclic ester) that exhibits robust nourishing repellence against numerous herbivorous pests, animals, and microbial pathogens is an example of sesquiterpenes (C15) that are being specified for their function in plant defense (Thimmappa et al. 2014). ABA, a sesquiterpene, largely regulates the onset of signaling pathways in a plant's reaction to environmental stresses, by changing cell membrane traits. It raises the cytosolic level of calcium and alkalinizes the cytosol. Abietic acid (C20) is a diterpene that occurs mostly in leguminous species. It is found in or alongside resins in plant stem resin channels. Once feeding bugs puncture the canals, the discharge of resins may directly restrict eating while also acting as a biological disincentive to ongoing assault. An additional chemical, phorbol (diterpene ester), is present in the Euphorbiaceae family and acts as an allergen and systemic poison in animals (Ashour et al. 2018). Moreover, gibberellins, a class of phytohormones, are also diterpenes that perform a variety of negative roles in a variety of plant stages of development. They are known for using specialised defence enzymes, whose synthesis affects the basic processes of translocation and gene transcription, to apply their numerous biological effects. Many sterols of the triterpenes (C30) group are significant components of the membranes of plant cells, where they act as control conduits while preserving porosity to smaller molecules by slowing fatty acid chain mobility (Herrera et al. 2015). Phytoecdysones possess a defense function against bugs by interfering with molting. Similar triterpene is limonoid, which is a collection of caustic molecules found in citrus fruits that operate as antiherbivore and antifungal chemicals (Kanda et al. 2017). Tetraterpenes and polyterpenes are higher terpenes. Plants include a number of molecules with dense molecular weight polyterpenes (C5)n as phytoanticipins.

10.4.1.2 Phenolics/Polyphenolics

Phenolics have a minimum of one aromatic ring having one or more hydroxyl groups linked and are found across the plant world. These have been shown to add to the coloration of many tissues as well as to protect from various biotic and abiotic stressors. Organic phenolics found in plant tissue are divided into two distinct categories: flavonoids and non-flavonoids. Coumarins are the simplest of the phenolic chemicals found in vascular plants that are shown to have numerous roles in plant defense systems against herbivorous bugs, bacteria, and fungi. They originate from the shikimic acid route, which is found in bacteria, fungi, and plants but not in vertebrates (Becerra-Moreno et al. 2012). Halogenated coumarin compounds have been revealed to suppress fungal proliferation quite well. Some variants of coumarin have greater stability and stronger antifungal efficacy toward a variety of soil-dwelling fungal pathogens than the primary coumarin molecules. Furanocoumarins are an additional kind of protective coumarins found in Umbelliferae species. Psoralen is a basic linear furanocoumarin recognized for its application in the management of fungal and viral defense (Stringlis et al. 2019). Flavonoids, another major family of plant phenolics, serve a variety of roles in plant systems, notably

coloration and defense. Flavonoids are polyphenolic chemicals having 15 carbons and 2 aromatic rings joined by a three-carbon bridge (C6 -C3 -C6). Flavonoids can be categorized as flavones, flavanols, flavanones, flavan-3-ols anthocyanidins, and isoflavones (catechins and their oligomers like proanthocyanidins), based upon their multifaceted structure (with roughly 10,000 structurally distinct compounds). The majority of flavonoids discovered naturally are glycosides. Chalcone synthase is an enzyme involved in the production of the flavonoid framework, catalyzing the sequential condensing of three acetate groups from malonyl-CoA with 4-coumaroyl-CoA to the intermediary chalcone (Du et al. 2010). There are several examples of their powerful significance in maintaining immunity against pathogens. The presence of flavonols quercetin and its glycoside rutin in the majority of fruits, particularly *Malus* species, made them impervious to their sensitive fungal infections. Likewise, flavan-3,4-diols and conjugated tannins are responsible for banana nematode tolerance. Flavonoids also have an important function in fruit and vegetable after-harvest protection (Panche et al. 2016). Unripe fruits have high levels of flavonoids, which protect them against infections. Ripe fruits, on the other hand, are more susceptible to fungal degradation because of lesser levels thereof (Ashfaq and Amit 2021). Flavones and flavonols are two other significant categories of flavonoids that occur in flowers to safeguard cells against ultraviolet (UV)B rays as they build up in epidermal layers that cover stems and leaves and capture light fervently in the UV-B part while allowing visible (PAR) wavelengths to pass through unrestricted (Ahmad and Gupta 2022). Furthermore, increasing UV-B rays exposure has been shown to promote the elevated biosynthesis of flavones and flavonols, implying a role against abiotic stressors (Agati et al. 2013). Furthermore, it appears that the production of these kinds of flavonoids is a viable method for combating oxidative stress. There also exist other types of non-flavonoids, the most common of which are phenylpropanoids, which have solely the C6C3 phenylpropane framework and are closely associated with phenylpropanoid (lignin) formation in vascular plants. Cinnamic acids and their analogs, including p-coumaric acid, chlorogenic acid, sinapic acids, ferulic acid, and stilbenes, are prominent instances (Agati et al. 2007). Isoflavones are distinguished by the attachment of the B-ring at C3 instead of C2 (Fig. 10.5). More than 1650 isoflavones have been identified to date, and the number is constantly rising. Isoflavonoids are generated from naringenin, a flavanone precursor found in all plants, and serve a crucial part in plant growth and defense mechanism. Genistein, daidzein, and glycitein are isoflavones that are synthesized by the phenylpropanoid cascade and accumulated in the vacuoles as glucosyl- and malonyl glucose conjugates. Following the interactions between plants and microbes, they act as progenitors for the formation of important phytoalexins and prevent pathogen assault (Csepregi and Hideg 2018). Tannins' protective capabilities are commonly linked to their capacity to attach to peptides and proteins. Protocatechuic acid (PCA) and chlorogenic acids are likely to play a unique role in establishing disease immunity in specific plants. They inhibit the growth of spores and proliferation of various fungi as well as prevent smudge in garlic and onions, an infection brought about by the fungus *Colletotrichum circinans* (Barbehenn and Peter 2011). Stilbenes are phenylpropanoid-procured chemicals

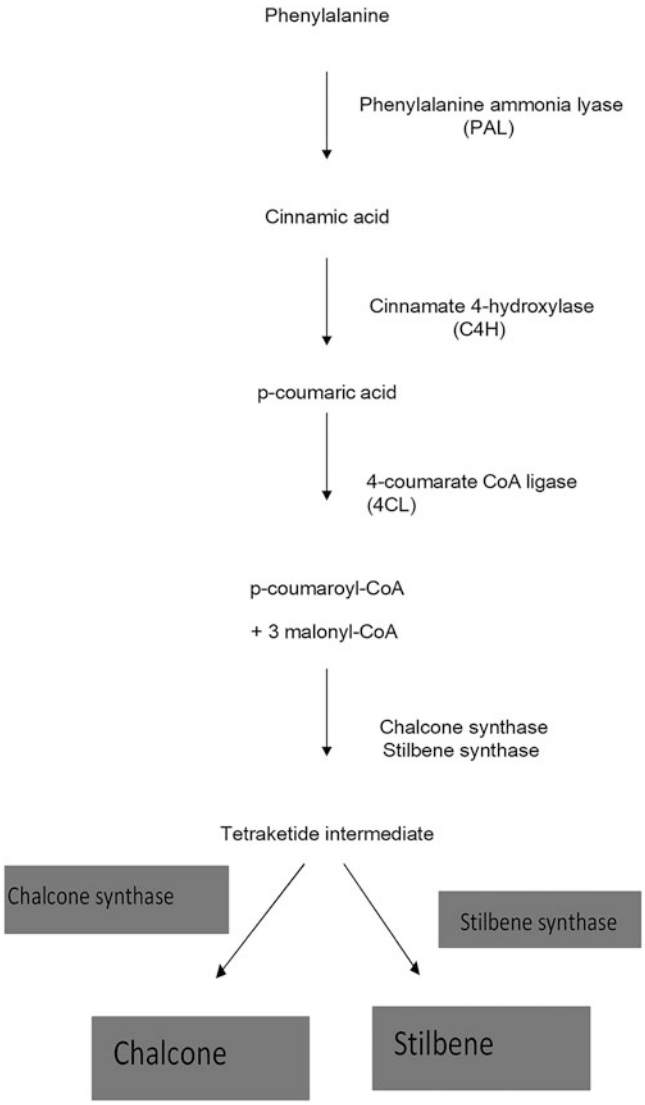

Fig. 10.5 Steps of stilbene biosynthesis

with a 1,2-diphenylethylene backbone (C6 -C2 -C6). Stilbenes occur in two stereo-isomeric configurations (E and Z) according to how the functional units are connected to each other. Stilbenes are a significant collection of organic compounds that are of special interest due to their diverse physiological applications. Commonly found stilbenes include resveratrol piceatannol, combretastatins, rhapontigenin, pterostilbene, and pinosylvin. Among them, combretastatins and resveratrol have received the most attention when it comes to human health and disease as well

(Chong et al. 2009). Above a thousand stilbenoid compounds have been determined as the consequence of various structural replacement strategies such as the glycosylation, isoprenylation, and methylation processes, as well as oxidant condensing of soloists into dimers and further condensate formation of these dimer structures. Although virtually all higher plants appear to be capable of producing malonyl-CoA and CoA-esters of cinnamic acid derivatives, only a small number of species of plants are capable of producing stilbenes because the stilbene synthase, the basic component of stilbene production, is found in just a few plant species (Fig. 10.5). Some plant species, including *Fallopia japonica*, pine, and grapevine, maintain substantial quantities of stilbenes persistently. Stilbenes may build up in tissues of plants to quantities sufficient to suppress fungal and bacterial development when these parasites invade (Zakova et al. 2018).

10.4.1.3 Sulfur-Containing Secondary Metabolites

Secondary metabolites comprising sulfur are a diverse category of phytochemicals found in Brassicaceae, with around 200 forms now known. The model bioactive chemicals include GSH, defensins, allicin, GSL, thionins, and another majority of plant phytoalexins, almost all of which are believed to be attributed to plant defense regarding pathogenic microbes and numerous of which have been proposed to be engaged in the systemic induced resistance (Burow et al. 2008). GSH constitutes one of the principal kinds of molecular sulfur in plant-soluble fractions and plays a key function as a moving reservoir of reduced sulfur in the development and growth of plants. Specialized cells, like trichomes, have significant enzyme activity that is responsible for the production of GSH and other phytochelatins required for heavy metal decontamination. GSH acts as an immediate antioxidant in addition to being a crucial tool in the defense against reactive oxygen species to reduce oxidative stress (Czerniawski and Bednarek 2018). Furthermore, GSH participates in the detoxing of xenobiotics and toxic substances by directing them to the vesicles. GSH is swiftly built up during a fungal invasion and may serve as a persistent messenger, delivering knowledge regarding the incursion to non-infested regions. GSL is a class of low-molecular-weight N- and S-possessing plant glucosides that degrade to form aromatic defense compounds that are poisonous or repellant to parasites. Whenever plant tissue is injured and GSL comes into touch with the plant enzyme myrosinase (EC 3.2.3.1), a beta-thioglucosidase enzyme, the glucose component, is removed, resulting in the creation of destabilizing intermediates such as isothiocyanates and nitriles. The resultant aglycons reorganize with the elimination of the sulphate to produce noxious and highly reactive chemical compounds, such as nitriles, which serve as herbivorous poisons, antibiotics, and repellents for insects, mites, bugs, etc. (Dorion et al. 2021). The vast majority of plant genera generate natural phytoalexins with broad chemistry forming a group while sharing an indole or related ring system and one S atom, such as sesquiterpenoids of *Solanaceae*, isoflavonoids of *Leguminosae*, and phytoalexins from *Brassica*. The greatest examples of S-possessing phytoalexins include furano-acetylenic variants, multiple forms of

phaseollin and glyceollins, trifolirhizin, ipomeamarone, pisatin, and orchinol, among others (Gerbirk et al. 2009). Additional S-rich non-storage protein molecules from plants that synthesize and concentrate following microbial assault include thionins, defensins, and lectins, all of which hinder the development of a wide variety of pathogens (Shah and Gupta 2022a, b).

10.4.1.4 Nitrogen-Containing Secondary Metabolites

Secondary metabolites having nitrogen comprise alkaloids, cyanogenic glucosides, and non-protein amino acids. All are of significant importance due to their key role in anti-herbivore defense and cytotoxicity to a diverse spectrum of microbial diseases. Alkaloids are a wide class of nitrogen-containing secondary metabolites present in around 20% of flowering species of plants. The majority of them, notably pyrrolizidine alkaloids, are poisonous to some extent and tend to act largely as a defense against bacterial assault. They are often derived from one of the several prevalent amino acids, specifically lysine, aspartic acid, tyrosine, and tryptophan (Singh 2018). The manner of exerting their effects on organisms is extremely varied at the cellular scale. Some interact with neurological elements, particularly neurotransmitters, while others influence transport through membranes, the formation of proteins, and other enzyme processes. Cyanogenic glucosides are nitrogen-holding defensive chemicals that produce the toxin HCN and are found in representatives of the *Rosaceae*, *Poaceae*, and *Leguminosae* families. They don't seem to be noxious on their own; however, when smashed they release highly volatile harmful chemicals such as HCN and H2S whose emission dissuades eating by bugs and other predators like slugs and snails (Yeats 2018). Amygdalin, a frequent cyanogenic glucoside that occurs in almonds, apricots, and peach seeds, and dhurrin, located in sorghum bicolor seeds, are not generally fragmented down in the undamaged tissues since the glucosides and enzymes that degrade them are segregated. Within typical circumstances, their segregation avoids disintegration. Nevertheless, under challenging circumstances, such as the invasion of necrotrophs and herbivores, the interiors of cells from various tissues blend and form HCN, a cellular respiration poison that connects to the Fe-possessing heme class of cytochrome oxidase and other respiratory proteins to suppress it (Yeats 2018). Numerous plants additionally include nonprotein amino acids, which are not integrated into proteins but exist in separate forms and operate as defensive defense chemicals. Canavanine and azetidine-2-carboxylic acid, for instance, are near analogs of arginine and proline, correspondingly. They are poisonous in a variety of ways. Some inhibit protein amino acid production or absorption, whereas others might be accidentally absorbed into proteins. Canavanine is recognized by the herbivore enzyme that typically binds arginine to the arginine transfer RNA component and so gets integrated into proteins in lieu of arginine after intake. The normal outcome is a protein that is no longer viable due to either its tertiary framework or its active site having been disrupted (Lee et al. 2016).

10.4.2 Instances of Important Secondary Metabolites Induced as Phytoanticipins and Phytoalexins Upon Microbial Invasions

It has been revealed that infections caused by fungi in plant tissues right away activate the gene expression of messenger-RNA, which codes for specific enzymes that catalyze the formation of phenolics in leaves and fruits. The buildup of flavonoid molecules, particularly anthocyanin, and catechin, was found to have increased substantially in rust-diseased leaves (Shah and Gupta 2022a, b). Due to biotic and abiotic stresses, the aggregate phenolic content in diseased peels of fruits was found to be five times higher than in the interior flesh. Fruit species accumulate more secondary metabolites in the skin compared to the meat, suggesting peels are the initial line of defense against pathogenic microbes like the epidermis for human beings (Sadef et al. 2022). Another research found that suppressing phenylalanine ammonia lyase, a key enzyme in phenolic synthesis, made the scab-tolerant *Malus* cultivar "Sir Prize" susceptible to scab, demonstrating the importance of phenolic compounds in scab resistance in apple (Sarkate et al. 2018). It was discovered that secondary metabolite-dependent defense toward apple infectious agent by demonstrating that fruits contaminated with scab had up to 7.6 times higher titer of hydroxycinnamic acids, 2.6 times greater flavan-3-ols, and 2.9 times more flavanol amounts than normal ones. The total amount of phenolic compounds was 1.5- to 2.5-fold greater in tissue with infection than in normal leaves and fruits (Mikulič Petkovšek et al. 2009). According to the results of a new investigation done by Ahmad & Gupta (2022), contaminated peel decoctures of *Malus domestica* var. delicious have greater total phenolic and total flavonoid levels than noninfected peel extracts. In these extracts FTIR and GC-MS analyses indicated quantifiable changes in secondary metabolites. Exclusively contaminated peel preparations were possessing compounds like cyanidin, ferulic acid, hyperoside 3-phosphoglyceric acid, aucuparin, eriobofuran, avicularin, cyanidin 3-o-galactoside, and reynoutrin, while healthy peel extracts were devoid of such compounds, suggesting that these compounds are synthesized de novo upon fungal invasion, or their concentration levels are raised to detectable concentrations. Based on these findings, it can be concluded that secondary metabolite foundations of *Venturia inaequalis* resistance exist significantly in *Malus* cultivars (Ahmad and Amit 2023). Efforts are being made to determine the compounds generated in the aftermath of microbial assault in a variety of other agricultural plants, and a variety of chemical structures have been discovered to meet the phytoalexin notion. Pisatin, a pterocarpan product formed by capsules of *Pisum sativum* infected with spores of the brown rot fungus, *Monilinia fructicola*, was crystallized and qualitatively characterized by Cruickshank and Perrin (1960). Following research, it was discovered that additional legumes, such as *Phaseolus vulgaris*, generated comparable pterocarpans (e.g., phaseollin) in response to fungal colonization (Hadwiger and Tanaka 2017). When the phytopathogenic fungus *Phytophthora megasperma* infects soybean, the fungal cell wall is attacked by β-1,3-glucanase, which is found in the host cells. As a result, an elicitor

(β-glucan) is produced, causing the plant to begin accumulating phytoanticipins and phytoalexins. The sensitivity of parsley leaves to *P. megasperma* is representative of this plant variety. The reaction includes hypersensitive cellular death, reactivation of defense-related genes, and the encoding of the phytoalexin furanocoumarin (Ahuja et al. 2012). Allixin (3-hydroxy-5-methoxy-6-methyl-2-pentyl-4H-pyran-4-one), the initial phytoalexin identified from garlic, is a molecule with a γ-pyrone framework (Kodera et al. 2002). Simultaneously, a review of chemicals extracted from invaded plants in other families revealed that phytoalexin generation is a trait of the *Orchidaceae* (orchinol in orchid bulbs) and Convolvulaceae (ipomeamarone in diseased sweet potatoes). Several compounds, including the sesquiterpenoid rishitin, were identified in the potato-blight interplay. One of the most abundant phytoalexins in the grapevine, resveratrol falls to a class of molecules known as stilbenes. Resveratrol and its dietary equivalents can be extracted from a variety of foods, including grapes, blueberries, peanuts, and red wine. Resveratrol's effect on world-wide DNA methylation trends in cells with breast cancer in humans highlighted the possibility for epigenetic treatment (Sirerol et al. 2016). Several organic resveratrol derivatives, notably pterostilbene and polydatin, are prevalent in grapevine cells. The melinjo plant, which is widely farmed and utilized in Indonesian cuisine, accumulates dimer-resveratrol gnetin C. Trans-resveratrol is a phytoalexin generated in *Vitis vinifera* that hinders the development of mycological diseases like *Botrytis cinerea*, and delta-viniferin is additional grapevine phytoalexin generated during *Plasmopara viticola* fungus infestations (Biais et al. 2017). Figure 10.6 depicts the chemical structure of model phytoalexins thoroughly studied to date. In other instances, Zafar et al. (2020) discovered an enormous rise in reserpine deposition in *Rauwolfia serpentina* callus after elicitor therapy. Increased vincristine and vinblastine production was additionally observed in *Catharanthus roseus* when an abiotic elicitor, sodium chloride, was used (Fatima et al. 2015). *Aspergillus flavus*, a biotic elicitor, boosted callus volume development and later increased alkaloids buildup in *Catharanthus* (Maqsood and Abdul 2017). Abiotic stimulants are widely employed in the hairy roots of *Panax ginseng* to promote development and the manufacture of ginseng saponin. Sakuranetin is a flavanone discovered in *Polymnia fruticosa* and the rice family, where it functions as a phytoalexin inhibiting *Pyricularia oryzae* spore growth (Stompor 2020). 6-Methoxymellein is a dihydroisocoumarin and phytoalexin that is produced by UV-C stress in carrots and provides immunity against *Botrytis cinerea* along with other bacteria (Talcott and Howard 1999). Danielone is a phytoalexin that has been identified in the pulp of papaya. This chemical demonstrated significant antimicrobial action toward *Colletotrichum gloeosporioides*, a papaya pathogen (Echeverri et al. 1997). Avenanthramides, being phytoalexins, are significantly generated by *Avena sativa* in reaction to the oat crown rust, *Puccinia coronate* (Li et al. 2019). Psoralene is a basic longitudinal furocoumarin recognized for its application in the management of fungal defense and is discovered in extremely low concentrations in SO_2-treated plants (Thakur et al. 2020). There are numerous instances, such as those mentioned above, that demonstrate the role of phytoanticipins and phytoalexins as phyto-secondary metabolites in plant health and illness. Plant secondary metabolites are

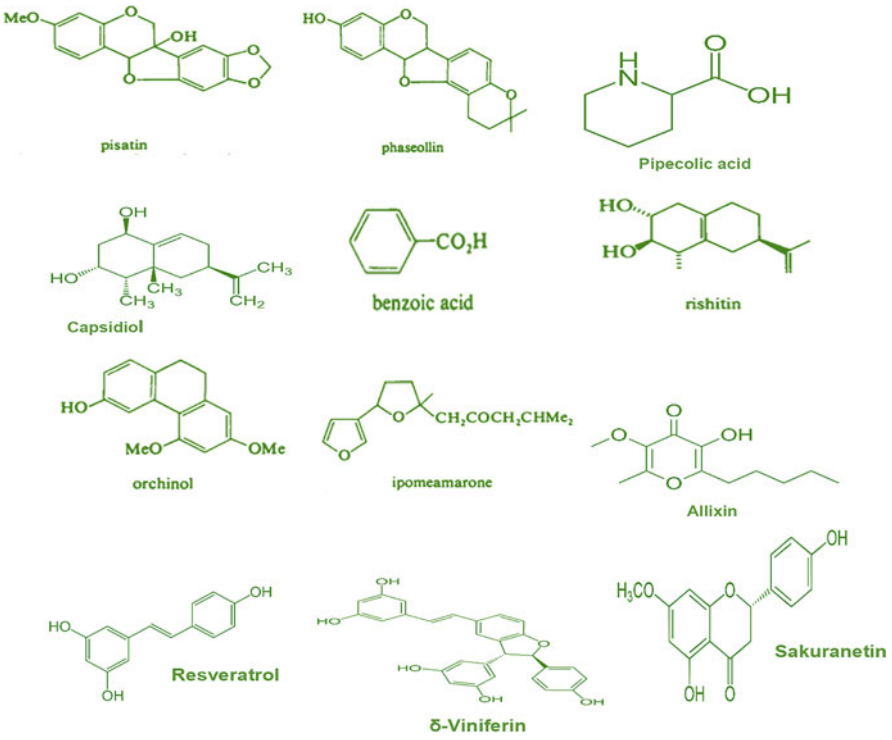

Fig. 10.6 Chemical structure of model phytoalexins thoroughly studied till date

attracting popularity and fascination among those involved with sustainable, secure, and holistic crop management techniques due to their pathogenesis inhibitory mode of action toward insects, microbiological diseases, and other parasites. As these chemicals provide innovative medicines in the treatment of refractory illnesses and medication resistances, they are also revealed to be benign in terms of human health and disease.

10.5 Mechanism of Secondary Metabolite Action

Secondary metabolic compounds such as flavonoids and phenylpropanoids are extensively dispersed in plants and have several modes of operation toward parasites. Hundreds of antimicrobial metabolites target only a few distinct pathways. They mostly function as mimics of cellular signal molecules or transporters. They have an impact on a variety of physiological processes and pathogen components, including biomembranes, enzyme inhibition, estrogenic characteristics, and DNA alkylation. The noxious derivatives of certain secondary metabolites suppress the respiratory process of their assaulters (Shah and Gupta 2022a, b). These compounds

often share numerous phenolic hydroxyl groups that can dissociate to form negatively charged phenolate ions. Proteins and peptides establish hydrogen and ionic connections with polyphenol hydroxyl groups. The more hydroxyl groups there are, the greater the abrasive and disintegrating impact (Zhang et al. 2022). Enzymes and other proteins are able to operate effectively when they possess the right three-dimensional framework, which is known as configuration. Conformational alterations in proteins modify their characteristics and can inhibit efficient interplay among proteins as well as among proteins and DNA or RNA. Most secondary metabolites engage with peptides in one or more ways, such as binding, complexing, or denaturing, and so change protein configurations (Wink 2015). Most secondary metabolites make covalent bonds with proteins, generally by adhering to free amino-, SH-, or OH- sites; for example, phenylpropanoids adhere to amino acid groups. Polyphenols (phenylpropanoids, quinines, catechins, tannins, flavonoids, lignans, and anthraquinones) communicate with proteins by creating hydrogen bonds with the electronegative atoms of peptide bonds and/or the positively charged ends of strands of basic amino acids (lysine, histidine, and arginine). A single one of these noncovalent ties is extremely sluggish. However, because multiple of them are generated concurrently when a polyphenol interacts with a protein, a shift in protein structure or diminished protein elasticity is likely to happen, which frequently results in protein or enzyme deactivation that could lead to cessation of invader activity right way (Anjali et al. 2023).

10.6 Conclusion

Current research suggests that exposing a plant to many stresses, both abiotic and biotic, might improve its efficiency by lowering vulnerability to stressors. The MAMPs-PRRs interaction causes crosstalk among their respective hormone signaling cycles, which will either encourage or antagonize other remodeling gene circuitry to raise defense response endurance. Reactive oxygen species load, nitric oxide generation, calcium, potassium, and proton fluctuations shifted quantities of salicylic acid and various other phytohormones, and stimulation of MAP kinases along with other specialized protein kinases is commonly elicited by excited receptors. These mechanisms consequently result in the alteration of proteins and enzymes that govern the transcription of defense-associated genes which finally leads to systemic resistance in part with the elevation of phytoanticipins and de novo synthesis of phytoalexins. The discovery of the processes generating secondary metabolite-linked SIR will be a significant step toward more sustainable agricultural cultivation since the need for fungicides will be reduced or abolished. As a result, SIR might become an essential tool for effectively controlling infections in organic farming systems. As a result, further study in the field of elicitor and effector development is required in the current context. In the long run, it will most likely be feasible to build gene cassettes for whole pathways, which might subsequently be employed in bioreactors to produce beneficial antimicrobial secondary metabolites

or for the metabolic manipulation of agricultural plants. This will increase their tolerance to herbivores, microbial diseases, pests, and other environmental challenges.

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Chapter 11
Importance of Medicinal Compounds from Traditional Plants for the Treatment of Endometriosis

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Abstract Endometriosis is a challenging gynecological disorder that causes pelvic pain and infertility attributed to the prevalence of ectopic endometrial tissue outside the uterine tract. Based on the patient's histology findings, it is typically identified by pathological lesions, endometritis, pyometra, and glandular cystic hyperplasia. Traditional medicines are thought to be the most reliable sources for the discovery of novel pharmaceuticals, notwithstanding recent developments in computational and chemical techniques. Endometriosis and other gynecological illnesses have been tested against a variety of therapeutic herbs and chemicals derived from plants. In healthcare areas with few resources, traditional medicine is still seen as the main treatment option, regardless of recent scientific progress and globalization. Plant-derived compounds were once thought to be a key source of modern medications and play a crucial biological role against some pathogenic organisms. Ever since the dawn of humanity, an increasing number of plants have been utilized as remedies. In communities all across the world, traditional medicine has been an essential resource for health for centuries, and it is still a cornerstone for some people who have unequal access to mainstream treatment, according to research conducted by the World Health Organization (WHO). This book chapter presents the activity profile of medicinal plants and their active components while highlighting the development of multitargeted endometriosis medicinal compounds.

Keywords Endometriosis · Traditional plants · WHO · Plant-derived compounds · Therapeutic herbs

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11.1 Endometriosis: An Enigmatic Disorder

Endometrial glands including stroma that exist outside of the uterine cavity, primarily but not solely in the pelvic space, are referred to as endometriosis. There is an overwhelming view that "endometriotic disease" is defined by an appearance of symptoms or lesion progression that necessitates investigation and possible treatment, but certain researchers believe that very few forms of endometriosis can sometimes be regarded as a para-physiologic or transient histologic thing (Vercellini et al. 2014). The three well-known phenotypes of endometriosis are ovarian endometriomas (OMA), deep infiltrated endometriosis (DIE), and superficial peritoneal lesions (SUP). The illness is linked to weariness and depression, which reduces work productivity and places a significant financial burden on society. Endometriosis should be viewed as a public health concern rather than a personal illness in light of these consequences (Chapron et al. 2019).

Most experts agree that endometriosis lesions develop through coelomic metaplasia, lymphatic dissemination, and retrograde endometrial tissue loss with menstruation in immunologically and genetics-sensitive people. Although the exact cause is unknown, it is probably complex and involves genetic and possibly epigenetic factors and possible exposure to environmental hazards (Johnson and Hummelshoj 2013).

Angiogenesis is the process by which endometriotic implants are established and grow. It is induced by an intricate web of locally produced hormones, cytokines, autacoids, and probably environmental contaminants. Recent research suggests that ectopic endometriotic implants use neuroangiogenesis to draw in particular neuronal and vascular resources. These developing nerve fibers in endometriosis implants are thought to affect dorsal root neurons in the brain, possibly causing patients to feel more pain. We anticipate and believe that a deeper comprehension of the control of neo-angiogenesis in such lesions may influence potential models of endometriosis etiology, medication development, and treatment trials for this widespread and crippling gynecological disease (Asante and Taylor 2011).

The disclosure of unique endometriosis cases prompts discussion on the pathophysiology and etiology of this widespread condition (Seydel et al. 1996).

Endometrial implants' capacity to endure in ectopic placements might be connected to an abnormal immune response. Several immune anomalies were discovered as a result of extensive research into the immune system's role in endometriosis. There is strong evidence to suggest that immunologic variables contribute to the development of this condition and infertility brought on by endometriosis. A woman's susceptibility to the implantation of exfoliated cells from the endometrium may depend on immunologic variables. Endometriosis development may also be significantly influenced by the peritoneal cavity's surroundings (Berkkanoglu and Arici 2003). In patients with protracted infertility, the idea of uninterrupted extended involvement with menstruation may be significant for the onset of endometriosis. A lengthy period of unbroken menstruation might have a causal role in the origin of endometriosis in vulnerable women (fertile and infertile).

Endometriosis may be a sign of another acquired or developing pathological condition that persists even after the endometriosis has disappeared and may be connected to infertility (Mahmood and Templeton 1991; Ranney 1975).

Despite surgery being the preferred method of controlling endometriosis, recurrence is a daunting obstacle. A current unmet medical requirement in the treatment of endometriosis is to prevent or delay recurrence. To do this, suggestions have been made to look into recurrent patterns, create recurrence biomarkers, and implement biomarker-based interventions. The identification of biochemical traits or facets derived from medical specimens that can be utilized for recognizing patients with a high risk of specific recurrence, which, ultimately, allows for intervention to delay or, better yet, get rid of recurrence, should be the key objective of establishing biomarkers for recurring of endometriosis (Guo 2009).

Endometriosis, one of the most prevalent benign gynecological disorders, is a crippling illness with negative implications on social, vocational, and psychological functioning. Patients with infertility are more likely to have this condition by up to 30%, while patients with persistent pelvic discomfort are more likely to have it by up to 45% (Mehedintu 2014).

11.2 History of Endometriosis

After this, Sampson's original theory that the split of an ovarian endometrioma caused superficial peritoneal endometriosis was likely revised after noticing how the unattached, superficial peritoneal implants behaved similarly to eutopic endometrium. The implants themselves were identified as being made of menstrual blood that had been reabsorbed into the cavity of the pelvis. As a result, adenomyosis externa, ovarian endometrioma, and peritoneal endometriosis were considered illnesses. In light of current research, it may be crucial to keep in mind this evolving concept of the diagnosis of what is now often referred to as pelvic endometriosis (Benagiano et al. 2014).

Rokitansky had a brilliant insight in the middle of the nineteenth century: endometrial glands and stroma might be associated with ovarian and uterine neoplasia. Yet, Cullen was the very first scientist to identify peritoneal endometriosis as an "adenomyoma" employing histological characteristics related to endometrial structure and activity. However, Rokitansky was the initial researcher to coin the term adenomatous polyp, which is a type of adenomyosis. At the latter part of the nineteenth century, ovarian endometriomas were first described as "hematomas of the ovary" or "chocolate cysts" (Benagiano and Brosens 1991).

Karl von Rokitansky's microscopic discovery of endometriosis in 1860 marked a turning point in the disease's history; nevertheless, the events leading up to that discovery have received little attention. Even though studying history may not be exact, clinical observations from the past can offer fresh viewpoints that would otherwise have gone completely unnoticed. In addition, it is clear from looking at the historical history of modern medicine that almost all of our current understandings of

complicated disease states are the culmination of centuries worth of observations (Nezhat et al. 2012).

11.2.1 Endometriosis During the Late Seventeenth and Early Eighteenth Centuries

Despite the highly specialized field of the history of medicine, historians know very little about this frequently obscure illness that still receives insufficient coverage today. According to the most recent research in this area, German researcher Karl von Rokitansky's 1860 account of the pathophysiology of endometriosis was the first comprehensive one. According to the Scottish physician Arthur Duff, it comes with adolescence and is characterized by simple menstruation by prolonged hot flashes, thirst, restlessness, and tension or discomfort in the vicinity of the groin. These observant eighteenth-century physicians were able to see the etiology of endometriosis in some complexity despite using less exact scientific language than is used now and without the histology data that was accessible in the late twentieth century. Additionally, this group of doctors from the eighteenth century was aware that only women were afflicted by this condition, which, in the words of the German doctor Carl Stoelzel, "definitely creates visceral experiences that developed at the moment of the first menstruation." This conclusion is supported by the knowledge that some doctors had in the eighteenth century that these organic inflammations did not just have the potential to interfere with and suppress menstruation but also, it appears, have the capacity to simultaneously produce leukorrhea (Knapp 1999).

It is challenging to retrace the steps that led to the discovery of the conditions known as endometriosis and adenomyosis today. The general picture of the key characteristics of these illnesses was developed with the help of several researchers (Benagiano and Brosens 2011). Endometriosis is a condition that affects women that manifests after the first cycles of menstruation and is linked to the uterus area, particularly with pelvic pain, infertility, and frequent miscarriages, according to scholars from England, Germany, Holland, and Scotland who studied autopsies in the middle of the nineteenth century (Acién and Velasco 2013).

11.2.2 Understanding of Endometriosis During the Late Nineteenth and Early Twentieth Centuries

The evolution of anatomical and clinical research before von Rokitansky's outline of endometriosis in 1860, as well as advances afterward through 1946, is covered in *A History of Endometriosis*. Anesthesia's advancement and the switch from autopsy to surgical pathology have made it possible to study disorders like endometriosis much better (Martin 2012). The term "adenomyoma" (and endometriosis) was first used to

describe a disorder distinctly referred to as "hemorrhagic ovarian cysts," and it wasn't until 1921 that the endometriotic genesis of this condition was acknowledged. Frankl described the physical picture of endometriosis in 1925, 2 years earlier Sampson coined the term "endometriosis," to refer to the existence of uterine mucosa islets in the cavity of the peritoneum (Benagiano and Brosens 2006).

Despite the fact that endometriosis was initially identified in the early twentieth century, its cause is still unknown. Although many explanations have been advanced, none completely explains how endometriosis developed in all of the studied sites. Endometriosis has been classified into three primary phenotypes: superficial endometriosis, endometrioma, and deep infiltrating endometriosis. Endometriosis is a heterogeneous, complex disease that is influenced by a variety of hereditary and environmental variables (Hudson 2022).

11.3 Etiology of Endometriosis

It's interesting to note that endometriosis is seven times more common and typically more severe among women whose first-degree relatives also have the condition. The information above points to a polygenic/multifactorial pattern of endometriosis transmission. Endometriosis' etiology is uncertain. If Sampson's theory regarding histogenesis—according to which endometrial cells spill onto the ovary and other sites in the pelvis as a result of the retrograde tubal flow of menstrual dump from the uterus via the fallopian tubes—is accepted, mechanical factors that result in transplantation of fragments of endometrium could be thought of as the primary etiologic factors in the onset of this disease (Dmowski and Radwanska 1984).

The basic biological research of the ectopic endometrium could be closely related to a woman's risk of getting endometriosis, according to recent research from numerous laboratories. However, the precise functions of different biological factors and different endometrial types of cells during the onset and after the advancement of endometriosis are still up for debate. Although surgical diagnosis and pathologic verification of endometriosis will continue to be crucial for meeting the requirements of women with the condition, a more effective noninvasive diagnostic test is anticipated to replace surgical visualization as the gold standard for treating it (Osteen et al. 2005).

There are two main ideas that attempt to explain the origins of endometriosis; one postulates that the disease is brought on by mechanical factors, while the other emphasizes the involvement of estrogen. Many experts think that retrograde menstruation, which results in minute pieces of healthy endometrium settling in the peritoneal cavity, is the source of endometriosis. Many medical experts have hypothesized that endometriosis depends on estrogen. For instance, the illness almost never occurs before puberty and is very rare after menopause (Barbieri 1990).

11.3.1 Genetic Predisposition

Uterine leiomyomata (UL), among the most common tumor of the female reproductive system with the main cause of hysterectomy, convey severe morbidity and an enormous financial burden. These findings expand our knowledge of the biology and genetic variables that contribute to the emergence of uterine leiomyomata and indicate that endometriosis and uterus leiomyomata may share hereditary forebears (Gallagher et al. 2019). An important advantage of the study of genetic linkages for endometriosis in different ethnic populations is the capacity to shed insight into the division of distinct ethnicities' disequilibrium links. This "trans-ethnic detailed mapping" method is essential for focusing the signal on causative genetic variants. This highlights how important it is to evaluate genetic variants in different cultures in order to characterize the endometriosis inheritance pattern and the scope of the impact of specific risk alleles in different racial groups (Angioni et al. 2020).

11.3.2 Hormone Dependence

Though an in-depth analysis of initial chemotherapeutic and hormone therapy at this time does not appear feasible, we believe that first-line endocrine therapy may be beneficial for some patients with hormonal substance receptor-positive endometrioid tumors. Progestins function in a finely balanced manner to counteract the proliferative effects of estrogens on the endometrium in the menstrual cycle. Endometrial cancer may develop if progesterone is insufficiently able to control the proliferative effects of excessive estrogen production (Bennett et al. 2022). The innermost layer of the womb lining, known as the endometrium, has the unusual capacity to regenerate or lose cells depending on the phases and hormonal levels of the menstrual cycle. Environmental factors that promote oxidative stress, interfere with hormonal balance, or change immune responses can raise the incidence of endometriosis (Terzic et al. 2021).

11.3.3 Confrontation to Progesterone

It was previously thought that endometrial stromal cells in ectopic endometrium do not respond to progesterone just like they would in eutopic endometrium because there is no bioactive progesterone there. Although PR-A isoforms were retained, suggesting some progesterone resistance, progesterone mRNA and protein levels have been shown to have declined in endometriotic lesions (Donnez and Dolmans 2021).

Progesterone and the signaling pathways that regulate it are all carefully managed to maintain regular menstruation and guarantee a safe pregnancy. The imbalance of

the intricate regulatory systems of estrogen and progesterone results in estrogen dominance and progesterone resistance. It is important to emphasize the mechanisms through which abnormal progesterone signaling processes and cellular responses to progesterone contribute to the development or progression of gynecological diseases, including endometriosis (Maclean and Hayashi 2022).

11.4 Diagnosis of Endometriosis

A physical examination might reveal a wide range of results. Sometimes, especially in situations of moderate endometriosis, a gynecologic exam may reveal nothing unusual. The examination should ideally be conducted while the patient has at least some symptoms, particularly during menstruation, as this may make it easier to spot and locate locations that may be home to endometriosis. There is currently not much proof regarding the selective use of test results for tracking endometriosis relapse and therapeutic follow-up in particular populations at risk (Spaczynski and Duleba 2003). The search for a minimally invasive endometriosis test continues in the twenty-first century. Today, diagnostic laparoscopy with an examination of the abdominal cavity and histological validation of suspect lesions, is the only method that can reliably diagnose endometriosis. However, the necessity of histological backing is still in question because not all macroscopically visible endometriotic diseases are histologically validated. Combination tests are more inclined than individual ones to be effective in the diagnosis (Kiesel and Sourouni 2019).

Combining several criteria improves the capacity to detect endometriosis non-surgically. When used in conjunction with symptoms, a patient's medical history, and/or physical findings, transvaginal ultrasonography enhances accuracy and can be a helpful adjuvant to clinical diagnostic procedures. Deep endometriosis and ovarian endometriomas can both be found with ultrasound (Agarwal et al. 2019).The total removal of all symptomatic severe lesions in a single surgical phase is the aim of the medical management of deep lesions of the endometrium. The degree of exeresis relies on the effectiveness of the surgical intervention. Although MRI can identify all endometriosis sites, ultrasonography is still the go-to imaging technique since it is available right away and is simple to use (Kinkel et al. 2006).

11.5 Treatment of Endometriosis

The hormonal manipulation of the menstrual cycle has been the focus of medical therapy for endometriosis in an effort to induce a false pregnancy, false menopause, or persistent anovulation. Endometriosis is usually treated with progestational medications. They lead to the decidualization and eventual atrophy of endometrial tissue.

Endometriosis-related infertility is only ever cured surgically, with the illness being removed and the pelvis' anatomical relationships being returned to normal. This medication may be used in conjunction with assisted reproductive technologies to speed up conception and maybe boost the likelihood of a successful pregnancy (Avid et al. 2001). There are several treatments for endometriosis pain alleviation, including laparoscopic ablation, excision, gonadotropin-releasing hormone analogs (GnRHa), leuprolide, buserelin, goserelin, and triptorelin. Analogs of the gonadotropin-releasing hormone and laparoscopic ablation or excision are linked to higher clinical pregnancy rates in endometriosis-afflicted women. Randomized clinical trials are required to ascertain if surgical procedures in endometrioma-affected women affect the ovary's ability to produce fertile egg cells, leading to a healthy pregnancy and live birth (Brown and Farquhar 2015).

11.5.1 Hormone Treatments

11.5.1.1 GnRH Antagonists

Progestins and GnRH agonists are preferably beneficial in lowering endometriotic lesions' severity and symptoms. Endometriosis treatment experiences over the past 10 years have demonstrated that the progression and remission of this condition are estrogen-dependent. The action of paracrine and endocrine factors, such as angiogenic and growth-promoting elements, which are hypothesized to be implicated in the pathogenic processes of endometriosis, is negatively impacted by the administration of a GnRH agonist. A novel therapeutic approach for the management of symptomatic endometriosis is made possible by the sequential delivery of the GnRH antagonist cetrorelix (Küpker et al. 2002). It has a favorable pharmacokinetic pattern for a single daily dosage. Linzagolix is a novel oral GnRH antagonist. With or without add-back therapy, this medication alters serum E2 suppression in a dose-dependent way. For the treatment of pain brought on by endometriosis and uterine myomas, linzagolix is currently undergoing late-stage clinical trials. According to the findings of earlier clinical trials, linzagolix appears to quickly and effectively reduce the symptoms of both gynecological illnesses while maintaining a high level of tolerability. In women who have limitations to other hormonal medications or who simply reject them, this medication may have the potential to be used as a standalone therapy option (Dababou et al. 2021).

Patients with symptoms that continue after receiving first-line therapies may be treated with GnRH agonists; however, long-term use of GnRH agonists is linked to frequent adverse events related to hypoestrogenism, so the combination with add-back medical care is essential. The most fascinating innovation for real therapeutic usage in the treatment of endometriosis in this setting is the GnRH antagonist elagolix. The oral formulation of this medication and its brief half-life (6 h), which enables quick clearance of elagolix from the system if treatment must be stopped for

any reason, are its two key advantages over traditional GnRH agonists (Alessandro et al. 2017).

11.5.1.2 Selective Progesterone Receptor Modulators

The endometrial (glands and stroma) that is present outside of the cavity of the uterus is known as endometriosis. Because it is estrogen-dependent, this illness is most common in women throughout their reproductive years. Progesterone receptor modulators (PRMs) have been recommended for the treatment of endometriosis because of their antiproliferative impact on the endometrium. Mifepristone appears to treat dysmenorrhea in endometriosis-affected women, and there is some evidence that it may also relieve dyspareunia; however, amenorrhea and hot flashes are frequent side effects. Data on dosage were equivocal; however, they do imply that mifepristone 2.5 mg may not be as effective as greater doses (Fu et al. 2017).

11.5.1.3 Selective Estrogen Receptor Modulators

Progestins can be added to the hormone replacement therapy regimen to successfully prevent endometrial cancer, which has been associated with decades of unrestricted estrogen usage. The majority of early-stage breast and endometrial tumors are ER-positive, and hormone therapy is the first line of treatment. Independent of breast cancer stage, tamoxifen, an ER inhibitor in the breast, is the usual medication used for the treatment of pre- or postpartum ER-positive patients. Tamoxifen is an ER partial agonist in the endometrium even though it is frequently used as an antiestrogenic medication to treat ER-positive breast cancer. In positive for ER ECC-1 endometrial cancer cells, the estrogen and antiestrogen regulation of either briefly transfected gene reporters or endogenous genes is assessed (Dardes et al. 2002). It has been established that all three endometrial cell lines—Ishikawa and ECC-1 from human endometrial adenocarcinomas and one immortalized from sheep endometrial stroma—responded to estrogen receptor E2 (estradiol) by upregulating PR (progesterone receptor) mRNA quantities. Indicating that E2 (estradiol) was likely working through the ER (estrogen receptor) protein on the transcriptional function of the PR (progesterone receptor) gene, this was consistently inhibited by SERMs (selective estrogen receptor modulators) (Farnell and Ing 2003).

11.5.1.4 Aromatase Inhibitors

AIs (aromatase inhibitors) have been utilized to treat pain related to endometriosis based on molecular studies of elevated expression of aromatase P450 in endometriotic tissues. Medication with aromatase inhibitors (AI) must be taken with additional medications to downregulate the ovaries since premenopausal women have a rise in FSH (follicle-stimulating hormone) following follicular

development. Even reproductive-age and postmenopausal women may find improvement in their chronic pelvic pain by using aromatase inhibitors (Pavone and Bulun 2012). Since the invention of GnRH-a in the 1980s, aromatase inhibitors seemed to represent the first advancement in the medical management of endometriosis. AIs (aromatase inhibitors) tend to provide significant pain relief for endometriosis patients who do not react to conventional therapies. Treatment options for premenopausal endometriosis that are innovative and promising include the administration of aromatase inhibitors together with an ovarian suppressor. The pain or infertility linked to endometriosis may also be treated with lower doses of aromatase inhibitors (AIs) (Attar and Bulun 2006).

First, aromatase inhibitors seem to be important candidate drugs for the treatment of endometriosis, independent of the cause. The fast absence of pelvic pain, in this case, serves as an example of the treatment's astonishingly excellent response. Strong aromatase inhibitors are potential treatments for endometriosis that are ineffective with conventional regimens (aromatase P450 mRNA) (Takayama et al. 1998).

11.5.2 Nonhormonal Treatments

11.5.2.1 Immunomodulators

For usage in the clinical environment, new medications that target the immunological abnormalities frequently observed in endometriosis patients are being researched. They are believed to function by reducing the immune system's response to illness. Although the majority of these substances have not yet been properly studied in people, preliminary research in rodent models seems encouraging. Loxoribine, IFN-2, and TNF inhibitors are among the many drugs that fall under this category. Another immunomodulator, IFN- 2, has been demonstrated to lessen endometriosis in tissue cultures and animal models. Intraperitoneal inflammation may be the target of new immune modulators, which could be advantageous (Rodgers and Falcone 2008). It has been proposed that using immunomodulators like IL-2 to increase the cytotoxic function of macrophages and killer cells can help endometriosis therapy options. In one investigation, endometriotic explants in rats treated with the selective COX-2 inhibitor rofecoxib, an immunomodulator, experienced regression. In a different rat trial, treatment with the COX-2 inhibitor celecoxib started on the day endometriosis was induced and stopped the early growth of ectopic implants (Mihalyi et al. 2006).

11.5.2.2 Antiangiogenic Agents

Antiangiogenic drugs disrupted the vascular supply, which prevented the development of explants in an in vivo model of endometriosis; this result is probably

applicable to human illness. Two VEGF-A antagonists, an antibody and the shortened inhibitory receptor sflt-1, were first demonstrated to have antiangiogenic effects in vivo using a rat sponge implant paradigm. They prevented the progression of human endometrial tissue transplanted into mice, according to later investigations. The blood arteries that support endometrial explants in females are largely immature. Anti-VEGF-A medications may therefore have the ability to alter the vasculature of female endometriotic lesions (Hull et al. 2003). In many laboratory and animal models, it has been demonstrated that administering antiangiogenic medications lessens the development, maintenance, and growth of endometriotic lesions. In particular, it would be wise to research the efficacy and safety of the antiangiogenic compounds in baboons, a nonhuman primate model of endometriosis. Antiangiogenic drugs' function in the management of endometriosis is yet unclear. Antiangiogenic medications don't seem likely to be able to treat the symptoms brought on by large endometriotic lumps that are primarily made of fibromuscular tissue (Ferrero et al. 2006).

11.6 Significance of Foods in Endometriosis Treatment

Social and environmental factors play a significant influence on the relationship between endometriosis and physical activity or perception of physical activity; thus, social and psychological support may be even more relevant in this case. The studies have shown that sessions of health education about altering lifestyle, specifically dietary health education, were effective and had led to an important rise in understanding and a reduction of pain associated with endometriosis with a noteworthy betterment in pain for a period of time after finishing of health education (Ghonemy and El Sharkawy 2017). Foods high in omega-3 fatty acids, which have anti-inflammatory properties, N-acetylcysteine, vitamin D, and resveratrol supplements, along with a greater number of vegetables and fruits (preferably organic), and whole grains, have a protective effect that lowers the risk of disease development and potential remission. The avoidance and management of endometriosis may benefit from dietary re-education (Halpern et al. 2015).

Endometriosis is more likely to develop in people with inadequate amounts of vitamin D, zinc, and vitamin E. Due to their antiangiogenic properties, magnesium, curcumin, resveratrol, and ECGC were advantageous in animal experiments. In human investigations, omega 3 fatty acids and alpha-lipoic acid reduced pain brought on by endometriosis. In research including both animals and people, supplements of curcumin, omega 3, NAC, vitamin C, and ECGC reduced the extent of endometriotic lesions. In animal experiments, it was discovered that curcumin, omega 3, and NAC stop the etiology or recurrent illness (Bahat et al. 2022).

11.7 Traditional Herbal Medicines for Overcoming Endometriosis

Despite technological advancements, there haven't been many breakthroughs in drug research. The investigations showed that endometriosis was significantly impacted by treatment with medicinal herbs and their phytoconstituents. The employment of medicinal herbs for treating endometriosis has expanded dramatically in recent years. Many plants have been traditionally used to treat various gynecological conditions, including endometriosis. Furthermore, numerous research has demonstrated the efficacy of ginsenoside, curcumin, puerarin, epigallocatechin gallate, and resveratrol as endometriosis treatments. Therefore, using medicinal plants and their phytoconstituents has been viewed as an innovative strategy for managing endometriosis and keeping a healthy lifestyle (Ilhan et al. 2018).

11.7.1 Studies Concerning Medicinal Plants for Endometriosis

From ancient times, people have used phytomedicine all across the world to heal or prevent human disease. Herbal extracts are multicomponent combinations that are quite complicated. Therefore, the combined effects of their distinct parts may be very advantageous (Meresman et al. 2021).

11.7.1.1 PFE (*Pueraria* Flower Extract)

Pueraria flowers were historically used as a vegetable, a component of tea, and an ingredient in jam. PFE (*Pueraria* flower extract) treatment activated an extracellular signal-regulated kinase (ERK)1/2, and ERK1/2 inhibitor PD98059 dramatically reduced PFE-inhibited cell movement in endometriotic cells. Additionally, PFE (*Pueraria* flower extract) dramatically reduced the development of endometriotic lesions in a mouse model. These findings imply that the *Pueraria* flower may have anti-endometriotic properties by preventing adhesion, movement, and MMP (matrix metalloproteinase) activity of endometriotic cells (Kim et al. 2017).

11.7.1.2 Hexane Extract of Aged Black Garlic

Human endometriosis prevention and treatment may be aided by the use of HEABG (aged black garlic extract in hexane). Our findings are particularly significant because they show that HEABG, a natural product, offers insightful information into the creation of novel and advantageous therapeutic modalities for treating endometriosis. This is critical because conventional hormone-based medications

have serious disadvantages when used for the long-term control of endometriosis. In TNF-induced HESCs (human endometrial stromal cells), HEABG dramatically reduced cell proliferation and cell cycle progression by inhibiting the ERK (extracellular signal-regulated kinase) and JNK (c-Jun N-terminal kinase) signaling pathways (Kim et al. 2013).

11.7.1.3 Viburnum Opulus Extract

A gynecological condition known as endometriosis is marked by the presence of endometrial tissue beyond the uterine cavity. Primary and secondary dysmenorrhea, ovarian cysts, and other gynecological problems have all been treated with the fruits of *Viburnum opulus L.* Biological activity of fruit extracts was determined using a rat model of surgically induced endometriosis. The administration of EtOAc (ethyl acetate) and MeOH (methanol) extracts was found to drastically lower the endometriotic volumes. It is necessary to undertake more thorough photochemical investigations to identify the constituents that are essential to the action (Saltan et al. 2016).

11.7.2 Usage of Phytoconstituents in Endometriosis Treatment

11.7.2.1 Resveratrol

Resveratrol's fundamental cellular and molecular processes have been the subject of much research. Resveratrol inhibits the proliferation of endometriotic lesions; induces apoptosis; reduces inflammation, angiogenesis, and oxidative stress; and blocks adhesion and invasion. These protective effects of resveratrol toward endometriosis are exerted by a network of numerous cell signaling pathways. Numerous clinical trials indicate that resveratrol is pharmacologically relatively safe (Kolahdouz Mohammadi and Arablou 2017).

11.7.2.2 Curcumin

In endometriosis, curcumin can reduce oxidative stress and inflammation. Curcumin can also have a direct impact on endometrial lesions' invasion, adhesion, apoptosis, and angiogenesis. For women's disease management and nutritional disease prevention, curcumin usage may be of importance (Vallée and Lecarpentier 2020).

11.7.2.3 Apigenin

Employing array-based comparative genomic hybridization, genomic abnormalities were discovered in endometrial cancer cells that had been exposed to apigenin and other phyto-estrogenic substances. In comparison to cancer cells treated with the same concentration of apigenin, more than 20% of the array of genes involved in insulin metabolism were altered in the ß-estradiol-treated cancer cells, indicating that it may be useful for the management of endometrial cancer and postmenopausal women (Patel et al. 2007).

11.8 Conclusion

Natural substances originating from plants are excellent possibilities for the creation of cutting-edge endometriosis management treatment plans. They frequently have a pleiotropic action profile that concurrently targets key elements of the intricate pathophysiology of the disease, including angiogenesis, apoptosis, inflammation, and cell proliferation. Since they are usually noticed in response to targeted therapy, they may therefore aid in preventing escape mechanisms. In vitro animal and human studies on phytotherapy, which includes medicinal plants, phytochemicals, and multicomponent herbal formulations, have shown encouraging outcomes. The majority of medicinal plants include phenolic chemicals that have an impact on endometriosis. Although encouraging, the information currently available is primarily on endometriosis in vitro and animal models, with a scant number of well-conducted clinical investigations. Consequently, in order to get more certain conclusions concerning the promising function of phytotherapy in the treatment of endometriosis, adequately designed clinical trials are essential.

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Chapter 12
Secondary Metabolites of Endophytic Fungi Against Candidiasis

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Abstract Plants have the ability to produce bioactive substances that aid in the treatment or prevention of disease. They also offer endophytes a special environment. The bulk of these endophytes are fungi that defend their hosts from infections through a mutualistic connection, yet occasionally they may also behave as opportunistic pathogens. Many endophytic fungi have been identified as sources of novel metabolites with potential for use in pharmaceuticals. Also, they have the capacity to produce a variety of bioactive metabolites that may be employed, either directly or indirectly, as therapeutic agents against a wide range of diseases and have drawn the interest worldwide due to their strong demand. Invasive fungal infections brought on by *Candida* species have emerged during the past three decades as a significant public health issue due to their high rates of morbidity and mortality in immunocompromised and hospitalized patients. Candida infections are difficult to diagnose and typically respond poorly to therapy. As a result, a variety of medicines that are now used to treat candidiasis commonly cause resistance in individuals, encouraging toxicity as a result of prolonged therapy. Therefore, a precise diagnostics and cutting-edge antifungals are of utmost importance in order to raise the quality of life and life expectancy of those affected with this infection. Several plant groups include a variety of bioactive secondary metabolites, including terpenoids, organosulfur compounds, isoquinoline alkaloids, flavonoids, fatty acids, lactone, naphthoquinone, phenolic compounds, etc. Numerous researchers have examined the inhibitory mechanisms of endophytic fungal bioactives against *Candida albicans* and other *Candida* spp.

Keywords Endophytes · Metabolites · Pharmaceuticals · Bioactive substances · Infections · Candidiasis

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12.1 Introduction

The phrases endophyte and endophytic fungus have been used extensively over the past 30 years to refer to the internal mycotic of living plants in the mycological literature. Notwithstanding the phrases' nineteenth-century roots, they now have a completely different meaning than when they were first coined (Large 1962; Larran et al. 2007). To describe a particular host type, a taxonomic group of hosts, or the type of tissue occupied, the phrases are frequently used in combination with modifiers (e.g., systemic grass endophytes, bark endophytes). The terms are not always used consistently today, and not all researchers agree with them (Pereira et al. 1993; Saikkonen et al. 1998). Yet, in general, the words refer to fungi that can asymptomatically occupy seemingly healthy plant tissue. Endophytic fungi, in the broadest sense, are fungi that invade living plant tissue without immediately manifesting any detrimental effects (Hirsch and Braun 1992). This definition essentially encompasses the full range of symbiotic relationships that fungi and plants engage in, including parasitism, commensalism, and mutualism.

Forest pathologists classify many of the fungi that are frequently described as endophytes as minor or secondary diseases. Their frequent appearances in both healthy and unhealthy tissues highlight how difficult it is to draw clear distinctions between endophytes, facultative pathogens, and latent pathogens. In fact, the behavioral differences between many fungi regarded as "endophytic" and those regarded as "latent pathogens" are negligible and may only be caused by variations in the length of the latent or quiescent phase and the severity of the host harm incurred during the fungus' active growth (Schardl et al. 1994).

Endophytes include a variety of commensal saprobic and mutualistic fungi that have cryptic, imperceptible patterns of host colonization, as well as pathogenic fungi that can occupy their hosts asymptomatically for part of the infection cycle, "quiescent infections," and strains with reduced virulence. This extended, unnoticeable time during which growth and colonization temporarily halt and then resume when the host undergoes a physical or maturational change is a hallmark of "endophytic" fungi. Whether endophytes are ultimately classified as commensal saprobes, latent pathogens, or protective mutualists, this periodic growth is a distinctive characteristic of them. The majority of fungal biologists agree that, despite the fact that such a definition might seem overly broad, the species composition of the internal mycobiota differs for different hosts, organs, and tissues (Fisher et al. 1992; Freeman and Rodriguez 1993). However, some endophytic infection species may also be present in the epiphytic or rhizosphere mycobiota.

As a result of recruitment, plants formed symbioses with a wide variety of soil microorganisms, some of which can live inside plant tissues and interact with the host endophytically. The endophytic fungi may first adhere to the surface of the roots and develop structures resembling appressoria (Yedidia et al. 1999). These associated fungi subsequently go into and populate the internal tissues of plants after penetrating the root systems' outer layers (Nogueira-Lopez et al. 2018; Viterbo and Chet 2006). The integrity of the cells was not compromised during the early

colonization of roots by the endophytic fungus *Trichoderma*, according to microscopic examinations. However, the necrosis of the penetration peg, the elevated chitinase activity, and the generation of fluorescent chemicals in the intercellular gaps were seen in cucumber roots that had been colonized by endophytes. This could be as a result of the extensive extracellular enzyme production by the endophytic fungus (Suryanarayanan et al. 2012). In actuality, rather than remaining static, the established endophytic interactions respond to real-time dynamic change. Citrus diseases, or the yellowing of the leaves, have been demonstrated in studies to influence foliar endophytic communities more than endophyte assemblages of healthy leaves. This shows that certain endophytes may grow more quickly when a leaf becomes yellow than others (Douanla-Meli et al. 2013). Microbial communities are influenced by an interaction between host genotype and abiotic conditions. By interactions between microbes, transmission of the impacts to the microbial community, and altering the architecture of plant microbial communities, these differences have a direct impact on a small number of closely related taxa and have a significant impact on communities (Agler et al. 2016). To counteract reduced plant photosynthesis and altered host nitrogen metabolism, endophytic fungus can alter the genetics and phenotypic expression of the host to increase resistance to diseases and herbivores (Mejía et al. 2014). Endophytic fungi retain symptomless survival and benefit their host plants thanks to this dynamic regulation. Hence, the precise control of host genes, phenotypes, and metabolism results in the relationship between endophytic fungi and their host plants.

Endophytes would create a variety of bioactive substances that would aid host plant growth while also assisting the host plants in coping with external biotic and abiotic challenges. Some endophytic fungi have the capacity to produce bioactive compounds that are identical or similar to those that come from the host plants. Paclitaxel, podophyllotoxin, camptothecin, vinblastine, hypericin, and diosgenin, which were also produced by their host plants, were created by the endophytic fungi. The applications of endophytic fungi in various fields are shown in Fig. 12.1.

The term "candidiasis" is used to refer broadly to cutaneous, mucosal, and deep-seated organ infections brought on by fungi of the *Candida* genus. These illnesses can strike at any age and are typically associated with readily observable risk factors for infection. A deep-seated infection such as an intra-abdominal abscess, peritonitis (inflammation of the peritoneum, the tissue covering the inner wall of the abdomen and abdominal organs), or osteomyelitis (infection of the bones), with or without candidemia, is referred to as invasive candidiasis (Chowdhary et al. 2017; Lockhart et al. 2017). A developing infection directly related to technological developments in medicine, invasive candidiasis is widely acknowledged as a leading source of morbidity and mortality in the medical setting (Clancy and Nguyen 2017). At least 15 different *Candida* species are capable of harming humans, although just five of them—*Candida albicans*, *Candida glabrata*, *Candida tropicalis*, *Candida parapsilosis*, and *Candida krusei*—are responsible for the bulk of invasive infections. *Candida auris*, a relatively uncommon microbe, has become a significant disease in several parts of the world (Magill et al. 2014; McCarty and Pappas 2016; Wisplinghoff et al. 2004).

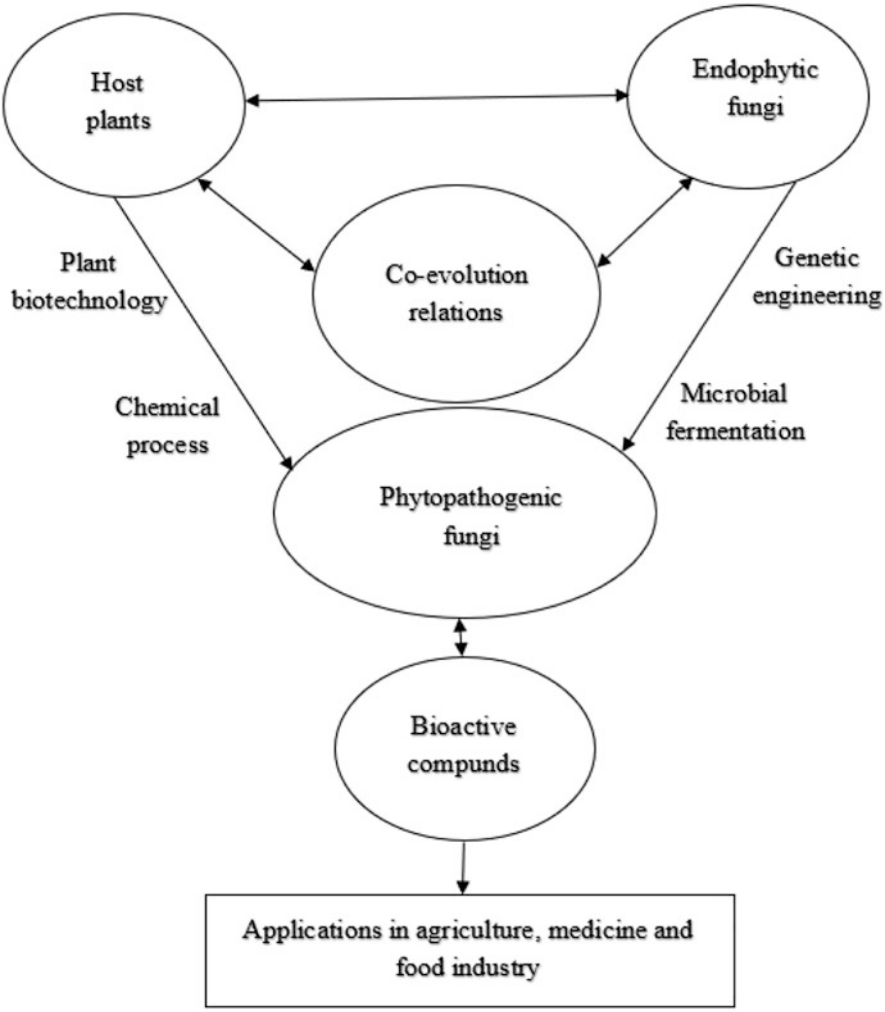

Fig. 12.1 Applications of endophytic fungi in various fields

Together with *Staphylococcus aureus*, coagulase-negative staphylococci, and *Enterococcus* species, *Candida* spp. are among the top three or four bacteria responsible for healthcare-associated bloodstream infections in many affluent nations. According to data from the National Healthcare and Safety Network, *Candida* spp. may be to blame for up to 22% of bloodstream infections connected to healthcare in the United States; it should be noted that this was a very selective patient cohort made up only of people using antibiotics (Magill et al. 2014). A point prevalence assessment of ICUs from throughout the world found that 18% of infections were caused by *Candida* spp. (Vincent et al. 2009; Playford et al.

2009). The complexity of sickness often linked to this infection is reflected by the fact that 50% of episodes of candidemia take place in the ICU. All patients in hospitals are at risk for developing invasive candidiasis, but those in the intensive care unit are more vulnerable due to well-described risk factors. Some risk factors originate from iatrogenic interventions, whereas others are inherent to the host or the disease state. Indwelling central venous catheters, broad-spectrum antibacterial exposure, prolonged ICU stays with or without assisted ventilation, recent major surgery, necrotizing pancreatitis, any form of dialysis, total parenteral nutrition, and iatrogenic immunosuppression are among the most prevalent individual risk factors (Wisplinghoff et al. 2004).

Age, local epidemiology, geographic location, and other variables all affect candidemia occurrence. Incidence rates of 3–5 per 100,000 people in the general population and 1–2% of all admissions to medical and surgical ICUs are reported in the majority of big national surveys (McCarty and Pappas 2016). A relatively recent discovery in the United States and other developed nations, community-acquired candidemia (i.e., acquired outside of a hospital) is a result of increased use of long-term intravenous access devices (such as peripherally inserted central catheters and tunneled intravascular catheters) and parenteral outpatient antimicrobial therapy. A more accurate estimate of the attributable mortality rate for all patients with candidemia is likely to be between 10 and 20%, with the risk of death being closely related to growing older, having higher APACHE II scores, having the infecting *Candida* species (e.g., *C. parapsilosis* is less virulent than other *Candida* species and is typically associated with lower all-cause mortality), and using immunosuppressive medications. According to reports, the attribute cost of candidemia is around US $40,000 per patient (Strollo et al. 2017).

12.2 Endophytic Fungal Diversity

The majority of endophytic fungi are *Ascomycota* members or their mitosporic fungus, together with a few species from *Basidiomycota*, *Zygomycota*, and *Oomycota* (Drew and Demain 1977). These endophytic fungi form symbiotic-to-pathogenic connections with the host while living inside the host's live tissues. These fungi colonize plants in a variety of ways, and some therapeutic plants are said to host more endophytes than others. It is recognized that the endophytes attached to medicinal plants have the capacity to manufacture the active ingredients for which the host is famous (Zaynab et al. 2019; Zheng and Jiang 1995; Perotto et al. 2002). Many endophytic fungi isolated from various groups of plants have been identified using both conventional taxonomic techniques and molecular taxonomy. It is well known that the isolated fungus creates a variety of chemicals that are both unique to the host and also universal to all hosts. In terms of characterization, species diversity, and bioactive compounds, research on the diversity of endophytic fungus is crucial (Huang et al. 2008; Verekar et al. 2014; Nadeem et al. 2012). Sequences of the 5.8S gene and the adjacent internal transcribed spacers (ITS1 and ITS2) of the rDNA,

18S, and 28S rRNA genes are utilized as molecular tools to research endophytic fungi. It is likely that many endophytic fungi cannot be cultivated due to the drawbacks of conventional isolation techniques (Garyali et al. 2013; Lucero et al. 2011). Molecular methods have also been used to identify endophytic fungi directly in the host tissues, overcoming any potential technical bias (Deckert et al. 2001).

12.3 Bioactive Metabolites of Endophytic Fungi

On our globe, there could be up to one million different types of fungi, according to estimates. Plant scientists have just started to understand that plants may act as a reservoir for countless numbers of endophytes or other microorganisms (Hawksworth and Rossman 1997). Some of these endophytes might be generating bioactive compounds that could have a role in the connection between the host and the endophyte. These metabolites may ultimately be found to be useful in medicine as a direct result of the role that they may play in nature. Endophytes are currently being isolated, and their natural products are being studied by scientists all over the world (Strobel 2003).

12.3.1 Primary Metabolites

Catabolic enzymes work in microbial cells to break down complex, high-molecular-weight carbon and energy sources as they expand. The finished goods of primary intermediates such amino acids, nucleotides, vitamins, carbohydrates, and fatty acids are produced as a result of catabolism. These biosynthetic intermediates are subsequently put together to form the intricate and crucial metabolites that give organisms their structure and biological function. The pathways that make up primary metabolism are made up of several mutually dependent catabolic and biosynthetic processes. The substances and enzymes of primary metabolism play fundamental, clearly defined, and frequently crucial roles in supporting microbial growth and reproduction (Drew and Demain 1977).

On the other hand, substances produced by secondary metabolism have hidden roles in the survival, growth, and reproduction of the generating organisms. Those substances and the specific enzymatic processes required for their synthesis seem to be of secondary importance to the organism and not necessary for growth. Although secondary metabolites differ from primary metabolites in terms of both structure and metabolic activity, this division tends to downplay the intricate interactions between primary and secondary metabolism. As the precursors for secondary metabolism are frequently provided by major metabolic pathways, we would anticipate that variables affecting primary metabolism would also affect secondary metabolism. It is clear that primary metabolism requires metabolic regulation. Efficiency plays a

significant role in microbial species survival, just like it does in all competitive processes.

A variety of microbial species can effectively compete in nature for the finite resources required for growth and reproduction due to the regulatory mechanisms that have developed to control the synthesis and function of the hundreds of enzymes involved in primary metabolism. Although our knowledge of these processes is fairly restricted, we may comment on some fundamental forms of regulatory activity. Primary metabolism is regulated by a number of different regulatory systems (Drew and Demain 1977; Zaynab et al. 2019).

When plants in a natural environment are exposed to a variety of diseases, they naturally create defense to preserve their fitness and reduce pathogenic harm. Research on the relationship between plants and diseases is becoming more important since they use primary metabolites as weapons. Pathogenesis and resistance are the ultimate outcomes of either defense strategy (Ferreira et al. 2007). Plant defense mechanisms can be divided into two categories: chemical, structural, and morphological defense and inducible and constitutive defense. A few defense mechanisms are quite active, but the majority are passive and only defensive against infections (Passardi et al. 2004). The significance of plant primary metabolites in immunity is attracting attention. Through signal transduction and pathogen identification mechanisms, primary metabolites carry out their role as molecules signaling to initiate a defense response. Primary metabolite production starts when the necessary nutrients are present in a growth medium during the active development phase (trophophase). Cell growth, development, and reproduction depend on primary metabolism (Penninckx et al. 1996). The primary metabolites participate in the primary response by controlling the synthesis of proteins, carbohydrates, and lipids in response to pathogen infection. Primary and secondary metabolism during pathogen infection affects plant growth and productivity. Supplying energy is necessary for the defense reaction (Gao et al. 2000). When there is a shortage of nutrients, pathogens can readily influence plant metabolism, which raises the requirement for nutrients to be assimilated. The reason metabolism slows down is that photosynthesis is reduced after a pathogen infection, which alters the growth of necrotic and chlorotic tissue and the sugar accumulation (Broekaert et al. 1995). There is very little information on the role of major metabolic pathways in the control of plant defense responses for growth and development (Kishimoto et al. 2002).

The host plant produces pathogenesis-related (PR) proteins, but these proteins are only activated in pathological conditions like bacterial, viral, or fungal diseases. The PR-9 family's peroxidase activity may contribute to cell wall fortification by catalyzing lignification, which would boost pathogen resistance. Families PR-12, PR-13, and PR-14 demonstrated antifungal properties. Proteins PR-15 and PR-16 produce hydrogen peroxide, which may be useful for enhancing plant defense or detrimental to intruders (Yamamoto et al. 2000). Two defensins (RsAFP1 and RsAFP2) were produced by *Alternaria brassicicola*-inoculated leaves. When pathogens attack, defensin inducibility has been seen in tobacco and *Arabidopsis* sepals. Several investigations have shown that plant defensins shield vegetative tissue against pathogen attack (Takahashi et al. 2005). By increasing defensin constitutive

expression, tobacco and tomato plants are defended against *Alternaria longipes* and *Alternaria solani*, respectively, which are fungal diseases (Rafin et al. 2000). By constitutively expressing *Brassica napus* defensin, resistance to the *Leptosphaeria maculans*-caused blackleg disease is increased. The potato exhibited a higher resistance to *Verticillium dahliae* due to defensins' constitutive expression (Tomoya et al. 2013). *Rhizoctonia solani* was reduced in canola and tobacco transgenic plants by overexpressing PR-3. Carrot and *N. sylvestris* were more resistant to *R. solani* when treated with tobacco chitinase. *Magnaporthe grisea*, *R. solani*, *B. cinerea*, *Uncinula necator*, and *Puccinia coronata* were all susceptible to rice chitinases' efficacy. *Fusarium oxysporum* in vitro activity of carbamic esters was assessed (Majumdar et al. 2017). Thionins with antifungal qualities have the ability to cause the formation of open pores on phytopathogens' cell membranes, which leads to the release of calcium and potassium ions from the cell and the antifungal activity of the Thi2.4 protein against *F. graminearum*. Maize's ZmPRms protein increases resistance to *Aspergillus flavus* (Majumdar et al. 2017). *R. solani*-induced tomato foot rot and the expression of defense-related genes including chitinase and peroxidase in the resistant and susceptible cultivars were assessed. The important aspect of a plant's basic defense mechanisms is the creation of physical barriers at places of attempted fungal penetration. These structures stop pathogen growth in plant tissues (Taheri and Tarighi 2012).

12.3.2 Secondary Metabolites

Organic compounds known as secondary metabolites are not necessary for an organism's regular growth and development. While primary metabolites play a crucial role in the survival of the species by actively participating in photosynthesis and respiration, the absence of secondary metabolites results in long-term impairment of the organism's survivability instead, which frequently plays a crucial role in plant defense. These substances are a hugely varied category of organic materials produced by a wide range of organisms, including plants, fungi, bacteria, algae, and mammals. The majority of secondary metabolites, including terpenes, phenolic compounds, and alkaloids, are categorized according to where they were created synthetically. Several medicinal, aromatic, colorant, and spice plants as well as some functional foods contain various classes of these chemicals, which are frequently linked to a small number of species within a phylogenetic group.

A transition from active growth to stationary phase typically results in the highest amounts of secondary metabolite production. The fact that the producer organism can develop without them suggests that secondary metabolism is not necessary, at least for immediate survival. According to a different theory, the genes responsible for secondary metabolism act as a "genetic playing field" on which natural selection and mutation can work together to fix new advantageous features. According to a third viewpoint, secondary metabolism is an essential component of cellular metabolism and biology. It depends on primary metabolism for the enzymes, energy,

substrates, and cellular machinery that it needs to function, and it helps ensure the producer's long-term survival (Roze et al. 2011).

Terpenes (such as plant volatiles, cardiac glycosides, carotenoids, and sterols), phenolics (such as phenolic acids, coumarins, lignans, stilbenes, flavonoids, tannins, and lignins), and nitrogen-containing chemicals make up a straightforward classification of secondary metabolites (such as alkaloids and glucosinolates). It has been claimed that a number of conventional separation methods using different solvent systems and spray reagents can distinguish between secondary metabolites using diverse adsorbents and eluents through column chromatography (CC) and thin layer chromatography.

12.3.2.1 Biological Activities

Numerous endophytic fungi have been identified as sources of new metabolites with potential medicinal use. Additionally, they have the capacity to produce a variety of bioactive metabolites, which may be employed directly or indirectly as therapeutic agents against a wide range of diseases. Endophytes have a variety of bioactive compounds that can be used to make essential medicinal medications commercially (Zhang et al. 2006). These bioactive compounds are primarily secondary metabolites and have been shown to have a variety of pharmacological effects. Many beneficial bioactive substances with antibacterial, cytotoxic, anticancer, antioxidant, antimalarial, antiviral, and antituberculotic activity have been successfully isolated from the endophytic mycoflora over the past two decades. From the endophytic fungus *Phomopsis longicolla* S1B4 isolated from a plant in South Korea, Lim et al. extracted various antibacterial chemicals, including dicerandrol A (1), dicerandrol B (2), dicerandrol C (3), deacetylphomoxanthone B (4), and fusaristatin A (5). The minimum inhibitory concentrations (MICs) of these substances against *Xanthomonas oryzae* KACC 10331 were 8, 16, 4, and 128 g/mL, respectively. Dicerandrol A also demonstrated antibacterial action against *Bacillus subtilis* KCTC 1021 and *S. aureus* KCTC 1916. Sim et al. isolated 24 endophytic fungi from *Garcinia mangostana* and *Garcinia parvifolia* and used filtered broth suspension to examine the antibacterial activity (Lim et al. 2010). On average, eleven isolates (or 46%) had antibacterial activity against one or more test organisms. An antimicrobial action was displayed by *Colletotrichum* sp., which was isolated from the medicinal herb *Lippia sidoides*. From the leaves and stems of *L. sidoides*, a total of 203 endophytic fungi representing 14 species of *Ascomycota*, *Coelomycetes*, and *Hyphomycetes* were isolated. The most typical fungus that was isolated was *C. gloeosporioides*, which was followed by *Alternaria alternata*, *Guignardia bidwellii*, and *Phomopsis archeri*. *S. aureus* and *B. subtilis* were resistant to the endophytic fungus *A. alternata*, *P. archeri*, *C. gloeosporioides*, and *Drechslera dematioidea's* antimicrobial effects (Sim et al. 2010; Ichikawa et al. 1971). The medicinal plant *Michelia champaca* produces a variety of secondary metabolites with a wide range of pharmacological characteristics. This plant's endophytic fungus exhibits antifungal, anticancer, and acetylcholinesterase (AChE)-inhibiting

properties. The activity of each extract was seen against two phytopathogenic fungi. The endophytic fungus *C. gloeosporioides* ethanol extracts produced eight known compounds, including 2-phenylethyl 1H-indol-3-ylacetate, uracil, cyclo-(S*-Pro-S*-Tyr), cyclo-(S*-Pro-S*-Val), 2-(2-aminophenyl)acetic acid, 2-(4-hydroxyphenyl)acetic acid, 4-hydroxy-benzamide, and 2-(2-hydroxyphenyl) acetic acid (Chapla et al. 2014). Numerous strains of *Fusarium* spp. produce enniatins (ENs), six-membered cyclic depsipeptides, as secondary metabolites. Meca et al. used reverse-phase low-pressure liquid chromatography (LPLC) on Amberlite XAD-7 to extract ENs A, A1, B, and B1 from *F. tricinctum*. Additionally, semipreparative liquid chromatography was used to purify ENs. MTT assays were used to conduct cytotoxicity tests. Cancer cell lines of human origin (epithelial colorectal adenocarcinoma cells, Caco-2) were only cytotoxicly affected by ENs A1 and B1 (Meca et al. 2010). The IC50 produced by EN A1 was 12.3 mM on Caco-2 cells, while the IC50 produced by EN B1 was 19.5 mM. Mycotoxins called enniatins have the potential to be cancer-fighting substances. The extracellular enzyme synthesis of amylase, lipase, pectinase, protease, cellulase, and laccase was examined in 50 endophytic fungi isolated from the medicinal herbs *Calophyllum inophyllum*, *Catharanthus roseus*, *A. calcarata*, and *Bixa orellana* (Sunitha et al. 2013). They stated that it was possible for these fungi to manufacture these enzymes. The numerous enzymes generated vary between different fungi and frequently depend on the host and other ecological elements. *Bacopa monnieri* is a medicinal herb that has been tested for its antibacterial properties against a variety of microbes by Katoch et al. It was researched how these endophytes interact ecologically with the host plant and their capacity to create the enzymes cellulase, protease, amylase, and lipase (Katoch et al. 2014). All endophytes displayed amylase activity. Notably, 98% demonstrated lipolytic, 28% cellulolytic, and 31% proteolytic activities. Devi et al. isolated the cellulase-producing *Penicillium* sp. endophytic fungus from the *Centella asiatica* plant (Devi et al. 2012).

12.3.2.2 Antifungal Properties of Secondary Metabolites

With cancer chemotherapy, allogeneic bone marrow transplantation, and organ transplantation, invasive fungal infections are significantly rising. Life-threatening fungal infections can be treated with a small selection of antifungal medications. Even though new antifungal medicines have been released on the market, the development of resistance to antifungal treatments is rising in patients receiving long-term treatment (Deshmukh and Verekar 2012). One of the most promising alternative sources for the separation of new metabolites for the treatment of fungal diseases is endophytic fungi. Endophytes from *Artemisia annua* were isolated by Liu et al., who then tested them against fungus known to damage crops, including *Gerlachia nivalis*, *Rhizoctonia cerealis*, *Helminthosporium sativum*, *Fusarium graminearum*, and *Gaeumannomyces graminis var. tritici* (Liu et al. 2001). The strongest antifungal activity was seen in ethanol acetate extracts. *A. indica*, *Holarrhena antidysenterica*, *Terminalia arjuna*, and *Terminalia chebula* are four

plants with significant therapeutic value that Tejesvi et al. identified as *Pestalotiopsis* strains. The maximum antifungal activity was demonstrated by their ethyl acetate extracts against six test species. A number of fungi were isolated and tested for their ability to inhibit a variety of pathogenic and saprophytic fungi (Tejesvi et al. 2007). These fungi produced six bioactive substances, including cerulenin, arundifungin, sphaeropsidin A, 5-(1,3-butadiene-1-yl)-3-(propene-1-yl)-2-(5 H)-furanone, and ascosterosides A and B. Arundifungin, 5-(1,3-butadiene-1-yl)-3-(propene-1-yl)-2-(5 H)-furanone, ascosteroside A, and ascosteroside B have antifungal properties on par with amphotericin, the standard treatment (Weber et al. 2007).

The medicinal plant *Michelia champaca* produces a variety of secondary metabolites with a wide range of pharmacological characteristics. This plant's endophytic fungus exhibits antifungal, anticancer, and acetylcholinesterase (AChE)-inhibiting properties. The activity of each extract was seen against two phytopathogenic fungi. Ethyl acetate extracts of the endophytic fungus *C. gloeosporioides* yielded one new compound, 2-phenylethyl 1H-indol-3-ylacetate, and seven known compounds, such as uracil, cyclo-(S*-Pro-S*-Tyr) (28), cyclo-(S*-Pro-S*-Val), 2-(2-aminophenyl) acetic acid, 2-(4-hydroxyphenyl)acetic acid, 4-hydroxy-benzamide, and 2-(2-hydroxyphenyl)acetic acid (Chapla et al. 2014).

Using NMR and X-ray crystallography, Li et al. isolated the endophytic fungus *Pestalotiopsis adusta* (L416) and discovered three metabolites that were derivatives of the chlorinated benzophenone, known as pestalachlorides A–C. *Fusarium culmorum* (CGMCC 3.4595), *Gibberella zeae* (CGMCC 3.2873), and *Verticillium albo-atrum* were three plant pathogenic fungi that the organic solvent extract from the fermentation broth significantly inhibited (CGMCC 3.4306). Their research also revealed that whereas pestalachloride B showed comparable action against *G. zeae*, pestalachloride A displayed strong antifungal activity against *F. culmorum*. *F. culmorum*, *G. zeae*, and *V. albo-atrum* were not resistant to the antifungal effects of pestalachloride C. Many endophytic fungi were isolated from the garlic plant by Shentu et al. (Ding et al. 2008). A significant antifungal efficacy against phytopathogens was demonstrated by these isolates. By using spectrum and mass data analyses, the bioactive metabolite generated by *Trichoderma brevicompactum* was identified as trichodermin as compared to the the compound's antifungal activity (Shentu et al. 2014).

12.3.3 Extraction of Metabolites from Endophytic Fungi

A vast range of secondary metabolites produced by filamentous fungi are known to be a rich source of biomolecules with potential medical uses. Secondary metabolites can be broadly categorized into four types based on their chemical structures and biosynthesis pathways: polyketides, non-ribosomal peptides, alkaloids, and terpenes. Plant endophytic fungi have the ability to produce a wide range of bioactive metabolites with different structural characteristics because of their unique living conditions. It has been shown that several of these metabolites have medicinal and

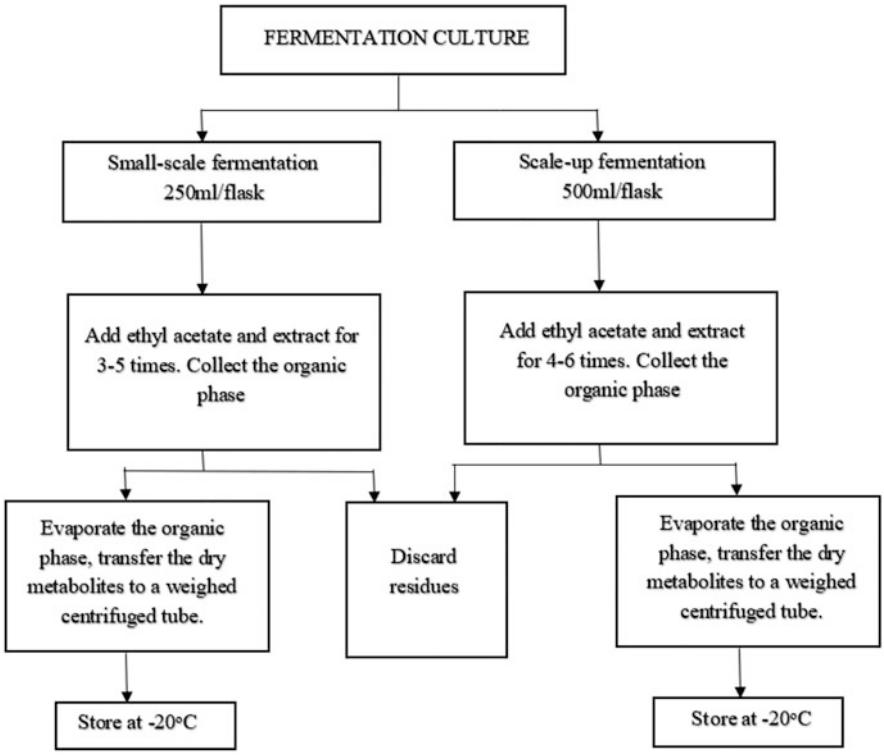

Fig. 12.2 Process of extraction of secondary metabolites from endophytic fungi

ecological relevance. Different techniques have been developed to isolate secondary metabolites from plant endophytic fungus utilizing high-performance liquid chromatography (HPLC) in order to find bioactive chemicals (Liu and Liu 2018) (Fig. 12.2).

The amount of spore pellet to be used determines how much 15% sterile glycerol (w/v) should be used; typically, a plate requires 100 L of glycerol. The fungus spores can survive for 2–3 years at −80 °C. A single colony is often obtained and used for subsequent tests after the fungal strains have been activated. Since different fungi have varying sporulation times, the incubation period for each can vary from day 5 to day 10. The majority of fungi typically undergo sporulation after a 7-day incubation period. The harvest time should be controlled in accordance with the production of desired metabolites since different fungi have varied growth rates and secondary metabolites may be produced either at the late stage of exponential growth phase or at the stationary growth phase. The majority of filamentous fungi are typically harvested after 7 days in order to extract secondary metabolites. However, in some circumstances, an incubation period of up to 20 days is used. The number of agar plugs that are incubated can be raised to 20–25 for fungi that develop slowly. In most cases, fungal growth enters the stationary growth phase after 48 h of liquid medium

fermentation. To make sure there is no contamination during fermentation, it is important to keep an eye on the fungal growth situation. A microscope can be used to observe contamination and determine its source. Typically, after 48 h of fermentation in liquid medium, fungal growth enters the stationary growth phase. For small-scale fermentation, the rice medium consists of 30 g of rice and 50 mL of double-distilled water. Following autoclaving for sterilization, the rice medium solidifies. After 30 days of fermentation, the rice medium and mycelia are mixed in solid-state fermentation. The solid-state cultures should be broken up into little bits for easier extraction. With a large pair of scissors or a pair of strong forceps, the cultures are minced in the fermentation flask. The colonies are given 100 mL of ethyl acetate for liquid-state fermentation. The amount of ethyl acetate needed for solid-state fermentation is around 100–150 mL; this ensures that the cultures are completely submerged. The ethyl acetate and culture mixture are put into a separatory funnel for liquid-state fermentation, where it is left to stand until the organic phase and aqueous phase are entirely separated. Pour out the organic phase for liquid-state fermentation once the lower phase has exited the separatory funnel. Since the mycelia and the rice remain in a solid state during solid-state fermentation, the organic phase can be drained off immediately. To prevent metabolite degradation, the water bath temperature for the rotary evaporator should be less than 40 °C. One of the fractions is that which was eluted using 30% EtOAc. This method can also be used to separate other fractions. We often use the maximum velocity to wash the column and collect the fractions because the flow rate is not constant. One drop is typically injected into the sample every 4–5 s. For additional analyses to determine the structure of the metabolites, the LC/MS and NMR are utilized. *Enterococcus* species, the second most common cause of hospital-acquired infections, are responsible for a wide range of illnesses, including infective endocarditis, bloodstream infections, and urinary tract infections. Vancomycin- and ampicillin-resistant bacteria include *Enterococcus faecium*. Additional fungi or bacteria are also an option. Depending on the goal of your research, choose several pathogenic bacteria or fungi. The initial concentration and subsequent dilutions of the metabolite might be downregulated depending on the solubility of the metabolite. The degree to which the color of the negative control changes determines the precise incubation period for bacteria and fungi. When the pink color vanishes is when it is best to act (Liu and Liu 2018).

A mycelial agar block taken from an active colony on a potato dextrose agar plate was used to inoculate 500 mL of potato dextrose broth with the endophyte for growth. The flask cultures were kept stationary for 2 weeks of incubation at 25 °C. Filtering the fungus broth culture allowed the cell-free supernatant to be extracted using ethyl acetate, chloroform, methanol, and hexane as solvents. An equivalent volume of solvent was added to the filter, and the mixture was violently agitated for 10 min. The sample was dried after the organic layer was separated using a separating funnel. For future usage, the dried residue was dissolved in dimethyl sulfoxide (DMSO) at a concentration of 1 mg/mL (Meenambiga and Rajagopal 2018).

12.4 Overview of Candidiasis

12.4.1 Virulence and Pathogenicity

Because of its virulence characteristics, candida actively contributes to the pathophysiology of infection's onset and progression. The initiation of an infection or colonization is caused by one set of virulence factors, while the spread of the infection is assisted by the other set (Deorukhkar and Roushani 2017). Due to changing circumstances, *Candida* spp. polymorphism suggests that it can transform from commensal to pathogenic form. It is distinguished by the morphological change from blastospores to hyphae, with pseudohyphae serving as the intermediary phase (Noble et al. 2017; Hanaoka and Domae 2021). One of the most important virulence factors of *C. albicans* is its capacity to develop true hyphae (San-Blas et al. 2000). Within the human microbiome, *C. albicans* can be found as yeast, and the change from yeast to hyphal form is a pathogenic transition (Mayer et al. 2013; Tsui et al. 2016). The hyphal form is invasive, and the cells penetrate the tissues of the host via active penetration which depends on the fungal activity and induced endocytosis that is achieved by hyphae invasion and is dependent on host activity. Hyphal development is influenced by a number of signaling pathways where the cyclic adenosine monophosphate protein kinase A (cAMP-dependent protein kinase A) is the most significant (Maza et al. 2017; Galocha et al. 2019; Lin and Chen 2018). A range of surface molecules of *C. albicans* interact with host ligands on the epithelial or endothelial surface that are crucial for mediating epithelial adhesion (Gale et al. 1998). Various factors may affect the adhesion process, such as types of proteins present in the cell wall, and cell surface's physiochemical properties. Adhesins on the yeast cell recognize ligands including sugar residues on human buccal epithelial cells and an array of extracellular matrix proteins such as fibrinogen, fibronectins, type I and type IV collagen, laminin, and the complement components like iC3b and C3d. Adhesins are the surface proteins that have a role in specific adhesion. A few well-studied adhesins of *Candida* spp. include those from the ALS (agglutinin-like sequence) family, Hwp (hyphal wall protein), EPA (epithelial adhesin) families, Int (integrin-like surface protein), and Mnt (-1-2-mannosyltransferase). The attachment of *C. albicans* to host epithelium is not entirely due to a single adhesin molecule, and other mechanisms of interaction with the host surface are hypothesized for this commensal organism (Deorukhkar and Roushani 2017; Hostetter 1994). It is a characteristic of *C. albicans* pathogenesis to produce biofilm. The majority of *C. albicans* infections result in severe morbidity and death due to the development of a biofilm on the host surface or on abiotic surfaces (implants) (Tsui et al. 2016). Although the biofilms produced by *C. albicans* are much denser and more complicated than those produced by other *Candida* spp., both *C. albicans* and *C. parapsilosis* attach effectively to the surface of catheters (Hawser and Douglas 1994; Ramage et al. 2005). Biofilm develops through several consecutive phases, beginning with the individual cells of *Candida albicans*, which form the basal layer. Cell proliferation and filamentation then occur, with hyphae

being the first step. In the maturation phase, an extracellular polysaccharide matrix is formed. Finally, the dispersion of non-adherent cells has the possibility of new biofilms and the dissemination in the tissue (McCall et al. 2019; Cavalheiro and Teixeira 2018). The extracellular matrix is composed of extracellular polymers and DNA involved in maintaining biofilm structure. DNA plays a vital role in binding the biofilm to the substrate, providing β-1,3-glucans which are essential for antifungal drug resistance. Biofilm channels facilitate cell supply with nutrients, air, and water, giving it new "multicellular" properties (Nett and Andes 2020; Talapko and Škrlec 2020; Li et al. 2020). Biofilm is caused by transcription factors such as Efg1, Bcr1, Tye7, cell wall proteins (Hwp1, Als3), and protein kinases (Cbk1, Ire1). These transcription factors are necessary for the expression of different genes for cell adhesion and filamentation in biofilms on abiotic surfaces. Adhesin Als3, the target of BCR1, plays a key role in the biofilm formation on abiotic surfaces (Ganguly and Mitchell 2011). Extracellular hydrolases are essential in the pathogenesis of candida, as they enable the invasion of host tissue by deranging the host cell membrane constituents especially in disseminated candidiasis. Phospholipases, secreted aspartyl proteinases, hemolysins, and lipases are mostly implicated in candida infections. The phospholipase enzyme in *Candida* spp. hydrolyzes the host cell membrane, revealing receptors to promote the adherence of yeast cells (Sardi et al. 2013; Ghannoum 2000).

12.4.2 Clinical Manifestations

The genus *Candida* has over 200 distinct species, but only a handful are opportunistic human pathogens which trigger infections once the host becomes emaciated or immunocompromised. Clinical manifestation of candidiasis may be superficial or invasive. Usually, superficial infections tend to affect the mucosal membranes and skin and can be effectively treated with topical antifungal medications. Invasive fungal infections, on the other hand, are frequently fatal, most likely as a result of inadequate diagnostic techniques and ineffective initial antifungal medications (Spampinato and Leonardi 2013). The most prevalent fungal infection of the oral cavity is candidiasis. Previous estimates suggest that 35–80% of the population carry oral candida. According to recent studies employing molecular detection techniques, the typical oral flora of all humans contains *Candida* spp. (Lewis and Williams 2017; Peters et al. 2017). *Candida albicans* is present in over 80% of oral fungal strains, making it the most common species in diseased and healthy mouths. *C. glabrata*, *C. parapsilosis*, *C. dubliniensis*, *C. tropicalis*, and *C. krusei* are also found in the mouth (Lewis and Williams 2017; Sav et al. 2020; Aslani et al. 2018). The highest incidence of diaper dermatitis in term newborns with 10% incidence and VLBW infants with 28% incidence occurs between the ages of 10–11 weeks and 7–9 months. Newborns who are diagnosed with diaper dermatitis showed intestinal colonization, with positive stool tests for a *Candida* species (Baley et al. 1986; Leyden 1986). Congenital candidiasis can be caused by a maternal infection or

massive exposure to maternal vaginal colonization during labor and delivery. Hematogenous dissemination, direct invasion of intact membranes, and ascending infection after ruptured membranes are key mechanisms for intrauterine infection. Although *C. albicans* is the species most frequently linked to congenital candidiasis, *C. glabrata* and *C. parapsilosis* have also been implicated with neonatal infections (Yadav and Prakash 2016; Whyte et al. 1982; Darmstadt et al. 2000). A distinct clinical condition called invasive fungal dermatitis has been identified in ELBW infants during the first 2 weeks of life. *Candida* species are commonly detected, but infection with other filamentous non-*Candida* fungal species, like *Aspergillus*, *Curvularia*, *Bipolaris*, and *Trichosporon*, produces analogous clinical array of lesions with erosive crusts (Rowen et al. 1995). In pulmonary candidiasis, although yeasts are frequently isolated from sputum or respiratory fluids, these isolates may not accurately reflect the pathogen that causes pulmonary infiltrates. It may be difficult to tell a deep-seated illness from a respiratory tract infection from positive cultures of specimens. Pulmonary candidiasis is either an impact produced by disseminated candidiasis or a localized infection brought on by aspiration, which is prevalent in patients (Meunier 1989). Esophageal candidiasis caused by *C. albicans* is the most prevalent kind of fungi-caused esophagitis. Patients with immunodeficiency and those with comorbidities are at increased risk of infection, which is either symptomless or manifested as acute odynophagia, dysphagia, and discomfort below the sternum (Mohamed et al. 2019; Rosołowski and Kierzkiewicz 2013). Yeast overgrowth within the gastrointestinal system is a primary cause of dissemination. In immunocompromised individuals, a novel clinical condition called focal hepatic candidiasis has been reported. Another kind of gastrointestinal candidiasis manifests as a fungus ball in the bile system, causing complete obstruction. Other rare types of candidiasis in patients with neoplastic disease are candidiasis peritonitis and intra-abdominal abscess (Meunier 1989). *Candida* species constitute a common cause of infant urinary tract infection, and renal candidiasis has been associated to it. Candidemia is frequently accompanied by renal parenchymal infiltration or fungus balls, which calls for systemic antifungal medication. Nonspecific symptoms of candida infection of the urinary tract in infants include fever, fatigue, apnea, abdominal distention, and significant gastric residuals. Acute renal failure is a typical clinical symptom of newborn renal candidiasis (Karlowicz 2003). *Candida* species invade the bones and joints as a result of hematogenous seeding or due to inoculation following trauma, intra-articular injections, a surgical operation, or injectable medication usage. Osteoarticular infections are frequently symptomatic months or even a year following a fungemia episode or a surgical operation (Johnson and Perfect 2007). *Candida* species are becoming more common reason for infections in neutropenic and non-neutropenic patients, increasing the risk of morbidity owing to invasive candidiasis and CNS involvement attributed to mycosis increase. Candida infections affecting the central nervous system are rare but occur most frequently in preterm newborns, who typically present with meningitis, micro- and macro-abscesses, and vascular and medullary damage (Henao and Vagner 2011). The growing frequency of invasive candidiasis in a variety of patient groups, including newborns, cancer patients, AIDS patients, and organ transplant

recipients, is a reason for worry in the medical community. The symptoms are varied, and distinct conditions such as localized and widespread infection must be evaluated independently. Similar disease manifestations may be caused by several yeast species.

12.5 Treatment for Candida Infections

12.5.1 Available Treatments

12.5.1.1 Therapeutic Application of Secondary Metabolites Against Candida Infection

Most *Candida* species are fluconazole-resistant and exhibit variable resistance to other antifungals, which has become a severe clinical challenge. Therefore, novel alternative antifungal strategies, including anti-candidal metabolites from endophytes and combination therapy, are required to improve patient outcomes due to the limited number of available treatment options and higher rates of therapeutic failures (Chowdhary et al. 2017). *Penicillium* sp., most likely *P. brevicompactum*, is one among the endophytic fungi identified within the inner bark of a yew tree in the Northwest Pacific that produced bioactive metabolites. The fungus was cultured and extracted with methylene chloride, to yield the compounds mycophenolic acid and ergosterol peroxide which showed activity against *C. Albicans* (Stierle and Stierle 2000). A study in 2007 isolated the fungi *Ascomycota* and *Basidiomycota* from their fruit bodies or from soil. In all, 1510 different fungus strains were cultivated in submerged culture, and then the extracts of the mycelium and culture filtrate were evaluated for resistance to *Candida albicans*. Five endophytic strains were chosen for the isolation of active principles due to the contribution endophytes provide to the overall fungal biodiversity and their bioactive compound production. The endophyte *Phomopsis* sp. produced a compound cerulenin which is a fatty acid and polyketide synthase inhibitor that showed anti-candidal activity. Another isolate identified as *Gnomoniaceae* produced ascosteroside A, which exhibited anti-candidal activity that is known to be attributed to the inhibition of b-(1,3)-glucan synthesis. Ascosteroside B analogous to the former was also obtained. Arundifungin, generated by the isolate *Amphisphaeriaceae* in *Xylariales* order, is structurally and functionally linked to ascosterosides which serve as b-(1,3)-glucan synthesis inhibitor. An endophyte isolated from an asymptomatic *Quercus ilex* leaf varied at only one position from *Gnomoniaceae* that produced sphaeropsidin A. Sphaeropsidin compounds are pimarane diterpenes that are phytotoxic and fungitoxic and have been implicated in the development of symptoms and the repulsion of competing phytopathogenic fungi, like cerulenin. The strain, which was isolated as an asymptomatic endophyte from *Cistus salviifolius*, belonged to the *Sarcosomataceae* (order *Pezizales*). Due to its anti-candidal efficacy, it was isolated and had similar activity identical as sphaeropsidin A (Weber et al. 2007).

For the first time, the metabolites of the endophytic fungus *Penicillium* sp. from *Hopea hainanensis* leaves have been described. Through bioassay-guided fractionation, the EtOAc extract of a solid-matrix stable culture from this fungus generated six metabolites, which were identified through a combination of spectral and chemical analysis to be monomethylsulochrin, rhizoctonic acid, asperfumoid, physcion, 7,8-dimethyl-isoalloxazine, and 3,5-dichloro-*p*-anisic acid. All six compounds were tested for in vitro bioactivity, including their ability to inhibit three human pathogens including *Candida albicans* species. The growth of *C. albicans* was suppressed by compounds physcion, rhizoctonic acid, asperfumoid, and 3,5-dichloro-*p*-anisic acid with minimal inhibitory concentrations (MICs) of 50.0, 40.0, 20.0, and 15.0 g/mL, respectively (Wang et al. 2008).

A cytochalasan derivative called phomopsichalasin has a unique ring structure with an isoindolone moiety attached to a 13-membered tricyclic framework. It was discovered in an endophytic *Phomopsis* species that was found on *Salix gracilistyla*, a willow plant. Cytochalasans are well-known actin-binding fungus metabolites. Phomopsichalasin has been demonstrated to have inhibitory properties towards the human pathogenic fungus *Candida tropicalis* and other bacterial pathogens. Another compound 1α-10α-Epoxy-7α-hydroxyeremophil-11-en-12,8-β-olide was found from *Xylaria* sp. BCC 21097, which was isolated from palm *Licuala spinosa*. It shares structural similarities with eremophilanolide sesquiterpenes. *Candida albicans* was successfully eradicated by the substance. The fungi *C. albicans* and *Candida utilis*, as well as the bacteria *S. aureus*, *B. subtilis*, *M. luteus*, *E. coli*, and *Pseudomonas aeruginosa*, were shown to be inhibited by the compound altersolanol A (Mousa and Raizada 2013). A study isolated two novel cytotoxic and antifungal compounds from the metabolites of the endophytic fungi *Dendrobium officinale*, (4S,6S)-6-[(1S,2R)-1, 2-dihydroxybutyl]-4-hydroxy-4-methoxytetrahydro-2H-pyran-2-one and (6S,2E)-6-hydroxy-3-methoxy-5-oxodec-2-enoic acid, and three other known compounds, LL-P880γ, LL-P880α, and ergosta-5,7,22-trien-3b-ol. Spectroscopic techniques were used to identify the chemical structures. Cytotoxicity and antifungal effects were assessed for all isolated compounds 1–5. All the pathogens evaluated, including *C. albicans*, exhibited considerable antifungal activity for compounds 1–4 with MIC of 50 g/mL) (Wu et al. 2015).

12.6 Conclusion

Endophytic fungi generate a wide range of secondary metabolites with intriguing applications in several industries. It has been demonstrated that a number of these metabolites exert antibacterial, anticancer, antifungal, and antioxidant properties. They have also been employed in the manufacture of pharmaceuticals and bioactive substances for the agricultural and pharmaceutical sectors. Another fascinating and important study field is the exploitation of these metabolites to control and to treat many fungal diseases including candidiasis. Candidiasis is a public health issue with high morbidity and mortality rates due to the growing frequency of invasive

candidiasis in a variety of immunocompromised patient groups, including newborns, cancer patients, AIDS patients, and organ transplant recipients. A range of metabolites that act against candida have been identified in the past few years, and currently a lot of novel compounds from endophytic fungi are screened both in silico and in vitro. As a result, it is anticipated that future research on endophytic fungi and their secondary metabolites would increase, leading to the identification of new bioactive substances and their uses.

12.7 Future Perspectives

One of the key sources for obtaining new bioactive substances is endophytic fungus. Endophytic fungi in their bioactive form have anti-candidal activity that is controlled by several pathophysiological processes associated with the development of candidiasis. Further research and explanation are required in studies relating to the safety concerns for the long-term use of endophytic fungi's bioactives and their interactions with other medications. Future study in this field is therefore required to confirm the efficacy of these medicinal substances derived from endophytic fungus and their products as prospective medications or nutraceuticals for the treatment of candidiasis. More research is needed to learn more about this underutilized resource for the creation and isolation of new molecules against candidiasis with therapeutic relevance and biochemical and pharmacological potential. The bioactive metabolites produced by endophytic fungus have been widely studied and published in recent years. These substances can be exploited to create brand-new natural products with therapeutic uses. Nearly 270,000 vascular plant species serve as the host for more than 1.5×10^6 endophytic fungi; therefore, new discoveries of their metabolites would be a viable course of action (Agrawal et al. 2022).

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Chapter 13
In Vitro Biotechnological Progressions in *Pueraria tuberosa* (Willd.) DC.

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Abstract *Pueraria tuberosa* (Willd.) DC., belonging to the family Fabaceae, is in great demand in Ayurveda because of its healing properties against weakness, diabetes, and fertility disorder. Indian kudzu tubers are rich in isoflavonoids like puerarin, daidzein, genistein, and genistin. It also possesses phytosterols, terpenes, and other phenolic compounds. The seeds of this plant have a low regeneration capacity, while the tuber takes a major role in propagation. *P. tuberosa* revives during the rainy season, and due to the substantial use of tuber for its medicinal properties, it is doubtful that it may be included in the endangered list. This review covers the problems associated with germination and the alternative techniques of in vitro regeneration to date and describes the future prospects of *P. tuberosa*. There is a need for the most advanced techniques of tissue culture with plant growth regulators and their implementation to successfully revive the plants in both in vitro and ex vitro conditions.

Keywords Isoflavonoids · Kudzu · *Pueraria* · Tuber

13.1 Introduction

People have historically used medicinal plants to cure a variety of chronic diseases in many indigenous communities. A large portion of the population in rural areas of the country is reliant on medicinal plants, which in the past were an important part of traditional Indian medicine. *Pueraria tuberosa* (Willd.) DC. (Indian kudzu) is an everlasting woody climber and is popularly mentioned as Vidarikand (Hindi) or Bhukushmandi (Sanskrit) (Chopra et al. 1956). Indian kudzu belongs to the

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Fabaceae family, and this important climber is in great demand in Ayurveda because of its healing properties against weakness, diabetes, fertility disorder (Pandey et al. 1998), and Parkinson's disease (Li et al. 2003). It acts as antiarrhythmic, hypothermic, and hypotensive as well (Kintzios et al. 2004). Kudzu tubers are rich in isoflavonoids like puerarin, daidzein, genistein, and genistin (Maji et al. 2012) along with phytosterols, terpenes, and other phenolic compounds (Venkata Ratnam and Venkata Raju 2009). Some Indian tribes use the tuber as a complementary food and a diabetic treatment. According to Ayurvedic practitioners, the tuber can be used to treat aging and reproductive issues and overall weakness (Pandey et al. 1998). According to Warrier et al. (1995), the tuberous roots have galactagogues, stimulants, and emollient qualities. There are many Ayurvedic formulations that contain it, including Nityananda rasa, Mahavishgarbha taila, Marma gutika, Shatavaryadi ghrita, Sarasvatarista, chyavanprasam, Goknura Vidari ghrita, macatmagupta sarpi, and Ashwagandharishta (Venkata Ratnam and Venkata Raju 2009). As a result, there is a substantial market demand for the plant's tubers. For phytochemical extraction, there is a widespread exploitation of tubers by pharmaceutical industries. As a consequence the natural habitat has restricted its reproduction and regeneration; therefore, the availability of this species is rare (Rathore and Shekhawat 2009). Due to the existence of physiologically active chemicals, there is an increasing need for plant-based medications, necessitating the selection, propagation, and characterization of significant medicinal plants for use in commerce. Additionally, in vitro procedures allow for the expansion and genetic improvement of desirable genotypes. Also its in vitro cell cultures proves to be the noteworthy reservoir of secondary metabolites. As a result, the immense use of its tubers-based bioactive compounds cannot be satisfied. The seeds of this plant have a low viability. Owing to these disadvantages, ex situ conservation and in vitro regeneration have been fulfilled by the plant tissue culture technique (Edson et al. 2008; Arya et al. 2003).

13.2 Distribution and Description

P. tuberosa is widely found in India, Pakistan, and Nepal. In India, it is disseminated in the Himalayas to Sikkim, tropical and subtropical regions that include hill forests and deciduous vegetation areas (Keung 2002). Kudzu is a woody creeper, with large tubers up to 35 kg and up to 0.75 cm in circumference; it is globular in shape, and the tuber pulp is white and gently sweet. The leaves are trifoliate (three leaves), whereas the leaflets are rounded-ovate in shape, conspicuously yellow in the dry season. The flowers are purplish in color, bisexual, and 1.5 cm across and bloom after the leaves tumble from December to February. The fruit pods align uniformly, measuring approximately 2–5 cm in length, and they enclose the seeds faintly. In one pod there are three to six seeds (Keung 2002; Jain and Choudhray 2016). Additionally, given that this species reproduces through tubers and seeds, nonscientific harvesting practices and other anthropogenic activities also pose a threat to its survival. Relatively little seed germination occurs. Vegetative propagation (through tuber) is

the most popular mode of multiplication, but it is insufficient to meet the demands of tribal populations and the pharmaceutical sector. The creation of a quick propagation technique is required to boost *P. tuberosa* output on a commercial scale (Rathore and Shekhawat 2009). In vitro plant cell culture has also been acknowledged as an important source of plant secondary metabolites. Additionally, the in vitro method offers chances for genetically desired genotypes and proliferation (Manisha et al. 2012). Plant tissue culture has been useful for off-site conservation and micropropagation (Prajapati et al. 2023). Thiem (2003) created a micropropagation technique for *P. lobata*, and the research by Korsangruang et al. (2010) indicates that *P. candollei* var. mirifica has also been effectively micropropagated. Kintzios et al. (2004) also reported on the production of puerarin from hairy root cultures made from *P. phaseoloides* leaf explants treated with *Agrobacterium rhizogenes* in bioreactors.

13.3 Medicinal Importance

Plants have a variety of defense mechanisms that serve as safeguards against physical, chemical, microbiological, or biological stimuli that might cause disruption or attack. After initiating the defense pathway's signal transductions, transcription factors control the synthesis of secondary metabolites using phytoalexins, low-molecular-weight compounds with a variety of advantageous biological effects (Endress 1994). Both biotic and abiotic elicitors can cause the formation, accumulation, and excretion of secondary metabolites in cell cultures (Barz et al. 1990; Endress 1994). Inorganic salts added to the culture medium in the form of heavy metals have been researched for their abiotic elicitation effects in plant culture systems. For instance, copper (Cu) is frequently used to stimulate the formation of secondary metabolites in plants as sulfate or chloride salts (Bhuiyan and Adachi 2003; Engelmann et al. 2009). Biotic elicitors' functions in isoflavonoid accumulation through a variety of elicitation processes have been the subject of extensive research. Studies on the interactions between plants and microbes frequently use yeast extract (YE) as a biotic elicitor. For instance, YE was used to clone the gene-coding enzyme chalcone synthase (CHS) in *P. lobata* cell suspension cultures (Nakajima et al. 1991). Because these substances can take the place of fungal elicitors during a pathogen attack, poly- or oligosaccharides are the signaling molecules with elicitation pathways that have been extensively studied.

Kudzu has a number of bioactive compounds, for instance, puerarin, daidzein, genistein, puerarone, tuberosin, quercetin, β-sitosterol, and stigmasterol (Maji et al. 2012; Sawale et al. 2013). The isoflavonoid puerarin shows antidiabetic (Wu et al. 2013) and antifertility (Saha et al. 2012) activities. Genistein influences the digestion of fatty acids, cholesterol, and steroids (Takahashi et al. 2009), in addition to improving transient spatial memory and diminishing oxidative stress (Bagheri et al. 2011). Daidzein lessens apoptosis and improves the antioxidant activity of the liver's enzymes (Choi and Kim 2009). Tuberosin shows antioxidant activity

(Pandey et al. 2007). The methanolic extract of tuber shows mitigating inflammatory potential in rodents (Pandey et al. 2013). It has been affirmed that the blend of daidzein and genistein invigorate wound relief in ovariectomized mice (Emmerson et al. 2010; Mukai et al. 2012). It has been accounted for that supplementation of daidzein and genistein deduced the high concentration of cholesterol and triglycerides in mice (Ae Park et al. 2006). Maintaining a coordinated effort to develop a successful propagation technique is essential to meeting market demand. According to Sudarat and Sanha (2006), *Pueraria candollei* var. mirifica callus culture could provide considerable amounts of isoflavonoids like genistein and daidzein. Over the past few decades, isoflavonoids have become increasingly popular. Cancer, postmenopausal symptoms, and cardiovascular diseases have all been successfully treated with isoflavonoids (Dixon and Ferreira 2002; Nestel 2004; Duncan et al. 2003; and Vitrac et al. 2004). *P. tuberosa* is frequently used because of its nutritional and therapeutic advantages despite its small germplasm base.

13.4 In Vitro Regeneration

Ordinarily, *P. tuberosa* is naturally reproduced through its tubers and seeds. The recurrence of multiplication is imitated because seed germination is poor and spread by means of tubers and the seeds are exclusively season dependent (Rathore and Shekhawat 2009). Another standard method is in vitro regeneration through plant tissue culture, which is considered the best technique aimed at the huge production by means of hereditary improvement of alluring genotype (Manisha et al. 2012). Tissue culture strategies give the uncommon opportunity to adjust the phytochemical profiling of the product, by controlling the physical and chemical properties, to make progressively important compounds for human use (Rady et al. 2018). Therefore, in this review, we have analyzed the micropropagation methods reported in *P. tuberosa*, such as the direct formation of multiple shoots, as well as in vitro production of secondary metabolites.

13.4.1 Choice of Explant

The initial step for in vitro regeneration is to select and collect suitable explants. The primary piece of plant tissue culture technique is explant, and the selection of explant relies upon the nature of the mother plant and season. *P. tuberosa* is a woody climber, and inspecting explants should be possible consistently, yet the seeds are fully grown in the months of April to June. In this way, the ideal time for the assortment of in vitro regeneration is considered to be between July and August (Rathore and Shekhawat 2009). A few sorts of explants such as nodal segments, stem, tuber, leaf, and germinated seedlings have been used for the callus initiation and in vitro multiplication as depicted in Table 13.1. In majority of the reports, the

Table 13.1 Factors involved and their influence on micropropagation and callus induction of *P. tuberosa*

Explant	Surface sterilization	Culture medium composition	Culture condition	Regeneration response	Acclimatization	References
Nodal	5% sodium hypochlorite for 10 min and rinsed five times with sterilized double-distilled water	MS + 1.5 mg/l BAP + 0.5 mg/l NAA	25 ± 2 °C, 16 h photoperiod with 50 μmol m^{-2} s^{-1} irradiance provided by cool-white fluorescent tubes with 60–65% RH	Maximum number of elongated shoots recorded is 4, 3.8 cm long	Wasn't carried out	Sharma et al. (2011)
Leaf	$HgCl_2$ for 3 min after several distilled water wash	MS + 1.5 mg/l BAP + 0.5 mg/l NAA	25 ± 2 °C, 16 h photoperiod at a RH of 55% with a light intensity of 3000lux	Best callus growth	Wasn't carried out	Bindu et al. (2018)
Nodal stem segment	0.1% $HgCl_2$ for 3 min rinsed 6–8 times with sterile water and kept in sterile and cold 0.1% of each of citric acid and ascorbic acid for 15 min	MS + 8.88 μM BA	12 h photoperiod of 30–40 μ Mol m^{-2} light intensity and 28 ± 2 ° C temp.	95% explant exhibited bud break with 2–5 shoots per node after 10–15 days	Regeneration of plantlets and acclimatization in black poly bags containing sand, black soil, and vermicompost in 3:1:1 ratio	Rathore and Shekhawat (2009)
Nodal stem segment	0.1% $HgCl_2$ for 3 min rinsed 6–8 times with sterile water and kept in sterile and cold 0.1% of each of citric acid and ascorbic acid for 15 min	MS + 4.44 μM BA	12 h photoperiod of 30–40 μ Mol m^{-2} light intensity and 28 ± 2 ° C temp.	Produced shoots were 7.42 ± 0.27 cm in length	Regeneration of plantlets and acclimatization in black poly bags containing sand, black soil, and vermicompost in 3:1:1 ratio	Rathore and Shekhawat (2009)
Nodal	0.1% $HgCl_2$ for 7–8 min washed four times with sterilized distilled water	MS + 1.0 mg/l NAA + 2.0 mg/l BAP	25 ± 2 °C temp. 16 h photoperiod	Brown-green compact callus	Wasn't carried out	Sadguna et al. (2014)
Nodal	0.1% $HgCl_2$ for 7–8 min washed four times with sterilized distilled water	MS+ 2.0 mg/l BAP + 2.0 mg/l KN	25 ± 2 °C temp. 16 h photoperiod	Nodular green friable callus	Wasn't carried out	Sadguna et al. (2014)

(continued)

Table 13.1 (continued)

Explant	Surface sterilization	Culture medium composition	Culture condition	Regeneration response	Acclimatization	References
Leaf	0.1% $HgCl_2$ for 7–8 min washed four times with sterilized distilled water	MS+ 1.0 mg/L 2,4-D+ 2.0 mg/l BAP	25 ± 2 °C temp. 16 h photoperiod	Light-green compact callus	Wasn't carried out	Sadguna et al. (2014)
Leaf	0.1% $HgCl_2$ for 7–8 min washed four times with sterilized distilled water	MS + 2.0 mg/l BAP + 2.0 mg/L KN	25 ± 2 °C temp. 16 h photoperiod	Green compact callus	Wasn't carried out	Sadguna et al. (2014)
Leaf	0.1% $HgCl_2$ for 7–8 min washed four times with sterilized distilled water	MS+ 2.0 mg/l BAP	25 ± 2 °C temp. 16 h photoperiod	Brown compact callus	Wasn't carried out	Sadguna et al. (2014)
Nodal	0.1%(w/v) $HgCl_2$ for 4 min followed by wash with sterile distilled water	MS + 0.5–1 mg/l BAP + 0.5–1.0 mg/l KN	The pH of the media was managed and maintained at 5.7	Maximum shoot formation and shoot induction 83%, but initiation was observed in 6 days of culture	Regenerated plantlets were acclimatized in soil and vermiculture in 1:2 ratio	Bindu et al. (2017)
Nodal	0.1%(w/v) $HgCl_2$ for 4 min followed by wash with sterile distilled water	MS + 0.5 mg/l BAP + 0.5 mg/l KN	The pH of the media was adjusted and maintained at 5.7, 25 ± 2 °C temp., 16 h photoperiod	Maximum number of multiple shoots in 40 days of culture	Regenerated plantlets were acclimatized in soil and vermiculture in 1:2 ratio	Bindu et al. (2017)

stage and age of the mother plants were not specified, which is the principal part during explant selection.

13.4.2 Surface Sterilization

When dealing with the sterilization of explants in any in vitro culture, the most pivotal step is inoculation in the media on account of its presence a high possibility of contamination collected plants from the wild or natural habitats. Three parameters are engaged in surface sterilization such as bactericide, their quantity, and period of growth. These variables must be standardized to minimize the sterilization hurdle. Without disturbing the regeneration ability of explants, the contaminants will be suspended. These three parameters rely on the explants' tissue in a larger number of models in contrast to mature and hard tissue needing an exhibition of lower levels of bactericide for a briefer timespan for softer or juvenile tissue. The surface sterilization of *P. tuberosa* can be completed using 0.1% $HgCl_2$ for 3 min, rinsing six to eight times with sterile water, and, later, dipping in cold 0.1% of each ascorbic acid and citric acid for 15 min (Rathore and Shekhawat 2009), which is noted in Table 13.1. Other alternative surface sterilants were also reported. Sharma et al. (2011) used 5% sodium hypochlorite for 10 min and washed it five times with sterilized double-distilled water.

13.4.3 Multiple Shoot Formation

There are only a few reports on the entire in vitro plant regeneration in *P. tuberosa.* Rathore and Shekhawat (2009) grew an entire in vitro plant from a nodal explant of *P. tuberosa* with a combination of 0.99 mg/l BA, 0.12 mg/l KN, and 0.099 mg/l IAA alongside 100.0 mg/l ascorbic acid, 25.0 mg/l PVP, and 0.02% activated charcoal in MS medium. Bindu et al. (2017) used a nodal segment from a 10-month-old plant maintained in a pot for in vitro regeneration of *P. tuberosa*. The explants were cultured in MS medium with a blend of 0.5 mg/l KN and 0.5 mg/l BAP or in BAP/BA plus kinetin in very low amounts, resulting in an increase in the shoot's multiplication. As indicated by Thiem (2003), MS medium fortified with 1.1 μM IAA plus 9.3 μM KN demonstrated 94% of growth frequency with an average of 3.6 shoots per explant of *P. lobata*. For further multiplication, these explants were re-cultured in MS medium having 0.44 μM BA alongside 5.8 μM GA, and the maximum elongated shoots were obtained. Other authors recorded that the explant of *P. lobata* acknowledged to IBA and BA by delivering numerous adventitious buds. The axillary buds could not elongate on MS medium containing 4.4 μM BA along with 5.8 μM GA (Liu et al. 1999). Subsequently, Hakamatsuka et al. (1994) documented that an increase in the concentration of BA led to a higher occurrence of callus formation. Cytokinin BA is routinely employed for in vitro

Fig. 13.1 Micropropagation of *P. tuberosa*: (**a**) seed germination after 15 days of inoculation in MS medium with 1.0 mg/l BAP and 1.0 mg/l GA, (**b**) in vivo tuber, (**c**) induction of node callus using a nodal segment from a seedling in MS medium with 1.0 mg/l BAP and 0.5 mg/l 2,4-D, (**d**) induction of cotyledonary callus using the cotyledon from a seedling in MS medium with 1.5 mg/l BAP, (**e**) induction of leaf callus using leaf explant from a seedling in MS medium with 1.0 mg/l 2,4-D, (**f**) induction of tuber callus from in vivo tuber s in MS medium with 1.5 mg/l BAP and 1.5 mg/l 2,4-D, (**g**) direct shoot initiation in a nodal segment from a seedling after 1 week in MS medium with 0.75 mg/l BAP and 0.5 NAA mg/l, (**h**) elongation of the shoots after 2 weeks in MS medium with 0.75 mg/l BAP and 0.5 NAA mg/l, (**i**) induction of multiple shoots after 21 days, (**j**) multiple shoot formation after 4 weeks, (**k**) multiple shoots ready for rooting, (**l–m**) in vitro root formation in MS medium with 1.0 mg/l IBA and 0.5 mg/l NAA, and (**n**) plantlet acclimatization

regeneration of legumes (Liu et al. 1999; Hakamatsuka et al. 1994). Thiem (2003) recommended that shoot elongation of *P. lobata* relies upon the synergistic effect of BA and gibberellic acid. Recently, we experimented successfully (not yet published) with the in vitro regeneration of *P. tuberosa* as shown in Fig. 13.1. This entailed the direct initiation of shoots through a nodal segment from a seedling after one week of growth in an MS medium containing 0.75 mg/l BAP and 0.5 mg/l NAA (Fig. 13.1g), elongation of shoots after 2 weeks in MS medium with 0.75 mg/l BAP and 0.5 NAA mg/l (Fig. 13.1h), induction of multiple shoots after 21 days (Fig. 13.1j), and multiple shoots formation after 4 weeks (Fig. 13.1i).

13.4.4 Callus Induction and Regeneration

There is just one report for in vitro regeneration of *Pueraria* plant from callus. Bindu et al. (2017) reported that a leaf explant of *P. tuberosa* showed creamy-yellow friable callus when 0.5 mg/l NAA plus 1.5 mg/l BAP was supplemented in MS medium. This medium brought about 84% callus formation just in 7 days of inoculation. Following 50 days of culture, the callus becomes harder and green, indicating the inception of buds. This callus was transferred on a higher concentration of 2.0 mg/l BAP with 0.5 mg/l NAA, and the buds have grown into five to six shoots per explants following 65 days of subculture. In our study (not yet published), the induction of node callus using a nodal segment from a seedling of *P. tuberosa* in MS medium with 1.0 mg/l BAP and 0.5 mg/l 2,4-D (Fig. 13.1c), induction of cotyledonary callus using the cotyledon from a seedling in MS medium with 1.5 mg/l BAP (Fig. 13.1d), induction of leaf callus using leaf explant from a seedling in MS medium with 1.0 mg/l 2,4-D (Fig. 13.1e), and induction of tuber callus from in vivo tubers in MS medium with 1.5 mg/l BAP and 1.5 mg/l 2,4-D were successfully observed.

13.4.5 Rooting and Acclimatization

In the tissue culture process, the last phase of the regeneration is the root formation, acclimatization, and establishment of regenerated plantlets in the natural habitat. The major medium used in in vitro rooting of *P. tuberosa* is MS medium. For the root regeneration, in vitro produced shoots were inoculated in half-strength MS media with 0.5 mg/l IBA, resulting in 3.54 roots/shoot of 2.96 cm length within 14 days (Bindu et al. 2017). Here, the roots were healthy in the presence of IBA. According to Rathore and Shekhawat (2009), half-strength MS salt medium with 0.02% activated charcoal and 9.84 μM IBA acquired 98% frequency of rooting in *P. tuberosa*. The roots obtained were healthy and strong; however, in a lower concentration of IBA, the shoots showed a slow response. As the concentration of IBA increases, the number of roots and the root length decline. Regenerated multiple shoots of *P. lobata* set on MS medium supplemented with 2.68 μM NAA along with 11.42 μM IAA and showed 10.9 roots per explant. Higher concentrations of NAA induced small and thicker roots, whereas low hormonal concentrations form thinner and long roots (Thiem 2003). In our study (not yet published), in vitro root formation was observed in MS medium with 1.0 mg/l IBA and 0.5 mg/l NAA (Fig. 13.1l, m).

Roughly 80% of in vitro regenerated whole plant was transferred to soil in polypots. Following 1 month they were planted in the fields (Bindu et al. 2017). As indicated by Rathore and Shekhawat (2009), in vitro rooted plantlets were moved in a bottle containing sterile soilrite with one fourth strength of MS medium. After the induction of the roots in a bottle, the plantlets were transferred to the greenhouse. This ensured an 85% of survival rate after 45–50 days of acclimatization.

13.5 Conclusion

The outlook of this discussion on in vitro regeneration after the explant collection and selection, surface sterilization, multiple shoot regeneration, induction of callus, and in vitro rooting of *Pueraria* species has been discussed in a rigorous manner in this review. Moreover, several contemporary techniques, such as seed encapsulation or the creation of artificial seeds, remain unexplored in this plant's context up to the present time. It's worth noting at this point that no genetic transformation technique has been documented for *Pueraria*. As its vegetative growth is limited to a specific season, it is pertinent to go for the protoplast fusion technique, or the incorporation of the desired genes through protoplast transformation could be a tool for the enhancement of secondary metabolites. However, due to the lack of use of this technique in *Pueraria*, there is sufficient scope for the establishment of *Agrobacterium*-mediated transformation technique to produce isoflavonoids. This review provides researchers with an opportunity to advance their investigations in the realm of *Pueraria*.

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Chapter 14
Secondary Metabolite Enhancement via In Vitro Techniques and Its Industrial Prospects

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Abstract Secondary metabolites are organic compounds produced by plants, microorganisms, and other organisms that are not directly involved in their growth or reproduction but play essential roles in ecological interactions, defense mechanisms, and other physiological processes. Many secondary metabolites are commercially valuable due to their pharmaceutical, agricultural, and industrial applications. In vitro techniques, which involve growing plant cells, tissues, or microorganisms in controlled laboratory conditions, can be used to enhance the production of secondary metabolites for industrial purposes. Industrial prospects of secondary metabolite enhancement via in vitro techniques are many such as increased production (in vitro techniques offer precise control over environmental conditions such as temperature, light, and nutrient availability, which can be optimized to stimulate the production of secondary metabolites); genetic manipulation (in vitro techniques also allow for genetic manipulation of plants, microorganisms, or cells to enhance the production of specific secondary metabolites); quality control (in vitro techniques provide precise control over the growth conditions, which can result in consistent secondary metabolite production with minimal batch-to-batch variation. This ensures high-quality secondary metabolites with consistent chemical profiles, which is critical for industrial applications that require standardized and reproducible products. Additionally, in vitro techniques allow for real-time monitoring of secondary metabolite production, enabling early detection and management of any

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contamination or quality issues); sustainability (in vitro techniques offer a sustainable approach to secondary metabolite production as they can reduce the dependency on traditional field cultivation, which often requires large amounts of land, water, and other resources. In vitro techniques can be more resource-efficient, requiring less space, water, and pesticides, and can be carried out year-round, irrespective of seasonal variations. This can lead to reduced environmental impact, improved sustainability, and conservation of natural resources); scale-up potential (in vitro techniques offer the potential for scalable production of secondary metabolites. Once an optimized in vitro production system is established, it can be scaled up to meet industrial demands. Bioreactor systems, which provide controlled and scalable environments, can be used for large-scale production of secondary metabolites. This enables the production of secondary metabolites in large quantities, which is essential for industrial applications); diversification of sources (in vitro techniques allow to produce secondary metabolites from diverse sources, including rare or endangered plant species that may not be feasible for field cultivation. This can contribute to the conservation of endangered plant species and the sustainable production of valuable secondary metabolites from alternative sources); and new product development (in vitro techniques also offer the potential for the development of entirely new products). In conclusion, in vitro techniques provide a promising approach for enhancing the production of secondary metabolites with significant industrial prospects. They offer advantages such as increased production, genetic manipulation, quality control, sustainability, scale-up potential, diversification of sources, and new product development. As technology continues to advance, in vitro techniques are likely to play an increasingly important role in the industrial production.

Keywords Secondary metabolites · In vitro · Ex vitro · Increase · Plant science

14.1 Introduction

14.1.1 The Importance of Secondary Metabolites in Medicinal Plants

Organic substances known as secondary metabolites play a crucial part in the plant's defense against biotic and abiotic stresses, even though they are not directly involved in the growth, development, or reproduction of the plant. These metabolites also give many plant species their therapeutic qualities, which have been widely applied in traditional medicine to treat a variety of diseases (Devi and Ganasoundari 2002; Sarker and Nahar 2013).

Medicinal herbs have been utilized to treat a range of diseases since the ancient times. There are several secondary metabolites in medicinal plants, and they have been used by many cultures across the world for their potential health benefits and have long been recognized as important components of traditional medicine (Sarker

Fig. 14.1 Different medicinal aromatic plants used for medicines

and Nahar 2013). Traditional medical practices, including Ayurvedic and Native American medicine, have utilized plant-based treatments to cure a variety of illnesses and problems, including diabetes, cancer, and arthritis (Debnath et al. 2019). These treatments have been handed over the years and are based on monitoring, experimentation, analysis of the real world, and scientific method. Additionally, the pharmaceutical sector has greatly benefited from secondary metabolites as a source of medications, with most of the medicines are now targeted from natural sources (Newman et al. 2000; Atanasov et al. 2015) and having a number of benefits over manmade medications as well, including lower toxicity, excellent specificity, and a wide range of pharmacological actions (Cragg and Newman 2013). They frequently have fewer negative effects than manmade medications and are more quickly absorbed, digested, and removed by the body. Number of plants are being used for medicinal purposes such as those given in Fig. 14.1. They can work together with various substances including minerals, vitamins, and other secondary metabolites to increase their medicinal effects (Farooqui et al. 2012).

In-depth research has also been done on the ecological functions of secondary metabolites. It has been discovered that some substances have allelopathic effects on other plants and that others serve as insect repellents or attractants (Wink 2018).

14.1.1.1 Types of Secondary Metabolites

Alkaloids, flavonoids, terpenoids, and phenolic metabolites are only a few of the several types of secondary metabolites. Alkaloids are nitrogen-containing substances that are extensively dispersed across the plant kingdom and have a variety of biological functions (Atanasov et al. 2015). Many flowers and fruits have colors that come from compounds called flavonoids. Moreover, they engage in a variety of biological processes, such as anti-inflammatory and antioxidant ones (Ferreira et al. 2017). Terpenoids are a broad class of chemicals that are created from isoprene units and have a variety of biological functions (Cragg and Newman 2013). Phenolic compounds exhibit a variety of pharmacological effects and are identified by the appearance of one or more hydroxyl groups connected to an aromatic ring (Atanasov et al. 2015). Secondary metabolites have an important role in plant medicinal effects. Many biological actions of these substances, such as antibacterial, antitumor, anti-inflammatory, and antioxidant characteristics, have been demonstrated for many of these substances (Atanasov et al. 2015). In fact, alkaloids have been used to treat infections, fevers, and pains. Moreover, they have been employed as tranquillizers, stimulants, and hallucinogens (Dzoyem and Kuete 2013). Many illnesses, such as cancer, cardiovascular disorders, and respiratory infections, have been treated using flavonoids (Ferreira et al. 2017). Terpenoids have been utilized to treat skin conditions, respiratory infections, and digestive problems (Cragg and Newman 2013). The antidiabetic, anticancer, and antioxidant effects of phenolic compounds have been demonstrated (Atanasov et al. 2015).

Hence, secondary metabolites are essential for the therapeutic qualities of plants. A vast source of molecules with a variety of biological activities can be obtained thanks to the diversity of chemical compounds found in medicinal plants (Cuong et al. 2017; Kumar and Pandey 2013). For the creation of new medications from medicinal plants, it is crucial to comprehend the chemistry and biological functions of secondary metabolites (Newman et al. 2000; Patil et al. 2012). The creation of novel medications from these molecules should be the key focus of future research, along with the identification and characterization of new chemicals from medicinal plants (Singh and Dubey 2019).

14.1.2 Significance of Genetic Manipulation in Enhancing Secondary Metabolite Production

As mentioned above, secondary metabolites, which are made naturally by plants but are not vital to them, have important uses in medicine and other sectors. With the aim of improving the yield, quality, and variety of secondary metabolites, genetic manipulation is a potent technique that enables scientists to add, alter, or remove particular genes from plant genomes.

The potential to increase the output and quality of target compounds is one of the most significant advantages of genetic modification for improving secondary metabolite production (Bhattacharyya et al. 2015). Secondary metabolite extraction from plants using conventional techniques can be time-consuming and expensive and frequently gives low yields. The number of secondary metabolites generated by the plant can be increased by altering the genes responsible for their production, making them simpler to extract and purify. Another benefit of genetic engineering is the ability to create novel plant species with increased levels of desired chemicals or new compounds with enhanced biological activity (Atanasov et al. 2015).

Several plant species have demonstrated the use of genetic engineering to increase the production of secondary metabolites. For example, it has been demonstrated that altering the terpenoid biosynthesis pathway can boost the production of the antimalarial medication artemisinin in the medicinal plant *Artemisia annua* (Ro et al. 2006). In a variety of medicinal plant species, genetic manipulation has also been used to increase the production of a number of different secondary metabolites, including alkaloids (Kumar and Sharma 2017), flavonoids (Kumar et al. 2020), and phenolic compounds (Kumar et al. 2018). Additionally, the genetic engineering of plants has made it possible to produce bioactive substances with increased specificity or efficacy for application in the pharmaceutical business.

Targeting the regulatory genes that manage their biosynthesis enables genetic engineering to increase the production of particular secondary metabolites (Liu et al. 2018a, b). For example, in *Coptis chinensis*, overexpression of the gene for the essential enzyme involved in berberine biosynthesis resulted in a significant increase in berberine production (Jing et al. 2018). Similar to this, the synthesis of significant flavonoids in plants, including quercetin, kaempferol, and isoflavone, has been increased through genetic alteration of metabolic pathways (Chen et al. 2018).

However, genetic modification for the improvement of secondary metabolites is not without its difficulties. Potential harm to plant development, growth, and ecological relationships is one of the main worries. It is crucial to make sure that the addition of additional genes does not cause the synthesis of carcinogenic or toxic substances or alter other plant properties, including shape, yield, and nutritional value. A further issue with genetic modification is the possibility of genetic contamination, which could result in the spread of transgenes from genetically altered plants to wild-type plants and have unfavorable effects on the environment (Hussain et al. 2010).

In summary, genetic engineering is a viable method for increasing the number of secondary metabolites produced by therapeutic species (Kjær et al. 2018; Kumar and Sharma 2017). It has shown enormous potential for accelerating the synthesis of particular target metabolites, boosting yield, and creating innovative metabolites (Katsuno et al. 2018; Jing et al. 2018; Chen et al. 2018). The possible hazards and difficulties of genetic alteration, including the possibility of unexpected effects on plant development and ecological relations, must be carefully considered (Hussain et al. 2010). More research is required to completely explain the effects of genetic manipulation and to assure the safeness and effectiveness of the products that arise

Fig. 14.2 Medicines are being made through RDT and genetic editing processes

from it; the medicines are being prepared from genetic change as well as RDT technique as shown in Fig. 14.2 (Kumar et al. 2020).

14.2 Methods of Genetic Manipulation

Genetic manipulation refers to the process of modifying an organism's genetic material (DNA or RNA) in order to achieve a desired trait or characteristic. There are several methods of genetic manipulation, including:

Recombinant DNA technology: This involves the insertion of a desired gene into the DNA of an organism, using techniques such as restriction enzymes, ligases, and vectors. Recombinant DNA technology is commonly used in genetic engineering to create genetically modified organisms (GMOs) that express a desired trait. CRISPR-Cas9: CRISPR-Cas9 is a revolutionary genetic editing tool that allows scientists to precisely target and modify specific genes within an organism's genome. It works by using a guide RNA molecule to target a specific DNA sequence and then using the Cas9 enzyme to cut the DNA at that location. The cell's natural DNA repair mechanisms can then be used to introduce specific genetic changes.

RNA interference (RNAi): RNA interference is a mechanism that allows scientists to selectively silence specific genes by introducing short, double-stranded RNA molecules that match the sequence of the target gene. These RNA molecules then interfere with the expression of the target gene, effectively "turning it off."

Gene therapy: Gene therapy is a technique that involves the insertion of a functional gene into an individual's cells to correct a genetic disorder or disease. This can be accomplished using a variety of methods, such as viral vectors or electroporation.

Transgenic technology: Transgenic technology involves the insertion of a foreign gene into the genome of an organism, resulting in the expression of a new trait. This can be accomplished using a variety of methods, such as microinjection, electroporation, or viral vectors. It's worth noting that genetic manipulation is a complex and often controversial field, and there are a range of ethical and safety considerations that must be taken into account when conducting research or creating genetically modified organisms.

14.2.1 Recombinant DNA Technology

Recombinant DNA technology, also known as genetic engineering, is a technique used to create artificial DNA molecules by combining DNA fragments from different sources. This technique allows scientists to create new genes, modify existing ones, and transfer genes between organisms.

The basic steps involved in recombinant DNA technology include:

Isolation of DNA: The DNA of interest must be extracted and purified from the organism in which it naturally occurs.

Fragmentation of DNA: The DNA is cut into smaller fragments using restriction enzymes, which recognize specific DNA sequences and cut the DNA at those locations.

Insertion of DNA fragments: The desired DNA fragments are inserted into a cloning vector, which is a DNA molecule that can carry foreign DNA and replicate inside a host cell.

Introduction of the vector into host cells: The vector is introduced into a host cell, such as a bacterium or yeast cell, where it can replicate and produce many copies of the recombinant DNA molecule.

Selection of recombinant cells: The cells containing the recombinant DNA molecule can be selected based on a marker gene that is inserted along with the DNA of interest.

Recombinant DNA technology has many practical applications, such as the production of therapeutic proteins, creation of genetically modified crops, and development of new vaccines. However, there are also ethical and safety concerns associated with the use of genetic engineering, particularly when it involves human embryos or genetically modified organisms (GMOs).

14.2.1.1 CRISPR-Cas9 Gene Editing Technology

CRISPR-Cas9 is a revolutionary gene editing technology that allows scientists to precisely modify DNA sequences in living cells. It is based on a natural defense mechanism used by bacteria to defend against viral infections.

The basic steps involved in CRISPR-Cas9 gene editing technology are:

Designing the guide RNA: A short RNA molecule is designed to match a specific DNA sequence of interest. This RNA molecule, called the guide RNA, is designed to direct the Cas9 enzyme to the correct location in the genome.

Introducing the guide RNA and Cas9: The guide RNA and Cas9 protein are introduced into the target cells, where they form a complex that searches for and binds to the target DNA sequence.

Cutting the DNA: Once the guide RNA and Cas9 complex has bound to the target DNA sequence, the Cas9 protein cuts the DNA at that location.

Repairing the DNA: The cell's natural DNA repair mechanisms then repair the DNA by either adding or deleting nucleotides at the site of the cut or by replacing the cut section with a new DNA sequence that has been provided.

CRISPR-Cas9 gene editing technology has many potential applications, such as the treatment of genetic diseases, the development of new crops, and the creation of animal models for disease research. However, there are also ethical and safety concerns associated with the use of CRISPR-Cas9 technology, particularly when it involves the editing of human embryos or the creation of genetically modified organisms (GMOs). As such, there is ongoing debate and regulation surrounding the use of CRISPR-Cas9 gene editing technology.

14.3 In Vitro Techniques for Secondary Metabolite Enhancement

Secondary metabolites are organic compounds that are produced by plants, bacteria, and fungi and have many important applications in medicine, agriculture, and industry. In vitro techniques can be used to enhance the production of secondary metabolites by manipulating the culture conditions and genetic makeup of the organisms that produce them. Some common in vitro techniques for secondary metabolite enhancement include:

Cell and tissue culture: Cells and tissues from plants, bacteria, and fungi can be grown in vitro under controlled conditions to enhance the production of secondary metabolites. This can involve the use of different types of culture media, growth hormones, and environmental factors such as temperature, light, and oxygen levels.

Elicitation: Elicitation involves exposing cells or tissues to specific chemical or physical stimuli to enhance the production of secondary metabolites. For

example, the addition of specific nutrients, hormones, or plant extracts can trigger the production of secondary metabolites in plant cells or tissues.

Genetic engineering: Genetic engineering techniques can be used to enhance the production of secondary metabolites by modifying the genes that regulate their biosynthesis. This can involve the overexpression of genes involved in secondary metabolite production or the introduction of new genes from other organisms.

Bioreactor technology: Bioreactors are specialized vessels that provide a controlled environment for the growth of cells and tissues. Bioreactor technology can be used to optimize the production of secondary metabolites by controlling factors such as pH, temperature, oxygen levels, and nutrient availability.

Metabolic engineering: Metabolic engineering involves the manipulation of cellular metabolic pathways to enhance the production of secondary metabolites. This can involve the modification of genes that regulate the synthesis of precursors or the enzymes involved in secondary metabolite biosynthesis.

Overall, in vitro techniques can be a powerful tool for enhancing the production of secondary metabolites and have important applications in the development of new drugs, agricultural products, and industrial processes.

14.3.1 Callus Culture

Callus culture is a type of tissue culture technique in which undifferentiated cells are induced to proliferate and form a mass of cells called callus. Callus culture can be used to study the growth and differentiation of plant cells and to produce plants with desirable traits through genetic engineering.

The basic steps involved in callus culture are as follows:

Selection of explants: Explants are small pieces of plant tissue that are used to initiate callus culture. They can be obtained from various parts of the plant, such as the leaves, stems, or roots.

Surface sterilization: The explants are sterilized to remove any surface contaminants, such as bacteria or fungi, that might interfere with the callus culture.

Culture initiation: The sterilized explants are placed onto a sterile culture medium containing plant growth regulators, such as auxins and cytokinins, that promote cell division and callus formation.

Callus growth: The cells in the explant divide and proliferate, forming a mass of undifferentiated cells called callus. The callus can be subcultured onto fresh medium to promote further growth.

Differentiation: The callus can be induced to differentiate into specific types of cells or tissues by altering the growth conditions, such as the type and concentration of plant growth regulators, or by genetic engineering.

Callus culture has many applications in plant biotechnology, such as the production of genetically modified plants with desirable traits, the regeneration of plants

from single cells, and the study of plant growth and development. It is also used in the production of secondary metabolites, such as alkaloids and flavonoids, which have important applications in medicine and industry.

14.3.2 Suspension Culture

Suspension culture is a type of tissue culture technique in which cells are grown in a liquid medium rather than on a solid medium like agar. The cells are suspended in the liquid medium and are kept in constant motion by stirring or shaking. Suspension culture can be used to study the growth and metabolism of cells and to produce large quantities of cells or secreted products such as proteins.

The basic steps involved in suspension culture are as follows:

Cell culture initiation: Cells are initially seeded into a liquid culture medium containing nutrients, growth factors, and other supplements that are necessary for cell growth and metabolism.

Suspension culture maintenance: The cells are maintained in suspension by continuously stirring or shaking the culture vessel to prevent the cells from settling. The culture medium is typically changed regularly to ensure that the cells have access to fresh nutrients and growth factors.

Cell growth and propagation: The cells will continue to divide and grow in suspension culture and can be propagated by transferring a portion of the culture into fresh medium.

Harvesting cells or products: Suspension culture can be used to produce large quantities of cells or secreted products such as proteins. The cells or products can be harvested from the culture by centrifugation or filtration.

Suspension culture has many applications in biotechnology and biomedical research, such as the production of recombinant proteins, monoclonal antibodies, and vaccines. It is also used in the study of cell growth and metabolism and in the production of cells for tissue engineering and regenerative medicine. Suspension culture can be scaled up to produce large quantities of cells or products, making it a valuable tool for industrial applications.

14.3.3 Explant Culture

Explant culture is a tissue culture technique in which a small piece of plant tissue, called an explant, is removed from a plant and grown in a culture medium under sterile conditions. The explant can be any part of the plant, such as the leaves, stem, root, or embryo. The goal of explant culture is to initiate cell division and generate a mass of undifferentiated cells, known as a callus, which can then be used for further experiments or for plant regeneration.

Fig. 14.3 In vitro cultures to achieve enhanced secondary metabolites

The basic steps involved in explant culture are as follows:

Selection of explants: The explants are selected based on the tissue type and the purpose of the culture. For example, leaf explants may be used to study photosynthesis, while embryonic explants may be used to regenerate whole plants.

Surface sterilization: The explants are sterilized to remove any surface contaminants, such as bacteria or fungi, that might interfere with the culture. This is usually done by soaking the explants in a solution of bleach or alcohol.

Culture initiation: The sterilized explants are placed onto a sterile culture medium containing nutrients, vitamins, and plant growth regulators, such as auxins and cytokinins, that promote cell division and callus formation.

Callus growth: The cells in the explant divide and proliferate, forming a mass of undifferentiated cells called callus. The callus can be subcultured onto fresh medium to promote further growth.

Regeneration: The callus can be induced to differentiate into specific types of cells or tissues by altering the growth conditions, such as the type and concentration of plant growth regulators. This can lead to the regeneration of whole plants from the callus. It is clearly monitored in many experiments, and it is observed that in vitro plants have high secondary metabolites as compared to ex vitro plants given in Fig. 14.3.

Explant culture has many applications in plant biotechnology, such as the production of genetically modified plants with desirable traits, the propagation of rare or endangered plant species, and the study of plant growth and development. It is also used in the production of secondary metabolites, such as alkaloids and flavonoids, which have important applications in medicine and industry.

14.4 Industrial Perspective on Secondary Metabolite Enhancement

Throughout ancient times, humans have relied on resources that are derived from nature for their requirements, including nourishment, medicine, construction materials, various industrial products, etc. Both traditional and modern medical practices have relied heavily on whole plants or their parts for their bioactive components. The oldest evidence of the use of plant-based components dates back to 2600 BC in Mesopotamia, while the use of natural components as drugs in Ayurvedic medicine dates back 5000 years. Traditional medical practices such as Unani, Ayurveda, Homeopathy, and Siddha all use plants as the primary therapeutic agent in practically all of their prescriptions. Furthermore, Chinese literature is also filled with various types of plants and their derived bioactive components (Kumar et al. 2019; Alves and Rosa 2007; Savithramma et al. 2011). About 80% of the world's population, or about 4 billion people, depends on compounds derived from plants to maintain good health and well-being. In recent years, the demand for such plant-based compounds has increased, as they are more inexpensive, less environmentally friendly, and more widely available than synthetic drugs. Growing demand for such plant-based compounds, which has attracted attention to research and the development of new and improved strategies for increasing such compounds without having an impact on the natural wildlife populations of the plants themselves (Chandran et al. 2020a, b; Ekor 2014). Plants are equipped to produce a plethora of secondary metabolites, which are an essential part of their defense mechanisms. Plant secondary metabolites are produced in response to biotic and abiotic stress, such as pathogen invasion, metal stress, interaction between plants and animals, or plants and humans that activate its defense mechanisms. There is enormous potential in the industrial, medicinal, and nutraceutical applications of plant secondary metabolites. Even so, the extraction and purification technique for these secondary metabolites from wild natural plants is complex and time-consuming, and the procedure also results in a relatively poor yield in comparison to the amount of raw material that is employed. For instance, plants under stress condition produce secondary metabolites that include alkaloids, terpenoids, phenols, carotenes, lipids, steroid, saponin, flavonoids, etc. to counter the unfavorable conditions (Chandran et al. 2020a, b; Anjitha et al. 2021; Zhang et al. 2011; Yeshi et al. 2022). As a result of the rising demand for these secondary metabolites, the generation of large quantities of secondary metabolites through in vitro culture is a process that has the ability to scale up production

and is independent of any seasonal or environmental conditions. The in vitro cell culture consists of different stages, which are required in order to achieve a high quality and quantity of natural products. It is essential to have primary cell lines that produce high yields of high-quality secondary metabolites and to determine the proper components required in the media for optimization, elicitation, and regulation of the culture environment. Gottlieb Haberlandt (1854–1945), the pioneer of plant tissue culture, is credited with developing the first callus root or embryo cultures around the turn of the twentieth century. Plant tissue culture technique has added benefits in terms of large-scale secondary production due to its independent approach to production without depending on any seasonal change, being devoid of any plant pathogens or contaminations, and allowing the production of high-quality secondary metabolites of choice, cost-effective genetic variability, and the generation of new plant varieties as well as rare and endangered species that can be grown using the techniques. An additional benefit of cell culture is the ability to modify the metabolites in both quality and quantity by either supplementing the culture with elicitors or subjecting the cells to circumstances that induce stress. Furthermore, the bioreactor-based large-scale generation of therapeutically useful secondary metabolites from plant cell cultures using callus cell culture, embryos, immobilized root and shoot culture, suspension cell culture, etc. is an efficient method for industries (Abdulhafiz et al. 2022; Efferth 2019; Malayaman et al. 2017; Motolinía-Alcántara et al. 2021; Nitzsche et al. 2004).

14.4.1 The Current Market for Secondary Metabolite

As consumers become more aware of their health and lifestyle, there has been a surge in the market for natural and organic goods. Recently, about 70–90% of the population preferred using ancient medicines obtained from plant extracts. Interestingly, more than half of all pharmaceutical drugs approved by the FDA are derived from natural ingredients (Anand et al. 2019). The global market for herbal medicine is growing fast, and the global trade of medicinal plants and their metabolites in 2000 was valued at 60 billion USD; estimates indicate that it will exceed 5 trillion USD (Chandran et al. 2020a, b).

Secondary metabolites from plants represent a number of medicinal compounds, which include alkaloids, terpenoids, phenols, cyanogenic glycosides, etc., and are the primary source for the production of various lifesaving drugs. Studies suggest that the secondary metabolites found in plants have been shown to be highly successful in treating a wide variety of diseases, including viral and microbial infections, diabetes, renal disease, lung disease, and cancer chemotherapeutics. For instance, the anticancer medicine paclitaxel (Taxol), which is made from the *Taxus* plant, is a prime example of a drug that is developed using cell culture techniques. The secondary metabolite taxol, which is a complex diterpene, is found in the bark of *Taxus brevifolia* Nutt. (Pacific yew tree). As the prevalence of cancer rises, so does the worldwide demand for medicine, which is also increasing. However, to cater to

such a demand for the taxanes, researchers have devised biotechnological methods involving the cultivation of *Taxus* sp. in cell suspensions. In addition, the demand for taxanes is enormous, with global sales that generate annual sales of up to $1 billion (Yue et al. 2016; Poddar et al. 2020; Hussain et al. 2012; Onrubia et al. 2013; Malik et al. 2011).

Similarly, the market for the essential oil industry is rapidly growing and is estimated to have a worldwide market value of $7.47 billion USD in 2018 (Sarkar et al. 2018; www.medgadget.com). Lavender essential oil is popularly used in perfume, medicine, and cosmetics. The demand for the oil extracted from the plant is of great importance. However, there are various factors that influence the harvest and the oil composition of the lavender plantation, such as climatic change, slow seed development, the composition and content of the volatile oil of the plant, etc. On the contrary, tissue culture techniques provide advantages over all these limitations and further facilitate the vegetative seedling propagation by producing more concentrated metabolites in huge quantities. Studies have revealed that using jasmonic acid treatment and stress as inducers resulted in the production of cinnamic acid, a precursor of metabolites like caffeic acid and rosmarinic acid. In addition, the study stated that the culture condition is a very crucial criterion that might influence the desired metabolites and might lead to the production of unwanted metabolites (Nitzsche et al. 2004; Korkunc 2018; Gonçalves and Romano 2013).

Vanilla is the second most valuable and expensive spice, as well as one of the most popular flavors. Vanilla belongs to the family Orchidaceous and is obtained from the vanilla bean. The flavors are used for various products, cakes, ice creams, perfumes, etc. Interestingly, less than one percent of the vanilla flavors used in the food sector originate from a completely natural source, which is the vanilla orchid. On the other hand, the remaining component is a synthetic vanillin that is primarily derived from petroleum. According to the projection, the worldwide vanilla market contributed 510 million US dollars in 2018, and it is expected to be around 735 million US dollars by 2026, representing an annual growth rate of 4.7%. Also, the increase in demand for vanilla across the globe is due to people demanding natural and organic vanilla flavors. Vanilla propagation is done by stem cutting, which is not an expensive or a time-demanding process. In addition, the removal of these stem cuttings from the mother plant has a negative effect on the plant and causes a cease in growth and development, which reduces the yields. A study conducted by Tan et al. (2011) stated the optimization of growing *Vanilla planifolia* in vitro for callus formation using the leaves and nodal segments. As a result, the study has provided techniques to boost the production of vanilla using in vitro procedures, which will allow for an adequate response to the demand for vanilla. The study further described that throughout the hardening procedure, which lasted for 4 weeks, when the plantlets were subjected to 80% shade, 90.0% of them survived. On the contrary, the number dropped to 50% when the plants were under 50% shade, and this was due to the radiation exposure. Moreover, a similar study also described that the vanilla plant can also be propagated in vitro using the shoot tip and nodal bud as explants (Ralandison 2021; Wulandari and Ardana 2021; Tan et al. 2011; Kalimuthu et al. 2006; Dörnenburg and Knorr 1995).

14.4.2 Challenges/Barriers Faced by the Industry in Producing Secondary Metabolite

Plants are the biofactories that are capable of the biosynthesis of a wide variety of secondary metabolites, including alkaloids, glycosylates, etc. Secondary metabolites are spread throughout the plant cells, including their site where the metabolites are biosynthesized; in addition, they are typically localized in one organ and then transported to other regions via vascular tissues or symplastic and apoplastic transport to their storage sites, respectively, depending on the polarity of the metabolite. These secondary metabolites are important components of various pharmaceutical products and lifesaving drugs. Yet, there is a disparity between the need for the metabolites and the supply from the plants that can provide them. The overuse of plants for their secondary metabolites also poses a persistent risk. Yet, the micropropagation method offers a way around the risks of overexploitation and environmental destruction in order to access these crucial biometabolites. Nevertheless, the production of secondary metabolites also faces several hurdles in terms of the genetic construction of the explant or the mother plant, as clonal fidelity or plant cell heterogeneity is an important criterion for in vitro culture that causes slow growth in the culture. Moreover, the scaling-up process of the in vitro plant tissue and organ culture also proves to be a limitation in the process of secondary metabolite production. Furthermore, a lack of adequate knowledge and skill also makes the in vitro production of secondary metabolites a challenging process. Furthermore, the in vitro process of secondary metabolite production also requires optimization of the culture media, including elicitors, and an adequate in vitro environment for a high yield of secondary metabolites (Tan et al. 2011; Sharma et al. 2014; Isah et al. 2018; Li et al. 2020; Bhaskar et al. 2022). In one of the study, production behavior instability was observed in the culture of *C. roseus* cell strains and was not exclusive to *C. roseus* cell strains but rather widespread among different cultures, although some cells showed stable behavior and have been reported to have produced secondary compounds in high yields for up to 14 years. On the contrary, high-yielding cell strains presumably contain the chemical instability and are prone to fluctuations in output since they were selected from a population of variant cells (Deus-Neumann and Zenk 1984).

14.4.3 Potential Solutions and Opportunities

Plants are an essential source of renewable resources and an essential provider of complex chemical compounds. Secondary metabolites are unique small organic plant compound molecules that are biosynthesized by plants in response to environmental stresses. Plants are equipped to synthesize a huge variety of secondary metabolites that are essential cornerstones for today's modern healthcare and pharmaceutical industries. These plants are the vital source of alkaloids, phenols,

terpenoids, food flavors, colorants, additives, etc. However, using raw plants for obtaining the compounds might lead to overexploitation of plants, leading to their extinction. In vitro culture of such plants further overcomes all such limitations but requires further intervention to improve the process. Furthermore, there is still a need to characterize many secondary metabolites from plants for bioprospecting in healthcare and pharmaceutical uses. The in vitro techniques for secondary metabolites further demand extensive research in the field to understand the mechanisms involved in the production of secondary metabolites by plants and their utilization in various sectors. The poor yield of plant secondary metabolites in plant cell cultures is one of the primary challenges that must be overcome. Because secondary metabolite production is induced in response to various abiotic and biotic stresses as a defense mechanism, it is a complicated process to mimic in vitro. Despite this, various developments and treatments have been made with regard to boosting the yields of secondary metabolites. They include elicitors, stresses from biotic factors, and signal molecules.

In addition, optimization and customization of basal media for certain species and genotypes are also crucial steps in in vitro plant tissue culture. Furthermore, overcoming the issue of contamination is also imperative to achieving a high quality and quantity of secondary metabolites. However, there has been a lot of work done to find solutions to the issue of secondary metabolite deficiency in entire plants, and the use of sterile plantlets is one such improvement. In addition, several taxa, especially woody plants, have proven challenging for tissue culture methods, and only a small number of species have been successfully produced in tissue culture. Developing species-specific protocols is a common practice (Tiwari and Rana 2015; Twaij and Hasan 2022; Akula and Ravishankar 2011; Giri and Zaheer 2016; Phillips and Garda 2019; Touchell et al. 2020). Also, in certain in vitro plant cultures, there are specific organs or tissues in the plant that are equipped to produce those particular secondary metabolites, and therefore, the generation of secondary phytochemicals needs highly differentiated micro plant or organ cultures. Despite the underlying fact that various sources of literature depict a successful procedure that has achieved yields of secondary metabolites in differentiated cell calls as well as organ culture, furthermore, there are specific conditions and stages of a plant where efficient production of secondary metabolites takes place. For instance, the study conducted by Al-Qudah et al. (2011) revealed the in vitro culture of *Teucrium polium* (wild germander) and further stated that in vitro grown plants had more β-caryophyllene than their in vivo grown counterparts, while oil production was higher from in vivo grown plants. It's possible that this is due to the presence of β-caryophyllene at a certain stage in the plant's development (Karuppusamy 2009; Al-Qudah et al. 2011).

14.5 Applications of Genetic Manipulation in Medicinal Plants

The plant has been a vital lifeline for human civilization since ancient times. In this regard, medicinal plants are of crucial importance because of their biocompounds, which hold the key to curing various diseases. The plants are equipped to produce a vast range of biometabolites that are the basis of various lifesaving drugs in today's world. The implications of plant tissue culture on medicinal plants have a major advantage in the contribution of various organic compounds in greater yield under a controlled environment. Furthermore, it can eliminate the potential for the overexploitation of medicinal plants, which could ultimately lead to their extinction (Siahsar et al. 2011; Hussain et al. 2012).

Genetic manipulation or transformation is a process that involves the introduction and subsequent expression of a foreign gene or gene of interest into the genome of a targeted plant and allowing it to express itself to produce the desired product (Gantait et al. 2021). However, genetic manipulation is also being used to decrease certain secondary metabolites in order to increase the production of certain metabolites. Most commonly, this technique is used to increase metabolite production in plants. So far, a very limited number of genes that are responsible for the production of certain secondary biometabolites have been explored (Veerpoorte et al. 2002). Research conducted by Wang et al. (2001) has reported that silencing a trichome gland-specific cytochrome P450 gene from the tobacco plant results in enhanced natural products and also aphid resistance (Wang et al. 2001).

One such prominent example of gene manipulation is hairy root culture, where the root-inducing plasmid present in *A. rhizogenes* induces the root hairs in the dicotyledonous plants. Root hair induction in dicotyledonous plants, known as hairy root culture, is a well-known example of gene manipulation. This phenomenon is triggered by the transfer and expression of T-DNA from the Ri plasmid into the plant nuclear genome. Similarly, in crown gall disease, the bacterium *Agrobacterium tumefaciens* carrying the Ti (tumor-inducing) plasmid is responsible for the unorganized, tumorous growth at the site of infection. This is because a T-DNA fragment, around 20 kb in size, gets integrated into the chromosomes of the infected plant cell from the Ti plasmid of the bacterium and expresses itself. In addition, at least four oncogenes have been reported inside T-DNA that code for enzymes involved in the synthesis of the plant hormones auxin and cytokinin in infected plant cells (Kowalczyk et al. 2020; Gelvin 1990). A study conducted by Yousefian et al. (2020) examined the hairy root induction of the medicinal plant *Mentha spicata*. However, the challenge is to extract, separate, and purify the secondary metabolites from the plant *Mentha spicata*. Moreover, the yield of secondary metabolites in the plant is also low in the differentiated tissues of medicinal plants. Therefore, the hair root culture in this medicinal plant provides a quick growth rate, easy preservation, and biochemical as well as genetic stability and enhances the biosynthesis capabilities of secondary metabolites in plant roots. Further research has revealed the successful synthesis of phenolic acid in the *Mentha spicata* plant (Yousefian et al.

2020). Another example of using transgenic cell suspension that facilitates the production of enhanced secondary metabolites is using transformed *Silybum marianum* cell suspension culture with the stilbene synthase gene from *Vitis vinifera* L. by enabling enhanced accumulation of t-resveratrol. Using transgenic cell suspension cultures is another excellent method of obtaining secondary metabolites. Stable transformation of *Silybum marianum* (L.) Gaertn. cell suspension cultures with the *Vitis vinifera* L. stilbene synthase gene (Hidalgo et al. 2017). Hence, genetic modification of medicinal plant species is an essential option for obtaining major secondary metabolites; nevertheless, additional intervention and research are still needed to comprehend the behavior and identify key genes of various medicinal plants.

14.6 Future Prospects

14.6.1 Potential for Novel Drug Discovery

Since time immemorial, plants have always played an important role in traditional medicine as well modern medicine (Chandran et al. 2020a, b). The health benefits of medicinal plants with the perks of safety and cost have picked the interest of consumers and industry over the last two decades (Ekor 2014; Thomford et al. 2018; Anand et al. 2019). The wide range and diverse groups of secondary metabolites, viz., alkaloids, flavonoids, phenols, and terpenoids, present in medicinal plants have been reported to exhibit broad-spectrum therapeutic potentiality and are the key source for the discovery of novel drugs to be used for the treatment of various diseases and infections. The present global issue of antibiotic resistance in the medical world due to the profuse usage of commercially synthesized antibiotics as well as the side effects and inefficacy of other chemotherapeutics cannot be ignored and has provoked scientists to find alternative ways to combat infections (Pandey and Gupta 2023). In such situation, the role and efficacy of medicinal plants as a healing agent have laid the foundation for researchers to further probe into the mechanism of potential bioactive compounds that is conferring the antimicrobial properties. Many scientists and researchers have started unlocking the vast potential of medicinal plants as alternative treatment by identifying, characterizing, and investigating the possible mode of action through which they function which will, in the future, direct our focus to herbal therapy. Thus, active research on bioactive compounds of medicinal plants will help in the elucidation of discovery of novel drug compound and will pave the way for alternative herbal therapy. Apart from its lifesaving entity, these bioactive secondary metabolites have their application in food and cosmetic industry as dyes, pigments, additives, perfumes, pesticides, etc. (Patel et al. 2022).

Secondary metabolites from plants, being more specific and biodegradable, present themselves as the best source for obtaining novel drugs (Tomoko et al. 2002). In addition to these, the functionality and structural diversity, which are

present in secondary metabolites, are highly desirable in the discovery of novel bioactive molecules and drugs (Veerpoorte et al. 2002). The different stages of potential drug discovery are as follows:

(a) *Identification of potential compounds*: The identification and extraction of targeted compound, the study of its physical properties, testing its potential under physiological stress condition at cellular level, and the study of its absorption, distribution, metabolism, and excretion.
(b) *Screening and preclinical trial*: The study of drug safety in human.
(c) *Clinical trials*: Safety and efficacy of drug and its reproducibility (Patel et al. 2022)

The technique of plant propagation exploiting the totipotency property under controlled aseptic condition is known as plant tissue culture. The recent advances in biotechnology coupled with tissue culture have provided a vast potential for commercial production of desirable drug in controlled condition with less space and a high scale-up capacity at low contamination risk resulting in less purification.

14.6.2 Integration with Traditional Medicine

Medicinal plants or herbs used in folk and traditional medicines are promising candidates for the search of natural remedies and novel drugs. Plant tissue culture utilizing bioreactors provides a cost-effective, sustainable, and well-controlled means for mass production of the bioactive compounds from rare and endangered medicinal plants (Zhou and Wu 2006). With limitation of land and natural resource, plant tissue culture (PTC) techniques provide an effective alternative for the biosynthesis and biotransformation of phytomedicines. In addition to that, PTC is also continuously utilized for the conservation of genetic diversity, mass propagation, and efficient production of pharmaceutically important compounds by enhancing genetic and phenotypic variabilities, misidentification, extract variability and instability, toxic components, and impurities (Rather et al. 2022).

Oplopanax elatus is a traditionally used medicinal plant in China, Russia, and Korea whose roots are used to treat various disorders such as chronic fatigue syndrome, cardiovascular diseases, diabetes mellitus, and rheumatism (Wawrosch and Zotchev 2021). Jian et al. (2017) studied the content of flavonoids, phenolics, and polysaccharides in *O. elatus* and observed that on application of an elicitor (200 μM methyl jasmonate), there was an accumulation of 53.87 mg/g DW flavonoids and 192.64 mg/g DW polysaccharides, while treatment with 100 μM salicylic acid led to the production of 30.10 mg/g DW phenolics (Jiang et al. 2017). Accumulation of nearly twofold chlorogenic acid derivatives as compared to control was observed in suspension cultures of *Gardenia jasminoides* when treated with 200 μM methyl jasmonate (Liu et al. 2018a, b). Camptothecin is a cytotoxic compound that is used as a starting material for the semisynthetic preparation of important antitumor drugs such as topotecan and irinotecan. It was originally isolated from *Camptotheca*

acuminata, upon which studies were carried out for cell line selection, nutrient medium, optimization, and elicitation with jasmonic acid, and it was found that camptothecin production increased from 0.06 mg/g DW in the original cell line to 1.12 mg/g DW (Deepthi and Satheeshkumar 2017).

Reports of many such studies and findings over the past years indicate the vast potential and importance of the production of bioactive compounds in vitro. The effects of elicitors and culture techniques appear to be the most important factors for the production of higher levels of relevant metabolites compared to control cultures. The market as well as clinical demand is prone to change, while ongoing research demonstrates the potential of plant in vitro cultures to produce bioactive secondary metabolites.

14.6.3 Impact on the Pharmaceutical Industry

Biotechnology is a powerful alternative approach to control the overexploitation of natural resource while producing secondary metabolite from medicinal plants at industrial level (Mohaddab et al. 2022). For this purpose, efforts have been made towards culture condition optimization for production as well as manipulating the synthesis of bioactive molecules by various approaches such as cell line selection, precursor feeding, and elucidation (Gaosheng and Jingming 2012) in order to meet the pharmaceutical industry demand while conserving nature (Gonçalves and Romano 2018; Guerriero et al. 2018; Mulabagal and Tsay 2004; Yue et al. 2016). Organ cultures act as a source of plant material for secondary metabolite production. See the list of some medicinal plants that produce secondary metabolites under callus and suspension type culture in Table 14.1. During the in vitro production of secondary metabolites, plant cells are induced to form callus by subjecting them to stress using triggering agents, which are then used to form suspension culture.

Studies have shown that in vitro production of plant secondary metabolites can also be enhanced by the addition of adjuvants or elicitors to the cell culture media acting as a trigger to activate the plant defense mechanism (Table 14.2). The application of jasmonic acid to the callus culture of *Salacia chinensis* increased the total phenol, flavonoid, and mangiferin content as reported by Chavan et al. (2021). Mahendran et al. (2021) also observed the highest accumulation of deacylgymnic acid and XVII gymnemic acid on the cell suspension culture of *Gymnema sylvestre* that was supplemented with 20 μM sodium nitroprusside.

Table 14.1 List of some medicinal plants producing secondary metabolites under callus and suspension-type culture. (Source: Mahendran et al. 2021)

Plant	Culture type	Bioactive component
Ageratina pichinchensis	Suspension	Artemesinol
Anethum graveolens		Carvone
Cayratia trifoliata		Stilbenes
Eysenhardtia platycarpa		Total phenolics
Gymnema sylvestre		Gymnemic acid
Ocimum basilicum		Chicoric acid, rutin, linalool, methyl chavicol, rosmarinic acid
Camellia sinensis	Callus	Catechin
Capparis spinosa		Rutin
Caralluma tuberculata		Total phenolics, total flavonoid
Cupressus sempervirens		Rutin, quercitrin
Gardenia jasminoides		Rutin
Phyllanthus acidus		Phyllanthusol
Pluchea lanceolata		Quercetin
Rosmarinus officinalis		Flavonoid, terpenoids
Labisia pumila		Total phenolics, total flavonoid

14.7 Conclusion

14.7.1 Summary of Key Findings

The use of plant tissue culture strategy along with progress in biotechnology has satisfied the question of availability and daily requirement for the burning demand of population and industrial commencement. The differential screening approach and application of plant genomics and genetic manipulation may help to a great extent in plant drug discovery. However, there are a number of critical factors which have to be considered in order to determine the commercialization of plant in vitro production systems such as market prices (both of the pure isolated compound and raw material used for extraction of the compound), economic feasibility of chemical synthesis, regulatory requirements, and, most importantly, consumer acceptance.

Table 14.2 Effect of biotic and abiotic elicitor on plant secondary metabolite production (Source: Mahendran et al. 2021)

Plant species	Elicitor	Product	Finding
Abiotic			
Chelidonium majus	Methyl jasmonate (MJ), salicylic acid (SA)	Chelidonine, sanguinarine	Expression of genes in the benzophenanthridine alkaloid biosynthetic pathway stimulated
Ocimum basilicum	Copper oxide (CuO)	Rosmarinic acid, chicoric acid, eugenol	Biosynthesis of the secondary metabolite stimulated
Ocimum basilicum	Salicylic acid (SA) + light regimes	Rosmarinic acid, chicoric acid, cyanidin, peonidin	Increase in content of phenolic compounds and flavonoids and also antioxidant activity
Coelogyne ovalis	Salicylic acid (SA)	Flavonoids, anthocyanins, phenolic compounds	Stimulated chalcone synthase expression and secondary metabolite production
Papaver orientale	Methyl jasmonate (MJ), salicylic acid (SA)	Thebaine, morphine, codeine	Expression of morphinan biosynthetic genes significantly upregulated, enhanced thebaine, morphine, and codeine biosynthesis
Crocus sativus	Ultrasonic waves	Safranal, crocin	Acts as stimulus on the production of secondary metabolites in suspension cultures
Gymnema sylvestre	Sodium nitroprusside (SNP)	Deacylgymnemic acid, gymnemagenin, gymnemic acid XVII	Increase in content of gymnemic acids in cell suspension cultures of *G. sylvestre*
Momordica charantia	Silver nanoparticles (AgNPs)	Hydroxybenzoic, hydroxycinnamic	Significant increase in bioactive compounds as well as pharmacological activities
Biotic			
Corylus avellana	*Chaetomium globosum*	Paclitaxel	44% increase in extracellular portion of paclitaxel
Bletilla striata	*Byssochlamys spectabilis*	Total phenolic content	Increased total of phenolic compounds
Panax ginseng	*Aspergillus Niger*	Ginsenosides	Enhanced accumulation of nitric oxide (NO), SA, and JA Significantly upregulated gene expression of terpenoid biosynthesis
Panax ginseng	*Alternaria panax*	Ginsenosides	Regulating ginsenoside synthesis
Trichosanthes cucumerina	Chitosan	Bryonolic acid	Higher levels of bryonolic acid in callus and suspension cultures than the ones in natural roots
Psammosilenet unicoides	Chitosan	Quillaic acid, gypsogenin, gypsogenin 3-O-?-D-glucuronopyranoside	Promotes triterpenoid saponin biosynthesis by enhancing antioxidant activities and differential gene expression

(continued)

Table 14.2 (continued)

Plant species	Elicitor	Product	Finding
Iberis Amara	Chitosan	Total phenol, flavonoid, flavonol, anthocyanin	Promotes phenolic compound biosynthesis
Plumbago zeylanica	Chitosan and yeast extract	Plumbagin	A 12-fold increase of plumbagin content

14.7.2 Significance of Genetic Manipulation in Medicinal Plant Research

Plant secondary metabolites are the byproducts released by plants for their survival and fitness and possess diverse properties which has led to its contribution in 25% of the world's pharmaceutical drugs used today (Bindu et al. 2018). The commonly used analgesic aspirin is derived from *Salix* and *Spiraea* species, the anticancer drug paclitaxel from *Taxus* species, and vinblastine from *Catharanthus* species. Eleven percent of the 252 drugs in the essential medicine listed by the World Health Organization (WHO) are solely of plant origin. Medicinal plants are high-value crops that can be cultivated in a small scale, providing resource base for traditional and herbal medicine as well as livelihood. However, despite its abundant availability in nature, destruction of natural habitats in order to meet the required quantity has posed a big threat to the environment. On the other hand, the synthesis of alternative chemicals may be possible, but the impact of chemicals on the environment will be too huge a price to pay. Therefore, breeding via genetic manipulation is preferred for mass production of chemically active metabolites and helps in overcoming environmental and agronomic problems. Genetic engineering combined with tissue culture has opened a new avenue for the production of plants with desired traits such as disease and pest resistance, increased nutritional value crops, increased level of desired pharmaceutical and nutraceutical compounds, and reduced unwanted compounds in food and fodder (Bindu et al. 2018). In order to genetically manipulate a plant, it is vital to know the biosynthetic pathway, genes encoding regulatory factors, and biosynthetic enzymes involved in the secondary metabolite production. The implementation of omics and high-throughput sequencing technology can be used for genome-wide association studies and for identifying genes that are responsible for a specific trait.

Wang et al. (2016) isolated a novel gene, MsYaBBY5, and functionally characterized it as a gene that is expressed in peltate glandular trichomes (PGT), which is a specialized structure for secondary metabolite production in spearmint (*Mentha spicata*). This gene was overexpressed in transgenic spearmint using RNA interference which resulted in the reduction in terpene level, and on silencing it, terpene production increased. Similarly, in maize *R* and *C1* are regulatory genes that interact in the biosynthesis of anthocyanins in the kernel. Upon expression in unpigmented maize kernels, biosynthesis and accumulation of anthocyanins were observed (Bindu et al. 2018). Some transcription factors (TFs) that regulate the expression of plant secondary metabolite are provided in Table 14.3.

Table 14.3 Transcription factors controlling plant secondary metabolite (Source: Gantet and Memelink 2002)

Transcription factor (TF)	DNA-binding domain	Metabolite
C1	MYB^{C} (*Arabidopsis thaliana*)	Anthocyanins
P		Phlobaphenes
TT2		Condensed tannins
PAP1		Anthocyanins
AtMYB4		Sinapate esters
CrBPF1		Alkaloids
R	bHLH	Anthocyanins
TT8		Condensed tannins
CrMYC2		Alkaloids
ORCA2	AP2/ERF (*C. roseus*)	Alkaloids
ORCA3		Alkaloids
CrGBF1	bZIP (*C. roseus*)	Alkaloids
CrGBF2		Alkaloids

Metabolic engineering is a dynamic technique developed which can interfere with the biosynthetic pathway yielding in increased secondary metabolite production by employing recombinant DNA technology in manipulating cellular functions: enzymatic, regulatory, and transport. An example is the *Rhizobium rhizogenes*-mediated transformation that transfers any foreign gene of interest that is placed in a binary vector into the plant genome. Increased secondary metabolites obtained from transgenic plants are shown in Table 14.4.

Transgenic plants not only offer new opportunities for the production of specific bioactive compounds through biosynthetic pathway manipulation but also greatly improve protein yield through recombinant protein expression system. CRISPR gene editing technology can improve the yield of medicinal plant by (1) overexpressing or knocking out genes involved in photosynthesis and (2) regulating the gene expression of functional key gene controlling the growth of a specific plant part, thus increasing yield (Guo et al. 2022).

14.7.3 Future Directions for Research

The primary issue that can affect the efficacy and safety of the herbal products is quality, which can range from very high to very low because of various intrinsic, extrinsic, and regulatory factors. Species differences, organ specificities, and diurnal and seasonal variations are the contributing factors that can intrinsically affect the qualitative and quantitative properties of chemical constituents actively present in the source medicinal plants. Other environmental factors such as collection methods, harvest, post-harvest transport, cultivation, storage, manufacturing practices, inadvertent contamination, substitution, and intentional adulteration are the main

Table 14.4 Increased secondary metabolites obtained from transgenic plants (Source: Kowalczyk et al. 2022)

Plant	Metabolite	Vector/construct	Culture type
Nicotiana tabacum L./Solanaceae	Triterpenoid saponins (dammarenediol-II, protopanaxadiol)	*Panax ginseng* dammarenediol-II synthase (PgDDS) and cytochrome P450 716A47 (CYP716A47)	Suspension culture
Leonurus sibiricus L./Lamiaceae	Phenolic acids	Anthocyanin pigment 1 (AtPAP1) transcription factor from *Arabidopsis thaliana*/ pCAMBIA1305.1-AtPAP1 vector	Roots
Senna obtusifolia (L.) H.S. Irwin & Barneby/Fabaceae	Pentacyclic triterpene (betulinic acid)	*Panax ginseng* squalene synthase 1 gene (PgSS1)/ pGFPGUSPlus-PgSS1 vector	Roots
Nicotiana tabacum L. cv. Petit Havana SR1/Solanaceae	Terpenoid indole alkaloid (geraniol)	A plastid-targeted geraniol synthase gene originally isolated from *Valeriana officinalis* L. (VoGES)/ pBIN2.4VoGES1 vector under the control of 35S promoter	Hairy roots
Centauries maritimum (L.) Fitch/Gentianaceae	Secoiridoid glycosides (swertiamarin (SM), gentiopicrin (GP), and sweroside (SW))	Plasmid with GUS construct integrated into TL region of pRiA4 plasmid/GUS construct contains uidA sequence under the 70S promoter (enhancer-doubled 35S CaMV promoter), followed by NOS polyadenylation sequence	Hairy roots
Curcuma zedoaria L./Zingiberaceae (Christm.) roscoe (rhizoma)	Sesquiterpenes (β-elemene)	3-Hydroxy-3-methylglutaryl coenzyme A reductase (HMGR), farnesyl-diphosphate synthase (FDS), and germacrene A synthetase (GAS), as well as terpene synthase (ST02C) driven by CaMV35S promoter, were separately introduced into *Agrobacterium* GV3101	Cell suspensions
Vinca minor L./ Apocynaceae	Monomeric eburnamine-type indole alkaloid vincamine	Tryptophan decarboxylase (TDC) and strictosidine synthase (STR) genes	Cell suspensions

external factors that affect the quality of herbal medicinal products. The focus, therefore, should be in producing medicines that have obtained scientific proof for quality, efficacy, and safety with active collaboration between pharmaceutical companies and researchers working on natural products. Another major emphasis to

focus on is the lack of clinical data which is unavailable in a majority of herbal products that are in the market along with adverse combinatory effects that can present when administered with conventional medicines (Pandey and Gupta 2015).

Over the past decades, researchers have discovered the vast reservoir of molecular diversity in herbal products that is a gateway to the development of novel drugs. However, the isolation and characterization of the active components from herbal extracts is time-consuming which is a major disadvantage during scale-up. There is, therefore, a need for systematic search of novel drugs which can be achieved by standardizing extraction and in vitro testing methods. Advances in biotechnology and genetic improvement, particularly in the area of tissue culture, have satisfied the cloud of doubt regarding the availability and ability to meet the daily requirement of the growing population at industrial level. An insight into the biosynthetic pathways of medicinally important compounds in plants is yet to be explored which is necessary to unravel information at molecular and cellular levels for upscaling at the industrial level (Patel et al. 2022). The rapid progress in biotechnology tools such as CRISPR gene editing technologies, sequencing technologies, transformation and regeneration technologies, and many other related techniques will continue to revolutionize medicinal plant biotechnology in the near future.

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Chapter 15
Traditional Herbal Medicines, Bioactive Compounds, and Plant Products as Therapeutic Approach Against Interstitial Lung Disease

Lovely Sinha and Saurabh Karmakar

Abstract Interstitial lung disease (ILD) encompasses a diverse range of respiratory disorders characterized by lung tissue inflammation and fibrosis. Its development can be influenced by multiple factors, and currently, there is no known cure for this condition. Despite advancements in medical treatments, ILD poses a significant health challenge with limited therapeutic options and varying outcomes. While synthetic drugs targeting specific pathways involved in ILD development are available to alleviate symptoms and slow disease progression, their usage has been associated with various side effects. It has been widely recognized for decades that medicinal plants and their compounds have been utilized worldwide in natural remedies for treating various diseases. This review article focuses on exploring the effects of different traditional herbal medicines, plant extracts, and their bioactive compounds. Specifically, it examines traditional Chinese herbal prescriptions, individual Chinese herbs containing bioactive compounds, and additional plant extracts that demonstrate prophylactic and therapeutic properties against ILD. These natural alternatives hold potential as substitutes for synthetic drugs and may help mitigate the side effects associated with their use in ILD treatment.

Keywords Interstitial lung disease (ILD) · Traditional herbal medicine · Plant extract · Bioactive compound

15.1 Background

Traditional medicines across the globe have incorporated the use of medicinal plants and their bioactive compounds due to their beneficial properties. This integration has occurred in response to the limitations of modern medicine in effectively treating chronic diseases and addressing the emergence of multidrug-resistant bacteria and

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parasites. Additionally, treating diseases with medicinal plant extracts is known for its cost-effectiveness and has fewer side effects. Research plays a crucial role in understanding the biological activity of plants and their compounds and their impact on health. Many plants are utilized in traditional medicine worldwide to treat various diseases, including lung diseases. Examples include *Papaver somniferum* for coughs and cramps, *Lobelia* spp. for asthma, *Ephedra* spp. for respiratory alignments, and *Coffea arabica* as a stimulant (Van Wyk and Wink 2004). Various plants, oils, and compounds are also employed in the treatment of various lung diseases (Van Wyk and Wink 2004).

The lungs are a vital and complex system of the human body responsible for gaseous exchange. Being constantly exposed to airborne chemical toxicants and infectious agents, they are highly susceptible to environmental insults. According to the Global Burden of Disease (GBD) study in 2019, respiratory diseases ranked third among the most common causes of death (Abbafati et al. 2020). Lung diseases can manifest with various symptoms depending on the affected part of the system. Interstitial lung diseases (ILDs) specifically involve the lung interstitium, which encompasses the supporting tissue surrounding the terminal airways and alveoli. ILDs are characterized by interstitial inflammation, cell growth, fibrosis, or a combination of these symptoms within the alveolar wall. They are classified as chronic fibrotic lung diseases, with an average survival duration of 3–5 years following diagnosis (Harari et al. 2020). ILDs are influenced by genetic and environmental factors and have been associated with various variables such as exposure to toxins and infectious agents, medications, hepatitis C virus, chronic obstructive lung disease, neoplastic disease, immunologic conditions, and a history of pneumonia or tuberculosis (Choi et al. 2018; Thierry et al. 2017). ILDs can be categorized into three subcategories: exposure-related ILD, systemic disease-related ILD, and ILD of unknown cause. In approximately 20–25% of patients, pulmonary fibrosis can occur, resulting in permanent lung dysfunction. Idiopathic pulmonary fibrosis (IPF), an interstitial lung disease (ILD), is known for its aggressive and progressive characteristics. It is characterized by an abnormal fibroproliferative response to multiple alveolar injuries and has a 5-year survival rate of only 30%.

Evidence from various sources suggests the involvement of oxidative stress in fibrotic lung disease. Additionally, depletion of glutathione, a prominent low-molecular-weight antioxidant, has been observed in the epithelial lining fluid (ELF) of patients with IPF, sarcoidosis, and hypersensitivity pneumonitis (HP). Numerous endogenous and exogenous factors associated with the development of pulmonary fibrosis can lead to an increase in reactive oxygen species (ROS) levels. Extrinsic sources of ROS include cigarette smoke, asbestos, silica, and bleomycin, all recognized as risk factors for pulmonary fibrosis. Endogenous ROS sources involve phagocytic cells that produce superoxide and hydrogen peroxide, as well as intra- and extracellular enzymatic systems such as members of the NADPH oxidase (NOX) family, the mitochondrial electron transport chain, and extracellular lysyl oxidase activity in collagen cross-linking (Schamberger et al. 2016).

Various disorders can arise during the wound healing process of damaged or scarred lung tissue, characterized by fibroblast differentiation, infiltration of

inflammatory cells, extracellular matrix remodelling, and collagen deposition (Higashiyama et al. 2007). The extracellular matrix (ECM) which primarily consists of collagen plays an important role in providing strength and integrity to the tissues. Amino acids like glycine, proline, and lysine play crucial roles in the formation of the ECM. Additionally, non-elastin components and proteoglycans, such as glycosaminoglycans, help to maintain the matrix's resilience, while enzymes and glycoproteins aid in tissue cohesiveness (Hay 1991). Epithelial-mesenchymal transition (EMT) is a contributing factor to lung tissue thickening, leading to impaired function and worsening fibrosis, ultimately resulting in shortness of breath. In addition to IPF, there are various associated conditions, including pulmonary hypertension, gastroesophageal reflux, coronary artery disease, malignancy, telomeropathy, pleuroparenchymal fibroelastosis, and hypoxemia (George et al. 2019). IPF increases the risk of developing lung cancer due to mutations in the p53 tumor suppressor gene, which plays a role in DNA repair, apoptosis, cell proliferation, and differentiation (Kuwano et al. 1996). Unfortunately, the treatment of IPF remains unsatisfactory, with limited therapeutic options available. In critical cases, lung transplantation is often the only curative measure (Selman et al. 2004).

Despite receiving treatment, lung function progressively declines as the disease advances, ultimately leading to respiratory failure and death. The median survival time from time of diagnosis is typically only 2–3 years (Xaubet et al. 2017).

Current treatment approaches for IPF include the use of glucocorticoids, immunosuppressive or cytotoxic medications, tyrosine kinase inhibitors, and antifibrotic drugs. However, clinical studies have revealed the potential inefficacy or risk associated with several of these medications (Behr 2012; Noth et al. 2012). Only pirfenidone and nintedanib have demonstrated effectiveness as disease-modifying therapies for IPF (King et al. 2014; Richeldi et al. 2014). Lung transplantation, in conjunction with pharmaceutical therapy, can prolong survival and improve the quality of life for IPF patients, with a 5-year survival rate exceeding 50% (Richeldi et al. 2014). However, due to the high cost and limited availability of donor organs, only a small number of patients meet the eligibility criteria for transplantation. In summary, there is an urgent need to discover novel therapies with demonstrated efficacy and minimal side effects for interstitial lung diseases. This book chapter aims to provide a comprehensive overview of herbal medicine and its bioactive compounds used in the treatment of interstitial lung diseases (ILDs). Additionally, it highlights the future direction and potential role of herbal medicine as a viable treatment option.

15.2 Interstitial Lung Diseases (ILDs)

Interstitial lung diseases (ILDs) are alternatively referred to as diffuse parenchymal lung diseases (DPLDs). They encompass a diverse range of disorders that are classified together due to similar clinical, radiographic, physiological, or pathological characteristics (Travis et al. 2013). The clinical manifestations of these diseases

stem from inflammatory infiltration of the interstitium and capillary endothelium. Under normal circumstances, the interstitium houses a small number of interstitial macrophages, fibroblasts, and myofibroblasts. Additionally, the interstitium contains matrix proteins such as collagen-related macromolecules, fibronectin, and laminin. The primary mechanism underlying the entire pathological process involves injury to the alveolar interstitium and capillary endothelium, leading to alveolar permeability and fibroblastic proliferation. This injury can result directly from the initial insult, the inflammatory cell response releasing pro-inflammatory and profibrotic cytokines, or the regenerative and reparative process occurring at the epithelial and endothelial surfaces. Consequently, repeated insults contribute to excessive collagen deposition and interstitial fibrosis, with or without involvement of the capillary endothelium. Interstitial lung diseases encompass numerous causes that damage the lung parenchyma and present similar physiological, clinical, and radiographic features. Some develop in conjunction with other conditions such as connective tissue disease (CTD) or drug use, while others are idiopathic. ILD is a chronic progressive disease that encompasses more than 200 disorders. It significantly impacts morbidity and mortality rates and represents a crucial health concern. Various risk factors are associated with ILDs, including genetic factors, autoimmune diseases, occupational exposures, environmental factors, certain medications, radiation, and chemotherapy. Additional known causes involve exposure to specific allergens or dust, prolonged occupational or environmental history, and occupations such as farming, bird fancier or breeder, and poultry work. The frequency and incidence of ILDs vary widely between nations due to various factors such as age, gender, ethnicity, and smoking habits. Over the past few decades, the diseases have gained clinical significance due to advancements in diagnostic capabilities. The prevalence of ILDs is higher in males compared to females (80.9 vs. 67.2 per 100,000). Likewise, the overall incidence of ILD is higher in males than in females (31.5 vs. 26.1 per 100,000 per year). Among all deaths, the prevalence of undiagnosed or preclinical ILDs was 1.8% (Coultas et al. 1994). The most common ILD reported in India, according to the Indian ILD registry, is hypersensitivity pneumonitis (47.3%), followed by CTD-ILD (13.9%) and idiopathic pulmonary fibrosis (IPF) (13.7%) (Singh et al. 2017).

15.2.1 Classification of ILDs

ILDs can be classified histologically, radiologically, or clinically. But clinical classification is used widely as given below and briefly described.

15.2.1.1 Idiopathic Interstitial Pneumonia

Idiopathic interstitial pneumonia (IIP) is a pulmonary disease characterized by identifiable histological features and an unknown etiology, primarily affecting the

lung interstitium. IIP encompasses various subtypes, including idiopathic pulmonary fibrosis, idiopathic non-specific interstitial pneumonia, respiratory bronchiolitis-interstitial lung disease (ILD), cryptogenic organizing pneumonia, desquamative interstitial pneumonia, acute interstitial pneumonia, rare idiopathic interstitial pneumonias, idiopathic lymphoid interstitial pneumonia, and unclassifiable idiopathic interstitial pneumonias. This classification system is based on the 2013 update of the American Thoracic Society/European Respiratory Society (ATS/ERS) classification, which builds upon the 2002 ATS/ERS classification (Travis et al. 2013).

15.2.1.2 Autoimmune Interstitial Lung Disease

Autoimmune interstitial lung disease (ILD) is a specific type of ILD that arises from autoimmune disorders, where the immune system mistakenly attacks the body. Examples of such autoimmune disorders include lupus, rheumatoid arthritis (RA), sarcoidosis, and scleroderma. Autoimmune ILD can be further classified into various subtypes, including rheumatoid arthritis ILD, interstitial pneumonia with autoimmune features, Sjogren's syndrome ILD, systemic lupus erythematosus ILD, polymyositis and dermatomyositis ILD, Mixed connective tissue disease (MCTD) ILD, systemic sclerosis ILD, and other connective tissue disease ILD.

15.2.1.3 Hypersensitivity Pneumonitis

Hypersensitivity pneumonitis is a condition that can develop as a result of exposure to various substances, including fungi, such as aspergillus, as well as moisture and bird feathers. Common symptoms observed during physical examinations include flu-like symptoms like fever, chronic cough, and dyspnea (shortness of breath).

15.2.1.4 Sarcoidosis

Sarcoidosis, also referred to as Besnier-Boeck-Schaumann disease, is a condition characterized by the formation of clusters of inflammatory cells known as granulomas. These granulomas can develop in various parts of the body such as the lungs, skin, or lymph nodes, as the disease progresses.

15.2.1.5 Other Interstitial Lung Diseases

The category of other interstitial lung diseases encompasses conditions such as lymphangioleiomyomatosis, Langerhans cell histiocytosis, drug-associated ILD, ILD associated with occupational exposures, vasculitis/granulomatosis interstitial lung diseases, and other less common interstitial lung diseases (Kekevian et al. 2014).

15.2.2 Pathogenesis

Interstitial lung diseases (ILDs) can be caused by several factors, including dust, allergens, autoimmune diseases, drugs, and prolonged occupational or environmental exposures of farmers, poultry workers, bird fanciers, and bird breeders. These factors initiate a series of pathological changes in the lung interstitium, resulting in pro-inflammatory and profibrotic cytokines. The regenerative and abnormal repair mechanism also contributes to injury and fibrosis, leading to defects in gaseous exchange and deranged lung functions (Bagnato and Harari 2015). Inflammation plays a significant but not essential role in lung remodelling and fibrosis since inflammation does not always lead to fibrosis. Primary and secondary ILDs have different pathogenesis. In the case of ILDs with no identifiable factors, cellular infiltration by alveolar macrophages, lymphocytes, neutrophils, plasma cells, and eosinophils results in pathological changes. Persistent and repetitive injury and aberrant repair mechanisms result in recruitment of fibroblasts and other inflammatory cells, ultimately leading to fibrosis. In ILDs with secondary causes, the pathology is mostly inflammatory in origin. Exposure to an agent (either intrinsic or extrinsic) causes activation of the immune inflammatory response, recurrent inflammation, granuloma formation, and fibrosis.

The pathogenesis of idiopathic pulmonary fibrosis (IPF) has been extensively studied, and besides the inflammatory theory, various hypotheses have been proposed:

1. Vascular hypothesis: Increased angiogenesis due to an imbalance between proangiogenic and anti-angiogenic chemokines.
2. Abnormalities of alveolar type II epithelial cell injury and repair mechanisms.
3. Matrix remodelling: Increased production of extracellular matrix molecules such as collagens, proteoglycans, tenascins, and glycosaminoglycans, among others.
4. Fibroblast activation and dysfunction.

15.2.3 Symptoms

A detailed medical history, along with the presentation of dyspnea, can help suggest a diagnosis of interstitial lung disease and provide information about its specific form or cause. The time course of symptom development can aid in differentiating between various types of diseases. Some interstitial lung disease, such as organizing pneumonia or acute interstitial pneumonia, may have a rapid onset, while others, like idiopathic pulmonary fibrosis, typically have a more gradual progression with breathlessness persisting for over a year. Age also plays a role, as certain interstitial lung diseases, such as hypersensitivity pneumonitis and connective tissue disease-associated interstitial lung disease, tend to be more prevalent in individuals under 50 years of age. Sarcoidosis commonly presents in individuals aged 25–40 years,

although it can occur in older individuals as well. Idiopathic pulmonary fibrosis is typically diagnosed in patients in their 60s or 70s (Morimoto et al. 2008).

Cough is a common symptom in interstitial lung disease and is often dry, possibly aggravated by reflux, which is implicated as a potential causative factor in the disease. Patients may experience general malaise and constitutional upset, particularly those with underlying connective tissue disease, vasculitis, or certain cases of sarcoidosis. Pertinent questions regarding connective tissue diseases may involve symptoms of Raynaud's phenomenon, sicca symptoms (dry eyes and difficulty swallowing), sclerodactyly, and arthralgia. Hemoptysis is uncommon and may suggest complications such as infection, pulmonary embolism, malignancy, or pulmonary vasculitis. Patients with known connective tissue disease and worsening respiratory symptoms should be referred to a respiratory specialist. Lung disease can also manifest de novo in patients without previous clinical features of connective tissue disease, and this possibility should be actively investigated, especially in patients showing an usual interstitial pneumonia morphology on computed tomography. Joint symptoms may develop later. Smoking is associated not only with idiopathic pulmonary fibrosis but also with respiratory bronchiolitis-interstitial lung disease, as well as rarer interstitial lung diseases like Langerhans cell histiocytosis and desquamative interstitial pneumonitis. Smoking may also contribute to coexisting emphysema. Retrospective trials suggest a potential link between gastroesophageal reflux disease and fibrosis pathogenesis (Lee et al. 2011). Patients should be specifically asked about symptoms related to gastroesophageal reflux disease, including nonacid reflux, which can potentially cause or exacerbate interstitial lung disease through recurrent episodes of aspiration.

15.2.4 Mechanism of Disease

The precise mechanism underlying the initiation of pathogenic processes in interstitial lung diseases (ILDs) is not fully understood. Approximately half of all ILDs have an unknown cause, and the exact mechanisms triggering lung injury and fibrosis remain unidentified (Delmonico et al. 2009). While the underlying causes are often unidentifiable, certain ILDs have been associated with occupational or environment exposures, as well as prolonged courses of certain medications (Delmonico et al. 2007; Morley et al. 2001). ILDs frequently exhibit varying degrees of inflammation, fibrosis, and abnormalities in the connective tissue structure (Leslie 2009). Exposure to causative agents in the respiratory tract can lead to lung inflammation, followed by scarring and fibrosis. Lung fibrosis can arise from a variety of factors, including both short- and long-term injuries or preexisting diseases. While some forms of fibrosis may be partially reversible, the majority are irreversible and can be fatal. The activation of fibroblasts and the accumulation of extracellular matrix (ECM) are believed to contribute to the initiation of fibrosis. The exact origin and activation mechanism of fibroblasts, however, remain poorly understood. One possible scenario is that when a drug, disease, or other causative factor affects the

lungs, it causes epithelial damage and inflammation. These inflammatory processes then generate signals that activate resident fibroblasts, leading to the thickening of the interstitium and the development of mild to chronic lung disease symptoms (Delmonico et al. 2007).

A pictorial representation of the mechanism is given in Fig. 15.1.

15.2.5 Conventional and Current Treatment and Management of ILD

15.2.5.1 Idiopathic Pulmonary Fibrosis

Current international guidelines recommend the use of either pirfenidone or nintedanib as treatment options for patients with IPF. Both medications have demonstrated the ability to slow down the decline of forced vital capacity (FVC) by approximately 50% after 1 year of therapy. Additionally, these drugs have shown improvements in other important outcomes. Pirfenidone reduces the incidence of respiratory-related hospitalizations, while nintedanib decreases the frequency of acute exacerbations. A pooled analysis suggests that both medications may also contribute to reduced mortality. In the past, prior to the availability of antifibrotic medications, the recommended therapeutic approach for IPF involved a combination of azathioprine (AZA), prednisone, and N-acetylcysteine (NAC). However, the PANTHER-IPF trial revealed that patients receiving this drug combination were at a higher risk of death and hospitalization compared to those receiving placebo. A secondary data analysis of the trial demonstrated that a significant proportion of patients (62%) had telomere lengths below the tenth percentile. Furthermore, in patients treated with combination of AZA, prednisone, and NAC, having a short telomere length was associated with an increased risk of adverse outcomes, including death, lung transplantation, hospitalization, or decline in lung function. These findings raise questions regarding the safety of using immunosuppressive therapy in patients with other types of ILDs that share clinical, genetic, and biological characteristics with IPF.

15.2.5.2 Rheumatoid Arthritis-Associated Interstitial Lung Disease

Immunosuppressive therapy is considered a crucial component in the management of patients with RA-ILD. Most of the evidence guiding the treatment of CTD-ILD and RA-ILD comes from studies conducted on populations of patients with scleroderma-related ILD (Scl-ILD). Prospective randomized clinical trials have demonstrated the safety and efficacy of immunosuppressive therapies, such as cyclophosphamide and mycophenolate mofetil (MMF), in Scl-ILD. However, the improvement in lung function associated with these medications remains modest. Retrospective studies have also shown stabilization or improvement in lung function

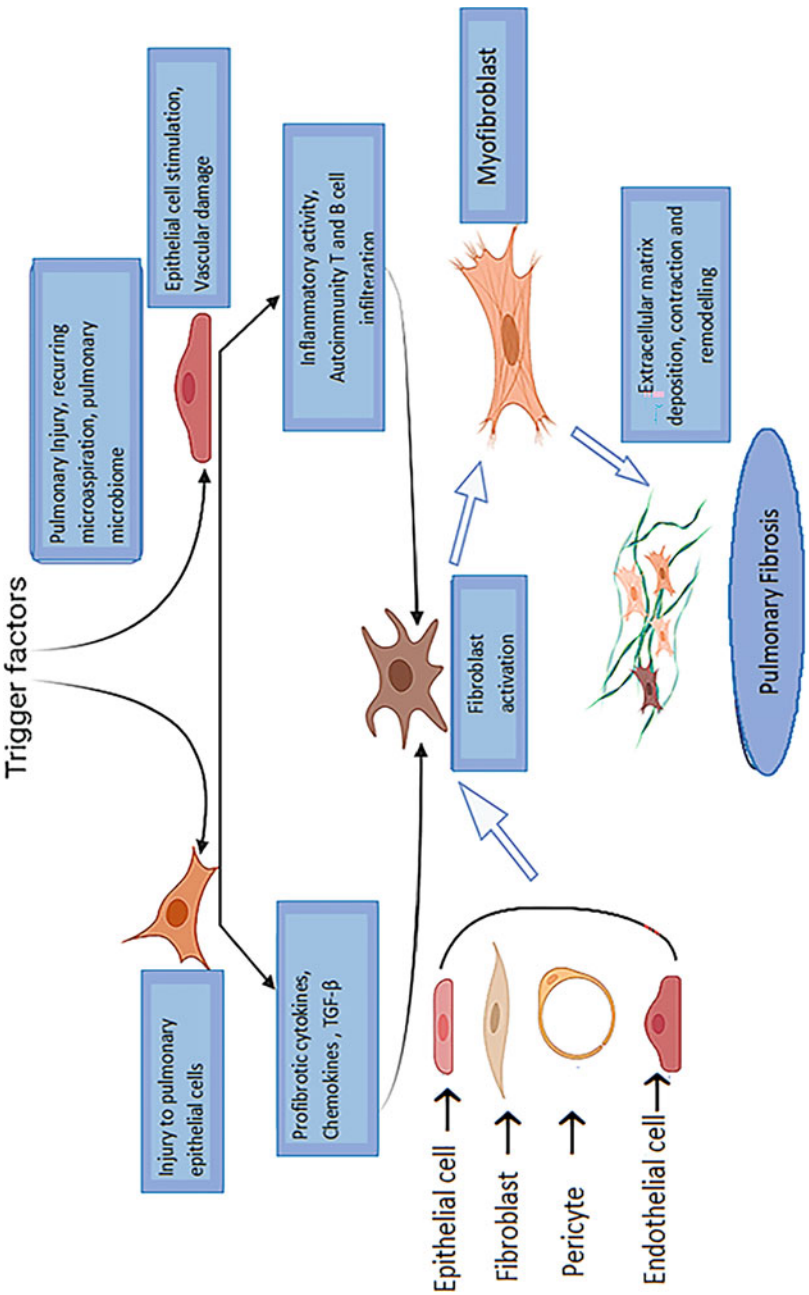

Fig. 15.1 Pictorial representation of pulmonary fibrosis in interstitial lung disease

and tolerability in patients with various types of CTD-ILD who received immunosuppressive medications. Despite these favorable results, many patients with RA-ILD still experience disease progression, which significantly impacts life expectancy. The optimal medical management of these patients remains unknown, highlighting the need for new therapeutic agents and a more comprehensive treatment algorithm. A recent phase 2 open-label study demonstrated the tolerability of pirfenidone in patients with Scl-ILD, paving the way for further research on the use and efficacy of antifibrotics in patients with RA-ILD and other CTD-ILD.

15.2.5.3 Chronic Hypersensitivity Pneumonitis

The mainstay of medical management for chronic hypersensitivity pneumonitis involves identifying and eliminating the casual antigen; however, some patients experience persistent symptoms or disease progression despite antigen remediation and may require additional therapy. Unfortunately, there is a lack of prospective studies evaluating different therapeutic approaches for hypersensitivity pneumonitis. In case of acute hypersensitivity pneumonitis, corticosteroids have been found to alleviate symptoms, although their long-term efficacy does not surpass that of antigen eradication alone. Managing chronic hypersensitivity pneumonitis possesses even greater challenges. Immunosuppressive agents like MMF and AZA may provide benefit and aid in stabilizing lung function, but further prospective studies are necessary to validate their effectiveness in patients with chronic hypersensitivity pneumonitis (Morisset and Lee 2019).

15.3 Role of Herbal Medicine with Their Bioactive Compound in the Treatment of ILD

Patients who have progressed to a malignant stage of ILD often find that lung transplantation is the only viable treatment option that significantly improves life expectancy by reducing the risk of death by 75%. Therefore, research on pulmonary fibrosis (PF) focuses on two primary objectives: gaining a better understanding of the underlying mechanisms leading to PF and improving its treatment. Notably, improving patients' survival remains a challenging objective due to the disease's heterogeneity, complexity, and the limited effectiveness of current treatments, which can be attributed to the incomplete understanding of PF's causes and development. Numerous tests have been conducted using pharmacological substances as well as plant extracts, with the latter showing promise as a potential therapeutic alternative due to their rich content of bioactive substances.

Plant extracts are derived from natural products found in plants and contain various bioactive compounds. Some of these compounds have demonstrated antifibrotic properties, suggesting their potential as candidates for pulmonary fibrosis

treatment. Moreover, plant extracts and bioactive compounds offer advantages over conventional drugs. They possess anti-inflammatory, antioxidant, and antifibrotic properties, making them of growing interest for the treatment of pulmonary fibrosis. These natural products are generally considered safe, exhibit fewer side effects, and are more cost-effective compared to conventional drugs. Additionally, they can be easily extracted from plants and standardized to ensure consistent quality and efficacy.

Nevertheless, further studies are required to fully comprehend the mechanisms of action and determine the optimal dosages and treatment durations of plant extracts and bioactive compounds. Despite this, natural products provide a promising alternative to conventional drugs for the treatment of pulmonary fibrosis. Several traditional herbal medicines, plant extracts, bioactive compounds, and plant products have been investigated for their potential in treating interstitial lung disease, as previously discussed.

15.3.1 Traditional Herbal Medicine Formulations

Many traditional herbal medicines have been used for centuries to treat various respiratory disorders, including ILD. In recent years, there has been an increasing interest in the use of these herbs for the treatment of ILD. Studies have shown that some of these herbs have anti-inflammatory and antioxidant properties that can help reduce inflammation and oxidative stress in the lungs.

Traditional herbal medicines have been used for centuries in different cultures for the treatment of various diseases, including lung disorders. Some of the traditional Chinese herbal medicines used as prescription for the treatment of ILD include:

15.3.1.1 *Danggui Buxue Tang*

Danggui Buxue Tang is a traditional Chinese herbal formula that combines two herbs, namely, the root of *Astragalus membranaceus* and the root of *Angelica sinensis*. According to pharmacological studies, this herbal formula has been found to possess various benefits such as promoting hematopoietic functions, enhancing cardiovascular circulation, preventing osteoporosis, boosting antioxidant activity, and stimulating the immune system. In a recent research study conducted on rats, the administration of total glucosides of *Danggui Buxue Tang* demonstrated a reduction in weight loss induced by Belomycin (BLM) and a decrease in the lung index [lung index = lung weight/body weight × 100%]. Histological evidence also supported the potential of *Danggui Buxue Tang* to alleviate BLM-induced lung fibrosis and consolidation. Furthermore, it exhibited a dose-dependent decrease in the activity of TNF-α and TGF-β1, as well as a reduction in the expression of type I collagen in lung tissues (Hosseini et al. 2018; Bahri et al. 2017; Wang et al. 2021a, b).

15.3.1.2 *Yin Chiao San*

Yin Chiao San (YCS) is a traditional Chinese medicinal prescription that is commonly used in clinical practice for patients with lung diseases. The prescription consists of ten crude drugs, which include the Fructus of *Forsythia suspensa* (Oleaceae), the Flos of *Lonicerae japonicae* (Caprifoliaceae), the Herba of *Lophatherum gracile Brongn.* (Gramineae), the Radix of *Platycodon grandiflorum* (Campanulaceae), the Herba of *Menthae Haplocalycis* (Labiatae), the Fructus of *Arctium lappa* L. (Compositae), the Radix of *Glycyrrhiza uralensis Fisch.* (Leguminosae), the Semen of *Glycine max (L.) Merr.* (Leguminosae), the Herba of *Schizonepeta tenuifolia Briq.* (Labiatae), and the Rhizoma of *Phragmites communis Trin.* (Gramineae). In an experimental study conducted on rats, the administration of *Yin Chiao San* at a dosage of 1000 mg/kg BW demonstrated inhibitory effects on collagen formation; reduced lung index, levels of MDA, hydroxyproline, and TNF-α; and exhibited antioxidant and anti-inflammatory activities when compared to the BLM-induced PF group. The study investigated the effects of *Yin Chiao San* on BLM-induced pulmonary injury (Hosseini et al. 2018; Bahri et al. 2017; Yen et al. 2007).

15.3.1.3 *Feitai*

Feitai is a combination formula composed of various herbs, including the Radix of *Scutellaria baicalensis Georgi* (Labiatae), Radix of *Glehnia littoralis Fr. Schmidt ex Miq.* (Umbelliferae), Fructus of *Trichosanthes kirilowii Maxim.* (Cucurbitaceae), Radix of *Stemona sessilifolia (Miq.) Miq.* (Stemonaceae), and Folium of *Eriobotrya japonica (Thunb.) Lindl.* (Rosaceae). This composite formula has been used in China as a traditional remedy for treating patients with pulmonary tuberculosis and has been reported to have a protective effect against lung fibrosis in rats. The main ingredient in Feitai is Radix of *Scutellaria baicalensis Georgi* (Labiatae), which contains beneficial components called flavonoids. Flavonoids exhibit various biological activities, including anti-inflammatory, antibacterial, antiviral, anti-allergic, immune-stimulating, and antioxidant properties. The other ingredients in Feitai also possess therapeutic effects such as anti-inflammatory, antibacterial, antitussive, and antipyretic properties. It is hypothesized that this formula may exert some effects on lung fibrosis. Consequently, a comparative study was designed to evaluate the effects of Feitai on pulmonary fibrosis induced by BLM in rats, comparing it with the traditional anti-inflammatory drug dexamethasone. Additionally, the study aimed to investigate whether Feitai could suppress collagen production and proliferation in the WI-38 human lung fibroblast cell line (Bahri et al. 2017; Gong et al. 2005).

15.3.1.4 *Qizhukangxian* Granules (QG)

A pilot clinical trial was conducted to evaluate the effects of *Qizhukangxian granules* (QG), a traditional Chinese medicine (TCM), on patients with idiopathic pulmonary fibrosis (IPF). The composition of the granules includes Huangqi (Radix *Astragali Mongolici*), Ezhu (Rhizoma *Curcuma phaeocaulis*), Danggui (Radix *Angelica sinensis*), Shanzhuyu (*Fructus Macrocarpii*), Ziwan (Radix *Asteris Tatarici*), Huangqin (Radix *Scutellaria baicalensis*), Zhebeimu (Bulbus *Fritillaria thunbergii*), and Gancao (Radix *Glycyrrhizae*). The trial results indicated that QG administration could be an effective treatment option for IPF, as it contributed to a delay in pulmonary function decline and alleviation of IPF symptoms compared to the control group. This improvement in symptoms led to an enhanced quality of life for the patients. Moreover, QG administration during a 48-week therapy term demonstrated a lower incidence rate of acute exacerbations without any noticeable adverse effects. These findings suggest that QG has the potential to be considered as a treatment option for IPF, effectively ameliorating pulmonary function, improving the quality of life, and reducing the occurrence of acute exacerbations (Sijia et al. 2020; Murthy et al. 2022).

15.3.1.5 *BuqiHuoxueTongluo* Formula

A TCM regime known as *BuqiHuoxueTongluo formula* (BHTF) has shown promise as a therapeutic agent for idiopathic pulmonary fibrosis (IPF). The composition of BHTF includes *Astragali* Radix (Huangqi, 30 g), *Lonicerae japonicae* Flos (Jinyinhua, 30 g), *Angelica sinensis* Radix (Danggui, 30 g), *Glycyrrhizae* Radix et Rhizoma (Gancao, 10 g), *Dioscoreae Nipponicae* Rhizoma (Chuanshanlong, 15 g), *Pyrrosiae Folium* (Shiwei, 15 g), *Fritillariae Thunbergii* Bulbus (Zhebeimu, 10 g), *Trichosanthis* Fructus (Gualou, 15 g), *Platycodonis* Radix (Jiegeng, 10 g), *Aurantii Fructus* (Zhiqiao, 10 g), and *Rhodiolae Crenulatae Radix* et Rhizoma (Hongjingtian, 10 g). Studies have indicated that BHTF exhibits anti-inflammatory effects in a bleomycin-induced IPF model, thereby alleviating fibrosis progression. In the pulmonary mesenchyme, BHTF has been found to reduce inflammatory cell infiltration, collagen deposition, and fibrosis. Moreover, BHTF suppresses the expression of TGF-β1 and α-SMA, which are key factors involved in the onset of IPF. Additionally, BHTF has shown potential in preserving the function of type II alveolar epithelial cells during IPF, as it influences pulmonary surfactant secretion (Murthy et al. 2022; Yu et al. 2018).

15.3.1.6 *Danlou* Prescription

The *Danlou prescription* (DLP) is a formulation derived from the traditional Chinese medicine formula *Gualou Xiebai Decoction*. It consists of ten herbs, namely,

Trichosanthes kirilowii Maxim. (Gualoupi), *Allium macrostemon Bunge*, *Puerariae lobatae Radix*, *Salvia miltiorrhiza Bunge*, *Astragalus mongholicus Bunge*, *Davallia trichomanoides Blume*, *Paeonia lactiflora Pall*, *Alisma plantago-aquatica L.*, *Ligusticum chuanxiong Hort*., *and Curcuma aromatica Salisb*. Modern research has shown that DLP possesses a wide range of pharmacological effects, including anti-myocardial ischemia, anti-inflammatory, antioxidant, and improvement of blood lipid metabolism. In a study by Shao et al., it was found that DLP could alleviate bleomycin (BLM)-induced idiopathic pulmonary fibrosis (IPF) by inhibiting TGF signaling, which is involved in myofibroblast differentiation and α-SMA expression. DLP also demonstrated the ability to regulate genes associated with endocytosis, alveolar macrophage activation, myofibroblast differentiation, and collagen secretion. Thus, DLP has the potential to simultaneously inhibit pro-inflammatory and profibrotic pathways, making it a promising treatment option for IPF (Murthy et al. 2022; Zhang et al. 2021; Shao et al. 2019).

15.3.1.7 *Yangqing Kangxian* Formula

The *Yangqing Kangxian Formula* (YKF) contains several main ingredients, including *Ophiopogon japonicus*, *Adenophorae Radix*, *Panax quinquefolius Radix*, *Trichosanthes kirilowii Maxim*., *Fritillaria thunbergii Bulbus*, *and Radix Paeoniae Rubra*. In a study by Li et al. (2017), it was observed that bleomycin (BLM)-induced lung injury in rats resulted in the infiltration of inflammatory cells, collagen deposition, and elevated levels of hydroxyproline (HYP). The administration of YKF was found to regulate the release of certain inflammatory factors, including a decrease in TNF-α and IFN-γ levels in the lungs of BLM-induced rats.

15.3.1.8 *Shenmai Kaifei San*

Shenmai Kaifei San (Shenks) is a traditional Chinese medicine (TCM) preparation known for its "tonifying qi and yin" properties. The ingredients of this formula include *Panax quinquefolius L.*, *Ophiopogon japonicus (L. f.) Ker-Gawl*., *Salvia miltiorrhiza Bunge*, *Gynostemma pentaphyllum (Thumb.) Makino*, *Perilla frutescens*, *Amygdalus communis Vas*, *Scutellaria baicalensis Georgi*, *Desmodium styracifolium (Osbeck) Merr*., and *Perilla frutescens (L.) Britt*. In a study by Chu et al. (2017), it was observed that Shenks could reduce the phosphorylation level of Smad3 and inhibit the activity of TGF-β signaling by suppressing Smad-binding element activity.

15.3.1.9 *Yupingfeng* (YTE) Extract

Yupingfeng extract, derived from a Chinese plant, and its *Yupingfeng* powder have been found to be effective in preventing respiratory tract diseases, including viral

infections and chronic bronchitis. When administered at a dose of 12 mg/kg BW, it has shown to decrease the levels of hydroxyproline and collagen I, as well as reduce the overexpression of TGF-β1 and α-SMA. Previous studies have demonstrated that the total glucosides from Yupingfeng exhibit anti-inflammatory and immunoregulatory activities (Bahri et al. 2017; Wang et al. 2021a, b).

15.3.1.10 *Gancao Ganjiang* Decoction (GGD)

Gancao Ganjiang decoction (GGD) is a Chinese medicinal formula originally used for the treatment of "atrophic lung disease" (Liu Miao et al. 2018). It is composed of two herbs, *Glycyrrhiza uralensis Fisch.* and *Zingiber officinale Roscoe*, both of which are listed in the *Pharmacopoeia of the People's Republic of China* (2020 Edition). *Glycyrrhiza uralensis Fisch.* is a widely used plant in Russian traditional medicine, known for its expectorant and emollient properties in the treatment of coughs, bronchitis, whooping cough, and asthma. Pharmacological studies have shown that GGD can improve pulmonary fibrosis induced by BLM in rats, leading to an improvement in forced vital capacity and protection of the alveolar structure in non-fibrotic areas. Additionally, GGD has been found to attenuate BLM-induced pulmonary fibrosis in rats by enhancing antioxidant defense and modulating SIRT1 and TGF-β1. Phytochemical analysis of the herbs used in GGD has identified various bioactive compounds, including flavonoids and alkaloids (Murthy et al. 2022; Wang et al. 2021a, b).

15.3.1.11 *Yiqi Huayu Hutan* Decoction

In a study conducted by Tian et al. (2019), it was found that the *Yiqi Huayu Hutan decoction* reduced the expression of the TGF-β/Snail pathway in mice with BLM-induced pulmonary fibrosis. The treatment group, particularly those receiving the "moderate concentration" decoction, showed a decrease in the protein levels of TGF-β, Snail-1, and fibronectin. These findings suggest that the *Yiqi Huayu Hutan decoction* has the potential to be a promising therapeutic agent for IPF, a form of interstitial lung disease characterized by pulmonary fibrosis (Murthy et al. 2022).

15.3.2 Single Traditional Chinese Herbal Drug

Some of the single traditional Chinese herbal drugs used to treat pulmonary fibrosis in interstitial lung disease are described below.

15.3.2.1 *Centella asiatica*

Centella asiatica, commonly known as Asiatic pennywort, is a perennial herb belonging to the Apiaceae (Umbelliferae) family. It is widely used in oriental traditional medicine and is distributed in warm and humid areas around the world, including China, America, Australia, Malaysia, and South Africa. This herb contains triterpenes, with up to 8% biosynthesized in its composition. One of the triterpenoids extracted from *Centella asiatica* is Asiatic acid (AA), which possesses various protective properties against inflammation, oxidation, and fibrosis. Studies have reported that *Centella asiatica* herb, administered at different doses (10, 20, or 40 mg/kg BW), can prevent the deposition of extracellular matrix, attenuate inflammation and oxidative stress, and reduce TGF-β1 overexpression in rats treated with bleomycin (BLM). Previous research has demonstrated the inhibitory effect of Asiatic acid on the progression of bleomycin-induced pulmonary fibrosis (PF) and its potential therapeutic role in treating scleroderma and idiopathic pulmonary fibrosis, with minimal side effects (Bahri et al. 2017; Xia et al. 2018; He et al. 2022; Dong et al. 2017).

15.3.2.2 *Allium sativum*

Garlic, *Allium sativum L.*, a member of the Alliaceae family, has been used as a traditional medicine for centuries, and scientific studies have shown that it can prevent thrombosis and inhibit inflammation and cellular oxidative stress. It contains many bioactive compounds with medicinal value. S-Allyl cysteine (SAC), a constituent of aged garlic extract, is stable and odorless with a low toxicity. It inhibits the release of cytokines/chemokines, inducing neutrophil recruitment and activation of key transcription factors including nuclear factor-κB (NF-κB) and activator protein-1, thereby augmenting inflammatory responses and tissue damage. It was also shown that S-allyl cysteine can play an antifibrotic role by attenuating myofibroblast differentiation through TGF-β1-mediated fibroproliferative processes (Bahri et al. 2017; Mizuguchi et al. 2006; Tsukioka et al. 2017).

15.3.2.3 *Rheum undulatum*

Rheum undulatum, a species belonging to the *Rheum* genus of the Polygonaceae family, is a Chinese medicinal herb. One of the constituents found in the root of this herb is rhapontin (3,3′,5-trihydroxy-4′-methoxystilbene-3-O-glucoside). Rhapontin has demonstrated various potentially beneficial effects, including anti-allergic, antidiabetic, and anti-inflammatory activities, as well as protective effects against acute colitis in mice. Additionally, studies have reported the antifibrotic effect of rhapontin on pulmonary fibrosis. Rhapontin has been shown to reverse extracellular matrix deposition in primary lung fibroblast cells and prevent lung fibrosis induced

by bleomycin (BLM) in mice by modulating AMPK activation and suppressing the TGF-β/Smad pathway (Bahri et al. 2017; Tao et al. 2017).

15.3.2.4 *Carthamus tinctorius*

Carthamus tinctorius L., commonly known as safflower, has been extensively used in Chinese medicine for the treatment of gynecological diseases and coronary heart disease. Safflower refers to the dried flowers of *Carthamus tinctorius*. Safflor yellow (SY) is the active ingredient present in the aqueous extract of safflower and has been used in the treatment of cardiovascular diseases. Hydroxysafflor yellow A (HSYA) is the main active ingredient in SY. Previous studies have demonstrated that HSYA can alleviate lung inflammatory response induced by lipopolysaccharide (LPS) or bleomycin in mice and attenuate the development of lung fibrosis induced by bleomycin in rats. HSYA has been found to alleviate the increase of TGF-β1, CTGF, α-SMA, and collagen I mRNA levels induced by bleomycin, inhibit the increase of α-SMA expression and Smad3 phosphorylation, and prevent morphological changes in lung tissue (Bahri et al. 2017; Jin et al. 2016).

15.3.2.5 *Garcinia hanburyi* Hook.f

Garcinia hanburyi Hook.f. is a plant species native to Southeast Asia that produces dry resin known as gamboge. The main active ingredient in gamboge is gambogic acid (GA). Research studies have demonstrated various beneficial effects of GA, including antitumor cell proliferation, anti-inflammatory, antibacterial, and neuroprotective effects. GA is considered a pure natural Chinese herbal medicine with the advantages of low toxicity, low residue, and a lower likelihood of developing drug resistance.

Qu et al. (2016) conducted a study and reported that GA can regulate the rate of VASH-/VASH-2 and inhibit TGF-β1 and $CoCl_2$-induced proliferation of HLF-1 cells in vitro. They observed a reduction in PDGF and FGF-2 levels. Based on these findings, GA shows promise as a new multitarget drug for the treatment of IPF, both in the early stages and during fibrosis progression (Bahri et al. 2017; Murthy et al. 2022; Zhang et al. 2021).

15.3.2.6 *Salvia miltiorrhiza* Bunge

Salvia miltiorrhiza, commonly known as Danshen, is a therapeutic traditional Chinese herbal medicine. It is a perennial erect herb belonging to the genus *Salvia* in the family Labiatae and is predominantly found in temperate to tropical regions. *Salvia miltiorrhiza* contains both fat-soluble and water-soluble compounds, with high levels of tanshinone IIA (TS-IIA) and salvianolic acid B.

Tanshinone IIA (TS-IIA) is a lipophilic diterpene extracted from the root of *Salvia miltiorrhiza Bunge*. Previous studies have demonstrated its potential in attenuating pulmonary edema, inflammation, and fibrosis. It has shown the ability to reduce the levels of TNF-α, IL-1β, IL-6, cyclooxygenase-2, prostaglandin E2, and MDA in experimental models of pulmonary fibrosis induced by BLM (He et al. 2015).

Salvianolic acid B (SAB) is another compound obtained from the roots and rhizomes of *Salvia miltiorrhiza Bunge*. It has been found to possess antioxidant properties and protective functions against brain damage, particularly in the cardiovascular system. SAB has shown inhibitory effects on cell infiltration, alveolar structure destruction, collagen deposition, and myofibroblast differentiation. It also regulates Smad-dependent and Smad-independent mitogen-activated protein kinases (MAPKs) signaling pathways and provides cellular protection against oxidative stress (Bahri et al. 2017; Murthy et al. 2022; Zhang et al. 2021; Li et al. 2022).

15.3.2.7 *Paeonia lactiflora*

Paeonia lactiflora Pall root, a renowned traditional Chinese medicine (TCM), has a history of more than 1200 years of use due to its anti-inflammatory and immune-regulatory properties. One of its main active components is paeoniflorin, which has been shown to possess therapeutic effects in attenuating pulmonary fibrosis. Paeoniflorin inhibits the activation of the TGF-β/Smad pathway, leading to a suppression of type I collagen synthesis. Additionally, it enhances the expression of IFN-γ (Bahri et al. 2017; Ji et al. 2013).

15.3.2.8 *Tripterygium wilfordii*

Tripterygium wilfordii Hook. F (TwHF) is a significant herb in traditional Chinese medicine with immunomodulatory, anti-inflammatory, and anti-allergic effects. It is commonly used for treating rheumatoid arthritis in China.

In China, *Tripterygium wilfordii Hook. F* (TwHF), also known as "Lei Gong Teng," is a traditional herbal medicine widely employed for the treatment of autoimmune diseases such as rheumatoid arthritis, systemic lupus erythematosus, and SSc. TwHF is typically taken orally at a dosage of 40–60 mg/day. It may cause side effects such as digestive symptoms (nausea, poor appetite) and reproductive side effects (menstrual disorders). TwHF has demonstrated anti-inflammatory and immunoregulatory effects and is considered cost-effective. Over 300 compounds have been identified from TwHF extracts, with triptolide, tripdiolide, and triptomide being the most active diterpenoids. These compounds have been investigated as potential treatments for autoimmune diseases and malignancies. A randomized clinical trial has shown that TwHF monotherapy is comparable to methotrexate in treating active rheumatoid arthritis, but data on its effect in SSc-ILD (systemic sclerosis-related interstitial lung disease) are lacking.

Triptolide (TPL), derived from *Tripterygium wilfordii Hook. F*, is another promising compound with anti-IPF properties. It exhibits anti-inflammatory and immunosuppressive effects. Recent studies have indicated that TPL inhibits epithelial-mesenchymal transition (EMT) of lung epithelial cells by directly binding to TGF-β, an EMT-inducing factor. This binding affects the activity of Smad3, E-cadherin, and vimentin, which are markers of EMT initiation. In vivo studies have also demonstrated the inhibitory effect of TPL on lung fibrosis in mice. Considering the close association between EMT and IPF, TPL shows potential as a therapeutic agent for IPF (Murthy et al. 2022; Li et al. 2021; Yang et al. 2020).

15.3.2.9 *Schisandrae chinensis fructus*

Schisandrae chinensis fructus (Wuweizi, Schisandra) is a widely used traditional Chinese medicine (TCM) specifically for lung-associated diseases like pulmonary fibrosis and bronchitis. A study conducted on a BLM-induced model demonstrated the protective effects of Schisandra in two phases: firstly, by improving lung structures and reducing inflammatory cell infiltration and, secondly, by reducing biomarkers associated with the M2 macrophage subtype. Previous studies have indicated that M2 macrophages contribute to fibrosis, suggesting that targeting M2 macrophages could be a potential therapeutic strategy for IPF. In vitro tests also confirmed that Schisandra decreased the M2 ratio, indicating suppression of M2 polarization (Murthy et al. 2022; Guo et al. 2020).

15.3.2.10 *Citrus* Extract

Citrus is a genus of flowering trees and shrubs from the Rutaceae family, known for their high content of vitamin C and flavonoids. Citrus extracts have been reported to exhibit various activities, including anti-inflammatory, neuroprotective, anti-amnesic, and antioxidative effects. Zhou et al. (2009) demonstrated that administration of citrus extracts at 100 and 200 mg/kg BW inhibited fibroblast proliferation in human embryonic lung, reduced TGF-β1 and hydroxyproline levels, and improved the fibrosis score in a rat model of pulmonary fibrosis induced by BLM. Additionally, an alkaline extract of *Citrus reticulata* administered at 32 mg/kg BW increased MMP-9 expression and inhibited TNF-α and TIMP-1 expressions in a rat model of BLM-induced pulmonary fibrosis. These findings suggest that citrus extract holds promise as a treatment for pulmonary fibrosis.

Furthermore, another Chinese herbal medicine used for lung-associated diseases is citrus alkaline extract (CAE), derived from the peel of *Citrus reticulata*. The therapeutic effect of CAE against IPF was investigated in a study using BLM-induced pulmonary fibrosis mice. The results indicated that CAE reduced collagen synthesis, cross-linking, and deposition, thereby alleviating BLM-induced pulmonary fibrosis. This effect was attributed to the downregulation of the TGF-β1/

Smad3 pathway. These findings suggest that CAE may serve as a potential therapeutic agent for IPF (Bahri et al. 2017; Murthy et al. 2022).

15.3.3 Plant Extract and Its Bioactive Compounds

Bioactive compounds are naturally occurring compounds found in plants that have therapeutic properties. Many of these compounds have been shown to have anti-inflammatory, antioxidant, and antifibrotic properties that can help reduce inflammation, oxidative stress, and fibrosis in the lungs. Bioactive compounds derived from plants, such as flavonoids, polyphenols, and terpenoids, have been studied for their therapeutic potential in ILD.

Some of the bioactive compounds that have been studied for their therapeutic potential in ILD include:

15.3.3.1 Quercetin

Quercetin, a flavonoid found in fruits and vegetables, has been shown to have anti-inflammatory and antioxidant properties. It has been shown to reduce lung inflammation and fibrosis in animal models of ILD. It can reduce the severity of lung fibrosis in animal models of IPF. Indeed, it is known that this flavonoid is capable of reducing LPS-induced levels of various pro-inflammatory cytokines including TNF-α and IL-8, two cytokines known to be elevated in sarcoidosis (Boots et al. 2011).

15.3.3.2 Resveratrol

Resveratrol, a polyphenol present in red grapes and berries, possesses anti-inflammatory and antioxidant properties. Its effectiveness in reducing lung inflammation and fibrosis has been demonstrated in animal models of ILD. Moreover, resveratrol has been shown to decrease the levels of malondialdehyde (MDA) in lung tissue and serum, indicating its ability to mitigate oxidative stress. Additionally, resveratrol treatment has been found to reverse the elevation in total cell count and neutrophil numbers in bronchoalveolar lavage fluid (BALF). In a separate study, resveratrol treatment was shown to normalize various biochemical markers affected by BLM, including a significant restoration of lung glutathione (GSH) levels and a notable reduction in myeloperoxidase (MPO) activity (Huo et al. 2023).

15.3.3.3 Epigallocatechin Gallate (EGCG)

EGCG is a catechin with anti-inflammatory and antioxidant properties. It has been shown to reduce lung inflammation and fibrosis in animal models of ILD (Bahri et al. 2017).

15.3.3.4 Curcumin

Curcumin is a polyphenolic compound found in turmeric, which has anti-inflammatory and antioxidant properties. It has been shown to reduce lung inflammation and fibrosis in animal models of ILD. It can reduce the production of pro-inflammatory cytokines and fibrotic markers in ILD (Bahri et al. 2017).

15.3.3.5 Matrine

Matrine, which is the principal active component of the alkaloids found in *Sophora flavescens*, possesses significant anti-liver fibrosis properties and has been widely used in the treatment of chronic hepatitis. Studies have shown a correlation between pulmonary fibrosis and the JAK-STAT signaling transduction pathways. Matrine has demonstrated the ability to alleviate alveolitis and pulmonary fibrosis severity and inhibit the proliferation of pulmonary fibrocytes by regulating the abnormal expression of JAK, STAT1, and STAT3. As a result, matrine has exhibited a remarkable therapeutic effect in the context of pulmonary fibrosis induced by BLM (Hosseini et al. 2018).

15.3.4 Plant Products

In addition to traditional herbal medicines and bioactive compounds, plant products have also been studied for their potential use in the treatment of ILD. Some examples of plant products that have been studied include honey, propolis, and royal jelly. Honey has been shown to have anti-inflammatory and antioxidant properties and has been studied for its potential use in the treatment of ILD. Propolis, a resinous substance produced by honeybees, has been shown to have anti-inflammatory and antioxidant properties and has been studied for its potential use in the treatment of ILD. Royal jelly, a substance produced by worker bees, has been shown to have anti-inflammatory and antioxidant properties and has been studied for its potential use in the treatment of ILD.

Several plant products have been identified as potential treatments for ILD. N-Acetylcysteine (NAC), a derivative of the amino acid cysteine, has been shown to have antioxidant properties and has been studied for its potential use in the

treatment of ILD. Omega-3 fatty acids, found in fish and fish oil supplements, have been shown to have anti-inflammatory properties and have been studied for their potential use in the treatment of ILD.

Plant products are products derived from plants that have been shown to have therapeutic potential in the treatment of ILD. Some of the plant products that have been studied for their therapeutic potential in ILD include:

15.3.4.1 Polyphenols and Flavonoids

They have demonstrated the ability to reduce lung inflammation and fibrosis in animal models of interstitial lung disease (ILD). These natural phenolic compounds have garnered increasing interest for their potential therapeutic applications in various conditions. Research has unveiled the antifibrotic and anti-inflammatory effects of polyphenols, highlighting their beneficial effects in pulmonary fibrosis (PF). For instance, hedysari radix, a Chinese herb rich in flavonoids, which are plant pigments, has been found to inhibit certain processes involved in PF. Polyphenols exhibit antioxidant activities by mitigating various processes, including the translocation of nuclear factor kappa Bp65 (NF-kBp65), downregulation of cyclooxygenase-2 (cox-2), and transforming growth factor-beta 1 (TGF-β1).

15.3.4.2 Carotenoids

Carotenoids are pigments found in plants with antioxidant properties. They have been shown to reduce lung inflammation and fibrosis in animal models of ILD.

15.3.4.3 Omega-3 Fatty Acids

Omega-3 fatty acids are polyunsaturated fatty acids found in fish oil and other sources. They have anti-inflammatory properties and have been shown to reduce lung inflammation in animal models of ILD.

15.3.4.4 Glycosides

Glycosides are natural compounds commonly found in plants and possess diverse therapeutic applications. They can be classified as alcohol, phenol, or sulfur compounds and are characterized by the presence of sugar molecules linked by specific bonds to one or more non-sugar components. Glucose is the most frequently encountered sugar in glycosides. Glycosides exhibit various biological activities, including anti-inflammatory effects. Recent research has demonstrated the impact of glycosides on pulmonary fibrosis (PF). For instance, fenugreek seed extract, which contains glycosides, has been shown to possess antifibrotic properties. This effect is

achieved through the upregulation of Nrf2, a transcription factor that subsequently downregulates the expression of interleukin-1 beta (IL-1β), interleukin-6 (IL-6), interleukin-8 (IL-8), and tumor necrosis factor-alpha (TNF-α). Additionally, fenugreek seed extract inhibits the expression of collagen I, transforming growth factor-beta (TGF-β), nuclear factor kappa B (NF-kB), vascular endothelial growth factor (VEGF), and Smad3 in a rat model of lung fibrosis induced by bleomycin (BLM).

15.3.4.5 Terpenoids

Terpenoids are a highly diverse group of natural compounds that find wide-ranging applications. They play a significant role in the defense mechanisms of plants. Terpenoids can be classified into different subclasses, including monoterpenes, sesquiterpenes, diterpenes, and triterpenes. Krishna et al. conducted a pioneering study on the therapeutic potential of plant triterpenes in the treatment of pulmonary fibrosis (PF). They specifically investigated the antifibrotic effect of PG-490-88, a water-soluble derivative of triptolide, in a mouse model of PF induced by bleomycin (BML). Subsequent studies have further explored the antifibrotic effects of terpenoid. These effects include the reduction of inflammatory cytokines and transforming growth factor-beta 1 (TGF-β1), as well as the inhibition of collagen deposition and other components of the extracellular matrix (ECM). Terpenoids have also been found to inhibit the Smad2/3/TGF-β1 signaling pathway (Hosseini et al. 2021).

15.3.4.6 Alkaloids

Alkaloids are a subclass of phytochemicals that are naturally present in various plants. The term "alkaloid" generally refers to basic substances, often with a cyclic structure containing one or more nitrogen atoms. These compounds are typically water-soluble in their protonated form under low pH conditions, while they exist in a lipophilic neutral form at high pH. This characteristic renders them advantageous as agents that can pass through membranes due to their solubility in water. The therapeutic effects of alkaloids in pulmonary fibrosis (PF) were initially investigated by Xiao et al. (2010), who explored the seed embryo of *Nelumbo nucifera* Gaertn. and identified a bisbenzylisoquinoline alkaloid called isoliensinine. This alkaloid demonstrated the ability to reduce elevated levels of hydroxyproline, malondialdehyde (MDA), tumor necrosis factor-alpha (TNFa), and transforming growth factor-beta (TGF β) in mouse models of lung fibrosis induced by BLM. Additionally, isoliensinine was found to increase the levels of superoxide dismutase (SOD), an antioxidant enzyme (Dudala et al. 2021; Hosseini et al. 2021).

Some of the single traditional Chinese herbal drugs, plant extracts and their bioactive compounds, and plant products are summarized in Table 15.1.

Table 15.1 Traditional herbal medicines, plant extracts with bioactive compounds, and plant products for interstitial lung disease treatment

S. no.	Plant extract	Main component/plant products/bioactive compound	Dose	Model	Role/target	References
1.	*Silybum marianum* (milk thistle)	Flavonolignan (silymarin)	100 mg/kg	Bleomycin-induced BALB/c mice	Induced GST and GSH and reduced MPO, TNF-α, IL-6, and MDA	Dudala et al. (2021)
2.	*Oxalis corniculate*	C-Glycosyl flavonoids (isoorientin, isovitexin, and swertisin)	200 mg/kg body weight	CCl4-induced female Sprague-Dawley rats	Rise in CAT, POD, GSH, GPx, and GST levels. No occurrence of edema and fibrosis	Dudala et al. (2021)
3.	*Tinospora cordifolia (Giloy)*	Isoquinoline alkaloids (berberine, palmatine, magnoflorine, and jatrorrhizine)	200 mg/kg body weight	Bleomycin-induced male Wistar albino rat	Inhibited NF-κB and iNOS and reduced TNF-α and TGF-β1	Kulkarni et al. (2022) and Dudala et al. (2021)
4.	*Phyllanthus emblica (amla)*	Polyphenolics, flavonoids (gallic acid, rutin, caffeic acid, kaempferol, pyrogallol)	200, 400 mg/kg	CCl4-induced Sprague-Dawley male rats	Rise in CAT, POD, GSH, GPx, and GST levels; disrupted alveoli; macrophages infiltration was reversed to normal	Tahir et al. (2016) and Dudala et al. (2021)
5.	*Eriobotrya japonica*	Triterpene acids (oleanolic acid, a-hydroxyoleanolic acid, arjunic acid, euscaphic acid, ursolic acid)	Triterpene acids (450 mg/kg)	Bleomycin-induced male Sprague-Dawley rats	Reduction of TNF-α and TGF-β1	Dudala et al. (2021), Wang et al. (2021a, b) and Yang et al. (2012)
6.	*Nelumbo nucifera Gaertn. (seed embryo)*	Isoliensinine (bisbenzylisoquinoline)	40 mg/kg BW	Murine model induced by BLM	Decrease the high level of hydroxyproline, MDA, TNF-α, and TGF-β and increase SOD level	Dudala et al. (2021) and Zhao et al. (2010)

7.	*Arenaria kansuensis*	β-Carboline alkaloids	100–150 mg/kg	Bleomycin-induced mice	TNF-α, TGF-β1 and IL-1β decreased; survival rate increased; macrophages decreased	Dudala et al. (2021) and Cui et al. (2021)
8.	*Mahonia aquifolium (stem bark)*	Isoquinoline alkaloids (berberine, berbamine, palmatine, magnoflorine, and jatrorrhizine)	200 mg/kg/day	Bleomycin-induced male Wistar rats	MPO, MDA, TGF-β1, NF-κB, and TNF-α are decreased; SOD, CAT, GPx, GSH, and vitamins A, C, and E are increased	Dudala et al. (2021)
9.	*Green tea extract (Camellia sinensis)*	Epigallocatechin gallate, caffeine	150 mg/kg BW	Murine model	Antioxidative, anti-androgen, and immuno-modulatory activities, decrease the high level of TGF-β, IL-1β, and histamine	Dudala et al. (2021) and Bahri et al. (2017)
10.	*Citrus extract*	Vitamin C, flavonoids	100 & 200 mg/kg BW	Rat model induced by BLM	Anti-inflammatory, neuroprotective, anti-amnesic, and antioxidative activities, inhibit fibroblast proliferation in human embryonic lung, decrease TGF-β and hydroxyproline, and increase MMP-9 expression and TNF-α and TIMP-1 expression	Bahri et al. (2017) and Murthy et al. (2022)
11.	*Grape seed extract*	Polyphenols such as proanthocyanidins	150 mg/kg BW	Rat model induced by silica	Antifibrotic, antioxidant, anticarcinogenic, and anti-inflammatory activities, inhibiting MMP-9 and TGF-β1 expression in the lungs	Bahri et al. (2017) and Murthy et al. (2022)

(continued)

Table 15.1 (continued)

S. no.	Plant extract	Main component/plant products/bioactive compound	Dose	Model	Role/target	References
12.	*Rosmarinus officinalis*	Rosmarinic and carnosic acids, polyphenols, and flavonoids	75, 100, 200 mg/kg BW	Rat model induced by BLM	Antioxidant, anti-inflammatory, and antitumor activities, inhibit excessive collagen I deposition & the upregulation of TGF-β expression by interfering TGF-β Smad signaling pathway activation on type 1 procollagen target gene	Bahri et al. (2017)
13.	*Fenugreek seed extract*	Plant glycosides	20 and 40 mg/kg BW	BLM-induced rat model	Anti-inflammatory and antifibrotic, Nrf2 induction which in turn modulates inflammatory molecule level (TNF-α, IL-1β, IL-6, and IL-8) and inhibits fibrotic ones (TGF-β, collagen I NF-kB, VEGF, and Smad3	Bahri et al. (2017)
14.	*Danggui Buxue Tang*	Plant glycosides		BLM-induced rat model	Decrease α-SMA, TGF-β, hydroxyproline, and type I collagen levels, to reduce the decrease of SOD activity and the increase in MDA level, through the suppression of NOX4 expression	Hosseini et al. (2018), Bahri et al. (2017) and Wang et al. (2021a, b)

15.	*Tripterygium wilfordii Hook. F*	Triptolide (diterpene triepoxide)			Antiproliferative, immunosuppressive, and antitumor properties, reduce myofibroblasts proliferation and TGF-β levels	Murthy et al. (2022), Li et al. (2021) and Yang et al. (2020)
16.	*Centella asiatica*	Madecassoside (triterpenoid saponin)	10, 20, or 40 mg/kg BW	BLM-treated rats	To prevent extracellular matrix deposition and to attenuate inflammation, oxidative stress, and TGF-β1 overexpression	Bahri et al. (2017), Xia et al. (2018), He et al. (2022) and Dong et al. (2017)
17.	*Hedysari radix*	Flavonoids			Inhibit the process of pulmonary fibrosis	Bahri et al. (2017)
18.	*Punica granatum*	Dilactone	400, 15 mg/kg BW	Bleomycin-induced rats	Reduced inflammatory cell infiltration and reduced collagen deposition; hydroxyproline MPO, GSH, and NO production is attenuated	Dudala et al. (2021)
19.	*Sophora plant*	Matrine	25 mg/kg BW	BLM-treated rats	Antifibrotic effect, by inhibiting JAK-STAT pathway	Hosseini et al. (2018)
20.	*Actinidia chinensis planch seed oil*	Unsaturated fatty acid	120 and 180 mg/kg BW	BLM-induced rats	Antioxidant and antifibrotic effects; increased SOD, catalase, and GSH-Px levels; and decreased MDA and hydroxyproline levels via activation of Keap1/Nrf2 pathway	Bahri et al. (2017)

(continued)

Table 15.1 (continued)

S. no.	Plant extract	Main component/plant products/bioactive compound	Dose	Model	Role/target	References
21.	*Pistacia lentiscus oil*			BLM-induced	Antioxidant and anti-inflammatory properties, decrease TGF-β level	Dudala et al. (2021)
22.	*Acnistus arborescens, Withania obtusifolia*	Steroidal lactone	2 mg/kg BW	BLM-induced mice	Decreased vimentin expression, collagen increased beclin 1 and LC3B expression	Dudala et al. (2021)
23.	*Nigella sativa*	Enzymes, vitamins, aromatic oils	1 mL/kg/day	Bleomycin-induced male Wistar rats	TGF-β reduction; inflammatory score 1.2 ± 0.45; fibrosis 1.83 ± 0.68	Dudala et al. (2021)
24.	*Linum usitatissimum*	Fatty acids	2 mL/kg BW	Bleomycin-induced male Wistar rats	Fibrosis 3.0 ± 1.05; inflammatory score 1.9 ± 0.87; SOD and CAT levels are raised. Here the fatty acid content is increased in cells	Dudala et al. (2021)
25.	*Tanacetum parthenium*	Sesquiterpene lactone	50 mg/kg	BLM induced mice	Inhibit TGF-β1, NF-κB, snail, IL-4, TNF-α, collagen deposition, vimentin, α-SMA and rise in MMP, E-cadherin	Dudala et al. (2021)
26.	*Passiflora edulis*	Flavonoids	100 mg/kg BW	BLM induced mice	Hydroxyproline deposition is suppressed, MPO levels are reduced, and SOD levels are increased	Dudala et al. (2021)

27.	*Pistacia chinensis bark (PCEB)*	Gallic acid and the flavonoid compound rutin, ascorbic acid, catechin, kaempferol, and tannin		CCl4 induced rats	Antioxidant	Dudala et al. (2021)
28.	*Yupingfeng extract (Chinese plant extract)*	Polysaccharide	350 mg/kg BW		Antifibrotic and anti-inflammatory effect, decreased the levels of hydroxyproline and collagen I, and reduced the overexpression of TGF-β and α-SMA	Bahri et al. (2017)
29.	*Ecliptae herba*	Ecliptasaponin	2.5 and 1.25 g/kg BW	BLM induced rats	Reduce oxidative stress and lung inflammation, reduce epithelial-mesenchymal transition	Hosseini et al. (2018)
30.	*Panax notoginseng saponins*		200 mg/L		Can inhibit EMT induced by TGF-β and can increase extracellular matrix degradation	Hosseini et al. (2018)
31.	*Rikkunshito*		1000 mg/kg BW	BLM injected mice	Can restore plasma concentrations of ghrelin	Hosseini et al. (2018) and Hosseini et al. (2018)
32.	*Yin Chiao san, a kampo*		1000 mg/kg BW	BLM induced	Antioxidant and anti-inflammatory activities, inhibit collagen formation, reduce lung index, MDA, hydroxyproline, and TNF-α levels	Hosseini et al. (2018)
33.	*Fufang Biejafeng*			Rats' lung	Antifibrotic effect by decreasing hydroxyproline level	Hosseini et al. (2018)

(continued)

Table 15.1 (continued)

S. no.	Plant extract	Main component/plant products/bioactive compound	Dose	Model	Role/target	References
34.	*Houttuynia cordata*	Quercetin, quercitrin, hyperoside, and rutin	1 g/kg BW	BLM induced PF in rats	Anti-inflammatory and antioxidant effect, decrease the levels of hydroxyproline, IFN-γ, TNF-α, SOD, and MDA	Dudala et al. (2021) and Hosseini et al. (2018)
35.	*Carissa opaca fruit extract*		200 mg/kg BW	CCL4 induced lung fibrosis in rats	Antifibrotic and antioxidant effect	Hosseini et al. (2021)
36.	*Tetrastigma hemsleyanum*	Quercetin, kaempferol, and caffeic acid			Increase the level of IL-6, IL-17A, IL 35, TGF-β, FOXp3, Th17/Treg cells	Hosseini et al. (2021)
37.	*Ligustrazine extract*				R educing the content of hydroxyproline and increasing weight	Hosseini et al. (2021)
38.	*Turmeric*	Curcumin			Inhibited TGF-beta-induced myofibroblasts, inhibited MMP-9	Murthy et al. (2022) and Hosseini et al. (2018)
39.	*β-Peltoboykinolic acid Astilbe rubra Hook. f. et Thomas*				Inhibited EMT and extracellular matrix production, inhibited Smad/snail signaling pathway	Murthy et al. (2022)
40.	*PM014 Chung-Sang-Bo-Ha-Tang (root of Rehmannia glutinosa, c ortex of Paeonia suffruticosa, fruit of*				Inhibited TGF-β1 pathway, suppressed EMT and fibroblast activation by targeting Smad-dependent and p38 MAPK pathways,	Murthy et al. (2022)

	Schisandra chinensis, root of Asparagus cochinchinensis, seed of Prunus armeniaca, root of Scutellaria baicalensis, root of Stemona sessilifolia)				exerted anti-inflammatory effect	
41.	*Tripterygium wilfordii* Hook.F	PG-490-88 (triptolide)	0.25 mg/kg BW	BLM induced murine model	Antifibrotic effect	Hosseini et al. (2018) and Krishna et al. (2001)
42.	*Grape extract*	Resveratrol		BLM-treated mice	Anti-inflammatory and antioxidant activities and in prevention/treatment of pulmonary fibrosis, by downregulating cyclooxygenase-2, reducing NF-kBp65 translocation	Hosseini et al. (2018), Bahri et al. (2017) and Huo et al. (2023)
43.	*Camellia sinensis*	Quercetin		BLM-treated mice	Exhibited anti-inflammatory and antioxidant activities, decreased sphingosine kinase 1 (SphK1)/sphingosine 1-phosphate (S1P) signaling	Murthy et al. (2022) and Hosseini et al. (2021)
44.	*Grape leaves extract*	Dihydroquercetin	10 mg/kg BW	BLM-treated mice	Anti-inflammatory and antioxidant activities	Bahri et al. (2017)
45.	*Sorbus aucuparia*	Aucuparin			Decreased inflammatory gene expression, macrophage activation-related	Murthy et al. (2022)

(continued)

Table 15.1 (continued)

S. no.	Plant extract	Main component/plant products/bioactive compound	Dose	Model	Role/target	References
					markers, and profibrotic marker gene expression, increased antifibrotic marker genes, and suppressed inflammatory cytokines and collagen synthesis	
46.	*Green walnut husks of Juglans mandshurica*	Juglanin			Reduced neutrophil infiltration and lung vascular permeability and reduced the expression of fibrotic markers such as TGF-β1, fibronectin, matrix metalloproteinase (MMP)-9, α-SMA, collagen I, and stimulator of interferon gene (sting)	Murthy et al. (2022)
47.	*Dried roots of Mongolian astragalus, also known as Astragalus membranaceus*	Astragaloside IV			Reversed EMT process, inhibited TGF-β1/phosphatidylinositol-3-kinase (PI3K)/protein kinase B (Akt)-induced forkhead box class O (FOXO)3a hyperphosphorylation, reduced degenerative alterations of alveolar epithelial cells, and r educed the	Murthy et al. (2022)

					levels of collagen III, laminin, hyaluronic acid, and hydroxyproline in the lungs	
48.	*Roots and rhizomes of Salvia miltiorrhiza Bunge*	Salvianolic acid			Inhibited cell infiltration, alveolar structure destruction, collagen deposition, and myofibroblast differentiation, regulated Smad-dependent and Smad-independent mitogen-activated protein kinases (MAPKs) signaling pathways, and protected cells against oxidative stress	Murthy et al. (2022)
49.	*Rheum palmatum L. and Eucalyptus robusta Smith*	Gallic acid			Regulated c-Jun N-terminal kinase (JNK) signaling pathway, tumor suppressor gene p53, and apoptosis, suppressed inflammation via TGF-β1/Smad2 signaling pathway, and balanced NADPH oxidase-4 (NOX4)/Nrf-2 ratio	Murthy et al. (2022)
50.	*Black tea extract*				Decreased collagen deposition, α-SMA, and TGF-β and e levated the antifibrotic molecule, interferon-γ	Murthy et al. (2022)
51.	*Flavonoids of Oxytropis falcata Bunge (FOFB)*	Flavonoids			Suppressed TGF-β/Smad signaling pathway	Murthy et al. (2022)

(continued)

Table 15.1 (continued)

S. no.	Plant extract	Main component/plant products/bioactive compound	Dose	Model	Role/target	References
52.	*Myrtus communis L.*	Flavonoid			Inhibited inflammation, lowered hydroxyproline levels	Murthy et al. (2022)
53.	*Primary component of Reynoutria japonica Houtt.* and *Rheum palmatum L.*	Emodin			Alleviated pulmonary edema, diminished collagen deposition, prevented invasion of myofibroblasts and inflammatory cells, and lowered tumor necrosis factor (TNF)-α, interleukin (IL)-6, TGF-β1, and heat shock protein (HSP)-47 levels in lung tissues	Zhang et al. (2021) and Murthy et al. (2022)
54.	*Andrographis paniculata*	Andrographolide			Reduced oxidative stress and malondialdehyde levels, increased glutathione/oxidized glutathione ratio, ameliorated MMP-1/tissue inhibitors of MMP-1 ratio alterations	Yin et al. (2015)
55.	*Schisandrae chinensis fructus*	*Wuweizi, Schisandra*			Protected against inflammatory cell infiltration and lung damages and also reduced and suppressed M2 macrophages	Guo et al. (2020) and Murthy et al. (2022)
56.	*Danshen*	*Cryptotanshinone*			Reduced inflammation and Stat3 phosphorylation and inhibited epithelial-to-mesenchymal transition (EMT)	He et al. (2015), Li et al. (2022), Zhang et al. (2021), Bahri et al. (2017) and Murthy et al. (2022)

57.	*Paeonia lactiflora*	Paeoniflorin	50 and 100 mg/kg BW		Suppresses collagen I synthesis, inhibits activation of the TGF-β/Smad pathway, and increases IFN-γ expression	Bahri et al. (2017) and Ji et al. (2013)
58.	*Salvia miltiorrhiza Bunge*	Tanshinone II A, a diterpene	15 mg/kg BW		Attenuates pulmonary edema, inflammation, and fibrosis and decreases TNF-α, IL-1b, IL-6, cyclooxygenase-2, prostaglandin E2, and MDA levels in an experimental model of pulmonary fibrosis induced by BLM	Bahri et al. (2017)
59.	*Extract of Garcinia hanburyi Hook.F., an Asian medicinal plant*	Gambogic acid	0.5 mg/kg and 1 mg/kg in vivo		Reduces the level of PDGF, fibroblast growth factor FGF-2, and human lung fibroblast proliferation. Prevent lung fibrosis by modulating VASH-2/VASH-1 and suppressing the TGF-β1/Smad3 pathway in BLM-induced pulmonary fibrosis model in rats	Bahri et al. (2017)
60.	*Carthamus tinctorius* L.	Flavonoids, hydroxysafflor yellow A	60 mg/kg BW	Bleomycin-induced male C57BL/6 mice	Alleviates BLM-induced increase of TGF-β1, CTGF, α-SMA, and collagen I mRNA levels and inhibits the increase of α-SMA expression, Smad3 phosphorylation, and the	Bahri et al. (2017)

(continued)

Table 15.1 (continued)

S. no.	Plant extract	Main component/plant products/bioactive compound	Dose	Model	Role/target	References
					morphological changes in lung tissue	
61.	*Paeonia suffruticosa*	Paeonol, the main phenolic compound	10 mg/kg BW		Paeonol has antioxidant, anti-inflammatory, and antifibrotic functions against bleomycin-induced pulmonary fibrosis in mice. These effects are mediated by the inhibition of the MAPKs/Smad3 signaling pathway	Bahri et al. (2017)
62.	*Rheum undulatum*	Rhapontin	50 and 100 mg/kg BW		Rhapontin reversed extracellular matrix deposition in primary lung fibroblast cells and prevented lung fibrosis induced by BLM in mice by modulating AMPK activation and suppressing the TGF-β/Smad pathway	Bahri et al. (2017) and Tao et al. (2017)
63.	*Aged garlic extract (0.15% S-allyl cysteine-containing diet)*	S-Allyl cysteine			S-Allyl cysteine can play an antifibrotic role by attenuating myofibroblast differentiation through TGF-β1-mediated fibroproliferative processes	Bahri et al. (2017), Mizuguchi et al. (2006) and Tsukioka et al. (2017)

64.	*Centella asiatica*	Asiatic acid, a triterpenoid	40 mg/kg	Bleomycin-induced mice	Asiatic acid downregulated inflammatory cell infiltration in bronchoalveolar lavage fluid and pro-inflammatory cytokine expression, inhibited TGF-β1 expression, and decreased the level of collagen I, collagen III, α-SMA and TIMP-1 in lung tissue treated by BLM in mice, via Smads/ERK1/2 inactivation	Xia et al. (2018), He et al. (2022), Dong et al. (2017) and Bahri et al. (2017)

15.4 Conclusion

Interstitial lung disease (ILD) is an interstitial inflammatory lung disorder with idiopathic etiology. Currently, with the use of conventional treatment, we can only impede disease progression and cannot reverse it. Further, the conventional treatment also has various side effects. Therefore, traditional herbal medicine and its bioactive compounds and plant products have been explored as alternative potential therapeutic options for ILD. Most of the herbal medicines investigated have been found to possess anti-inflammatory, antioxidant, and antifibrotic properties along with suppression of TGF-β that could be beneficial in reducing lung inflammation and fibrosis in ILD patients. Bioactive compounds, such as resveratrol and quercetin, have also been investigated for their potential therapeutic effects on ILD and have shown promising anti-inflammatory and antifibrotic effects in mouse models of pulmonary fibrosis. However, the pharmacological effects of these drugs are not well established, and further research is needed to fully evaluate the safety, clinical efficacy, and optimal dosage and route of administration of these alternative and complementary therapies for ILD. Overall, the exploration of alternative and complementary therapies for ILD offers hope for improved treatment options for this debilitating disease.

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