Mohd Sayeed Akhtar
Mallappa Kumara Swamy *Editors*

Natural Bio-active Compounds

Volume 3: Biotechnology, Bioengineering, and Molecular Approaches

Springer

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This book is dedicated to

Allama Shibli Nomani (1857–1914)

A great scholar, educationist, social reformer, and statesman of the nineteenth century, and founding father of the Shibli National College, Azamgarh, Uttar Pradesh, India.

Foreword

Natural bio-active compounds play a crucial role in pharmaceutical industries in designing and developing high-value products that help overcome human and animal health problems. These bio-active compounds are isolated from a wide variety of plants, microbes, algae, and several others. Due to their high therapeutic potentials and nutritional values, they are extensively used in the preparation of pharmaceutical drugs and functional foods. In view of this, comprehensive studies on various natural bio-active compounds for their potential pharmacological actions, including the identification, isolation and extraction, quality control, studies on the biological activities and mechanisms of action and clinical applications are becoming an exciting field of study in contemporary natural medication. Lately, biotechnological tools have been used in this connection. The application of such tools in natural product studies has helped in obtaining the desired compounds on a large scale. Deciphering the structure and functions of different classes of genes and enzymes involved in the biosynthetic pathways of bio-active compounds has also complemented the production of these compounds on a large scale. Similarly, molecular approaches, including genomics, transcriptomics, proteomics, and metabolomics for screening natural bio-active compounds too have augmented the discovery of new lead molecules and their large-scale production. The application of different strategies of metabolic engineering to modify existing pathways in plants and microbes have confirmed the impending prospect of producing high levels of natural bio-active compounds. Advances in technology are assisting us to a large extent in discovering new natural compounds, their biosynthesis and bioactivities. However, due to various reasons, the supply of natural bio-active compounds is still limited. On the other hand, the consumer demand is increasing progressively. Hence, there is great need to apply biotechnological and bioengineering strategies to meet the current growing demand for natural bio-active compounds.

This volume in the series titled "Natural Bio-active Compounds: Volume 3-Biotechnology, Bioengineering, and Molecular Approaches" includes 13 well-articulated chapters by academicians, scientists and researchers from different parts of the world. Chapter 1 discusses the role of bio-active peptides in plant growth and defense, whereas Chap. 2 focuses on the omics approaches related to the use of medicinal plants in human health applications. Chapter 3 presents the application of biotechnology in producing plant bio-active compounds. Chapter 4 discusses about the utility of transgenic plant cell cultures for the production of secondary

metabolites. Chapter 5 describes the biotechnological approaches for the improved production of secondary metabolites from the medicinal aquatic plant, *Bacopa monnieri*. Chapter 6 highlights the prospect of plant cell culture as alternatives to produce secondary metabolites. Chapter 7 discusses the biotechnological exercises in the production of secondary metabolites and its significance in health care practices. Chapter 8 presents the biotechnological interventions in *Crocus sativus*, while Chap. 9 discusses recent advances in extraction, characterization and potential use of citral. Chapters 10 and 11 provides an update on hairy root cultures as an alternative source for the production of high-value secondary metabolites and their role in the production of secondary metabolites. Chapter 12 explains the strategies of metabolic engineering in the production of bio-active compounds from medicinal plants, while Chap. 13 describes the role of biotechnological approaches and metabolic engineering in the enhancement of rosmarinic acid content.

This volume is unique in nature. It covers various aspects of biotechnological production of high-value natural bio-active compounds and provides a deep knowledge of modern natural product research focused on producing vital native bioactive compounds of pharmaceutical importance. It also covers crucial information on the recent progress in using modern methodologies for biotechnological production of natural compounds.

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Preface

Secondary metabolites are a unique group of compounds produced by plants to protect against various biotic and abiotic factors (diseases, pests, pathogens, herbivores, environmental stresses, etc.). These compounds, however, do not influence the primary metabolic activities, such as growth and reproduction of plants. The major classes of secondary metabolites include phenolics, alkaloids, tannins, saponins, lignins, glycosides, and terpenoids. Some of these compounds have become an integral part of plant–microbe interactions toward adapting to environmental irregularities. They regulate symbiosis, induce seed germination, and show allelopathic effect, i.e., inhibit other competing plant species in their environment. Moreover, these compounds induce adverse physiological activities, such as reduced digestive efficiency, reproductive failure, neurological problems, gangrene, goiter, even death, and also possess high toxicity. The discovery of such unique compounds has inspired many scientific communities to explore their potential applications in various fields including agriculture and biomedicine. For instance, plant secondary metabolites are utilized to manufacture eco-friendly bio-pesticides and as drug sources in medicine. Due to numerous health-promoting properties, these compounds have been widely used as a source of medication since ancient times. The assessment of plant secondary metabolites for their wide-ranging therapeutic potential has led to the discovery of many drug leads in recent times. Therefore, this field of research has become a significant area for researchers interested to obtain understanding of the chemistry, analytical methodologies, biosynthetic mechanisms, and pharmacological activities of these plant secondary metabolites.

The use of natural bio-active compounds and their products are considered as most suitable and safe as an alternative medicine. Thus, there is an unprecedented task to meet the increasing demand for plant secondary metabolites from flavour and fragrance, food, and pharmaceutical industries. However, their supply has become a major constraint as their large-scale cultivation is very limited. Moreover, it is difficult to obtain a constant quantity of compounds from cultivated plants as their yield fluctuates due to several factors including genotypic variations, geography, edaphic conditions, and harvesting and processing methods. In addition, medicinal plants have become endangered due to ruthless harvesting in nature. Alternatively, plant tissue culture approaches can be well explored to produce secondary metabolites without practicing of conventional agriculture, which requires more land space. In vitro cell and tissue cultures require less space and are grown

under the controlled lab conditions, and hence offer advantages of producing the desired compounds continuously without affecting their biosynthesis and quality. Furthermore, these cultures can be scaled up to produce metabolites in very large bioreactors and also, using genetically engineered cells/tissues, novel products can be obtained. The proper knowledge and exploration of these in vitro approaches could provide an optional source to produce plant secondary metabolites from many medicinal plants in large scale.

Natural Bio-active Compounds: Volume 3-Biotechnology, Bioengineering and Molecular Approaches is a very timely effort in this direction. This book volume with 13 contributions from Germany, India, Iran, Israel, Malaysia, New Zealand, Oman, Spain Turkey, and UK discusses on the Biotechnology, Bioengineering and Molecular Approaches in related to natural bio-active compounds. This book will undoubtedly encourage researchers, academicians and pharmaceutical industries towards the large-scale production of desired bio-active natural compounds using biotechnology and bioengineering approaches. Also, it will facilitate the discovery of new drugs or formulations with an improved efficacy and safety. Moreover, it is very useful for graduate students of medicinal chemistry, biotechnology and bioengineering streams, while also benefiting scientists who are keen to explore natural bio-active compounds for medical applications.

We are highly grateful to all our contributors for readily accepting our invitation and sharing their knowledge and research outcomes to compose the chapters and enduring editorial suggestions to finally produce this venture. We greatly appreciate their commitment. We are also thankful to Professor Munir Ozturk Hakeem for his suggestion and for writing the foreword for this volume. We also thank the team of Springer International, especially Dr. Kapila Mamta and Raagaipriya Chandrasekaran for their generous cooperation at every stage of the publication.

Shahjahanpur, Uttar Pradesh, India — Mohd Sayeed Akhtar
Bengaluru, Karnataka, India — Mallappa Kumara Swamy

About this Book

This book provides an updated and scientifically refined information about the production of several natural bio-active compounds obtained from microbes, plants, and algae, etc., through biotechnological, bioprocess and bioengineering approaches. The latest evidences on plant cell, tissue, organ, root culture and their utilization in the production of bio-active compounds are highlighted. Scale-up procedures using different types of bioreactors and their designs, optimization of culture conditions, the genetic and biochemical stability of biocompounds, the feasibility of using transgenic microbes and plants to enhance the production of targeted bio-active compounds are discussed in detail. Moreover, this book discusses on the possible explorations of metabolic pathway manipulations to produce bio-active compounds. Some of the modern high-throughput technologies, such as genomics, transcriptomics, proteomics, epigenomics, etc., to identify the genes and proteins involved in the biosynthesis of important bio-active compounds are discussed. Overall, the information provided in this book will undoubtedly encourage researchers, academicians and pharmaceutical industries towards the large-scale production of desired bio-active natural compounds using biotechnology and bioengineering approaches. Also, it will facilitate the discovery of new drugs or formulations with improved efficacy and safety to be effectively used in the future to counter the ever-growing challenges presented by diseases and infectious agents. This text could be useful for graduate students of medicinal chemistry, biotechnology and engineering streams. It also benefits scientists, who are keen to explore natural bio-active compounds for medical applications.

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About the Editors

Dr. Mohd Sayeed Akhtar (PhD) is Assistant Professor in Gandhi Faiz-e-Aam College, Shahjahanpur, U.P., India. He received his PhD degree from Aligarh Muslim University (AMU), India, in 2008, prior to conducting postdoctoral research at the Botanical Institute, University of Basel (BIB), Switzerland (2008–2010), and the Chonbuk National University (CBNU), Republic of Korea in 2011. He was Assistant Professor, Jimma University, Ethiopia (2011–2014), and a fellow researcher at the Institute of Tropical Agriculture, Universiti Putra Malaysia (UPM) (2014–2015). Dr. Akhtar has more than 15 years of research and 10 years of teaching experience in soil microbiology, applied microbiology, environmental microbiology, molecular biology, plant pathology and plant nanobiotechnology. He is author and co-author of more than a hundred articles in peer-reviewed journals, conference proceedings, and book chapters, and has edited ten books with international publishers. He serves the scientific community as editorial board member and reviewer of several high-impact international journals. His current research is focused on the rhizospheric plant–microbe interactions and their molecular biotechnology, bioremediation, biomineralization, nano-fertilizers and nanobiotechnology.

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Pvt. Ltd., India. He also serves as the editorial board member and reviewer for several high-impact international journals. His current research is on cell and tissue culture technology for bio-active compound production, phytochemistry, phytocompounds isolation, production and their biological evaluation, and on nanotechnology for medical applications.

Bio-active Peptides: Role in Plant Growth and Defense

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Sharadwata Pan, Dominic Agyei, Jaison Jeevanandam, and Michael K. Danquah

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Abstract

The emerging beneficial characteristics of bio-active peptides have made them suitable candidates for a wide range of applications. While their usage as potent nutraceutical and pharmaceutical agents has been well-documented, applications of bio-active peptides in addressing sustainable agricultural challenges relating to biotic and abiotic stresses, plant disease control, and nutrient use efficiency have not received much attention. Bio-active peptides are specific fragments of proteins with amino acid structures capable of enhancing molecular signaling in the rhizosphere to promote nodulation, nutrient uptake, and stress management. Bio-active peptides can be formulated with agrochemicals and assimilated through the leaf system in foliar treatments to achieve a wide range of plant benefits including coloring, nutrient delivery under drought conditions, plant health, and crop protection. Harnessing the maximum potential of bio-active peptides in sustainable agriculture is a rational contemplation, since the current years have witnessed a radical upsurge in the manufacturing scale of bio-active peptides under optimum economy. The present chapter discusses the unique potential of bio-active peptides in promoting sustainable agriculture. Moreover, the molecular mechanisms of bio-active peptides in influencing plant stress relief, disease control, and nutrient assimilation efficiency and signaling routes are also elaborated. Additionally, a few advanced standpoints pertaining to optimal utilization of bio-active peptides in advancing agricultural productivity are also discussed.

Keywords

Bio-active compounds · Cyclic peptides · Disease control · Stress relief · Sustainable agriculture

1.1 Introduction

Biologically active short fragments (typically 2–20 amino acids) of parent proteins are currently at the forefront of active research and development, both under the purviews of academic and commercial domains. This is predominantly due to the profound impacts of bio-active peptides to two major domains of active lifestyle: food and health sectors. Till date, from the repertoire of literature available on the subject, the major focus of the food sector is an attempt to extract and optimize maximum benefits of bio-active peptides as nutraceuticals. On the other hand, the health sector has been busy trying to systematically elucidate and characterize the pharmaceutical characteristics associated with bio-active peptides, with an objective to fully trap the extraordinary range of health benefits associated, such as cytoregulatory, antimicrobial, antidiabetic, and antihypertensive actions, among others. On the latter perspective, the widespread critical acclaim levied on these small molecules of tremendous capabilities is a direct consequence of their outstanding capabilities to scavenge the detrimental actions of the free radicals like reactive oxygen

species, which are perceived as the principal perpetrator in manifestations of a range of derogatory health complications. All these and many more insightful assertions, deliberations, comments, discussions, and recommendations are available in a wide volume of recent and past studies, both research and reviews (Agyei et al. 2015, 2016, 2017a, b, 2018; Sarethy and Pan 2017; Gnasegaran et al. 2017), to which interested readers may refer to. Although the active solicitations of bio-active peptides in medicine and food sectors are imminently noticeable and have been quite a long-standing initiative, the same cannot be ascertained regarding its applications in sustainable agriculture. This is aptly reflected in Fig. 1.1, which clearly reveals the insufficient investigations of bio-active peptide benefits in sustainable agriculture. In fact, if the current articles and news feeds are to be believed, the trial has just begun, and a "vast empty land lies ahead to be grazed." Probably the appeal of the bio-active peptides lies in their easier absorption by the host plants as compared to the free amino acids, which consequently hints at their high-class biological effectiveness and dietetic usefulness, as compared to the free amino acids. With the extension of the mechanized level of biologically active peptides and comparatively restricted manufacturing expenses, the opportunities for a widespread application in agro-based production fields are immense (Malaguti et al. 2014; Prasad et al. 2017). This is aided by the substantial benefits associated with the bio-active peptides. One of the most significant advantages of the bio-active peptide administration strategy in a sustainable agriculture is its "green and natural perspective" and the nonmandatory feature to introduce and incorporate tiresome and lengthy crossbreeding procedures to produce transgenic plants (Scheible 2018). Additionally, other benefits

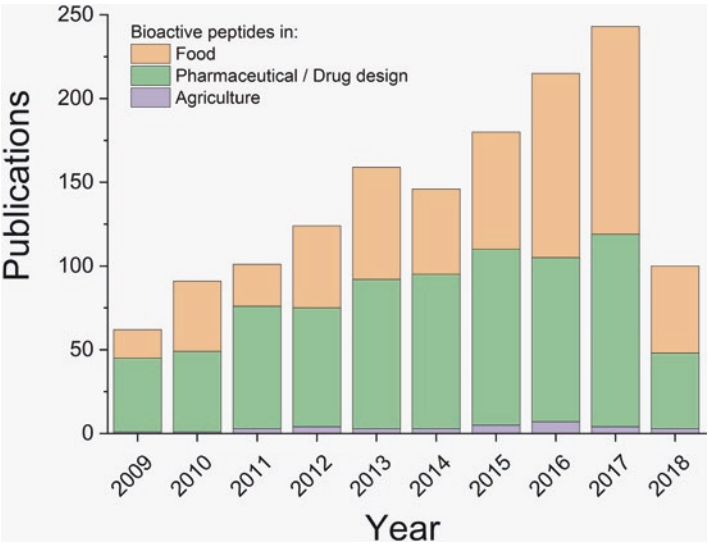

Fig. 1.1 Recent publications in the SCOPUS database (http://www.scopus.com) from 2009 to 2018, using the terms "bio-active peptides" and "food/pharmaceutical or drug design/agriculture" in the scientific literature

such as ability to synthesize peptides that mimic the original host peptide sequences, large-scale manufacturing capabilities, ability to show bioactivity even at a minuscule concentration, and an overall positive effect on plant development have only augmented the desire to achieve a mammoth-scale application of bio-active peptides in viable agriculture (Malaguti et al. 2014; Scheible 2018). On this front, Malaguti et al. (2014) have analyzed the agronomical, clinical, and biochemical viewpoints with reference to bio-active peptides and concluded that extensive trials and stringent validation of links between benefits of bio-active peptides and agronomical deliberations are necessary. Furthermore, robust quality control and assurance protocols need to be reinforced for bio-active peptides on the production front, as has been demonstrated in case of herbal medicines (Pan et al. 2013). This necessitates a careful attempt to review the current situation. Thus, the present chapter discusses the unique potential of bio-active peptides in promoting sustainable agriculture. Moreover, the molecular mechanisms of bio-active peptides in influencing plant stress relief, disease control, and nutrient assimilation efficiency and signaling routes are also elaborated. Additionally, a few advanced standpoints pertaining to optimal utilization of bio-active peptides in advancing agricultural productivity are also discussed.

1.2 Stress Sustenance Mediated by Bio-active Peptides

1.2.1 Biotic Stress Relief

One of the most significant advantages of bio-active peptides in sustainable agriculture could be their abilities to counter stress generated from invasion of foreign (mainly microbial) pathogens: bacteria, viruses, or fungi. It is no secret that failures to sustain and counter stresses from these biotic agents denounce crop productivity, mainly in terms of yield. Consequently, understanding the molecular mechanisms of biotic stress tolerance in crop plants via active involvement of biologically active proteins and peptides could be a continuous effort (Besseau et al. 2012; Scarpeci et al. 2013; Ramegowda and Senthil-Kumar 2015). In this context, functional-, biochemical-, and molecular-level investigations on plant-microbe communications have been studied which reveal significant influence of microbial associations toward biotic stress sustenance (Farrar et al. 2014). Furthermore, the interactions at the level of individual molecules and even genes have been facilitated through the synthesis of biological data founded on multi-omics strategies (Kissoudis et al. 2014). Interestingly, past studies focused on combined effects of biotic and abiotic stresses have indicated elicitation of unique responses (either positive or negative, or being liable or lenient) from the affected plant species (Ramegowda et al. 2013a, b; Ramegowda and Senthil-Kumar 2015). This is even more practical, considering the ever-changing ecological conditions and perpetual threats of global warming. The bottom line is that both biotic and abiotic stress sustenance mechanisms are interconnected to a considerable degree. Purely from the perspective of biotic stress tolerance, the implications of abscisic acid (ABA) stand above the rest, via laying

out either a substantial barricade or downregulating specific signaling pathways to counter the pathogen-induced responses (Ramegowda and Senthil-Kumar 2015). The bio-active agents derived from foreign pathogens may trigger immune responses in the host plants which bear resemblance to contagions, which may ultimately assist in enabling stress sustenance (Rasmussen et al. 2013). Past studies have laid claims to evidences of host plants, with prior reactions to drought stress, to have significantly altered pathogen reactions (Ramegowda et al. 2013a). Prasch and Sonnewald (2013) demonstrated that plants of *Arabidopsis* species, subjected to viral infections, trigger an ensemble of gene-encoded small bio-active peptides with stress-protective features. Recombinant DNA technology studies have confirmed the involvement of bio-active agents to counter biotic stress through mediation via either salicylic or jasmonic acid and/or ethylene signaling pathways (Besseau et al. 2012; Scarpeci et al. 2013; Chen et al. 2013). The involvement of several bio-active agents, including biologically active peptides, over-manifestation and optimum manifestation of transcription factors, and upstream monitoring of genes and even enzymes, in countering biotic stress, have been aptly highlighted (Ramegowda and Senthil-Kumar 2015 and references therein).

1.2.2 Abiotic Stress Relief

A wide array of abiotic stress factors is responsible for affecting agricultural crop yield: salinity, heat, drought, temperature, and precipitation, among others (Meena et al. 2017). The authors emphasize on some crucial aspects in order to achieve a desirable abiotic stress counter effect, including recognizing the profusion of metabolic routes, detection of characteristics linked to stress reactions and subsequent associated genetic markers, and novel gene pullout approaches that could optimize the stress alleviation approaches. Abiotic stress relief involving microorganisms has also received considerable attention (Nadeem et al. 2014; Souza et al. 2015; Meena et al. 2017). The volume of data and past studies, targeted toward involvement of biologically active agents, including small proteins and peptides, toward alleviation or mitigation of abiotic stress in agricultural crops, is much broader as compared to the biotic stress sustenance. Till date, diverse strategies have been employed to decipher and extract bio-active peptides with beneficial features, including high-throughput chromatographic techniques and innovative computational biology tools (Sagar et al. 2012; Torrent et al. 2012). This is logical, since downregulation or alleviation of abiotic stress responses, which is the leading restrictive aspect for agricultural throughput, has been focused on a wide range of agricultural research initiatives. Much of these studies have been directed using genetic-level investigations in the plant *Arabidopsis thaliana*. For instance, Vie et al. (2017) have recently showed that IDL6 and IDL7, the inflorescence deficient in abscission or IDA-LIKE bio-active peptides, adversely regulate the abiotic stress reactions in these host plants. Contextually, minor signaling peptides with resemblances to IDA-LIKE peptides have also been implicated earlier to aid in the process (Vie et al. 2015). The implications of novel bio-active peptides such as OSIP108, obtained from *A.*

thaliana, have been highlighted in countering the menaces of oxidative stress by scavenging various types of reactive oxygen species or free radicals and by conferring protections against agents like hydrogen peroxide (De Coninck et al. 2013). Their work is laudable, from the perspective of identification of biologically active peptides with potent free radical scavenging properties; those may only be programmed by minor open reading frames (ORF) in the host plant genome. This actually advances the work by Brand et al. (2012), who have reported similar efforts, except that the bio-active peptides could only be encoded in the plant proteome. Several other bio-active peptides, such as plant elicitor peptide At Pep1, phytosulphokine (PSK) peptide, C-terminally encoded peptide (CEP-3), and cysteine-abundant peptide AtCAPE1, among others, have shown promise toward alleviation or mitigation of abiotic stress in plants (Yamaguchi et al. 2010; Delay et al. 2013; Chien et al. 2015). Very recently, Meena et al. (2017) have comprehensively reviewed the Omics approaches involving microbial associations and subsequent countering of abiotic stresses in plants. The review is noteworthy, since the reader is directed toward detailed, insightful discussions regarding several unique strategies, like proteomics, genomics, metabolomics, and transcriptomics, from the perspective of an initial introduction to the concepts, and later the justification and rationale surrounding these techniques to assimilate, scrutinize, and infer real-time cellular information that could be effectively transferred from the lab to the field.

1.3 Bio-active Peptides in Plant Disease Control

Plants are the important source of energy for herbivorous animals, birds, and humans. Diseases in plants will disturb the continuity in food chain and also affect the economy that depends on agriculture. Generally, plant diseases are caused by microbes such as bacteria, fungi, and viruses. Thus, efficient antimicrobials are highly in demand to control the spread of microbial infection with antibiotic resistance in plants. Bio-active peptides are proved to possess enhanced potential as antimicrobials to eradicate microbe-mediated plant diseases (Gomes et al. 2018).

1.3.1 Antibacterial Response of Bio-active Peptides

Defensive peptides are produced in organisms as antimicrobial peptides (AMPs), which are the new class of antibiotics that are formed, when microorganisms or extraneous materials encounter with the surface of host organism. Ribosomes are the significant precursors which consist of 10–60 amino acid residues that help in the production of AMPs via C-terminal amidation, cysteine pairing, and amino acid isomerization. These AMPs possess antibiotic and antiendotoxic activities against fungi, bacteria, viruses, and some parasites as well as boost innate immune systems (Ovando et al. 2018). New strategies to produce transgenic plants that are expressing AMP genes via recombinant DNA techniques facilitate the bioactivity of AMPs against bacterial and fungal plant pathogens (Wang et al. 2018). Among

antibacterial peptides, cecropin B is an important peptide obtained from *Hyalophora cecropia* and *Bombyx mori* that shows antibacterial response against several Gram-positive and Gram-negative bacteria (Zou et al. 2017). Recently, cationic lytic peptide cecropin B was proved to possess antibacterial efficacy against two major pathogens of tomatoes such as *Ralstonia solanacearum* and *Xanthomonas campestris*, and also in vivo studies in transgenic tomato plants with these peptides demonstrate significant resistance to bacterial spot and wilt diseases (Jan et al. 2010). Likewise, bacteriocins, defensins, peptaibols, cyclopeptides, and pseudopeptides (Breen et al. 2015; Borriss 2016; Camó et al. 2017; Gwinn 2018) were also used to control bacterial-mediated plant diseases.

Among these wide variety of bio-active antibacterial peptides, bacteriocins, cyclopeptides, and pseudopeptides can be subclassified into further types. Bacteriocins that are produced by actinobacteria are classified into type 1 lantibiotics, which include microbisporicin and planosporicin; type 2 lantibiotics, namely, variacin, michiganin A, cinnamycin group, and actagardine; and labryinthopeptins and NAI-112 which are categorized under type 3 lantibiotics (Gomes et al. 2017). These bacteriocins help in controlling plant diseases such as tomato bacterial wilt (Konappa et al. 2015), vegetable diseases by plant growth-promoting rhizobacteria (Rizvi et al. 2017), citrus canker (Canteros et al. 2017), and Stewart's wilt of corn (Javandira et al. 2013) and also help as microbiota regulators and promote plant growth (Drider et al. 2016). Similarly, cyclic peptides or cyclotides are classified into homodetic, heterodetic, and complex based on their type of bonds within the rings (Claro et al. 2018). Cyclotides such as iturin, gramicidins, and lipid peptides help to control fire blight diseases (Habbadi et al. 2017); tailed lipid cyclotides, namely, polymyxins, putisolvins, and corpeptins inhibit the growth of bacteria that causes wilt, spot, speck, and canker disease in tomato (Panneerselvam et al. 2015) and fire blight diseases (Sonawane et al. 2015). Meanwhile, pseudopeptides are used to control bacterial growth in plants to avoid spreading of diseases such as fire blight disease (Patel et al. 2017), blackleg disease in potato (Dutkiewicz et al. 2016), citrus canker (Dutkiewicz et al. 2016), and nosocomial infections (Montesinos et al. 2012). Also, bio-active peptides are beneficial in controlling other bacterial infections in plants such as bacterial crown gall (Frikha-Gargouri et al. 2017), foliar diseases (Ali et al. 2016), leaf blight (Shi et al. 2016), soft rot disease (Charkowski 2015), and root and postharvest diseases (Rahman 2016).

1.3.2 Antifungal Response of Bio-active Peptides

Similar to antibacterial effect, bio-active peptides also possess antifungal properties toward various fungal infections. Peptides such as alfAFP, Pn-AMP2, CEMA, MSI-99, and polyoxins from various sources possess antifungal activity against several phytofungal diseases (Keymanesh et al. 2009). Plant sources, namely, *Medicago sativa* and *Pharbitis nil*, help to fabricate antifungal peptides such as alfAFP and Pn-AMP2 that help to control fungal species such as *Verticillium dahliae* (Maróti et al. 2011) that causes wilt disease (Ilyas et al. 2017). Pn-AMPs are hevein-like

peptides that are also used to control the growth of phytopathogenic fungi that causes disease in *Lycopersicum esculentum* (tomato) (Slavokhotova et al. 2017). CEMA and MSI-99 peptides are originated from synthetic sources such as hybrid chimeric form of cecropin-melittin (Li et al. 2015) and magainin analog (Białkowska et al. 2017), respectively. CEMA peptides possess enhanced antifungal property that helps to control fungal-mediated plant diseases such as huanglongbing (HLB, citrus greening), canker (Dutt et al. 2015), Pierce's disease (Li et al. 2015), and *Verticillium* and *Fusarium* wilt in cotton (Zhang et al. 2016). Likewise, MSI-99 peptide helps to control phytofungal diseases such as blue mold and sour rot diseases in citrus fruits (Wang et al. 2018), rice blast fungus (Wang et al. 2015), fungi that attack *Brassica juncea* (Rustagi et al. 2014), and aflatoxigenic fungi in maize (Schubert et al. 2015). Other novel peptides such as cathelicidin (Scarsini et al. 2015), 14-helical β-peptides (Raman et al. 2015), histatin 5-halocidin hybrid (Han et al. 2016), human β-defensin 3-C15 (Lim et al. 2016), and ABP-dHC-cecropin A (Zhang et al. 2015) also possess potential antifungal ability. These peptides help to control plant diseases such as dollar spot, brown patch disease in tall fescue, powdery mildew, root rot of kidney beans, and gray mold diseases (Zhou et al. 2016; Kusch and Panstruga 2017; Tian et al. 2017; Tong et al. 2017).

1.3.3 Antiviral Response of Bio-active Peptides

Viruses, especially bacteriophages, also cause wide variety of diseases in plants (Tepfer et al. 2015). Peptides such as entry blocker (Datta et al. 2015), RRKKLAVLLALLA, P1 (NDFRSKT), FluPep (Mendoza-Figueroa et al. 2014), N-modified peptide with palmitic acid (Aronin et al. 2015), and retrocyclins (Chen et al. 2014b) are proved to possess antiviral properties (Skalickova et al. 2015). Lactoferricin is an important peptide that possesses enhanced antiviral activity against viruses such as tomato yellow leaf curl virus (Mendoza-Figueroa et al. 2018) and potato virus X (Taha et al. 2015). Also, polysaccharide peptide (PSP) (Zhao et al. 2015), anthrax peptides (McComb et al. 2015), and RhoA peptide (Ortega-Berlanga et al. 2016) help to control the growth of famous tobacco mosaic virus. Also, plant elicitor peptides, aracins, and other novel peptides help to control plant diseases that are caused by insects and other pathogens (Huffaker 2015; Toopaang et al. 2017). Table 1.1 is a summary of different bio-active peptides that are used to control plant diseases. Thus, the peptide-based transgenic plants are highly in demand as they reduce the risk of pathogenic diseases which affect agriculture, economically (Lucht 2015). However, disruption of biodiversity and blockage in the food chain are the major drawbacks of using these transgenic plants (Abiri et al. 2015). These drawbacks, which can be unveiled by using formulation of bio-active peptides and nanomaterial encapsulated peptides to treat plants, instead of transgenic plant development, will reduce their environmental impact (Subbarao et al. 2015).

Table 1.1 Bio-active peptides as control agents for pathogen-mediated plant diseases

Peptides	Source	Benefits in controlling plant disease and pathogens	Reference
Antibacterial peptides			
Cecropin B	Giant and domesticated silk moth	*Ralstonia solanacearum*	Jan et al. (2010)
		Xanthomonas campestris	
		Controls bacterial wilt and spot disease	
Bacteriocins	Actinobacteria	Tomato bacterial wilt	Javandira et al. (2013), Konappa et al. (2015), Canteros et al. (2017), and Rizvi et al. (2017)
		Vegetable diseases by plant growth-promoting rhizobacteria	
		Citrus canker	
		Stewart's wilt of corn	
Defensins	Vertebrates and invertebrates	Alfalfa crown rot	Sasaki et al. (2016), Hsiao et al. (2017)
		Snow mold	
		Wilt disease	
Peptaibols	Fungi and bacteria	*Botrytis cinerea*	Vos et al. (2015), Bisen et al. (2016), and Hamid and Wong (2017)
		Basidiomycetes	
		Trichoderma species	
Cyclotides	Plants	Fire blight diseases	Panneerselvam et al. (2015) and Habbadi et al. (2017)
		Wilt, spot, speck, canker disease in tomato	
Pseudopeptides	Bacteria, fungi, and plants	Fire blight disease	Montesinos et al. (2012), Dutkiewicz et al. (2016), and Patel et al. (2017)
		Blackleg disease in potato	
		Citrus canker	
		Nosocomial infections	
Antifungal peptides			
alfAFP	Alfalfa, *Medicago sativa*	Wilt disease	Ilyas et al. (2017)
Pn-AMP2	*Pharbitis nil*	*Verticillium dahliae*	Maróti et al. (2011) and Slavokhotova et al. (2017)
		Diseases in tomato	
CEMA	Hybrid chimeric form of cecropin-melittin	Citrus greening	Dutt et al. (2015), Li et al. (2015), and Zhang et al. (2016)
		Canker	
		Pierce's disease	
		Verticillium and *Fusarium* wilt in cotton	

(continued)

Table 1.1 (continued)

Peptides	Source	Benefits in controlling plant disease and pathogens	Reference
MSI-99	Magainin analog	Blue mold and sour rot diseases in citrus fruits	Rustagi et al. (2014), Schubert et al. (2015), Wang et al. (2015, 2018)
		Rice blast fungus	
		Fungi that attack *Brassica juncea*	
		Aflatoxigenic fungi in maize	
Antiviral peptides			
Lactoferricin	Milk	Tomato yellow leaf curl virus	Mendoza-Figueroa et al. (2014) and Taha et al. (2015)
		Potato virus X	
Polysaccharide peptide (PSP)	Plants, animals, bacteria, and fungi	Tobacco mosaic virus	McComb et al. (2015), Zhao et al. (2015), and Ortega-Berlanga et al. (2016)
Anthrax peptides			
RhoA peptide			

1.4 Effects of Bio-active Peptides on Nodulation and Nutrient Utilization

1.4.1 Promoting Biofertilizer Actions

As a general notion, biofertilizers are envisaged as vigorous agents assisting in maintaining the ecological area surrounding soil abundant in both macro- and micronutrients by promoting inherent cycles of nitrogen fixation, secretion of plant growth regulators (PGR) or hormones, mineral assimilation and volatilization, and synthesis of active compounds with antimicrobial activities (Sinha et al. 2014). For a detailed discussion on the range of microbes, both fungi and bacteria, that have been used as potent biofertilizers, including *Azotobacter*, *Rhizobium*, *Phosphobacter*, *Azospirillum*, and *Rhizobacter*, among others, readers are encouraged to see the review by Bhardwaj et al. (2014). The authors have outlined probable solicitations of microbial biofertilizers in sustainable agriculture, their proposed high degree of optimization to improve the crop profile and productivity, and commented on possible mechanisms of biofertilizers actions. Past studies have highlighted the involvement of bio-active compounds and ligands in promoting nodulation and nutrient utilization in crop plants, including proposed machinery that aids in such processes. For instance, bio-active ligands known as Nod or Myc factors have been reported to activate the secretion of calcium ions in cytosol as a consequence of activation of signaling routes from the rhizosphere, mediated by intermediate receptors in *Rhizobium* and mycorrhiza (Bonfante and Genre 2010; Roberts et al. 2013). A hypothetical schematic depicting this action is reproduced from Bhardwaj et al. (2014) in Fig. 1.2. The onset of calcium release is also known to be facilitated by the

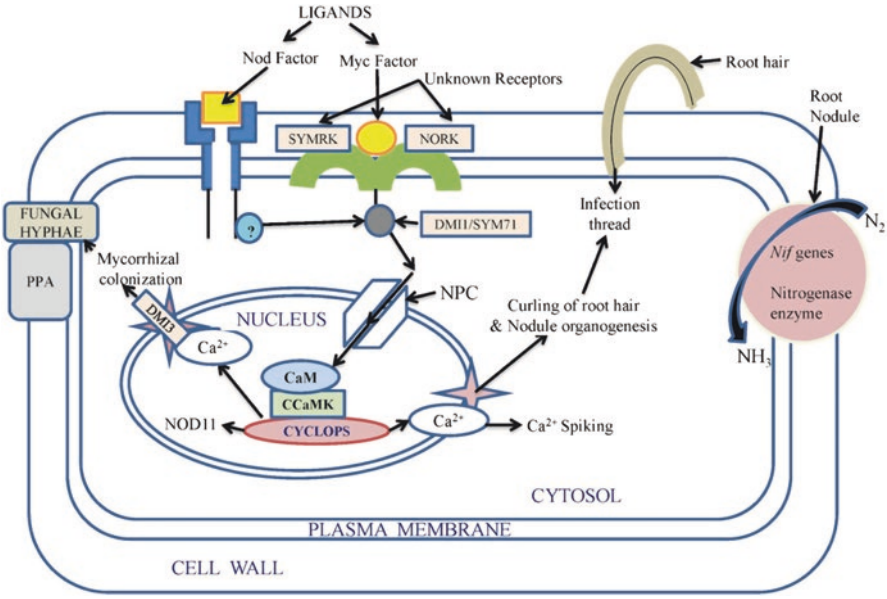

Fig. 1.2 Action mechanisms of bio-active ligands in a plant root cell: a conjectural representation. (Reproduced from Bhardwaj et al. (2014) with the Creative Commons Attribution License (http://creativecommons.org/licenses/by/4.0))

participation of nuclear pore complex and related proteins and kinases and kinase-associated proteins like SYM71 and DMI (Sieberer et al. 2009; Maillet et al. 2011). The nodulation process has also been linked to be benefited from the involvement of the enzyme calmodulin-dependent protein kinase or CCaMK (Maillet et al. 2011). A stimulating discussion related to the various factors and active molecules involved in nitrogen fixation and nodulation has been collated in the review by Zhuang et al. (2013). For instance, the authors report that nodulation and nitrogen fixation are promoted by bio-active molecules like exopolysaccharides and lipochitooligosaccharides such as Nod factors, mainly sourced from *Rhizobium* sp. Additionally, bio-active compounds such as lysophosphatidylcholine have been known to assist in phosphate acquisition (Drissner et al. 2007).

1.4.2 Protein Hydrolysates as Biostimulants

Protein hydrolysates represent a cohort of mixed sequences of amino acids and oligo- and polypeptides generated via fractional hydrolysis of source proteins (Schaafsma 2009). Bio-active peptides in the form of protein lysates, especially of plant origins, have been reported to offer service as "biostimulants," mainly due to their role in facilitating sprouting, improved yield and quality of agronomic crops, and laying a positive impression for countering abiotic stresses such as drought, heavy metal contaminations, and/or salinity (Colla et al. 2017). Contextually, the

biostimulant actions of protein hydrolysates, in the form of overall growth promotion and nitrogen acceptance, derived from corn and tomato plants, have been recently demonstrated by Colla et al. (2014). The authors report that the increased rate of nitrogen uptake could be due to a strong auxin- or gibberellin-like response, the widespread root machinery development, and enhanced nitrogen acclimatization procedures, as shown by the protein hydrolysates. The improved nitrogen incorporation may be a consequence of enhanced secretion of distinct enzymes such as glutamine synthetase and nitrate reductase, as observed previously (Ertani et al. 2009). The beneficial effects of bio-active peptide solicitations have been reflected in the form of laudable nitrogen contents in the leaves of vegetable crops (Liu and Lee 2012; Tsouvaltzis et al. 2014). Since this may optimize the efficacy of nitrogen consumption, the bio-active peptides in the form of protein hydrolysates could also be considered as active plant growth promoters. Incidentally, the roles of biologically active intrinsic peptides such as systemin, CLE, phytosulfokine, SCR/SP11, etc., in advancing cellular split and differentiation, including abilities to counter proteases, have been well-documented (Colla et al. 2014). Very recently, Colla et al. (2017) have systematically reviewed the biostimulant activities of protein hydrolysates and their effects on general plant functioning. The authors have collated recent references which highlight the positive and direct influence of plant-isolated protein hydrolysates, including their strong implications in facilitation of carbon and nitrogen absorption; in regulation of the activities of key enzymes like malate and isocitrate dehydrogenase, and citrate synthase, which are central to nitrogen uptake process; and in general advancement of root and foliar growth (Matsumiya and Kubo 2011; Colla et al. 2014, 2015; du Jardin 2015; Lucini et al. 2015; Nardi et al. 2016. It may be noted that although the phyto-protein hydrolysates have been associated with a wide variety of advantages, their animal counterparts may not be granted similar distinction and have been linked with growth clampdown and phytotoxic outcomes (Cerdán et al. 2009; Lisiecka et al. 2011).

1.5 Role of Bio-active Peptides in Phyto-signaling Pathways

Aside their roles in stress mitigation and disease control, bio-active peptides also play a crucial role in the metabolic signaling network of plants. In all life forms, signal transduction is important in a cellular communication, and without it metabolic processes which give rise to growth, defense, and survival will not occur (Banerjee and Sengupta 2011). Signal transduction involves the initiation and transmission of molecular events in the form of chemical or physical signals leading to a cellular response. In the field of botany, the role of signal transduction mediators, such as plant hormones and integrin-like receptors, are well described in the literature, and are always treated as chemicals, i.e., organic acids (e.g. salicyclic acid, jasmonic acid, indole-3- acetic acid), polyhydroxysteroids (e.g., brassinolide), hydrocarbons (e.g., ethylene), and lactones (e.g., 5-deoxystrigol). Interestingly, an increasing number of studies have shown that certain biologically active peptides also act as signaling molecules, hence the name “plant peptide

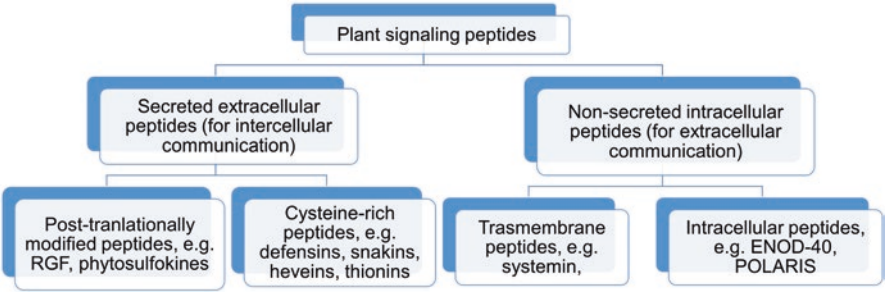

Fig. 1.3 Classification of signaling peptides or plant-based peptide hormones

hormones" (Pearce et al. 2001a; Marshall et al. 2011; Matsubayashi 2014; Oh et al. 2018). The sequencing of the *Arabidopsis* genome has shown that there are over 1000 potential signaling peptides (Oh et al. 2018), but the bulk of these has not been characterized biochemically (Czyzewicz et al. 2013). These peptides are involved in a number of plant processes and mechanisms, the most important being cell division, defense, and reproduction (Lindsey 2001). A structural classification of peptide phytohormones is given in Fig.1.3. The non-secreted peptides are located inside the cells, but their target functions could be either extracellularly or intracellularly (Guo et al. 2015; Xu et al. 2018). The secreted peptides differ based on the time of processing strategy that precedes the proteolytic cleavage used to release the matured peptide. It is either the peptides undergo posttranslational modifications (PTM) (such as sulfation) giving PTM peptides or intramolecular disulfide bond formation giving cysteine-rich peptides (Matsubayashi 2014; Oh et al. 2018). This section focusses on some of the characteristics and functions of signaling peptides responsible for processes such as root and foliar development. The distinct courses of bio-active peptide actions in effective crop management and a holistic development are captured in Fig. 1.4.

1.5.1 Root Signaling Machinery

Root development and growth were described in detail for the first time using *Arabidopsis* as a model. The process is precise and consists of a set of rapidly dividing stem (or "initial") cells that surround another set of infrequently dividing cells. The rapidly dividing initial cells include the ground tissue cells (cortex/endodermis), the central portion of root cap (columella), and the outer portion of root cap (epidermal or lateral root cells). In contact with the abovementioned initial cells are the quiescent centers which consist of nondividing cells (Scheres et al. 2002; Scheres 2013). The development, growth, and differentiation of root cell are mediated by several signaling molecules, some of which are peptides. The root meristem growth factor (RGF) is one example of such peptide. It is encoded by a family of 11 genes to polypeptides with conserved C terminal that are processed into

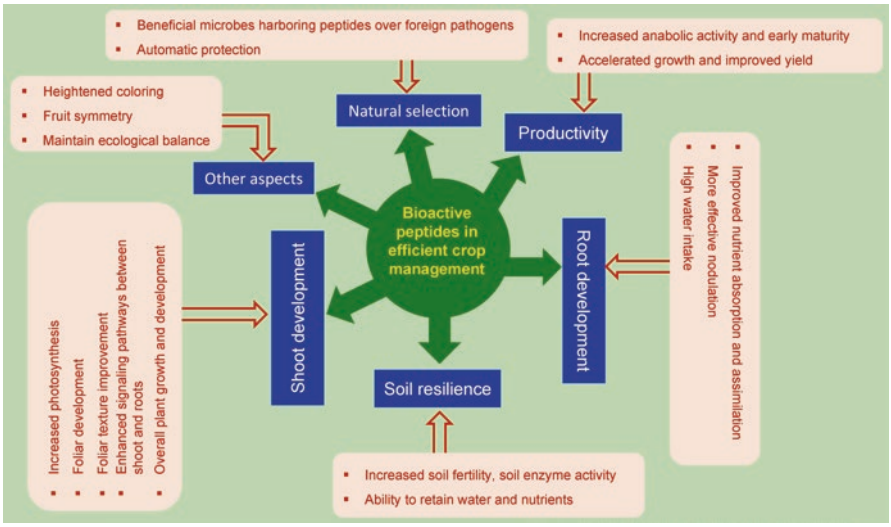

Fig. 1.4 Efficacy of biologically active peptides in overall crop management

RGF1–RGF11. RGF1 is a trideca-peptide hormone that is responsible for the post-embryonic development and maintenance of root meristem stem cells (Matsuzaki et al. 2010), but overexpression of RGF peptides results in wavy roots (i.e., a series of undulation shapes of roots) (Matsubayashi 2014). RGF is posttranslationally modified to contain a sulfonated tyrosine unit and works via the PLETHORA (PLT) stem cell transcription factor pathway (Shinohara et al. 2016). Receptors for the RGF peptides have not been identified, but the peptides share C-terminal sequence similarity with CLE18-type A, another class of secreted peptide hormones. CLE peptides are a group of secreted posttranslationally modified peptides with functions that fall into two categories, namely, the development of shoot and roots (type A) and control of xylem differentiation (type B) (Matsubayashi 2014). An example of type A CLE peptides that slows down root growth is CLE-RS (CLAVATA3/embryo surrounding region-related) peptides. CLE-RS peptides are a 13-amino acid glycopeptide that perform root-to-shoot signaling for the control of nodulation in plants. CLE-RS shows some degree of structure-function relationship as the arabinosylation of the hydroxyproline residue is vital for the hormonal functions of this peptide (Okamoto et al. 2013). Another group of plant peptide hormones with similar functions are phytosulfokines (PSK) and plant peptide containing sulfated tyrosine 1 (PSY1). PSKs are disufalted pentapeptides found in all higher plants (Sauter 2015). PSY1 on the other hand is an octadecapeptide secreted as a sulfated glycopeptide and responsible for cell growth. PSK is an autocrine growth factor, which controls root cells elongation in the elongation/differentiation zone (Oh et al. 2018). There are two receptors that recognize PSK, namely, PSKR1 and PSKR2, both of which are leucine-rich repeat receptor kinases (LRR-RK). PSY1R is also a LRR-RK and serves as the receptor for PSY1 (Mosher and Kemmerling 2013). PSK and

PSY1 perform physiological functions ranging from growth to defense (Mosher and Kemmerling 2013; Sauter 2015). In fact, PSK peptides have bio-active properties that control the formation and growth of lateral roots (Oh et al. 2018).

Early nodulin (ENOD-40) is another peptide growth factor with a high mitogenic activity and therefore responsible for the early stages of nodule development, as well as controlling sucrose metabolism by regulating the activity of the enzyme sucrose synthase (Farrokhi et al. 2008; Germain et al. 2006). ENOD-40 peptides are usually 10–13 amino acids long and is synthesized as an intact peptide and not in the form of a large precursor which needs to be processed to release the active peptide (as in the case for PSK) (Schaller 2001). Aside from root nodule formation, ENOD-40 is also responsible for other physiological processes in plants such as the formation and differentiation of vascular bundles. The existence and functions of ENOD-40 have been demonstrated in genetic studies, but their biochemical structural characteristics are yet to be deciphered (Lindsey 2001), and it is still debated whether ENOD-40 should be classified as a signaling peptide or an allosteric regulator of sucrose synthase (Germain et al. 2006). 5 kDa rapid alkalinization factor (RALF)-like peptides have also been isolated from tobacco, tomato, and alfalfa leaves and found to perform a negative regulatory role in the growth and development of lateral roots and pollen tube elongation (Pearce et al. 2001b; Murphy and De Smet 2014). RALF peptides monitor cell division in the pericycle and initiate the development of lateral roots by mediating a temporal increase in cytoplasmic calcium ion concentration with concomitant effect of rapid increase in alkalinity of extracellular space. The extracellular alkaline pH is picked up by cell surface receptors mitogen-activated protein kinase (MAPK) which signals the DNA in the nucleus to halt root development through the (Raf/MEK/ERK) pathway (Murphy and De Smet 2014). Other plant peptide growth factors not described in details in this chapter are POLARIS (for root growth, leaf vascular patterning, auxin and ethylene transport) (Casson et al. 2002) and inflorescence deficient in abscission (IDA) peptides which are responsible for floral abscission and cell separation during lateral root development (Matsubayashi 2014).

1.5.2 Foliar Development and Influence on Photosynthesis

The Devil/Rotundifolia (DVL/ROT) family of peptides have been shown to play a significant role in the proliferation of leaf cells and development of socket cells and trichomes. To date, about 24 DVL/ROT peptides which are between 41 and 145 amino acids have been discovered (Czyzewicz et al. 2013). These peptides have been shown to be nonmobile and do not use the usual plant secretary pathway (i.e., via endoplasmic reticulum-Golgi apparatus); thus, the signaling mechanism of these peptides is not fully understood (Germain et al. 2006; Valdivia et al. 2012). A few distinct peptides involved in plant signaling pathways are listed in Table 1.2.

Table 1.2 Peptides with phyto-signaling functions

Name of peptide families	Number of amino acids, sequence, and/or molecular weight	Receptor(s)	Function	Reference
Root meristem growth factor (RGF1)	Asp-Tyr(SO_3H)-Ser-Asn-Pro-Gly-His-His-Pro-Hyp-Arg-His-Asn	Unknown	Maintenance of root stem cells	Matsuzaki et al. (2010)
CLE-RS	Arg-Leu-Ser-Hyp-Gly-Gly-[Ara3] Hyp-Asp-Pro-Gln-His-Asn-Asn	Hypernodulation aberrant root formation (HAR1) rector kinase	Controlling of root nodulation	Okamoto et al. (2013)
Phytosulfokines	Tyr(SO_3H)-Ile-Tyr(SO_3H)-Thr-Gln-OH)	PSKR1 and PSKR2	Proliferation of plant cells; control of immune system in response to pathogens	Matsubayashi and Sakagami (1996)
PSY1	Asp-Tyr(SO_3H)-Gly-Asp-Pro-Ser-Ala-Asn-Pro-Lys-His-Asp-Pro-Gly-Val-[Ara_3] Hyp-Hyp-Ser	PSYR	Proliferation and expansion of cells	Oh et al. (2018)
Systemin	18, Ala-Val-Gln-Ser-Lys-Pro-Pro-Ser-Lys-Arg-Asp-Pro-Pro-Lys-Met-Gln-Thr-Asp	Systemin receptor 160 (SR160)	Wound response	Pearce et al. (1991)
IDA	*E*xtended *p*roliferating cell nuclear antigen *i*nteracting *p*rotein (EPIP) domain oligopeptide	HAE and HSL2	Lateral root development and control of floral abscission	Matsubayashi (2014)
RALF-like peptides	5 kDa polypeptide; 49 amino acids	Unidentified	Cell expansion	Czyzewicz et al. (2013)

(continued)

Table 1.2 (continued)

Name of peptide families	Number of amino acids, sequence, and/or molecular weight	Receptor(s)	Function	Reference
DVL/ROT peptides	41–145 amino acids		Cell proliferation in leaves and trichomes	Czyzewicz et al. (2013)
Early nodulin (ENOD-40) peptides	10–13 amino acids	Not identified	Regulation of nodule development and sucrose metabolism	Schaller (2001)
POLARIS	36-amino acid precursor peptide with predicted MW of 4.6 kDa	Unidentified	Root growth, leaf vascular patterning, auxin and ethylene transport	Casson et al. (2002)

1.6 Antimicrobial Peptides in Viable Agriculture

Applications of antimicrobial peptides, typically comprised of ~12 to 50/100 amino acid residues, in sustainable agriculture have been well reviewed in past studies (Keymanesh et al. 2009; Meng et al. 2010). A wide range of diverse sources have been reported for these types of biologically active peptides, including both Gram-positive and Gram-negative bacteria, mammalian tissues, insects, and fishes, among others, which are concomitantly revealed through a detailed collation of numerous patents on the foundation and solicitations of antimicrobial peptides (Meng et al. 2010). Several applications of these bio-active peptides pertaining to viable agriculture have been reported. For instance, implications of bio-active antimicrobial peptides have been documented in the development of hybrid or transgenic plants that demonstrate disease resistance and stress sustenance (Keymanesh et al. 2009; Meng et al. 2010; Maruyama et al. 2011) and in regulation of pests and postharvest deteriorations (Keymanesh et al. 2009; Meng et al. 2010). Transgenic crop production to facilitate human consumption with positive human health effects is an attractive area, since high degree and optimum utilizations of the seed storage proteins are possible, which are supposed to be powerhouses of bio-active peptides (Maruyama et al. 2011). The authors report that the introduction of bio-active peptides may be possible in either of the two distinct domains within the seed packing proteins: conserved or disordered regions. Although simulations pitch in favor of the conserved domains for incorporation of bio-active peptides, in spite of being highly rigid, past studies have favored more the disordered domains as a preferred site (Prak et al. 2006; Nishizawa et al. 2008). This even positively affects by playing a substantial role in deterrence of diseases which are so undeniably linked with daily life.

Antimicrobial bio-active peptides have also been linked positively with plant disease regulation and active resistance against pathogen-mediated stresses (Sarika et al. 2012; Montesinos et al. 2012 and references therein). Both natural and synthetic antimicrobial peptides, including cyclic decapeptides (CYC10) and linear undecapeptides (CECMEL11), have been documented using conventional standardization strategies including enforcing only minor cleavage characteristics against the proteases, with varied orders of maintenance and stability, production schemes and associated expenses, and diverse ranges of microbe-dependent antimicrobial actions (Montesinos et al. 2012). The authors additionally noted, despite presenting proofs of concept in favor of the technologies, the high-end economic concerns (mainly high costs) associated with the antimicrobial peptide synthesis technology. The protective roles of antimicrobial bio-active peptides against microbial pathogens are also duly acknowledged. For a detailed review of different sources based on hosts, either prokaryotic or eukaryotic (both invertebrates and vertebrates), net charges, and a comprehensive listing of various state-of-the-art databases available to employ computational biology and biotechnological tools to full utilization in development of novel bio-active peptides with potent actions facilitating instinctive host immunity, readers are directed to the review by Sarika et al. (2012).

Very recently, bio-active peptides with antimicrobial activities derived from the phylum *Actinobacteria* have been held in high esteem, which could be a consequence of the high G+C content in the constituent Gram-positive bacteria fitting to this taxon (Gao and Gupta 2012; Gomes et al. 2017). These classes of peptides, namely, thiopeptides, linaridins, lanthipeptides, etc., labeled as "lantibiotics" under a common umbrella term, could be classified either as thermo-resistant or thermos-labile and are typically around 10 kDa in molecular weight (Gomes et al. 2017). Different classes of lantibiotics have diverse and distinct action modes mediated by the actions of biologically active small polypeptide fractions. Type I lantibiotics like microbisporicin or NAI-107, containing 24 amino acids and isolated from strains of *Microbispora corallina* and *Actinoallomurus* spp., have been associated with potent cell wall synthesis blockages actions (Castiglione et al. 2008; Maffioli et al. 2014; Cruz et al. 2015). Type II lantibiotics such as variacin (25 amino acids) and actagardine (19 amino acids) have been implicated in downregulating the actions of a wide range of Gram-positive bacteria that are responsible for food decay (Gomes et al. 2017). Particularly, actagardine and its derivatives like NCIMB41362 and NVB333 have been reported to exert a special cell wall development impeding activity mediated via downregulating the transglycosylation response (Boakes et al. 2016; Gomes et al. 2017). Type III lantibiotics like labyrinthopeptins (18–21 amino acids) are unique since they do not possess MeLan and Lan residues, but characterized by dual Cys residues joined together by a disulfide bond, and have been known to exert strong antiviral reactions toward deadly viruses such as HIV and HSV (Meindl et al. 2010; Sambeth and Süssmuth 2011; Gomes et al. 2017).

1.7 Cyclic Peptides in Viable Agriculture

Cyclic peptides, or more commonly termed as cyclotides, are a comparatively lately found cohort of plant-derived small proteins (~30 amino acids), rich in disulfides, which may be traced to diverse plant tissues such as flowers, roots, leaves, and stems. These have received considerable attention in recent times due to their extremely high stability as a result of their unique structural conformation known as cyclic cysteine knot or CCK (Craik et al. 1999). Contextually, both ribosomal and non-ribosomal lineages of the sequential development of cyclotides have been reported (Gao et al. 2012; Arnison et al. 2013). Although initially the cyclotides were mainly conceived as agents that would facilitate and hold forth the plant defense against foreign pathogens, of late discoveries pertaining to their wide spread, potential applications in designing pharmaceutical agents and viable agriculture are noteworthy advancements. For detailed discussions and insightful deliberations of general structural features and functional mechanisms, chemical and biological assortments, evolutionary aspects, and general and specific applications, readers may refer to recent reviews (Craik et al. 2010; Anke and Laatsch 2018).

It is to be noted that reports of successful identification and subsequent extraction of cyclic peptides with potential biologically active characteristics are not limited to plants alone but extended to other kingdoms such as sponges (Almeida et al. 2016), cyanobacteria (Welker and von Döhren 2006), fungi, and bacteria (Anke and Laatsch 2018 and references therein). Particularly, kingdom fungi has received much interest in the endeavors corresponding to obtain cyclotides of varied applications and origin, i.e., cyclic depsipeptides and general cyclic peptides in addition to diketopiperazines (characterized by two amino acids joined by two peptide bonds) and siderophores (strong iron binding agents), with their biological and chemical diversities ranging from general bio-active features to the construction slabs (Bara et al. 2013; Chen et al. 2014a; Ebada et al. 2014; Hu and Dong 2015; Kawahara et al. 2016; Akone et al. 2016; Anke and Laatsch 2018). Much of the attention, as revealed from the past studies, has been focused on the bioactivities and host defense characteristics offered by the cyclotides. This is also directly related to their widespread solicitations in viable agriculture. One of the most exhaustively investigated defense mechanisms pertaining to the cyclotides is their protective properties against the attack of insects (see Craik et al. 2010 and references therein). Additionally, much effort has been employed to carefully decipher the actions of cyclic peptides against adventitious agents such as microbes, nematodes, molluscs, and cellular toxic agents. However, instances of additional substantiation against antimicrobial abilities of cyclotides do remain (Tam et al. 1999).

Although several illustrations of isolation of cyclic peptides from a wide range of hosts have been well-documented, clear-cut discussions of the active solicitations of cyclic peptides in viable agriculture are scarce. Agricultural applications are limited to expression of gene sequences belonging to the cyclotides in crop plant species, mainly from the perspective of offering pest tolerance (Craik et al. 2010). Interestingly, there is a thin line of demarcation between the pharmaceutical applications and agriculture solicitations, with strong linkages to establish a common

ground between these two broad domains. For instance, knowledge to practice active molecular pharming can be facilitated with the outcomes of expression of pest-tolerant gene sequences and vice versa. This aspect is also the source of potential interest in the domain of sustainable agriculture which may manifest through an elaborate production scheme associated with the production of transgenic crop plants with desirable attributes. There have been recent suggestions over the applicability of cyclodepsipeptides and cyclopeptides, through their representation as secondary metabolites, in sustainable agriculture (Anke and Laatsch 2018). Especially, the review by Scherlach et al. (2013) deserves special mention, who has systemically reviewed several aspects of multifaceted communications between endophytic bacteria and fungi. It is no secret that the cyclic peptides demonstrate wide varieties of biologically active properties as well as present unique environmental benefits. For instance, siderophores have been reported to confer oxidative stress relief and assist in plant sexual and asexual development and have been linked to the enhancement of iron assimilation (Eisendle et al. 2006). On one hand, while this represents an exciting opportunity of examination of the structure-function interplay, on the other hand, it may lead to the discovery of novel objectives and principal configurations that would advance sustainable agriculture. Arguments derived from these studies open up doors for future investigations with cyclotides, since it is clear that much needs to be done to properly establish the road map toward an optimum utilization of their potential toward a robust agricultural productivity (Craik et al. 2010; Anke and Laatsch 2018). The vulnerability of the bacterial strains toward cyclic peptides, with potential contributions in phyto-pathogenesis, has not been comprehensively investigated. Questions linger in the domain of action mechanisms and hierarchical evolution, with a quest to understand the basic principles within a unified framework. Several perspectives need to be understood before such framework may be convincingly founded, for instance, whether the events preceding cyclisation affect their subsequent properties and the exact spread of cyclic peptides over the entire plant kingdom, whether nucleotide or peptide screening presents a better strategy to understand the cyclotides function mechanism, and so on. Although there is promise, elaborate and accurate elucidation of benefits of cyclic peptides in sustainable agriculture needs more efforts.

1.8 Conclusions and Future Prospects

Bio-active peptides have the ability to counter undesirable stresses from pathogens and harsh environmental conditions, abilities to withstand the attack of microbial invasions, abilities to facilitate efficient nutrient assimilation and nodulation, and the capabilities to lay an overall beneficial effect on the growth and development of crops. Furthermore, the applications of cyclic and antimicrobial peptides in viable agriculture represent an interesting perspective, especially from the point of view of easiness of production and lower manufacturing costs. Thus, the bio-active peptides are one of the most promising opportunities in the sustainable agriculture. However, the potency of bio-active peptides as efficient biofertilizers has been ascertained, so,

more studies are desired on their functionalities, mechanisms, yield, and sustenance. Although several past studies targeted the abiotic stress counter mechanisms in case of host-crop plants mediated by bio-active peptides, the same can't be established regarding protection from foreign pathogens. In the future, more studies are desired on the role and efficacy of bio-active peptides in sustainable agricultural system.

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Linking Omics Approaches to Medicinal Plants and Human Health

2

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Abstract

People have an intimate association with plants since the onset of civilizations on this planet for various purposes, such as food, shelter, dye and healing. The use of plants for medicinal purposes in ancient times involved trial-and-error methods for their bioactivities. The therapeutic potential of medicinal plants has been well acknowledged in recent times with increasing experimental proofs. This has led to increased interest in their use for nutraceutical as well as medicinal purposes. It is not surprising that many of the modern medicines used currently are derived from plants. For this reason, there is an increased interest in the bioprospecting, usage and drug formulations of medicinal plants because of the presence of specialized metabolites in them. This upsurge in the interest on the usage of medicinal plants is attributed to their easy availability, developments in the recent high-throughput omics approaches, increased disease burden and increased participation of pharmaceutical companies in the business of producing phytomedicinal products for delivering plant-based healthcare services. To date, the primary focus of research with regard to medicinal plants has been in the areas of phytochemistry, pharmacognosy and horticulture. However, recent breakthroughs in high-throughput approaches have revolutionized this area of research and shifted the focus towards omics approaches, such as genomics, transcriptomics, proteomics, metabolomics, epigenomics, trichomics and ionomics. Thus, the present chapter discusses the high-throughput omics approaches in identifying genes, proteins and metabolites of medicinal plant species.

Keywords

Fingerprinting · Ionomics · Medicinal herbs · Metabolic profiling · Pharmacognosy

2.1 Introduction

Medicinal plants are in use for healing purposes, since ages across continents and among diverse civilizations (Briskin 2000; Suárez and Chávez 2018). They are being used tremendously by people, since they are easily available and affordable. Most of the medicinal plants are acting as natural chemical factories. They produce a set of small metabolites referred to as primary metabolites which are important for the housekeeping functions of the plants. Apart from primary metabolites, they also produce a vast array of secondary metabolites, which are termed as specialized metabolites. The secondary metabolites (SMs) are found to be useful for the plants, such as defence functions, such as deterrence to herbivores and counteracting the attack of pathogens (Kaufman et al. 1999; Wink and Schimmer 1999; DeLuca et al. 2012; Swamy et al. 2016a, b; Mohanty et al. 2017). It is the presence of these compounds which makes them suitable for medicinal purposes. However, the SMs are not only important for the plant's defence signalling pathways, but they are also known to have biological activities. Several important modern medicines have been

obtained from such medicinal plants that are being used in different traditional medicinal systems of the world for treating human illnesses and diseases since ages. The uses of medicinal plants have been investigated from prehistoric times using indirect approaches, such as radiocarbon dating, archaeogenomics and chemical analysis of the artefacts of human ancestors (Wadley et al. 2011). More than 80% of the world's population is still dependent on plants or plant-derived extracts/compounds for their major healthcare needs (Ekor 2013; Swamy et al. 2016a, b; Kaushal et al. 2018; Ahmed et al. 2018). Interestingly, more than 25% of the prescribed drugs today have at least one compound from plant origin (DeLuca et al. 2012). Many people are exploring the use of herbs and other alternative medicines to the Western medicine. Many of plant-based medicines are sold over the counter (OTC) as dietary supplements, which highlights the importance of medicinal plants in alleviating hunger-related issues apart from acting as medicines. The ten bestselling OTC medicines along with their sources are presented in Table 2.1. The direct harvesting and use of medicinal plants is not encouraged as it poses a huge pressure on such medicinal plants, undermining their biodiversity and erosion of important genetic stocks (Hamilton 2004). Further, the use of direct medicinal plants also poses serious health risks because of the presence of heavy metals (Hamilton 2004).

The present advancement in the use of high-throughput approaches for prospecting and identifying the bio-active compounds has led to realize today that the plants, which were used thousands of years ago for the treatments, possess various active principles in them. Screening plants for their potential medicinal compounds is interesting, but it is an immense task as there are more than 250,000 species of flowering plants being available (Akerele 1992; Padulosi et al. 2002). Therefore, scientists are taking the help of traditional healers and tribal communities to identify plants that might be medicinally important, and they are carrying out investigations

Table 2.1 List of ten bestselling OTC (over-the-counter) herbal medicines and the source of their production (Modified from Bent 2008)

Herbal drug	Botanical name	Major medicinal uses
Echinacea	*Echinacea* species	For treating upper respiratory tract infections
Garlic	*Allium sativum*	For treating cardiovascular diseases and cancers
Goldenseal	*Hydrastis canadensis*	For treating cancers, diabetes, upper respiratory tract and gastrointestinal tract infections
Ginseng	*Panax* species	For lowering blood sugar and cholesterol levels
		To relieve stress. For treating diabetes, cancers
		To manage sexual dysfunction in men
Gingko	*Ginkgo biloba*	For treating dementia
Saw palmeto	*Serenoa repens*	For the treatment of benign prostatic hyperplasia
St. John's wort	*Hypericum* species	To overcome depression
Kava kava	*Piper methysticum*	Treating anxiety
Chamomile	*Matricaria recutita*	For treating insomnia and gastrointestinal problems
Peppermint	*Mentha* × *piperita*	For treating irritable bowel syndrome

at the genome level to identify and characterize the genes responsible for producing plethora of bio-active compounds. More recent high-throughput approaches, such as genomics, proteomics, metabolomics, ionomics and metabolic engineering, are being exploited for the identification, characterization and large-scale production of plant-derived medicines both in vitro and in vivo. The omics approaches have assisted in investigations of complete genomes, transcriptomes, proteomes and ionomes of medicinal plants and the animal models in response to their treatment with either crude extracts or purified compounds (Mehta and Hasija 2018; Chakraborty 2018). Such investigations have helped in the identification of genes and proteins involved in the biosynthesis of important medicinal compounds. Understanding these gene-protein-metabolite networks have provided better opportunities for scaling up of production of important specialized metabolites through conventional as well as molecular breeding techniques (Hirai et al. 2004, 2005; Swamy et al. 2018a, b). The identified metabolites further help in the discovery of new drugs and their designing. These current omics approaches are also being used considerably to understand the responses of cells and animal body to a particular metabolite. Similarly genomics approaches are being employed to decipher transcriptional responses to these metabolites in the cells or animal models. The transcriptional responses in cells and humans further refine our understanding of the mode of action of these prospective drugs (Bora and Parihar 2018). Thus, the present chapter discusses the high-throughput omics approaches in identifying genes, proteins and metabolites of medicinal plant species.

2.2 Brief History of the Medicinal Plants

In the ancient times, the knowledge on usages of plants for healing purposes was transmitted orally from one generation to another. Although there are very few written records from the early civilizations, recent studies have used direct and indirect approaches, such as archaeological, archaeobotanical and archaeogenomics studies to unravel the medicinal role of plants. Several plants detailed in the prehistoric documentations when evaluated for their phytochemical profiles suggested the potential pharmacological activities. Some of these plants included species belonging to the genera *Achillea*, *Centaurea*, *Senecio*, *Muscari*, *Althea*, etc. These results suggest that plants might have been intentionally used for healing purposes in the ancient times (Lietava 1992; Sommer 1999). Wadley et al. (2011) discovered the 73,000-year-old remains of bedding material from South Africa wherein the leaves of a plant, *Cryptocarya woodii*, were also found. The chemical analysis of these plant leaves showed the presence of a-pyrones, cryptofolione and goniothalamin that are insecticidal and larvicidal in nature. These plants are still used in the present time for repelling mosquitoes in South Africa. The analysis of the fossil teeth of Neanderthals from the north Spanish site of El Sidrón provided the evidence in favour of the use of plants for medicinal purposes. The presence of certain

chemicals, such as chamazulene, dihydroazulene, 4-methylherniarin and herniarin, was found in yarrow plants, namely, *Achillea millefolium*, and *Matricaria chamomilla* (Hardy et al. 2012). Such prehistoric records are a testimony to the potential uses of medicinal plants since times immemorial. These records along with the help of traditional tribal communities have been playing a crucial role in the bioprospecting of more and more medicinal plants in recent times.

Most of the studies mentioned above are indirect and are obtained from the archaeological and archaeobotanical data subjecting the plant materials to further phytochemical and pharmacological analysis. The written records of the use of the medicinal plants date back to 35,000–4000 years ago in Sumerian and Egyptian cultures, where they used a wide range of medicinal plants, including *Papaver somniferum*, *Thymus* species, *Glycyrrhiza glabra*, *Salix alba*, etc. The traditional Chinese medicine is one of the oldest systems of healthcare systems, which exploited the use of medicinal plants, animals and minerals for treating a wide range of diseases. Similarly, the Indian Ayurvedic system of medicine, which is around 5000 years old, uses more than 2000 plant species (Duke 1993). In sub-Saharan Africa, the ratio of traditional healers to the population is far greater than medical doctors which signify the importance of these medicinal plants for the people of the region. Aztec and Maya Indian communities of Mexico and Central America also have a long history of the usage of natural curing substances. The historical description about the medicinal plants of the world is highlighted in Table 2.2.

2.3 The Present Status of the Medicinal Plants

According to the estimation by the organizations, i.e. the International Union for Conservation of Nature and the World Wildlife Fund, more than 50,000 species of medicinal plants are being used worldwide for medicinal purposes (Schippmann et al. 2002; Chen et al. 2016; Kumar et al. 2018). Many such traditionally used plants have been explored scientifically to obtain several significant life-saving medicines of today. For example, traditionally the crude extracts of the leaves of *Catharanthus roseus* (Madagascar periwinkle) were used as antidiabetic. The pharmacological and biochemical investigations lead to the discovery of the role of the plant extracts with antitumour activity. Lately, the antitumour activity was attributed to the presence of alkaloids, namely, vinblastine and vincristine (Noble 1990). The synthesis of these alkaloids is restricted to the leaves only, and it is also found to be fungicidal (Roepke et al. 2010). This is just an example of one such important anticancer plant used traditionally by indigenous communities, which was proved to be a potential source of anticancer drugs used in the modern medicines (Swamy et al. 2018a; Lee et al. 2018). Several such plants are being discovered for medicinal purposes, and some of the most important plants that have been in use since earlier times and have been proved useful for treating diseases are given in Table 2.3.

Table 2.2 History of the medicinal plants from around the world

Historical evidences on usage of medicinal plants	Place and usage of plants	Some of the plants mentioned
Sumerians (5000 years ago)	Oldest written records of around 250 plants used for medicinal purposes in a Sumerian clay slab at Nagpur	Poppy, henbane and mandrake
Shen Nung, Chinese emperor (2700 BC)	Shen Nung, Chinese emperor, discovered the medicinal properties of marijuana	Marijuana
Pen Tsao, China (2500 BC)	*Pen T'sao*, a Chinese book on the medicinal usage of roots and grasses, mentioned about 300 plants	Camphor, *Podophyllum*, ginseng, cinnamon bark and ephedra
Indian Vedas	Vedas: the Sanskrit manuscripts of India also have documented many medicinal plants	Nutmeg, pepper, clove
Egyptian Ebers Papyrus (1550 BC)	An Egyptian papyrus Ebers Papyrus has mentioned about 700 medicinal plants	Pomegranate, castor oil plant, aloe, senna, garlic, onion, fig, willow, coriander, juniper, common centaury
Bible and Jewish Talmud	*Talmud*, a Jewish holy book, has mentioned many plants for various treatments including aromatic plants and those used in rituals	Myrtle and incense
Homer's Epics, an ancient Greek author (800 BC)	Homer, in his epics, i.e. *The Iliad* and *The Odysseys*, has mentioned the usage of 63 plant species	*Inula helenium*, *Artemisia*
Zend Avesta, Ancient Persian Text (700 BC)	*Zend Avesta* mentioned about the usage of around 10,000 medicinal plants	*Hemp*
Herodotus (500 BC)	He mentioned some medicinal plants castor oil, garlic	Castor oil, garlic
Hippocrates (459–370 BC)	Work of Hippocrates has mention about 300 medicinal plants. He classified the plants based on their physiological action	Garlic, opium, henbane, Asparagus, oak
Theophrastus, father of botany (371–287 BC)	The books *De Causis Plantarium*, *Plant Etiology* and *De Historia Plantarium-Plant History* were published by Theophrastus. He classified more than 500 medicinal plants	Cinnamon, iris rhizome, false hellebore, mint, pomegranate, cardamom, fragrant hellebore, monkshood
Celsus (25 BC to 50 AD)	In the book *De Re Medica*, Celsus has mentioned about 250 medicinal plants	Pepper, flax, poppy, cardamom, etc.
Pen Tsao Ching (1 AD)	A Chinese medicinal text recommended the usage of marijuana for around 100 ailments	Marijuana
Dioscorides, father of pharmacognosy (65 AD)	Dioscorides, in his *Materia Medica*, wrote a practical text of 500 medicinal plants	500 medicinal plants

(continued)

Table 2.2 (continued)

Historical evidences on usage of medicinal plants	Place and usage of plants	Some of the plants mentioned
Pliny the Elder (23–79 AD)	Pliny wrote a book, *Historia Naturalis*, and mentioned the usage of 1000 medicinal plants	Diverse plants
Galen (131 AD–200)	Galen compiled the list of drugs with similar or identical action	Diverse plants
222 AD	Tea mentioned as a substitute for wine, and in a ca. 350 AD Chinese dictionary. By the third century AD tea was being presumed to be refreshing and healthy	Tea
Tang dynasty (618–906 AD)	They made the tea famous and it became the national tea of China	Tea

Modified from Petrovska (2012)

2.4 Applications of Modern Omics Approaches for Research in Medicinal Plants and Human Health

Omics employ high-throughput technologies, such as transcriptomics, proteomics, metabolomic and ionomics to analyse various kinds of molecules at a large scale. These high-throughput technologies are used to purify, identify and characterize DNA, RNA, proteins and other molecules at a large scale (Blankenburg et al. 2009; Swamy et al. 2018b). These methods are automated allowing rapid, accurate and precise analysis of very large numbers of samples in a very short period of time (Porter and Hajibabaei 2018). These technologies have enabled the study of medicinal plants relatively easier. The genomes and transcriptomes can be sequenced within a minimum time and cost by employing bioinformatics approaches (Ulrich-Merzenich et al. 2007; Saito and Matsuda 2010; Sato et al. 2011; Saito 2013). Such technologies have not only revolutionized the gene and metabolite discovery regimes but also enabled us to study their indigenous effects within the cell lines or in vivo models.

With increase in the burden of human diseases and burgeoning population of the world, it is imperative to discover new medicinal plants and their bio-active compounds for improving human health. For providing better healthcare services, it is an important step to first screen the medicinal plants for bio-active compounds and to establish gene-metabolite links. The identification and characterization of underlying genes responsible for the production and modification of metabolites is necessary step to further scale up the production of metabolites or engineer the genes in heterologous or homologous systems (Pickens et al. 2011). With the advent of genomics and transcriptomics approaches, it has now become easy to profile thousands of genes at a time. It has now become possible to even understand the phytochemical constituents of medicinal plants and their relative presence in different

Table 2.3 Most commonly used medicinal plants at present; the name of the plant, family, common use, active compound (s), mode of action (activity) and citations are provided

Medicinal plant (family)	Active compound (s)	Usage	Activity	References
Papaver somniferum (Papaveraceae)	Codeine and morphine	Anticancer Cough suppressant	Arrests mitosis and promotes microtubule polymerization	Chen et al. (2015) and DeBono et al. (2015)
Capsicum chinense, *C. frutescens*, *C. annuum* and *C. assamicum* (Solanaceae)	Capsaicin and dihydrocapsaicin	Antidiabetic, antioxidant and anticancer	Capsaicin stimulates hepatic conversion of cholesterol to bile acids	Whiting et al. (2013), Sricharoen et al. (2016), and Srinivasan (2016)
Artemisia annua (Asteraceae)	Artemisinin	Antimalarial	Still not clear (Lipid peroxidation of the *Plasmodium* parasite through reactive oxygen species and depolarization of the plasma membrane and mitochondrial membrane)	Krishna et al. (2008) and Muangphrom et al. (2016)
Cinchona officinalis (Rubiaceae)	Quinone	Antimalarial	Toxic to nucleic acid metabolism, ROS generation and disruption of cellular phospholipid membranes	Lown (1983)
Taxus brevifolia (Taxaceae)	Taxol	Anticancer	Stabilizes microtubule formation	Oberlies and Kroll (2004)
Panax ginseng (Araliaceae)	Ginsenosides	Neuroprotective effect, immunomodulation, anticancer, antioxidant	Release neurotransmitters, ROS production	Lu et al. (2009) and Jia and Zhao (2009)
Camptotheca acuminate (Cornaceae)	Camptothecin	Antitumour	Inhibits DNA topoisomerase I	Oberlies and Kroll (2004) and Pommier (2006)

Catharanthus roseus (Apocynaceae)	Vinblastine and vincristine	Anticancer	Inhibits tubulin formation	Noble (1990) and Roepke et al. (2010)
Ephedra sinica (Ephedraceae)	Ephedrine	CNS stimulant, antiasthmatic, vasoconstrictor and bronchodilator	Stimulation α- and β-adrenergic receptor	Abourashed et al. (2003)
Podophyllum peltatum (Berberidaceae)	Etoposide	Antitumour	Inhibits topoisomerase II	Baldwin and Osheroff (2005)
Rauvolfia serpentina (Apocynaceae)	Reserpine	Antihypertensive, tranquilizing drug	Release of serotonin	Brodie et al. (1957)
Digitalis purpurea (Plantaginaceae)	Digoxin	A heart medicine	Inhibition of the Na+/K+ ATPase	Orrego (1984)
Dioscorea mexicana, *D. villosa* and other species (Dioscoreaceae)	Diosgenin, dioscin and prosapogenin A	Antitumour	Induction of apoptosis	Corbiere et al. (2004)
Echinacea purpurea (Asteraceae)	Cichoric acid and echinacoside	Immunostimulant	–	Barrett (2003) and Senchina et al. (2011)
Ginkgo biloba (Ginkgoaceae)	Ginkgolides and bilobalide	Antioxidant, antidepressant, hepatoprotective	–	Smith and Luo (2004)

geographical locations at different times; this has led to emergence of relatively newer subdiscipline of genomics termed ecogenomics (Olivas 2016). The discovery of the large number of regulatory molecules, such as small RNAs (siRNAs and miRNAs), long noncoding RNAs (LnRNAs) and circular RNAs, is also crucial in understanding the gene regulatory networks that play role in regulating the metabolite synthesis and modifications (Yang and Qu 2013; Lasda and Parker 2014; Wang and Chekanova 2017; Zhang et al. 2018a).

2.4.1 Integration of Omics Approaches for Identification of Genes and Metabolites

Customarily, several techniques (e.g. precursor feeding, gene overexpression and inhibition, mutant selection or differential gene expression) are being available for the identification and characterization of genes involved in some yet to be elucidated pathways. Of lately, there has been a paradigm shift towards the use of integrated omics approaches, which essentially involve readily accessible next-generation sequencing (NGS) technologies (Wilson and Roberts 2014).

2.4.1.1 Genomics

Since the advent of the twenty-first century, there has been a tremendous increase in genomic data; more and more plants are being sequenced (Michael and Cristobal 2013) through whole-genome sequencing and RNA-sequencing with the aim to characterize the genes and elucidate their functions in the regulation of various aspects of cellular and metabolic processes. Whole-genome sequencing of a number of medicinal plants has been accomplished so far. *Salvia miltiorrhiza* is an important medicinal plant traditionally used in Chinese traditional medicine particularly for hydrophilic phenolic acids and tanshinones; the whole-genome sequencing of *S. miltiorrhiza* has predicted 30,478 protein-coding genes, many of which are validated through RNA-sequencing analysis (Xu et al. 2016). This whole-genome sequencing has been followed by a number of studies which has resulted in understanding the roles of individual genes as well as gene families (Li et al. 2018; Zhang et al. 2018a, b). Using an ab initio and evidence-driven gene annotation pipeline, 18,197 high-confidence genes have been annotated for *Calotropis gigantea*, which produces important anticancer and antimalarial cardenolides (Hoopes et al. 2018). Upadhyay et al. (2015) attempted sequencing of *Ocimum tenuiflorum*, an important medicinal plants used in Ayurveda. This study has identified key genes responsible for its medicinal properties. *Capsicum annuum* along with several other species is an important source of capsaicinoid complex (capsaicin and dihydrocapsaicin) which has been sequenced by two independent groups in 2014; they have identified candidate genes involved in the biosynthesis of capsaicin as well as dihydrocapsaicin (Qin et al. 2014; Kim et al. 2014). Urasaki et al. (2017) have reported draft sequence of *Momordica charantia*, an important vegetable as well as medicinal

plant. Their study has identified ca. 45,859 protein-coding gene loci. *Glycyrrhiza uralensis* and several other species have been widely used in Chinese traditional medicine and Indian Ayurvedic system because of the presence of glycyrrhizin and other important metabolites. To understand the metabolic pathway and their underlying genes, Mochida et al. (2017) reported its draft genome sequence paving the way for the identification of genes responsible for glycyrrhizin and other important metabolites. Yan et al. (2015) have reported de novo assembly of *Dendrobium officinale*, an important medicinal plant used in Chinese traditional medicine. Their study has reported important genes responsible for alkaloid biosynthesis. Many more such whole-genome sequencing projects are still being carried out with the aim to understand and elucidate genes and other noncoding elements such as transposons.

2.4.1.2 Transcriptomics

Transcriptome profiling enables the identification of candidate genes involved in important metabolite biosynthesis through differential gene expression analysis. Next-generation sequencing (NGS) technologies are increasingly being employed to profile transcriptomes of medicinal plants. The large-scale analysis of transcriptomes enables identification of key candidate genes involved in specialized metabolites and their role in specific cellular processes and responses (Strickler et al. 2012). Liu et al. (2017) analysed the transcriptome of an important traditional Tibetan medicinal plant, *Swertia mussotii* Franch. Their findings lead to the generation of expression profiles of 39 candidate transcripts encoding the key enzymes for secoiridoid biosynthesis. These results are an important step to understand the regulation of genes involved in this pathway. *Andrographis paniculata* is an important medicinal plant containing various bio-active terpenoids and flavonoids. De novo transcriptome analysis has helped in the annotation of 5606 transcripts which could be involved in ca. 140 pathways including terpenoids (Cherukupalli et al. 2016). Transcriptome profiling of *Withania somnifera* has suggested the differential expression of certain genes that could be useful in elucidating the withanolide biosynthesis pathway (Gupta et al. 2015).

Based on the huge data sets obtained from RNA-seq studies, a number of databases are being established for comprehensive annotation, visualization and analysis of transcripts. Van Moerkercke et al. (2013) have established a detailed metabolic pathway database, CathaCyc, using RNA-Seq data sets from Madagascar periwinkle (*Catharanthus roseus*). CathaCyc (version 1.0) contains 390 pathways and 1347 enzymes involved in both primary and secondary metabolisms and is under continuous curation. Based on NGS data available for all 75 plant species, Xiao et al. (2013a, b) have established a web-based BLAST server which allows easy access to the public (www.phytometasyn.ca).

Besides these, several other databases such as PlantGDB, Medicinal Plants Genomic Resource and PLEXdb are also evolving which form an important resource for identification of genes involved in metabolic pathways of various metabolites in plants. These data sets can play crucial role in the elucidation of uncharacterized

metabolic pathways. The information obtained from such databases would essentially help in the discovery of many enzyme variants that could be used to engineer in either heterogeneous or homogeneous systems for scaling up the production of SM's for they are present in very low quantity in the plants' cells and tissues. Combining metabolome data with the proteome and the transcriptome would help in further improving our knowledge of such SM's (Higashi and Saito 2013). Geu-Flores et al. (2012) have illustrated the role of short-chain reductase and its role as cyclase for the synthesis of iridoids in medicinal plants. Such discoveries using transcriptome analysis combined with genetic engineering provide for large-scale production opportunities using such alternative pathways. All these transcriptome-metabolome and genome data sets complement each other and assist in linking the genes with metabolomes and vice versa. An ambitious project has been initiated by Medicinal Plant Genomics Consortium which aims to integrate the genome, transcriptome and metabolome data sets of 14 key medicinal plants (http://medicinal-plantgenomics.msu.edu/) (Tables 2.4 and 2.5). Data from these studies have been

Table 2.4 Important medicinal plants selected by Medicinal Plant Genomics Consortium for integrative analysis using omics approaches

Medicinal plant	Important compounds	References
Atropa belladonna	Atropine, scopolamine, hyoscyamine	Ulbricht et al. (2004)
Camptotheca acuminata	camptothecin	Lorence and Nessler (2004)
Cannabis sativa	Tetrahydrocannabinol (THC), cannabidiol (CBD)	Andre et al. (2016)
Catharanthus roseus	Vinblastine and vincristine	Rischer et al. (2006)
Digitalis purpurea	Digoxin	Sharma and Purkait (2012)
Dioscorea villosa	Diosgenin	Marker et al. (1940)
Echinacea purpurea	Alkamides, caffeic acid derivatives and polysaccharides	Manayi et al. (2015)
Ginkgo biloba	Flavonoids, ginkgolide A, ginkgolide B	Guo et al. (2015)
Hoodia gordonii	P57 (an oxypregnane glycoside), hoodigogenin A, calogenin glycosides, hoodistanal and dehydrohoodistanal	Roza et al. (2013)
Hypericum perforatum	Hyperforin	Nahrstedt and Butterweck (1997)
Panax quinquefolius	Ginsenosides	Lu et al. (2009)
Rauvolfia serpentina	Indole alkaloids	Pathania et al. (2015)
Rosmarinus officinalis	Rosmarinic acid, camphor, caffeic acid, ursolic acid, betulinic acid, carnosic acid and carnosol	Bai et al. (2010)
Valeriana officinalis	Valerenic acid and valepotriates	Upton (1999)

Table 2.5 Metabolic engineering of genes for the production of specialized metabolites in various systems (yeast, microbes and plant cell cultures). Modified from DeLuca et al. (2012)

Drug name	Natural sources	Cells used for metabolic engineering
Resveratrol	*Vitis vinifera*	Yeast and bacteria
Reticuline	*Lindera aggregate*, *Annona squamosa* and *Ocotea fasciculata*	Bacteria and yeast
Artemisinic acid	*Artemisia annua*	Yeast
Vanillin	*Vanilla planifolia*	Yeast
Magnoflorine	*Sinomenium acutum* and *Pachygone ovata*	Yeast
Scoulerine	*Opium poppy* and *Croton flavens*	Yeast
Taxadiene	*Taxus brevifolia*	Yeast
Indole glucosinolates	Members of family Brassicaceae	Yeast
Dhurrin	*Sorghum bicolor*	*Arabidopsis* cell culture
Glucoraphanin	Broccoli and cauliflower	*Nicotiana benthamiana* cell culture

made publicly available which has revolutionized the elucidation of biosynthetic pathways for pharmaceutical compounds. Rotenoid, obtained from *Mirabilis himalaica*, is having a special medicinal value. A recent study using RNA-sequencing TOF-MS technologies has identified 522 candidate compounds responsible for rotenoid biosynthesis (Gu et al. 2018).

2.4.1.3 Metabolomics

The term metabolomics involves comprehensive, non-biased, high-throughput analyses of complex metabolite mixtures from plant extracts (Hall et al. 2002). In metabolomics the main purpose is comprehensive and simultaneous analysis of the metabolites produced in cells and organisms. It is an effective approach for the analysis of various chemical compounds occurring in plant cells (Pichersky and Gang 2000). Plant secretory trichomes are considered as important repositories of important metabolites. For this reason, an integrated omics database, TrichOME (http://www.planttrichome.org/), has been developed to pool the data and increase the understanding of trichome metabolome analysis. The database also provides for mining of trichome-specific genes and is a valuable source for plant trichome research (Dai et al. 2010). The database is helpful to elucidate unique characteristic features of plant trichomes that produce a large number of important metabolites by linking metabolites with their respective genes. Wang et al. (2018) have developed an important herbal medicine omics database (HMOD) (http://herbalplant.ynau.edu.cn/) with the aim to provide a reliable omics resource of herbal medicine plants for all researchers. This database is useful for the genome sequence viewing and BLAST search option. Medicinal Plant Metabolomics Resource (MPM) (http://metnetdb.org/mpmr_public/), another important database representing 14 important medicinal plants, has been developed, and it provides a platform for identifying the genes involved in the regulation and synthesis of important metabolites. It enables understanding metabolic networks that lead to specialized metabolites. The

database is publicly available and can be accessed by researchers from diverse disciplines such as medicine and plant biology (Wurtele et al. 2012). Different species of plants belonging to Zingiberaceae family have important medicinal values. To decode their metabolites present in different parts, Barbosa et al. (2017) attempted untargeted metabolite profiling to compare the metabolic composition of leaves and rhizomes. They demonstrated that different species show differential expression of few metabolites, whereas few of them could be used as potential markers among the members of Zingiberaceae family. Establishing metabolite maps for individual plant species and families and their compilation into databases is an important and emerging tool for pharmaceutical industry, which provides the plant-based solutions to treat diseases. The metabolite profiles are also increasingly used to study their biosynthesis and quality control studies of many medicinal plants.

2.4.1.4 Proteomics

Proteomics involves a large-scale analysis of the complete proteome of cells, organs and tissues. It is an integral part of omics technologies and assists in the investigation of changes in the proteome profiles of plants in response to external factors. The development of proteome analytical tools, such as one-dimensional polyacrylamide gel electrophoresis and two-dimensional electrophoresis coupled with tandem mass spectrometry, enables the systematic profiling of whole proteomes (Hussain and Huygens 2012). The proteomics approaches can assist in the identification of particular protein changes at a large scale in cells, tissues and organs in response to the administration of plant-based formulations or purified compounds of plant origin. Understanding the spatial localization of proteins in different tissues can provide clues about the biosynthesis of specialized metabolites (Martinez-Esteso et al. 2015). In recent years, proteomics study has been successfully employed to identify candidate proteins/enzymes that are involved in the synthesis of specialized metabolites in medicinal plants (Rai et al. 2017). The transcriptome and proteome analysis of opium poppy cell cultures have provided important inputs regarding alkaloid metabolism (Desgagne-Penix et al. 2010). The proteome analysis of *Artemisia annua* and its comparison with the genetic map (Graham et al. 2010) provided information of an enzymatic pathway which plays important role in the synthesis of artemisinin (Bryant et al. 2015). Likewise, Champagne et al. (2012) performed proteome mining of cultured *Catharanthus roseus* cells which provided important insights in elucidating terpenoid indole alkaloids. Further, their study identified 63 enzymes having potential role in secondary metabolism, 22 enzymes involved in monoterpenoid indole alkaloid biosynthesis and 16 of them predicted to be transporters. Oldham et al. (2010) demonstrated the identification of proteins from *Eschscholzia californica*, a medicinal plant using shotgun proteomics. These proteins were found to be important for the biosynthesis of benzophenanthridine alkaloids. A comparative proteome analysis of different *Cannabis sativa* plant tissues displayed differential patterns of proteome profiles. Further, the Western blotting experiment helped to identify a polyketide synthase, which is believed to be

involved in cannabinoid biosynthesis (Raharjo et al. 2004). The proteome profiling of *Andrographis paniculata* led to the detection of 44 proteins, some of which were induced in response to salt stress which provides an opportunity for breeding stress-tolerant varieties of *A. paniculata* (Talei et al. 2014).

2.4.1.5 Ionomics

The ionomics study has emerged tremendously as a new omics technique in the last decade, and it has become one of the most important pillars of functional genomics. The ionome is defined as "the mineral nutrient and trace element composition of an organism and represents the inorganic component of cellular and organismal systems" (Salt et al. 2008; Baxter 2009). So far, ionome profiling has been done for any plants, such as *Arabidopsis thaliana*, *Oryza sativa* and *Brassica* (Lahner et al. 2003; Broadley et al. 2008; Hammond et al. 2009). Many of the medicinal plant formulations in the traditional medicinal systems around the world have been proved to be rich in many toxic elements posing adverse physiological effects (Kohzadi et al. 2018; Yang et al. 2018). To avert the adverse physiological effects of heavy metals, it is a mandatory requirement to ascertain the level of such heavy metals in the traditional medicinal formulations. The ionomics rely on the use of high-throughput analysis technologies such as inductively coupled plasma optical emission spectroscopy (ICP-OES), X-ray fluorescence (XRF), inductively coupled plasma mass spectrometry (ICP-MS) and synchrotron-based micro-X-ray fluorescence which enables large-scale analysis of minerals and other elements in plants and their association with the genes (Ouedraogo et al. 2012).

2.4.2 Eliminating the Harmful Effects of Certain Metabolites: Genetic Engineering for Reducing the Expression of Genes and Metabolites

Toxicogenomics involves the study of interaction between exogenous agents with the genome and their biological effects (Bishop et al. 2001). This is based on the assumption that the toxic effects of external agents on biological systems are generally expressed at the cellular level, and they can be elucidated using transcriptomics, metabolomics and proteomics approaches. Some of the medicinal plants also contain compounds that apart from displaying positive effects may also cause negative effects to the genes by mediating cellular processes. The identification of target genes in the humans is an important step to further avert the deleterious effects of such compounds. Also, eliminating the compounds/metabolites, which are genotoxic to the humans, is necessary in drug development and medicinal plants research. Since the specialized metabolites are not uniformly produced in all the cell types, they are subjected to variation even within the same species under different environmental conditions. Moreover, these plant metabolites may have mixture of the chemical compounds in their cells, and they more often exert their effects in

combinations. So, there is an increasing challenge for the identification and isolation of the specific compounds for their specific roles. Under such considerations, there is an increasing demand for the production transgenic plants with altered metabolites having either more effectiveness or less harmful compounds. The production of decaffeinated coffee is an example of eliminating the harmful effects because of the stimulatory effects of the caffeine. Presently, decaffeinated coffee is produced industrially, and it involves a lot of expenditures with poor flavour. The synthesis of caffeine involves the successive addition of methyl groups to xanthosine which is brought about by three N-methyltransferase enzymes CaXMT1, CaMXMT1 (theobromine synthase) and CaDXMT1 (caffeine synthase). RNA interference (RNAi) technology was utilized for the production of the decaffeinated coffee wherein expression of one of the genes (theobromine synthase) was repressed. These transgenic plants showed 70% reduction in caffeine content (Ogita et al. 2003). Such methodologies can be applied in the other plants as well. In certain cases, metabolite in one form may be harmful to the humans, or in other cases, it may be less effective in its native form (natural product). Applying the genetic engineering techniques, these native metabolites can be converted into either non-harmful form or to more effective chemical derivative.

2.4.3 Large-Scale Production of Metabolites Through Metabolic Engineering for Drug Development

The production of the metabolites is very limited in the plants. So, a large-scale production for enhancing the metabolites needs metabolic engineering, wherein pathway genes are transformed into other organisms, and these transgenic organisms can be fed with the precursors for producing the desirable natural products or modified natural products. Traditionally, microbes are being used for the production of modified natural products by feeding alternative biosynthetic precursors. Though the microbes are successfully utilized for the production of simple compounds, there is not much success in the production of complex natural products because of the lack of enzymes in the microbes. This often requires the transformation of microbes with multiple genes. Because of these limitations, nowadays, plants and plant cell cultures are also being used for the production of the plant secondary metabolites (Effendi et al. 2009).

2.4.3.1 Metabolic Engineering Using Microbes

A large-scale production of secondary metabolites is being carried out by recombinant technology using microbes. Plant polyphenols have been demonstrated to be very important molecules for human health exerting a plethora of health-promoting benefits. There is a keen interest on the increased production of these polyphenols using heterologous models, such as bacteria and yeast (*Saccharomyces cerevisiae*) cells. Resveratrol, a member of the class of polyphenol compounds, is successfully

produced in increased quantity using yeast (Wang et al. 2011) as well as *Escherichia coli* (Lim et al. 2011). The multidrug-resistant strains of malarial parasite, *Plasmodium falciparum*, are one of the reasons for the most number of death occurrence in the world. The drug artemisinin obtained from *Artemisia annua* is produced in very less concentration. To increase the concentration of artemisinin, Ro et al. (2006) successfully demonstrated the scale-up production of artemisinic acid using yeast as a heterologous system. The high compartmentalization of plant system often limits the production of metabolite production. To overcome this, Mirza et al. (2016) engineered genes responsible for glucoraphanin synthesis in *E. coli* by expressing genes involved in methionine chain elongation part of glucoraphanin pathway.

2.4.3.2 Metabolic Engineering Using Plants and Plant Cell Cultures

Plant cells are used to produce metabolites, which otherwise cannot be produced in the heterologous systems. Because of the low concentration of metabolites in heterologous systems, transgenic plants are being generated for the specific metabolite synthesis. The use of engineering target genes in plants has significantly increased the production of the metabolites. The entire pathway genes involved in synthesis of cyanogenic glycosides, dhurrin, have been successfully transferred from *Sorghum bicolor* to *A. thaliana*. The transgenic *A. thaliana* plants stored large amounts of dhurrin and showed resistance to herbivore, *Phyllotreta nemorum* (Tattersall et al. 2001). Glucosinolates are a class of specialized metabolites restricted to the Brassicaceae family (cabbage, broccoli, cauliflower, mustard, etc.). These glucosinolates are ascribed to function as anticancer functional foods. The transfer of genes involved in one of the glucosinolates, glucoraphanin, into *Nicotiana benthamiana* plants scaled up the production of glucoraphanin up to considerable amounts (Mikkelsen et al. 2010). Further improvements in the production of the glucoraphanin were carried out recently by the co-expression of two other genes (large subunit of the heterodimeric isopropylmalate isomerase and bile acid transporter 5) (Crocoll et al. 2016). Paclitaxel, which is an important anticancer compound, is originally obtained from *Taxus brevifolia* (Vongpaseuth et al. 2007). There have been efforts to enhance production through plant cell cultures which has resulted in sustainable consumption-production patterns of medicinal plants and avoiding the overharvesting of medicinal plants. Paclitaxel is produced through a complex biosynthetic pathway; recently as many as 19 putative steps within its biosynthesis pathway have been fully characterized (Huang et al. 2001; Croteau et al. 2006; Nims et al. 2006; Vongpaseuth et al. 2007; Meng et al. 2011; Lenka et al. 2012). Plants are also being used as natural chemical factories for the production of unnatural derivatives of metabolites with varying specificities or improved medicinal properties. Reengineering of the plants with strictosidine synthase having altered substrate specificity and cocultivation with commercially available precursors produced modified monoterpene indole alkaloids (Katherine and Bradley 2009; Runguphan and O'Connor 2009; Runguphan et al. 2010).

2.5 Omics, Drug Discovery and Models (In Vitro and In Vivo)

The medicinal plants have an immense potential for treating a plethora of not only human diseases but also other animals'. There are plants that act as insecticides, and then there are plants that act as deterrence to herbivores and possess antimicrobial activity against a plethora of harmful microorganisms. The pharmaceutical industry has been searching for ways to make use of omics approaches for the drug discovery to cut down the time required as compared to the conventional technologies (Pelkonen et al. 2012). Certain models are being developed for testing the effectiveness of these plant-derived drugs. Using omics approaches to these models helps in ascertaining the targets of such drugs and their possible mechanisms of action. To investigate the bioactivity of natural products against the myotonic dystrophy type I [DM1], human cell model and mouse model were used. Several alkaloids of natural origin such as β-carboline harmine and the isoquinoline berberine were found to cure certain aspects of the human DM1 in myoblast cell line. The myoblast cell lines used contained a CTG1300 repeat in the 3′ UTR of the DMPK (dystrophia myotonica-protein kinase) gene (Herrendorff et al. 2016). To investigate the antidiabetic role of *Carica papaya* leaf extract, streptozotocin-induced diabetic Wistar rats were used. The experimental diabetes was induced by streptozotocin. To confirm the hyperglycaemia, the tail vein blood glucose was measured with an Accu-Chek Sensor Comfort glucometer (Juárez-Rojop et al. 2014). To develop the cell lines for testing the phytochemicals from atherosclerosis, Orekhov and Ivanova (2016) used cultured human aortic cells for testing the anti-atherosclerotic potential of a number of natural plant products. Important metabolites lodged in the plants and their importance for human health require the integration of approaches involving traditional knowledge with the modern high-throughput approaches especially the omics approaches (Figs. 2.1 and 2.2).

2.6 Conclusions and Future Prospects

The plants and their products are tremendously being used throughout the world since time immemorial, and these plants will continue to support the humans and better their health. The climate change poses serious threat not only to the survival of important medicinal plants, but it also impacts the spread and development of human diseases. The plants need to be screened for their valuable compounds, and their sustainable harvesting along with conservation steps needs to be strengthened. New strategies are needed to preserve the plants growing at high altitudes and the extreme environments. The models for testing these drugs before applying it to the humans are of considerable interest in the present scenario. Traditional knowledge and modern approaches need to be integrated for bioprospecting the new medicinal plants. Common people involved in the bioprospecting or conservation must be fairly and equitably compensated for the benefits arising out of the utilization of the

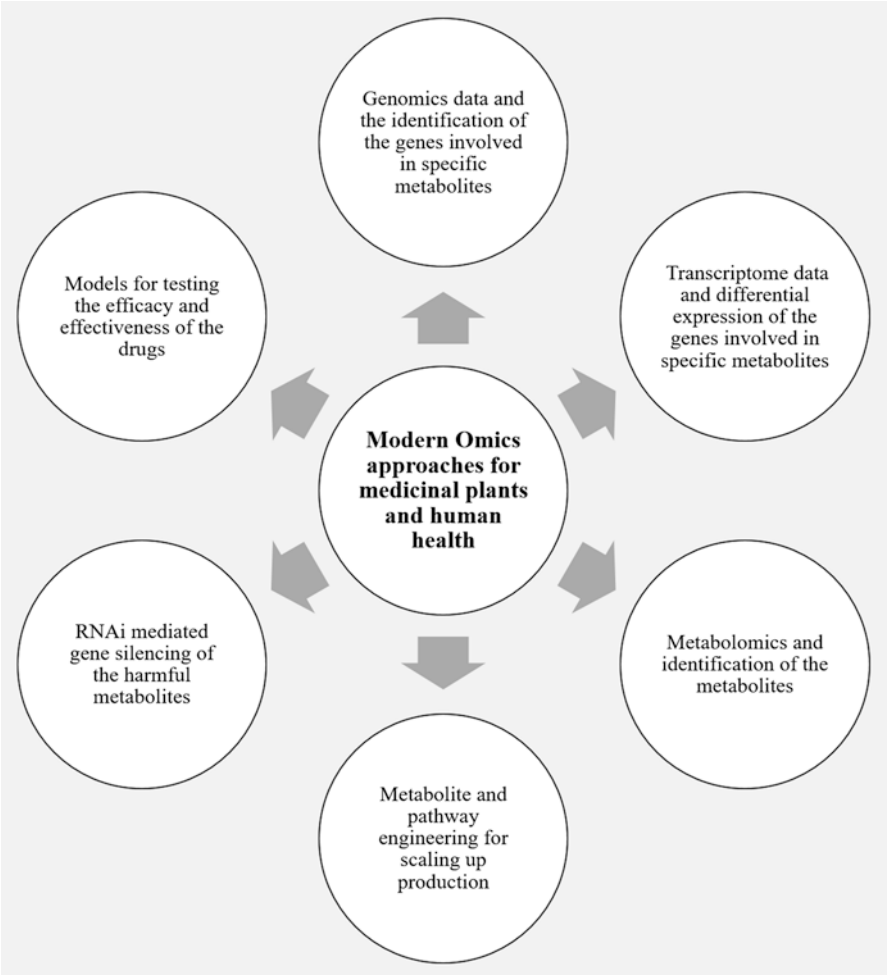

Fig. 2.1 Modern approaches for research in medicinal plants and human health

genetic resources. Overharvesting of natural medicinal plants is an important challenge during the present century, which leads to loss of species. This necessitates the integration of interdisciplinary approaches to ensure a sustainable management of valuable plant resources. Approaches such as traditional knowledge, folklore and zoopharmacognosy need to be employed for sustainable harvesting and optimum usage of medicinal plants. The future of medicinal plants for human health lies in the application of multidisciplinary approaches right from basics of biology, ethnobotany and conservation biology to the modern omics.

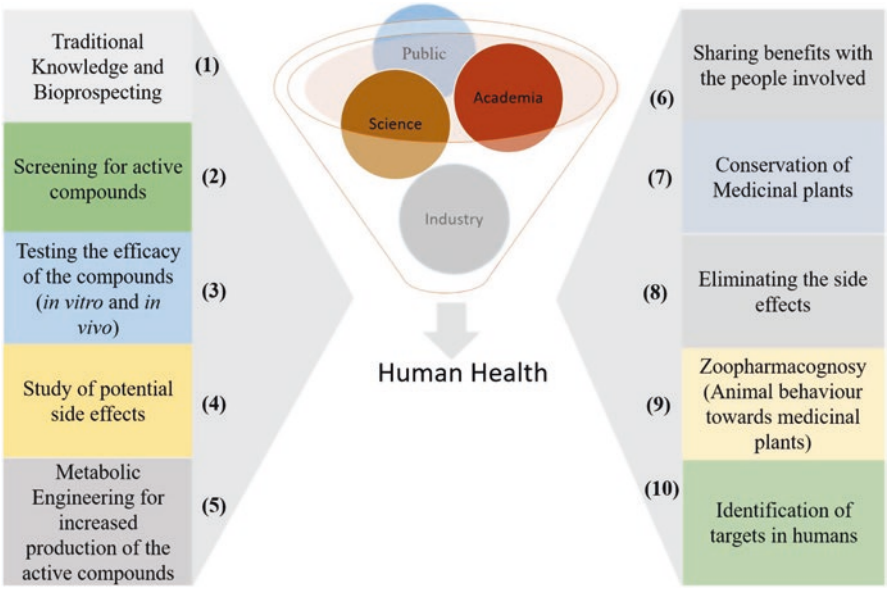

Fig. 2.2 Future of the medicinal plants and the human health

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3 Application of Biotechnology in Producing Plant Bio-active Compounds

Glaucia C. Pereira

Contents

Abstract

In the new global economy, technological advancement, increasing population density, ageing and widely reported sanitary problems intensified by social underdevelopment have justified an increasing need for sustainable and effective measures to alleviate some of the most common burdens the society has suffered from, as broadly reported by the World Health Organization (WHO). The global health has increasingly become a central issue in promoting individual wellbeing, which directly affects collective progress. Over the past decade, pharmaceutical companies have massively invested in new technologies, aiming at discovering new chemicals and progressing knowledge in synthetic compounds and in high-throughput workflows. In plant science, high-throughput screening (HTS) strongly supports drug discovery by accelerating the screening of biologically

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diverse samples, which at its very basic stages involves the isolation of biota samples and the determination of the structure of the underlying bio-actives. The resulting automation chain brings about fast-paced effective production of medicinal molecules, excelling in performance conventional approaches. Nevertheless, HTS is one single example of how technology has positively contributed to drug discovery. Since the beginning of civilisation, medicinal plants have offered the fundamental means to humankind for fighting diseases. In the current literature, it has been reported that more than 200,000 plant derivatives, among which key natural products, are being used in therapies for treating severe health conditions such as congestive heart failure and cardiac arrhythmias. However, production yield and compounds toxicity are still among the fundamental barriers of compounds production and drug discovery. Gene editing is another fruitful example of biotechnology-driven production of compounds. A growing body of literature has reported on CRISPR-Cas 9 modifying gene expression, whose phenotype is fundamental in modulating the biosynthesis of bio-active compounds. Ultimately, artificial intelligence (AI) is arriving strongly, potentially to stay, and impacting the pharmaceutical sector. Several consortiums have been formed and companies founded, over the last few years, with the purpose of applying AI technology in molecular design, drug screening and genotype-phenotype analysis for predicting drug activity in genetically engineered species. However, we have constantly argued on the need for increasing efforts towards improving plant metabolites production, via biotechnological resources, mainly recombinant-DNA technology: first, because certain compounds are scarce in nature, and second, because new bio-actives could bring about genetically engineered plant cells. Hence, the aim of this chapter is to review recent progress in the production of plant bio-active compounds promoted by biotechnological advancement.

Keywords
CRISPR-Cas9 · Drug discovery · Synthetic biology · Gene editing · Genomics · Omics · Plant bio-active compounds · Artificial intelligence · High-throughput screening

3.1 Introduction

Evidences suggest that plants are among the most important current sources of medicinal compounds. Plant bio-actives have been a rich source of successful therapies (Guerrero et al. 2002; Atanasov et al. 2015; Rawat et al. 2016; Domínguez and González Muñoz 2017; Sobhani et al. 2017). The structural diversity found in plant metabolites is much wider than that of standard combinatorial chemistry. However, both producing plant compounds' yield in large scale (Dhanani et al. 2017; Xu et al. 2018) and exploring the vast uncharted sources of medicinal compounds (Hunt and Vincent 2006; Vo and Kim 2010) for drug discovery are still challenging. There is a growing body of literature that recognises the importance of genetic engineering, in

drug discovery and compound production (Key et al. 2008; Elfahmi et al. 2014). First, because certain compounds are scarce in nature and the application of genetic engineering together with synthetic biology could result in increasing production yield. Second, because new bio-actives could bring about genetically engineered plant cells. In times of fast-paced technological progress, among the major challenges in production of plant bio-actives are limitations on yield due to extraction mode, pollutant residuals and toxicity, costs and complexity of existing isolation and compound characterisation techniques, design of high-throughput pipelines, advancement in compound characterisation libraries, and sustainable harvesting of natural resources.

Data from several studies suggest that genetic engineering could provide the means for enhancing production by creating plant cells' variants, showing a combination of desirable traits (Liang et al. 2015; Barrangou and Doudna 2016; Yang et al. 2017; Song and Palmiter 2018; Xin et al. 2018). Indeed, gene editing and recombination, united with the definition of molecular markers to screen and design allele-based germplasm, should bring about novel compounds, whose design matches pharmacological needs. Artificial intelligence would specialise the process by providing a simulation environment for genotype-phenotype design and testing, anticipating the implementation of laboratorial assays (Pereira 2017). This might provide an extra step, for quality control, resulting in stable phenotypic populations, in a well-regulated cost-effective production workflow. Omics play a crucial role, providing the knowledge on metabolites characterisation, proteins activity and other responses used to construct compound characterisation libraries. Once more, artificial intelligence methods play a fundamental role in genome sequencing, sequence-structure functionality matching, supporting omics databases construction and test and strengthening the existing screening approaches. Therefore, this chapter examines the relationship between new technologies and challenges in producing plant bio-actives, addresses the role of high throughput pipelines; and highlights the relevance of both genetic engineering and synthetic biology, in drug discovery and compounds' yields enhancement. Overall, the challenge seems to be still related to finding a compromise between existing modes of production, adding the right element of innovation.

3.2 Coping with Pharmaceutical Challenges in Plant Bio-active Production, in the Era of Biotechnology

3.2.1 Underlying Challenges in Sustainable Enhancement of Plant Bio-active Production

3.2.1.1 Conventional Modes of Production and Their Drawbacks: Extraction

A considerable amount of literature has been published on standard modes of extraction of medicinally active plant compounds, relaying mostly on usage of selective solvents (Trusheva et al. 2007; Sasidharan et al. 2011; Domínguez and González Muñoz 2017; Soquetta et al. 2018). Numerous technique-based strengths were, to date, pointed out, e.g. use of ethanolic and hydroalcoholic derivatives

resulting in high extraction yield from *Psidium guajava* and enhancing variability of phytoconstituents (Arya et al. 2012). However, limitations on the resulting yield have significantly challenged research in the field (Dhanani et al. 2017; Zhang et al. 2018a). As a rule of thumb, the major purpose of any extraction method is to separate soluble plant metabolites from residue, potentially reducing the need for post-extraction purification, which is occasionally complex and time consuming. The resulting mixture may include alkaloids, glycosides, phenolics, terpenoids and flavonoids. There have been several investigations on the causes of potentially reducing volume of the above-mentioned active agents, and evidences have pointed out to solvent types significantly influencing production, in methods like maceration, microwave-assisted extraction (MAE), sonication and accelerated solvent extraction (ASE) (Spigno et al. 2007; Do et al. 2014; Dvorackova et al. 2015; Dhanani et al. 2017). Arguably, those studies also indicated that solvent's volume might have no or little influence in the process, regarding final yield (Trusheva et al. 2007).

It has been reported that maceration is among the simplest and more cost-effective extraction methods. However, the process has the disadvantage of generating a significant amount of pollutant chemical residue. A common approach for mitigating the problem consists in adjusting temperature and solvent type, to reduce the volume of solvent needed and, consequently, the resulting amount of pollutants. Soxhlet is another technique that requires reduced volumes of solvent. Nevertheless, both toxic emissions and the handling of hazardous fluidised organic solvents make the process disadvantageous and less popular. Furthermore, the process is not cost effective, because of the required high purity of the chosen solvents, with the additional inconvenience of being highly sensitive to temperature and solvent-sample ratio. Methods like MAE and Sonication enhance the solvents' activity by exposing the solutes' surface. In MAE, microwaves promote surface material polarisation, stimulating conductive heat transfer throughout the solutes' surface. Simultaneously, hydrogen bonds are broken. This facilitates the solvents' action (Xu et al. 2018). In Sonication, the utilisation of ultrasound ranging from 20 to 2000 kHz results in acoustic cavitation, enhancing solutes' surface permeability. Hence, mechanically induced mass transport promotes optimal phytochemical extraction (Domínguez and González Muñoz 2017). The main constraint in microwave-assisted extraction, however, is its limited applicability to small-molecule phenolic compounds, e.g. phenolic acids, because these molecules remain stable at a microwave-induced temperature of about 100 °C, for solvents activity time reaching nearly 20 min. Alike, numerous other molecules like *anthocyanins* tend to degrade at high temperatures. Sonification suffers from a different drawback, phytochemicals' altered activity, due to formation of free radicals (Handa 2008).

Plethora of extraction methods exists, e.g. solid-phase micro-extraction, supercritical fluid extraction and pressurised liquid extraction. These methods improve the extraction and analysis of plant medicinal compounds, raising extraction efficiency and selectivity, by both reducing organic solvent consumption and sample degradation and eliminating additional pre-chromatographic steps (Huie 2002). However, the most critical issues discussed above persist, and finding a sustainable way to optimise plant compounds' yield remains work in progress.

3.2.1.2 Conventional Modes of Production and Their Drawbacks: Isolation and Characterisation

In producing plant-derived bio-active compounds, isolation, characterisation and recombination are fundamental. The molecular diversity in plant is vast, and often, plant extracts also comprise of phytochemicals with different polarities (Fig. 3.1). The extracted compounds are commonly purified via chromatographic techniques and characterised, to derive purpose-based mixtures, at the right proportions. Isolation and purification separate toxic constituents from those with therapeutic effect. Hence, it could conceivably be stated that assays are designed for combining therapeutic compounds showing coactive functionalities, in a reproducible and accurate manner. Phytochemicals' separation might be laborious, entailing different techniques for handling specific molecules (Handa 2008; Sasidharan et al. 2011), e.g. immunoassay, phytochemical screening assay, Fourier-transform infrared spectroscopy, high-performance liquid chromatography, Sephadex chromatography, thin-layer chromatography, column chromatography and flash chromatography. The resulting bio-actives are screened for structure and activity determination, often relying on compound characterisation libraries, for structure-functionality matching.

Number of natural products	
With nitrogen	
•Alkaloids (1)	12,000
•Non-protein amino acids (2)	700
•Amines (3)	100
•Cyanogenic glycosides (4)	60
•Glucosinolates (5)	100
•Alkamides	150
Without nitrogen	
•Monoterpenes (incl. Iridoids) (6)	2,500
•Sesquiterpenes (7)	5,000
•Diterpenes (8)	2,500
•Triterpenes, Saponins, Steroids (9)	5,000
•Tetraterpenes	500
•Phenylpropanoids, coumarins, lignans	2,000
•Flavonoids (10)	4,000
•Polyacetylenes, fatty acids, waxes (11)	1,000
•Polyketides (12)	750
•Carbohydrates	>200

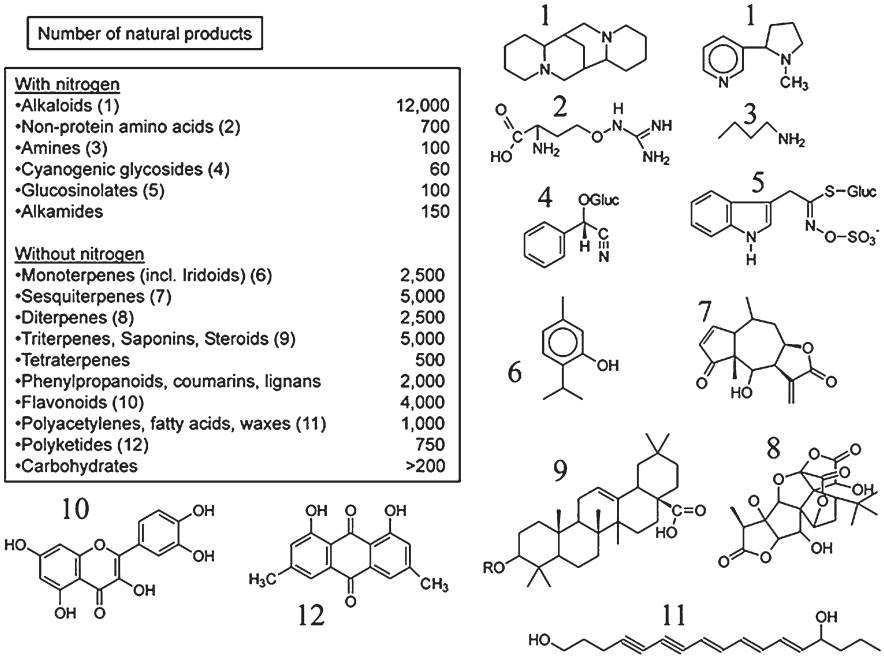

Fig. 3.1 Structural diversity of plant secondary metabolites (Extracted from Wink 2003, with copyright permits)

Among the formerly cited separation methods, high-performance liquid chromatography (HPLC) and gas chromatography (GC) are the most commonly used ones. High-specificity, high-sensitivity gas chromatography is broadly applied to analyse volatile constituents, enabling rapid separation of multiple volatile compounds, due to the high selectivity of the capillary columns. However, mass spectrometry-based variations of the method are still restrictive when applied to the separation of highly polar compounds (Groneman et al. 1984; Iwasaki et al. 2012), because these plant constituents degrade at high temperatures. HPLC is a robust phytochemical and analytic chemistry technique used to identify, quantify and purify low-yield combined bio-actives. Although a body of research might criticise its cost and speed, the technique is still suitable for high-throughput production methods. In HPLC, compounds with different migration rates are easily separated in particular columns, and separation rate is controlled via the synergetic combination of both the stationary and the mobile phases (Waksmundzka-Hajnos and Sherma 2010; Zhang et al. 2018b).

Regarding compound characterisation, high-throughput screening (HTS) for bio-active components relies heavily on the quality of compound characterisation libraries. These registers may contain either crude or semi-purified extracts, pure bio-active-based chemicals and their combination. While broadly used, crude libraries suffer from the complexity of bio-active constituents' identification, which is directly proportional to the chemical complexity of the extracts. Semi-purified extract libraries, under the form of partially fractionated mixtures, deriving from multichannel preparative methods like the HPLC, reduce complexity, by containing less compounds per fraction. Additionally, this increases the compounds' concentration in a mixture, facilitating functional analysis, if compared with crude libraries handling. Furthermore, as indicated above, HPLC systems are ideal for HTS, because in a pre-fractioned library context, compound characterisation requires fewer separation steps, significantly speeding plants bio-active discovery and production. Ultimately, construction and characterisation of a pre-fractioned library can be semiautomated, if HPLC hyphenated systems are in place, providing that multiple analytical detectors and computer-assisted data processing are used (Eldridge et al. 2002; Wagenaar 2008). The main inconvenient in using pre-fractioned libraries results from increasing library size during splitting, for a fixed number of extracts. Besides the drawbacks, in the last decade, highly specialised assays for profiling, isolation and structure elucidation of bio-actives have provided the community with highly purified natural product libraries, achieving purity levels beyond 80% (Bindseil et al. 2001). Libraries based on pure bio-active chemicals and their combination require a reduced hit detection process to that of synthetic libraries. On the top of that, crude extract-based registers offer much richer structural diversity, because in pure bio-active libraries, trace components are removed during separation. Hence, the challenge in compound characterisation and library construction might be associated with finding a compromise between all the above strategies.

3.2.2 Exploring New Resources in Drug Discovery and Finding Alternatives for Endangered Phyto Species: The Marine Ecosystem

3.2.2.1 Reported Challenges in Harvesting Unexplored Marine Microenvironments and the Potential for Plant Bio-active Discovery

There have been several investigations on the characterisation of marine microenvironments and their potential for drug discovery (Smit 2004; Anantharaman et al. 2009; Demunshi and Chugh 2010; Thomas et al. 2010; Vo and Kim 2010; Waters et al. 2010; Pereira and Costa-Lotufo 2012). As indicated in both Pereira and Costa-Lotufo (2012) and Tittensor et al. (2010), the vast majority of the sea ecosystem is unexplored, while accounting for c. 70% of the earth surface. This means that plethora of chemical compounds potentially leading to novel therapies might be hidden from the human eyes. An experimental demonstration of this was carried out by Rinehart et al. (1990). In this piece of work, the authors reported on several secondary metabolites produced by a sea squirt, which were proven to be prominent antitumour agents. Additionally, Smit (2004) reviewed the state of the art in marine algae metabolites-derived products with pharmacological effects, discussing drawbacks in harvesting unexplored micro-systems, searching for novel compounds. Conceivably, marine drug discovery shares many limitations with pharmaceutical innovation based on other sources of chemical compounds. Indeed, the production of new marine bio-active deriving from the plant kingdom suffers from the poor resolution of certain isolation and structural elucidation techniques. On the top of it, the sustainability of the entire process, from resources harvesting to drug production, still remains both crucial and controversial, in marine drug innovation (Pereira and Costa-Lotufo 2012).

To exemplify the referred controversy, to date, little evidence has been found associating exploitation of marine natural resources and conservation threatening. Indeed, the authors state that (i) marine harvesting occurs at low dimensions, with very few sample organisms extracted, per species; (ii) production does not commonly rely upon small populations; and (iii) following characterisation, the development of novel compounds might not require further recollections. We might not further agree with the last statement. However, the underlying drawback might be relieved by synthetic biology approaches, once the new compounds are discovered. Indeed, this would also alleviate the pressure on environmental policies, on rare or restrictedly distributed species, and provide – in certain cases – alternative solutions for production scalability, in terms of yield. Furthermore, the existing compulsory collection protocols and policies on environmental impact assessment are driving the way in the sustainable extraction of marine products. However, whether all this is diligently applied in practice may remain to be seen. Indeed, the matter might rely upon the means local authorities have in place for enforcing the existing regulations. Finally, over the last years, literature has emerged that offers vast material and discussion on viability-based technical requirements for exploiting the marine environment. One example is found in (OD and IO 2016), where discussions on the impact of marine exploitation on the

local ecosystem are combined with the description of specialised diving techniques and infrastructure needed for successfully exploring hostile hard-access natural environments. Overall, diligent resources harvesting made possible via new technologies might revitalise plant science, providing the scientific community with vast material for drug discovery. Combined with synthetic biology-based production, these new sources of bio-actives might extenuate the pressure for increasing production yield, in the highly demanding pharmaceutical industry.

3.3 Technological Innovation Promoting Progress in Biomedicine

3.3.1 Genetically Engineered Species and Computer-Assisted Pharmacology

3.3.1.1 Genetically Engineered Plant Cells, CRISPR-Cas9 and Omics Encouraging Biotechnological Transformation and Increasing the Need for Proper Governance

Comparable with the food industry, the pharmaceutical sector has increasingly become more demanding, with regard to sustainable alternatives for low production yield, endangered species-based production and lacking high-throughput pipelines. Genetically engineered species are in focus (Badenes et al. 2016; Lee et al. 2016; Osakabe et al. 2016; Gascuel et al. 2017; Ishii and Araki 2017; Chen et al. 2018). It is not only the need but the availability of technological resources for achieving the end, which promotes biotechnological transformation. From guided-gene-programmed knockout, via CRISPR-Cas9 technology and cre-loxp-based site-specific recombinant DNA (Hochrein et al. 2018; Kopertekh et al. 2018; Song and Palmiter 2018; Xin et al. 2018), to de novo mutations' insights-based gene design, metabolomics, transcriptome and general synthetic biology methodologies have increasingly appearing in basic research (Belhaj et al. 2015; Abbai et al. 2017), a plethora of studies with high potential to migrate towards both translational research and clinics (Rai et al. 2017). However, many scholars hold the view that genetically engineered species might represent a sanitary risk. Since the introduction of the first genetically modified product, in the United States market, the Flavr Savr tomato (*Solanum lycopersicum*); several countries have adopted genetically engineering driven crop enhancement approaches. According to Ishii and Araki (2017), the leading nations in the industry are the United States, Brazil, Argentina, India, Canada, China and Paraguay, while the most conservative markets are in Japan, Russian Federation, Republic of Korea, New Zealand and parts of the European Union – except Romania, Spain, Slovakia, Portugal and the Czech Republic. As indicated above, not limited to the food industry, major concerns are about potential adverse effects on the environment and human health, resulting from non-reported phenotypic changes in transgenes (Ishii and Araki 2017). However, in well-controlled regulated scenery, the prominence of genetically engineered species might leverage critical challenges in medicinal compound production.

Phenotypic plant breeding is the conventional approach, selecting plant germplasm with desirable characteristics from a population of species created by using crosses and mutagenesis. This approach is entirely based on yield and other phenotypic characteristics and does not directly assess the corresponding genotype aiming at designing cell lines with the intended characteristic, prior to migrating to production environment. Selecting the right profile can be both time and resources intensive, relying on cultivation of potentially large populations to be phenotyped, harvested and profiled.

Genome editing (e.g., CRISPR-Cas9 systems) relies upon creating DNA double-strand breaks (DSBs) at target sites. This is followed by an exogenous gene insert or a copy variant based on homology-directed repair (HDR). The resulting insertions and deletions (indels), with or without a DNA template, are often used for either silencing genes or, more generally, to reprogram the genome. If well designed, e.g. using an AI-based simulation environment, these systems can both build new compound variants via engineered plant cells technology and trigger yield enhancement, for the existing ones, at genotypic level, mitigating the main issues associated with phenotypic breeding and the inherent lack of pre-production design. The benefits are already observed in the food industry (Hartung and Schiemann 2014; Kanchiswamy et al. 2015), which is a strong indicative of success in biomedicine. Latest results have shown promising perspectives associated with genome editing-mediated breeding resulting in plant gems that are considered transgene-free. Once more, the main concern is on both how the resulting mode of production will be governed, if the resulting species fall outside existing regulations and non-target phenotypic effects (Kosicki et al. 2018), in genetically engineered species. In this sense, the key for assuring allelic stability may lie on both knowledge gained via the assessment of *de novo* mutations and the analysis of a set of transcripts present in a certain cell, organism or population and how the targeted transcriptome varies with environmental conditions, on the basis of gene expression triggered at a given time. Regarding governance, Brazil, being in 2012 the second major producer of genetically modified organisms (GMO)-based crops, can also be considered a pioneer in regulatory law in the field. The country had c. 30 million hectares occupied by GMOs, while the first world producer the United States dedicated 69 million hectares to GMO production. In general lines, Brazil's Biosafety Law No. 11105 of 24 March 2005 introduced the principles followed in biotechnology research, in the country, regulating ethics and defining safety standards involving GMOs and their derivatives (Subchefia para Assuntos Jurídicos 2005).

Characterising biosynthetic pathways of plant metabolites might be limited by insufficient insights on the underlying genotype-phenotype relations and on how these relations might trigger adjacent signalling networks. Regarding methodology, while short-term studies do not necessarily show significant changes in the current approaches, high-resolution high-throughput methods are a critical part of the story (Kellenberger et al. 2011; Macarron et al. 2011; Wetzel et al. 2011; Ymele-Leki et al. 2012; Eggert 2013; Schenone et al. 2013; Harvey et al. 2015). Figure 3.2 illustrates two different chemical screening approaches, highlighted as a description of a fundamental part of high-throughput pipelines. Enriching the indicated approaches

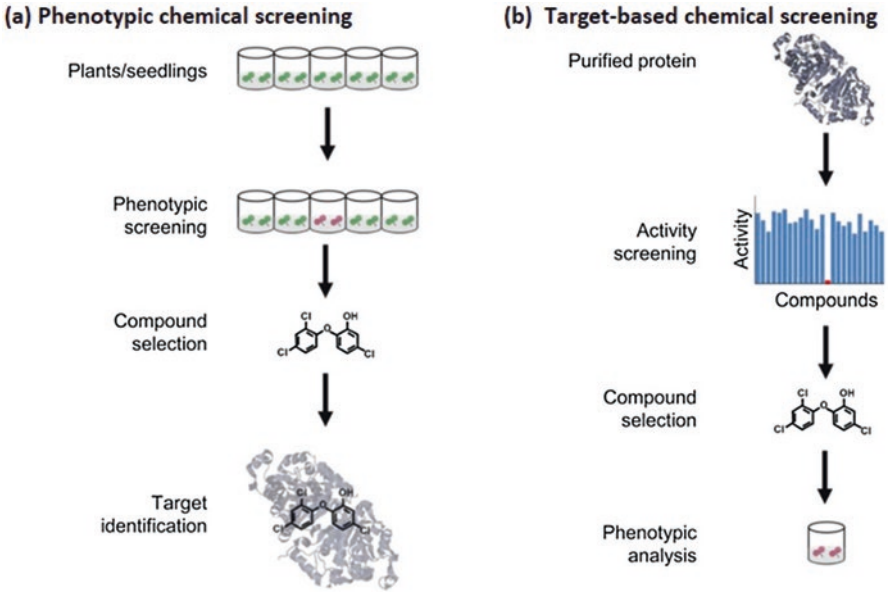

Fig. 3.2 Comparison of forward and reverse chemical screening. **(a)** The goal of phenotypic or forward chemical screening is to identify from an arrayed library of chemicals, a (selective) bioactive compound causing a phenotypic alteration, usually in a microplate format. Once a selective compound is found, the molecular target is identified, either by a genetic approach or some type of biochemical purification strategy. **(b)** The goal of target-based or reverse chemical screening is to identify a compound that modulates the activity of a selected protein. Subsequently, the chemical is used to determine the phenotypic consequences when applied to plants (Extracted from Serrano et al. 2015, with copyright permits)

by introducing state-of-the-art omics might result in decreasing costs and increasing performance. Therefore, benefitting from diverse specialised plant metabolites with important pharmacological properties might require exploring high-throughput large-scale analytical methods, unveiling key metabolic biosynthetic pathways.

Mapping metabolic responses rely upon tracing proteins activity (proteomics) and how small-molecule metabolites' characterisation may orchestrate the process (metabolomics) and vice-versa. The combined knowledge might support generating unique chemical fingerprints for specific cellular processes, resulting in the definition of relevant biomarkers characterising disease progression and, ultimately, defining optimal drug's composition, to be sustainably replicated in the synthetic biology domain (Kumar et al. 2014; Wishart 2016; Cuperlovic-Culf and Culf 2016). We believe that the future of biotechnology consists in combining conventional methods, varied omics strategies and marker-assisted selection (MAS). Understanding the DNA and the use of molecular markers to screen and design allele-based germplasm might provide stable phenotypic populations, via well-regulated cost-effective modes of production.

3.3.1.2 Disruptive Artificial Intelligence (AI) Technology: What Is It All About and How Can AI Enrich the Existing Modes of Plant Bio-active Production?

It is no novelty to see artificial intelligence (AI) methodologies highlighted in the news. The games industry is benefitting from deep learning, adversarial networks and reinforcement learning, used in algorithms forming the basis of platforms like the Google AlphaGo (DeepMind) (Gibney 2016) and the IBM's Watson embedded machines simulating a Jeopardy competitor. We argue on the criteria setting the measure of success. It is no rocket science to set standards when either no exact solution is given or no good techniques are implemented, so far, for dealing with precisely the same problem definition nor are benchmarks set for performance check. With the growing popularity of AI models, it is becoming widespread to base success on comparability with human performance – e.g. AlphaGo technology has bet the world champion in the game. In other medias, the author discussed the intrinsic power of AI, stressing the fact that all the novelty and the rapid increase in its applications are founded on mathematical concepts dating from the fourteenth to the seventeenth centuries (Press 2016), when conceptual correlation and combinatorics were idealised. More importantly, the foundations of the method of Archimedes for deriving the volume of different geometric structures via correlation could represent the first insights towards problem decomposition and complexity reduction.

Archimedes decomposed the initially n-dimensional problem into subproblems, which were less complex in nature, with the volume of the resulting figures being potentially known. We can associate the idea with the 'divide and conquer' paradigm, in its very basic premises, being broadly applied in efficient recursive algorithms and inductive rule learning processes. "*Since its foundation, the AI paradigm has grown and matured and are nowadays intrinsically immersed in almost all the corners of disruptive technology. AI methods are transforming the way we generate, analyse and use data in insights discovery, automatically searching for relevant patterns on which to support both learning – e.g. in machine learning – and knowledge generation in natural language processing (NLP). Voice analysis, intelligent searches and time series generation have projected the cognitive automation industry. Smart assistants, designed to follow voice commands, perform searches, respond to queries and make recommendations, are built on a hard core of AI algorithms, which, in the state of the art, connect patterns learned, award successful decisions and classify inputs to control behaviour – reinforcement learning.*" (https://www.t-impact.com/ai-in-the-age-of-digital-transformation-artificial-recall-driving-digital-transformation/)

In biomedicine, the figures could not be more promising. The IBM Watson Platform is being applied by Pfizer, searching for immuno-oncology drugs (Japsen 2016). Genentech (Roche) adopted GNS Healthcare AI system in oncological drug discovery (GNS Healthcare 2017). Another story of success started in the 1990s, with text to voice and gesture to voice synthesisers, which have supported several individuals, around the world, who have suffered from degenerative diseases compromising neuromotor and speech capabilities (Hubbard et al. 2009) – e.g. Stephen Hawking. Over the last decades, consortiums have formed, and companies founded

on the basis of AI technology applied in molecular design, drug screening and genotype-phenotype analysis, for predicting drug activity in genetically engineered species, e.g. Atomwise, Sirenas and Bristol-Myers Squibb and Engine Biosciences.

Adding to the above-mentioned efforts, in 2018, the author developed a predictive model based on gradient boost for improving logistics for the NHS Blood and Transplant (UK). The goal was to improve logistics in organ donation, increasing the number of successful transplantations. Allocating the limited number of specialised teams for retrieving donated organs is a challenge, because, to the best of our knowledge, to date, there is no consistently accurate and reproducible way of determining terminal donor's asystole time leading to donation – clinical death. This implies no precise metrics for deciding on how to allocate both the available professionals and the NHS infrastructure, during organ donation process. Asystole time is also a key measure of success in organ transplantation, because under reduced supply of nutrients and oxygen, long wait times for organ retrieval could result in tissue dysfunctionality, compromising the health of the donated organ, which would directly affect the recipient. Therefore, the accurate determination of asystole time also determines whether a donated organ would be given to a recipient. The model developed by the author was trained, tested and validated on both donor data (subjected to the NHS Information Governance approval – c. 11,000 records) and synthetic data created to mimic the discovered patterns. The tuned model showed high accuracy and generalisation capability, reaching 84% accuracy during test and about 75% accuracy during validation, without accounting for arrest variables, and c. 88–83% accuracy when arrest variables were included. The question on whether 88% accuracy is an acceptable threshold for a predictive algorithm used in biomedical automation stands still. Indeed, assessing the performance of specialised teams currently allocating retrieval teams manually would give a good insight on the capability of the developed AI model. Very recently, in June 2018, a group of Chinese researchers reported on the performance of an AI system, the BioMind AI, compared with tumour diagnosis made by 15 senior physicians. In brief, the AI platform achieved 87% accuracy during validation, compared with 66% accuracy in physicians' diagnosis (Yan 2018). This is not an isolated case. Pragmatically, AI models have performed well in different clinical domains – e.g. cardiovascular (Strickland 2017; Hutson 2017), oncological (Galeon and Houser 2016) and in general practitioners general assessments (Olson 2017). However, the question posed here is 'how can AI enrich the existing modes of plant bio-active production'. Plethora of options are found in the current literature (Fleming 2018; Scudellari 2018). The AI capabilities described above can empower natural organism libraries (NOLs), general omics databases and varied nonstructured data sources – e.g. text found on Internet – providing high-speed automatic insights generation and data mining based on data analysis (Mears et al. 2017), database validation and error elimination based on compound characteristics matching and compound disease matching in drug discovery and biomarker characterisation. It is broadly disseminated that artificial intelligence techniques rely upon massive data for excelling in performance. However, while supervised learning commonly works on the bases of massive

labelled records, the AI domain is populated with state-of-the-art techniques for knowledge generation based on limited and noisy data sets, combining exploration of unknown insights and exploitation of, occasionally, limited data-based background. On top of that, adversarial networks are rising, introducing in the AI domain powerful knowledge generation capabilities, allowing paired systems to learn via mutual interaction and adversarial knowledge generation. All that, translated into better usage of available information and insights discovery, can enhance the efficacy of existing compounds profiling methodologies and high-throughput screening, in production of plant bio-actives. Figure 3.3 illustrates how the NOL hosted by the Bioinformatics Institute of Singapore (BII) is formed via four different screening approaches. In that, AI technology may be a key contributor in genome sequencing; in improving omics databases, as suggested above; in both extracts and compounds bioassays testing; and in elucidating molecular structures, strengthening all the existing screening and compound characterisation approaches.

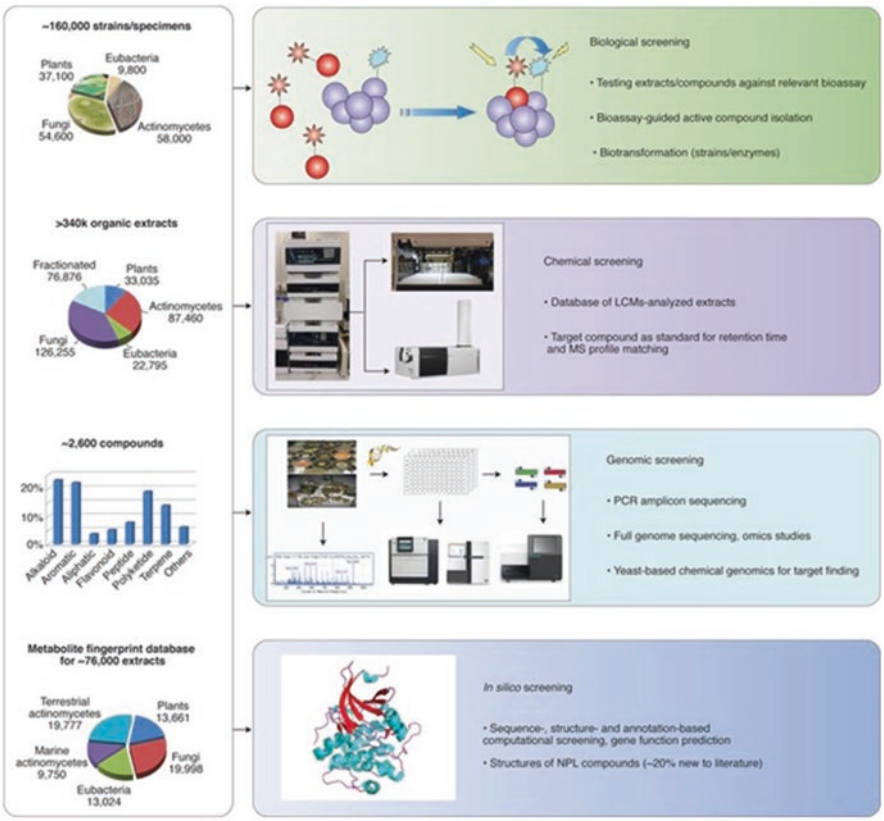

Fig. 3.3 Composition of NOL at BII and its exploitation using four screening approaches (Extracted from Ng et al. 2018, with copyright permits)

Simple AI techniques are being actively used to create sophisticated bioinformatics, contributing with progress in gene expression methodologies, in large scale. This helps elucidating biomolecular mechanism-driven responses to genotype-phenotype pairs; enriching genomics, metabolomics and proteomics studies; and shedding light to key signalling pathways associated with overcoming low yield in production of plant secondary metabolites. Finally, AI can also enrich genome reprogramming methodologies, providing a simulation environment for pre-laboratorial design and test (Pereira 2017). Recently, it was reported in the literature undesired changes in DNA strands, resulting from CRISPR-Cas9 editing (Kosicki et al. 2018; Ledford 2018), because the deletions created by the Cas9 enzyme are not fully repaired, due to faults in the DNA repair mechanism. As other members of the scientific community, the author shares the view that this should not invalidate the technique but promote better mechanisms for accessing the quality and viability of the resulting genotype-phenotype pairs. Again, AI might strongly support this process, as a pre-laboratorial step, forecasting the probability of success, reducing experimental costs associated with undesirable potentially unstable allele-based germplasm (Pereira 2017), on one hand. On the other hand, further quality assessment should be in place, as to assure that the designed genotype-phenotype pair was achieved.

3.4 Conclusion and Prospects

This chapter was designed to explore technology as a promoter in advancing plant bio-actives production. The field of biotechnology is growing fast and steady. We envisioned the underlying set of new materials and methods having a strong impact mitigating a series of reported drawbacks in plant bio-actives production. The most obvious finding to emerge from the correlation of new technologies and challenges in plant metabolites production is that alternative modes of production might overcome low yield, better attending the demanding pharmaceutical industry, either eliminate or reduce pollutant residue, reduce costs associated to post-extraction purification, promote high-throughput screening and compound categorisation, support progress in compounds databases and assure sustainable harvesting. Taken together, results found in the current literature suggest that final yield of bio-actives rely heavily upon the choice of selective solvents separating plant metabolites from residue. Moreover, certain extraction modes suffer from significant volume of pollutant chemical residue, and both the cost and complexity associated to post-extraction purification are still critical to production. This is partially due to the common need to combine numerous separation techniques, synergistically. Here, we highlighted the pros and cons using the most popular extraction, isolation and characterisation techniques. However, much more is discussed in the literature. Some conventional methods increase extraction efficiency and selectivity, reducing organic solvent consumption and sample degradation. Nevertheless, design of high-throughput pipelines, compounds' yield and sustainability do require further

attention. Compound characterisation defines structure and functionality, being followed by assays for the synergetic combination of bio-actives, i.e. showing coactive functionalities, in a reproducible and accurate manner. However, it is not all about production. Assuring sustainability in resources harvesting influences the whole process. Here, we discussed some critical issues in marine sampling collection. It is well accepted that marine microenvironments are a rich source of novel compounds, accounting for about 70% of the earth surface. Moreover, in its majority, the marine ecosystem is unexplored, hiding plethora of chemical compounds with potentially high potential for effective therapies. This also implies that vast volume of compounds could derive from marine species. However, the technical requirements for effective and sustainable exploitation of the marine environment are a challenge. Again, technology, providing the infrastructural means to achieve the end, might add huge value towards unveiling new resources, supporting drug discovery. Coming back to production, we discussed the benefits of using new technologies and methodologies, addressing design of high-throughput pipelines and production yields (e.g. HTS, AI, omics, genetic engineering and synthetic biology). As indicated in former sections, compound characterisation libraries are the foundations of effective HTS. In this regard, multichannel preparative methods like the HPCL reduce complexity and are used for deriving robust semi-purified extract libraries, facilitating functional analysis, and implying fewer separation steps speeding bio-actives discovery and production, being an attractive method to be part of high-throughput screening. However, the challenge is still on compromising the existing methods, because each one has its benefits and limitations, which affect compound characterisation and libraries construction. Gene editing is another state-of-the-art approach that production of plant compounds can benefit from. A growing body of literature has reported on CRISPR-Cas9 modifying genotype-phenotype-driven plant compounds biosynthesis, which can potentially be used in gene reprogramming and generation of plant cells, expressing certain proteins, driving the production of key bio-actives, at a certain concentration level, which might increase production yields. Furthermore, metabolomics, proteomics and other omics disciplines, combined, might support discovering chemical fingerprints for important signalling pathways, in biomarkers characterisation, influencing decision making in drug's composition. All that, to be fully replicated via synthetic biology approaches. Ultimately, artificial intelligence-driven methodologies are warming the pharmaceutical sector. Successfully applied in plethora of disciplines, from retail to bioinformatics, this field of knowledge is becoming popular, addressing several challenges in drug discovery and assisted production of medicinal compounds. Numerous business and research teams are investing in AI transformation, and major consortiums have formed. Here, we discussed omics and gene editing and, more importantly, how existing assays can benefit from AI technology. We suggested AI as a key mechanism in compound characterisation libraries design, quality and reliability check, and fast automatic compounds-activity matching, leading to high-throughput pipelines. AI can also enrich genome reprogramming methodologies, with pre-laboratorial design and test for breaches

in recombination, deletion and insertion, using a simulation environment. We believe that the future of biotechnology consists in combining conventional methods, omics, marker-assisted selection, novel infrastructure for sustainable harvesting and AI technology in assisted compounds design and production.

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Transgenic Plant Cell Cultures: A Promising Approach for Secondary Metabolite Production

4

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Abstract

Plants are an important resource for many novel bio-active compounds. As plant-derived compounds exhibit wide-ranging therapeutic and pharmaceutical properties with limited side effects, they are widely used for treating several diseases. Today, a variety of distinct plant secondary metabolites (SM) are serving as essential drugs, widely used around the globe. In addition, plant SM are used as pigments, natural dyes, flavors, food preservatives, fragrances, and as modern biopesticides. Some if the challenges of isolating metabolites include the wild species loss, low metabolite yield, and variations in phytochemical content with respect to habitat, method of extraction, etc. Alternatively, the use of biotechnological approaches will be very advantageous. In this regard, transgenic plant cell culture technology can be a reliable way for the large-scale production of plant-based products under controlled conditions. Besides, the potential to use this method for the production of various pharmaceutical compounds and SM is enormous. This is because transgenic cells can be manipulated in vitro to increase the accumulation of desired compounds and their productivity. The present chapter emphasizes on the application, scale-up methods, and current and future prospects for the production of valuable SM through transgenic plant cell culture approaches. Also, technical challenges involved in SM production are highlighted. The increased production of SM using transgenic plant cell cultures certainly benefit several sectors, such as the herbal, flavor, cosmetic, and pharmaceutical industries.

Keywords

Medicinal plants · Pharmaceuticals · Plant cell culture · Secondary metabolites · Transgenic plants

4.1 Introduction

The chemical compounds synthesized by plants through primary or secondary metabolism are considered as phytochemicals. They commonly have biological activities in the plant host and play a major role in plant growth and development and defense aligned with pathogens, competitors, or raptors. Most of these chemical compounds or plant-derived products are the secondary metabolites (SM) which do not contribute in plant metabolism or in any physiological functions; however, they are secreted in response to external environment or pathogen attack to protect plants against pests, herbivores, and pathogens (Swamy et al. 2016a; Sakamoto et al. 2018). The past few decades evidenced an increased exploration of plants for obtaining several SM having pharmaceutical significance. Some of these discoveries have led to the development of novel drugs that are available in the modern medicines (Chavez

et al. 2015). Though more than one million natural compounds have been identified and their explorations still in progress, only around 25% of them have been found to be biologically active. Of these, about 60% are explored from medicinal plants. Research projects are initiated exponentially around the world to isolate and identify such novel plant SM with bioactivities considering the current human health concerns which are increasing rapidly. These plant-based compounds are relatively effective and possess limited or no adverse side effects, and hence, they are highly preferred source for the development of modern drugs with therapeutic significance (Mulabagal and Tsay 2004; Ekor 2014; Swamy et al. 2016b). In addition to serving as the drug source, SM also find their applications in various fields, such as flavor and fragrance, cosmetics, natural dye, and agrochemical industries (Fig. 4.1). However, the intensities of SM synthesis in plants are affected by numerous factors, such as plant physiology, insects, pests, and environmental changes. Moreover, alongside the issues stated above, lack of wild plant resources, habitat-dependent phytochemical variations, difficulties involved in extracting the specific compounds, lack of extraction/purification methods, and increased manpower charges toward the extraction and purification of plant SM have forced the use of in vitro methods for their active synthesis in large scale (Srivastava and Srivastava 2007).

In vitro plant cell/tissue culture approach is one of the superior ways for the large-scale production of SM. This is because in vitro culturing can be done in large vessels (bioreactors) throughout the year under continuously monitored conditions, and it provides an option of modifying the culture conditions such as media, temperature, pH, nutrients, etc. (Patra and Srivastava 2018). In this process, adding elicitors in the culture medium can further enhance the chances of improving the SM production. Moreover, it allows an easy recovery of the desired phytocompounds in large quantities (Jiao et al. 2018b). Various plant SM such as coloring agents, coumarins, isoflavonoid, phytoalexins, sesquiterpenoid phytoalexin, terpenoid indole alkaloids, etc. have been successfully produced through in vitro culture

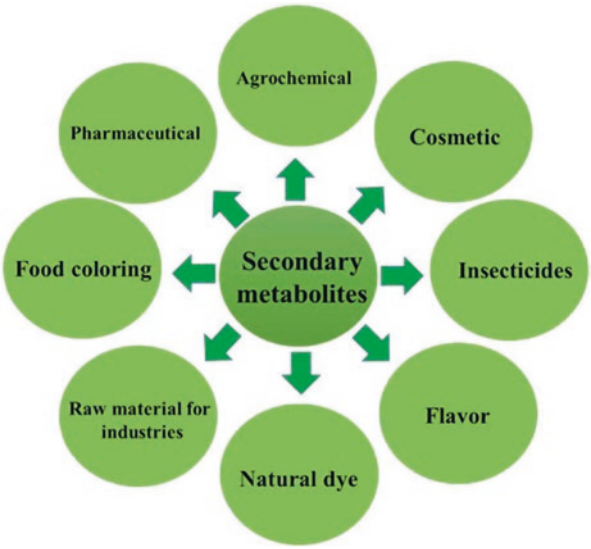

Fig. 4.1 Major applications of secondary metabolites in various fields

approaches (Zhou et al. 2009; Hussain et al. 2012; Siddiqui et al. 2013). However, the yield of the quality plant product is still low and requires adopting improved approaches. In this regard, the transgenic plant cell culture (TPCC) method is highly appreciated for obtaining plant compounds in large scale. Transgenic medicinal plants can be produced by infecting plant parts with *Agrobacterium rhizogenes* or *A. tumefaciens*. The plant tissues infected with *A. rhizogenes* containing either a gene of interest or not produces hairy roots. Likewise, *A. tumefaciens* inserted with a gene of interest produces a stable transformant. Transgenic plants are produced for specific purposes and the transgenic plants developed through *A. tumefaciens* system are mainly to overcome the problems of domestic cultivation, i.e., to enhance herbicide tolerance, pathogen resistance, etc., while *A. rhizogenes*-mediated transformants are used for producing root-oriented plant SM in large scale. In addition, genetics transformation is required for metabolic engineering approaches. The manipulation of plant genome results in the production of desired compounds in large scale. Also, transgenic plants have the ability to retain constant level of producing SM without additional intervention (Hussain et al. 2012; Yue et al. 2016). Thus, transgenic approach has been widely employed for improving/producing plant metabolites in recent times. TPCC techniques are characterized by the feature of in vitro regeneration methods and large-scale fermentation process of plant cell having totipotency. However till date, TPCC has only a limited success commercially due to lack of knowledge and understanding about how these SM are synthesized in plant cells. The present chapter emphasizes on the application, scale-up methods, and current and future prospects for the production of valuable SM through transgenic plant cell culture approaches. Also, technical challenges involved in SM production are highlighted.

4.2 Strategies Used for Increasing the Secondary Metabolite Production

Synthesis of novel SM can stimulate progresses in various strategies used to attain their practical research for biological assessment (Kuroda et al. 2018). Ever since the SM in medicinal plants are present naturally at very lower levels, and several active compounds are complex to be synthesized. When considering many strategies for the production of natural compounds from plants growing in extreme environmental conditions, numerous considerations have to be taken into report. The influx of the genomic interlude premeditated a fundamental change in the advancement toward discovering innovative natural products. Advanced research on the appliance of elicitors has been intensified into the induction system, including the study about signal molecules and functional genes involved in this strategy (Wang et al. 2017b). The number of familiar chemical compounds and structures is predicted to be approximately fourfold higher than that in the microbial population (Rao and Ravishankar 2002). About 3500 new chemical structures are analyzed in the year 1985, of which 2600 compounds are derived from the higher plants. Worldwide, about 121 clinically valuable prescription drugs are synthesized from

plants (Brakhage 2013). The plant species are habitually slow growing; some of their populations are preventive; the quantity of the target compound is highly variable and consistently present at very small concentrations (Ochoa-Villarreal et al. 2016). Researchers develop various new methods to induce the expression of transforming genes in plant tissues to enhance the synthesis of SM from plants by supplementing some elicitors into the culture medium. Several active SM from various types of medicinal plant species produced through biosynthesis, cell and hairy root induction methods, and adventitious root synthesis overplant tissue culture system or using bioengineering techniques has been reported in the recent review by Wang et al. (2017b). Higher CO_2 and O_3 frequently increase plant SM including total phenolics, flavonoids, and condensed tannins (Robinson et al. 2012; Yan et al. 2018). Quality control of various commercial products and compounds containing SM is essential as the quality openly affects their prospective activity (Sakamoto et al. 2018). As reported by Cragg and Newman (2013), about 34% of the currently used medicines are prepared from natural products. However, only a small fraction of this enormous chemical space has been excavated to date; thus, several epic compounds await utilization (Ochoa-Villarreal et al. 2016). Hence, there is a robust need to improve novel, effective, and cost-effective strategies to synthesize valuable plant SM (Xue and He 2015).

Basic plant tissue culture methods were proposed for in vitro synthesis of phytochemicals from various plant species since 1957. Plant cell culture method is an alternative to conventional and synthetic cell culture techniques. Mostly the small quantity of active compounds in plant source is also a possible brake on synthesis (Gurnani et al. 2014; Ochoa-Villarreal et al. 2016). Discoveries of cell cultures are accomplished for the production of metabolites in large-scale level than the intact plant; novel and useful compounds can be produced by providing same environmental culture conditions on in vitro. Until today, for in vitro production of SM in cell cultures, several strategies were developed such as parameters for providing the similar environmental culture conditions, selection of high-yielding clones, precursor feeding, and elicitation methods. Plant tissue culture method is a well-established system for the production of natural compounds, and various SM produced in vitro has been listed in Table 4.1.

4.3 Plant Tissue Culture Techniques for Secondary Metabolite Production

SM are obtained from different culturing techniques such as organ culture, callus culture, cell suspension culture, and hairy root culture. In the last two decades, the increasing trade prominence of the SM has resulted in a great concern in the possibility to change the production of natural bio-active compounds through plant cell culture technology (Mulabagal and Tsay 2004). These culturing techniques are used to obtain bio-active compounds for pharmaceutical and cosmetics, food additives, hormones, proteins, enzymes, antigens, and natural pesticides from the harvest of the cultured cells or tissues (Mathe et al. 2015). In vitro organ culture is a promising

Table 4.1 Details on valuable secondary metabolites synthesized from various medicinal plant species

Secondary metabolite	Source of plant	Reference
Callus culture		
Alkaloids	*Scopolia parviflora*	Tabata et al. (1972)
	Papaver somniferum	Furuya et al. (1972)
Saponins and sapogenins	*Panax ginseng*	Furuya et al. (1973)
L-DOPA	*Mucuna pruriens*	Brain (1976)
Isoquinoline alkaloids	*Corydalis ophiocarpa*	Iwasa and Takao (1982)
Saikosaponins	*Bupleurum falcatum*	Wang and Huang (1982)
Tropane alkaloids	*Hyoscyamus niger*	Yamada and Hashimoto (1982)
Caffeine	*Coffea arabica*	Waller et al. (1983)
Tropane alkaloids	*Duboisia leichhardtii*	Yamada and Endo (1984)
Betacyanin	*Portulaca grandiflora*	Schroder and Bohm (1984)
Alliin	*Allium sativum*	Malpathak and David (1986)
Sterols and phenolics	*Eucalyptus tereticornis*	Venkateswara et al. (1986)
Thebaine	*Papaver bracteatum*	Day et al. (1986)
Flavonoids	*Glycyrrhiza echinata*	Ayabe et al. (1986)
Cryptosin	*Cryptolepis buchanani*	Venkateswara et al. (1987)
Naringin, limonin	*Citrus paradisi*	Barthe et al. (1987)
Dihydrofuro [2,3–b] quinolinium (alkaloid)	*Ptelea trifoliata*	Petit-Paly et al. (1987)
Cephaeline and emetine	*Cephaelis ipecacuanha*	Jha et al. (1988)
Solasodine	*Solanum elaeagnifolium*	Nigra et al. (1989)
Plaunotol	*Croton sublyratus*	Morimoto and Murai (1989)
Triterpenes	*Glycyrrhiza glabra*	Ayabe et al. (1990)
Pyrethrins	*Chrysanthemum cinerariaefolium*	Rajasekaran et al. (1991)
L-Canavanine	*Canavalia ensiformis*	Ramirez et al. (1992)
Acridone and furoquinoline	*Ruta bracteosa*, *R. chalepensis*, and *R. macrophylla*	Baumert et al. (1992)
Alkaloids and coumarins		
Secoiridoid glucosides	*Gentiana* spp.	Skrzypczak et al. (1993)
Phenylpropanoid glycosides	*Tecomasam bucifolium*	Pletsch et al. (1993)
Lithospermic acid B and rosmarinic acid	*Salvia miltiorrhiza*	Morimoto et al. (1994)
Camptothecin-related alkaloids	*Ophiorrhiza pumila*	Kitajima et al. (1998)
Saponin	*Agave amaniensis*	Andrijany et al. (1999)
Altamisine	*Ambrosia tenuifolia*	Goleniowski and Trippi (1999)

(continued)

Table 4.1 (continued)

Secondary metabolite	Source of plant	Reference
Saponins	*Polygala amarella*	Desbene et al. (1999)
Phenolics	*Scutellaria columnae*	Stojakowska and Kisiel (1999)
3-Oxo-rhazinilam	*Rauvolfia serpentina*	Gerasimenko et al. (2001)
	Rhazya stricta	
Triterpenes	*Eriobotrya japonica*	Taniguchi et al. (2002)
Camptothecin	*Nothapodytes foetida*	Thengane et al. (2003)
Rosmarinic acid	*Coleus blumei*	Bauer et al. (2004)
Sennosides	*Cassia senna*	Shrivastava et al. (2006)
Capsiacin	*Capsicum annuum*	Umamaheswari and Lalitha (2007)
Rosmarinic acid	*Satureja hortensis*	Tepe and Sokmen (2007)
Reserpine	*Rauvolfia serpentina*	Nurchgani et al. (2008)
Stevioside	*Stevia rebaudiana*	Janarthanam et al. (2010)
Psoralen	*Psoralea corylifolia*	Parast et al. (2011)
β-sitosterol and caffeic acid	*Sericostoma pauciflorum*	Jain et al. (2012)
Polyphenol	*Inula crithmoides*	Bucchini et al. (2013)
Suspension culture		
Diosgenin	*Dioscorea deltoidea*	Heble and Staba (1980)
Furoquinoline alkaloids	*Choisya ternata*	Sejourne et al. (1981)
Cardenolides	*Digitalis purpurea*	Hagimori et al. (1982)
Tetrahydroanthracene glucosides	*Aloe saponaria*	Yagi et al. (1983)
Alkaloids	*Cinchona*	Koblitz et al. (1983)
Shikonin	*Lithospermum erythrorhizon*	Curtin (1983)
Isoquinoline alkaloids	*Fumaria capreolata*	Tanahashi and Zenk (1985)
Chrysanthemic acid and pyrethrins	*Chrysanthemum cinerariaefolium*	Kueh et al. (1985)
Anthraquinones	*Cinchona* spp.	Wijnsma et al. (1985) and Khouri et al. (1986)
Alkaloids	*Ailanthus altissima*	Anderson et al. (1987)
Berberine	*Coptis japonica*	Fontanel and Tabata (1987)
Rosmarinic acid	*Nicotiana tabacum* and *Eschscholzia californica*	Brodelius et al. (1989)
Canthinone alkaloids	*Bruceajavanica*	Liu et al. (1990)
Theanine	*Camellia Sinensis*	Orihara and Furuya (1990)
γ-Glutamyl derivatives		
Capsaicin	*Capsicum annuum*	Johnson et al. (1990)
Ginkgolide A	*Ginkgo biloba*	Carrier et al. (1991)

(continued)

Table 4.1 (continued)

Secondary metabolite	Source of plant	Reference
Quinoline alkaloids	*Cinchona ledgeriana*	Scragg (1992)
Indole alkaloids	*Catharanthus roseus*	Moreno et al. (1993) and Zhao et al. (2001)
Polyphenols	*Cornus kousa*	Ishimaru et al. (1993)
Diosgenin	*Dioscorea doryophora*	Huang et al. (1993)
L-ephedrine	*Ephedra* spp.	O'Dowd et al. (1993)
D-pseudoephedrine		
Anthraquinones	*Cruciata glabra*	Dornenburg and Knorr (1996)
Ginsenoside saponin	*Panax quinquefolium*	Zhong et al. (1996)
Robustaquinones	*Cinchona robusta*	Schripsema et al. (1999)
Rosmarinic acid and cryptotanshinone	*Salvia miltiorrhiza*	Chen and Chen (2000)
Catharanthine	*Catharanthus roseus*	Zhao et al. (2001)
Chlorogenic acid	*Eucommia ulmoides*	Wang et al. (2003)
Ajmalicine	*Catharanthus roseus*	Lee-Parsons et al. (2004)
Terpenoid indole alkaloid	*Catharanthus roseus*	Lee-Parsons and Royce (2006)
Catharanthine	*Catharanthus roseus*	Ramani and Jayabaskaran (2008)
Azadirachtin	*Azadirachta indica*	Devi et al. (2008)
Azadirachtin	*Azadirachta indica*	Sujanya et al. (2008)
Rosmarinic acid	*Coleus blumei*	Qian et al. (2009)
Ampelopsin, piceid, resveratrol, and viniferin	*Cayratia trifolia*	Roat and Ramawat (2009)
Caffeoylquinic acids, echinacoside, phenylethanoid glycosides	*Echinacea angustifolia*	Guarnerio et al. (2012)
Phenolics	*Artemisia absinthium*	Ali and Abbasi (2014)
Psoralen	*Psoralea corylifolia*	Ahmed and Baig (2014)
Phenolics and flavonoids	*Artemisia absinthium*	Ali et al. (2016)
Hairy root culture		
Cuscohygrine	*Calystegia sepium*	Jung and Tepfer (1987)
Indole alkaloids	*Catharanthus trichophyllus*	Davioud et al. (1989)
Indole alkaloids	*Amsonia elliptica*	Sauerwein et al. (1991)
Phytoecdysteroids	*Ajuga reptans*	Matsumoto and Tanaka (1991)
Tropane alkaloids	*Anisodus luridus*	Jobanovic et al. (1991)
Tropane alkaloid	*Brugmansia candida*	Giulietti et al. (1993)
Anthraquinone	*Cassia obtusifolia*	Ko et al. (1995)
Fusicoccin	*Armoracia lapathifolia*	Babakov et al. (1995)
Polyacetylenes	*Campanula medium*	Tada et al. (1996)
Aconites	*Aconitum heterophyllum*	Giri et al. (1997)
Essential oil	*Artemisia absinthium*	Nin et al. (1997)

(continued)

Table 4.1 (continued)

Secondary metabolite	Source of plant	Reference
Isoprenylated flavonoids	*Glycyrrhiza glabra*	Asada et al. (1998)
Rosmarinic acid	*Salvia officinalis* and *S. fruticosa*	Kintzios et al. (1999)
Methylputrescine and conjugated polyamines	*Hyoscyamus muticus*	Biondi et al. (2000)
Cryptotanshinone, tanshinone I, tanshinone II A, and tanshinone IIB, rosmarinic acid, and lithospermic acid B	*Salvia miltiorrhiza*	Chen et al. (2001)
Azadirachtin, nimbin, 3-tigloylazadirachtol, salannin, and 3-acetyl-1-tigloylazadirachtinin	*Azadirachta indica*	Allan et al. (2002)
p-Sitosterol, ursolic acid	*Salvia cinnabarina*	Savona et al. (2003)
New diterpenes	*Salvia broussonetii*	Fraga et al. (2005)
Diterpenoid tanshinones	*Salvia miltiorrhiza*	Ge and Wu (2005)
Apigenin, total flavonoids	*Salvia involucrata*	Li et al. (2006)
Tanshinone	*Salvia miltiorrhiza*	Shi et al. (2007)
Asiaticoside	*Centella asiatica*	Kim et al. (2007)
Tanshinone	*Salvia miltiorrhiza*	Wu et al. (2007)
Artemisinin	*Artemisia dubia* and *Artemisia indica*	Mannan et al. (2008)
Resveratrol	*Arachis hypogaea*	Kim et al. (2008)
Deoursin	*Angelica gigas*	Xu et al. (2008)
Tropane	*Brugmansia candida*	Marconi et al. (2008)
Terpenoid indole alkaloid	*Catharanthus roseus*	Goklany et al. (2009), Binder et al. (2009), and Li et al. (2011)
Psoralen	*Psoralea corylifolia*	Baskaran and Jayabalan (2009)
Anthraquinone, phenolics, and flavonoids	*Morinda citrifolia*	Baque et al. (2010)
Plumbagin	*Plumbago indica*	Gangopadhyay et al. (2011)
Allelochemicals	*Fagopyrum tataricum*	Uddin et al. (2012)
Withanolide A	*Withania somnifera*	Praveen and Murthy (2012)
Betuligenol	*Atropa belladonna*	Srivastava et al. (2013)
Flavonolignans and lipoxygenase	*Silybum marianum*	Khalili et al. (2009)
Rosmarinic acid and surface flavonoids	*Dracocephalum kotschyi*	Fattahi et al. (2013)
Tanshinones	*Salvia miltiorrhiza*	Wang et al. (2013b)
Solasodine	*Solanum trilobatum*	Shilpha et al. (2015)
Rosmarinic acid	*Salvia wagneriana*	Ruffoni et al. (2016)
Plumbagin	*Plumbago rosea*	Jose et al. (2016)
Bacoside A	*Bacopa monnieri*	Largia et al. (2016)

(continued)

Table 4.1 (continued)

Secondary metabolite	Source of plant	Reference
Alkannin and Shikonin	*Arnebia hispidissima*	Singh and Sharma (2016)
Steroidal glycoalkaloids	*Solanum lycopersicum*	Abdelkareem et al. (2017)
Flavonoids	*Isatis tinctoria*	Jiao et al. (2018a), (b), (c)
Triterpenoid saponins (ginsenosides)	*Panax quinquefolium*	Kochan et al. (2018)
α–L–iduronidase	*Brassica rapa*	Cardon et al. (2018)
Organ culture		
Pyrrolizidine (root)	*Senecio vulgaris*	Hartmann and Toppel (1987)
Alkaloids (root)	*Cephaelis ipecacuanha*	Teshima et al. (1988)
Corydaline (shoot)	*Corydalis cava*	Rueffer et al. (1994)
Saponin (root)	*Saponaria officinalis* and *Gypsophila paniculata*	Fulcheri et al. (1998)
Saikosaponins (root)	*Bupleurum falcatum*	Kusakari et al. (2000)
Hypericin (shoot)	*Hypericum perforatum*	Santarem and Astarita (2003)
Asiaticoside	*Centella asiatica*	Kim et al. (2004)
Lupeol, rutin (shoot)	*Hemidesmus indicus*	Misra et al. (2005)
Umbelliferone (shootlet)	*Ammi majus*	Krolicka et al. (2006)
Hypericins (shoot)	*Hypericum perforatum*	Kornfeld et al. (2007)
Rosmarinic acid (shoot apex)	*Zataria multiflora*	Francoise et al. (2007)
Stevioside	*Stevia rebaudiana*	Dheeranapattana et al. (2008)
Vasine (shoot)	*Adhatoda vasica*	Shalaka and Sandhya (2009)
Isoflavones (shoot)	*Psoralea corylifolia*	Shinde et al. (2009)
Gymnemic acid	*Gymnema sylvestre*	Praveen et al. (2014)
Ajmalicine, catharanthine, and vindoline (meristem)	*Catharanthus roseus*	Zhou et al. (2015b)
Ascorbic acid, flavonoids, tocopherol, and phenols	*Brassica juncea*	Ahmad et al. (2016)
Rosmarinic acid and volatiles	*Thymus leucotrichus*	Bekircan et al. (2018)

method to induce somaclonal variations in SM production, and it also overcomes the dependency of SM from the natural medicinal plants. This also helps to select high SM yielding clones. Organ cultures show low sensitivity to shear stress and have high degree of heterogeneity in biomass production. Organ culture methods have been developed for production of SM in various plant species (Giri and Narasu 2000; Verpoorte et al. 2002; Murthy et al. 2008; Baque et al. 2010).

4.3.1 Callus and Cell Suspension Cultures

Callus is an unspecialized, unorganized, and growing dividing mass of cells. The cells are compact and aggregated. Friable kind of callus also formed. They are softer

and easily breakable. For the development of callus, the cells should not be provided with any auxin or cytokinin, and source of light should be minimal. It has a benefit that depends on the secondary compound root, or only shoots can be regenerated. The undifferentiated mass of cell can be after induced for root or shoot induction in appropriate media which requires auxin or cytokinin in prescribed concentration which depends on the plant species as well as explant. The whole plant can be regenerated in multiple copies from the callus. Even in suspension culture, callus cells are accurately separated and allow it for SM production. Somatic embryogenesis can also be achieved. It is easy to extract the SM directly from the developed shoot or root from the callus. Callus tissue is a good source to produce plants which are completely genetically variable from the parent plants (Ma et al. 2003). This will help to produce the required secondary compound in high concentration. *Sericostoma pauciflorum* plant has been used against diabetes and cancer, also known to be health promoter. The bio-active SM, viz., β-sitosterol and caffeic acid, was synthesized from *S. pauciflorum* callus cultures (6 weeks old) and identified through TLC behavior, color reaction, and IR spectrum technique (Jain et al. 2012). Polyphenol isolated from callus cultures initiated from leaf sections of *Inula crithmoides* showed significant antimicrobial, antifungal (*Alternaria solani* and *Phytophthora cryptogea*), and antioxidant activities (Bucchini et al. 2013). Anticancer compound podophyllotoxin is produced in *Linum album* by the callus culture.

In cell suspension cultures, callus or hairy roots are inoculated into a liquid media with appropriate plant growth stimulants. The production of SM in cell suspension culture is based on the biosynthetic totipotency of the plant cell. It denotes that all the cells in the suspension culture can able to produce the same range of compounds which found in the whole plant. Cell suspension cultures have immediate potential for large-scale production of SM, and the product will be continuous and reliable. The main challenge of synthesizing SM through plant cell/tissue suspension cultures system is that SM are typically synthesized only by specific type of cells at unique developmental stages (Mukundan et al. 1997; Srivastava and Srivastava 2007). It is more advantageous that addition of some other nutrients like yeast extract and cork pieces to the culture is possible for the enhanced production of required secondary compound. But this method is highly investible and laborious. In some occasion, after several passages, the productivity may reduce due to lack of nutritional availability and optimum condition. So, proper investigation is important. Suspension cultures are carried out by transferring the callus into liquid medium. Clumping of callus should be avoided. The broth pH and density should be appropriate. To avoid clumps pectinase can be used, and polyvinylpyrrolidone can prevent the browning of culture (Matkowski 2000; Sidhu 2011). Plant cells in suspension culture will undergo genetic variation often due to the increasing concentration of SM. This variation is considered as somaclonal variation. But in some cases, it will be advantageous. Optimization of medium, feeding level, and mathematical model for extraction of intracellular metabolite will significantly improve the production rate.

4.3.2 Hairy Root Cultures

Root cultures are an important source of medicinal compounds (Wang et al. 2017b). Hairy root culture system is considered as a hormone-independent approach for the production of SM, and it shows the plagiotropic growth. The root cultures' growth will take longer period, i.e., the root growth in higher plants are slower and harvesting the roots is also much difficult. Hence, hairy root culture system is an alternative to organ (root) culture, and it is suitable for biochemical and pharmacological studies (Deepthi and Satheeshkumar 2017). The hairy root system will be an outstanding approach for genome engineering and molecular studies in which the development of transgenic plants is not necessary (Chen et al. 2018). Hairy root culture is initiated by the infection of *Agrobacterium rhizogenes*, a promising method for SM production in plants. The fragment of T-DNA of *Ri* plasmid from *A. rhizogenes* is inserted into the infected plant through the wound, and it controls auxin and cytokinin biosynthesis. T-DNA carries oncogenes and opine catabolism which supports neoplastic growth in transformed plants (Jung and Tepfer 1987; Satish and Ramesh 2017). *A. rhizogenes*-mediated transformation is also used for transgenic hairy root culture method. It has an advantage of inserting a foreign gene into the hairy root clone in an effective manner through binary vector. Hairy root culture is checked in root nodule studies for plant SM production, and the transgenic hairy roots obtained using hypocotyls with aerial shoots demonstrate as an improved approach for root nodules (Chen et al. 2018). Through this method, possibilities are there to alter the SM for our own purpose. Transformed roots for in vitro production of SM are also widely studied (Bonhomme et al. 2000; Tiwari et al. 2007; Kim et al. 2008). Genetically transformed hairy root cultures are useful for the production of tissue-specific SM. The main advantage of hairy roots cultures is that they frequently show about the similar or better biosynthetic ability for SM production as associated to their mother plants (Kim et al. 2002). Important things to be considered in this method are bacterial strain (opine type of *A. rhizogenes*), culture media, plant growth hormone type and combinations, culture conditions such as light and temperature, optical density of *Agrobacterium* culture, acetosyringone concentration, infection time, duration of cocultivation, and proper antibiotics for selection (Shilpha et al. 2015). Based on the type of opine, the bacteria can be classified into five different lines such as octopine, agropine, mannopine, nopaline, and cucumopine. Among all, agropine has more induction capability (Valdimirov et al. 2015). Hairy root cultures can also be developed from various parts of plants like leaf, stem, stalk, protoplast, root, and shoot tip. However, the explant may vary based on the type of SM as well as species, and age of the explant is also important to notice. The explant should be cocultivated or inoculated with *A. rhizogenes* separately for inducing hairy roots. Addition of elicitors and oxygen supply will enhance the SM production in in vitro cultures. Usually SM are acting as a defense system for plants and which is activated by elicitors in the plants. Elicitation is the induction by either biotic or abiotic approaches. While comparing normal SM products through PTC, elicitor-induced commercial products are optimized and controlled development. Most of the elicitors may be a killed form of any pathogen or

non-pathogen to the plant and chemicals. Microbial infection on the intact plant also helps to enhance a particular SM.

4.4 Transgenic Plant Cell Cultures (TPCC): An Alternative Secondary Metabolite Production Method

Transgenic plant cell culture (TPCC) is a potential method for the rapid propagation of medicinal plants with a great potential for producing SM in large scale, though the frequency of genetic deficiencies at high rates and some somaclonal variations in plants regenerated through tissue culture technique are critically limiting the production of SM in this regard. Consequently, it is highly important to optimize the culture conditions and several other parameters for transformation and genetic manipulation for each medicinal plant species to increase quality of plantlets for its commercial assessment. Various therapeutic proteins, growth factors, like antibodies, blood products, mammalian enzymes, cytokines, and vaccines have been expressed preferably in transgenic plants (Ma et al. 2005; Ono and Tian 2011). The first pharmaceutically important protein produced in plants was human growth hormone, which was expressed in transgenic tobacco plants (Barta et al. 1986).The production of various SM through TPCC is an alternative and best method which offers remarkable potential for transferring additional genes along with the T-DNA genes (Giri and Narasu 2000). However, limitations and procedures are enforced on the transgenic plant approaches because of the possibility of gene transfer to wild species over cross-pollination by closely related plant species (Ono and Tian 2011).

4.4.1 Transgenic Plants: Production and Advantages

Plants contain stably integrated expressed foreign genes that are useful to advance the quality and quantity of the invention which is called transgenic plants. Transgenic technology is advantageous for the genetic improvement of higher plants. Commonly in medicinal plants, an *Agrobacterium*-mediated gene delivery system has been developed and widely used (Karuppusamy 2009; Hussain et al. 2012). Several reports are available to control secondary metabolism in pharmaceutically important medicinal plants by various transgenic applications (Saito et al. 1992). Several studies have been reported since 1985 for the production of specific SM. *A. tumefaciens* contain *Ti* plasmid which causes crown gall disease in plants (Krens et al. 1982). The crown gall-derived cell suspension cultures trigger the wild-type *Ti* plasmids which have been used for production of specific SM (Saito et al. 1992). Similarly, *Ri* plasmid present in *A. rhizogenes* induce hairy root disease in dicot plants, and the induced hairy roots will grow rapidly in culture medium without addition of phytohormones (Giri and Narasu 2000). Most pharmaceutical proteins have been produced in transgenic *N. tabacum* plants, because it has a long history as a model plant and strong expression constructs are accessible. However, there is increasing interest in the use of other species, particularly cereals, legumes, fruit,

and vegetables. Although it is advantageous to focus on a lesser number of platform tools for the large-scale production of bio-active compounds, the transfer of recombinant vaccines in edible plant tissues is unique since it would be beneficial to use widely grown plants for vaccination processes (Fischer et al. 2004). In last decade, potatoes have been used for the production of novel vaccine candidates, tumor necrosis factor α, human serum albumin, and antibodies (Fischer et al. 2004).

4.4.2 *Agrobacterium rhizogenes*-Mediated Transgenic Plants

Plants act as chemical factories for their ability to produce industrially and pharmaceutically important phytochemicals. The major disadvantage of extracting phytochemicals from normal plants in the environment is significantly reducing their growth and yield. Hairy root cultures are an alternative source of phytochemicals because of their genetic stability, biosynthetic capacity, and biomass production (El-Esawi et al. 2017). Hairy roots highly accumulate phytochemicals than cell/callus cultures that contain undifferentiated cells and act as biocatalysts to transform substrates into products of high value. These are the major SM produced in hairy root cultures including terpenoids, alkaloids, and phenolics, and therapeutic proteins, such as vaccines, antibodies, and mammalian enzymes, have been expressed in transgenic plants (Desai et al. 2010; Ono and Tian 2011).Thus the hairy root culture method is a reliable system for use with *A. rhizogenes*-mediated genetic transformation and could be used when growth media and containment environmental space are comparatively inexpensive (Lee and Ko 2017). The elevated expression frequencies of the recombinant proteins in the hairy root culture systems resembles those of transgenic plants and still also afford a more defined and homogenous culture system, two attributes integral to recombinant protein production (Ono and Tian 2011). Production of recombinant proteins through this system and their release into the plant cell culture medium facilitate the purification system and proliferation of protein yield (Pham et al. 2012; Ochoa-Villarreal et al. 2016). Thaumatin I (the sweet-tasting protein) was expressed and successfully secreted from hairy root cultures of *Nicotiana tabacum* by supplementing polyvinylpyrrolidone and sodium chloride into the hairy root culture medium (Pham et al. 2012). *A. rhizogenes*-mediated genetic transformation of *Glycine max* predominantly focused on the development of composite plants in field cultivation with hairy roots formed using hypocotyl explants (Kereszt et al. 2007). A new way for enhanced SM synthesis through TPCC system is done by transforming a desirable gene into plant species with the natural vector system *A. rhizogenes* (Bourgaud et al. 2001; Srivastava and Srivastava 2007). The *A. rhizogenes*-mediated genetic transformation has been widely used to produce SM through transgenic hairy root induction in various medicinal plants (Karuppusamy 2009; Hussain et al. 2012). The ability to grow hairy root cultures in different media through optimized in vitro conditions has laid the establishment for several innovative and fundamental technologies for SM production in the field of plant biotechnology. This genetic transformation method leads to the occurrence of primary hairy roots at the site of *A. rhizogenes* infection

in the explants, and the transformed hairy root cultures are proficient to vast growth in culture media free of plant growth stimulants (Shanks and Morgan 1999; Srivastava and Srivastava 2007). Strategies for the elicitation of hairy root cultures are the massive depositories of plant phytochemicals that has been attained for the improved synthesis of SM in medicinal plants. In various medicinal plants, *A. rhizogenes* transformation method along with the elicitation procedure was established to be a highly effective technique for increasing the SM synthesis rapidly (Shilpha et al. 2015; Largia et al. 2016). A significant elevation in the biosynthesis of hydroxycinnamic acids, flavonoids, pectins, saponins, and protopectins in *Nitraria schoberi* hairy root cultures was procured by transforming seedlings' primary leaves with a wild *A. rhizogenes* strain (Zheleznichenko et al. 2018). Hairy root cultures can also serve as a tool to study gene silencing using RNA interference methods (Patra and Srivastava 2018). One of the major biotechnological applications of TPCC is producing beneficial compounds, together with SM as well as different recombinant proteins. Hence, the focus of discussion is positioned on elicitation, and a hairy root culture for SM production is enclosed. An important benefit of using TPCC for SM synthesis is its capability to carry the transformed transgene into next generations with no gene silencing necessary for active biological functions of the gene. Cultured plant hairy root samples also possess a number of advantages over non-transgenic samples, and they grow rapidly with no contaminations in appropriate culture conditions within a confined environment.

Since the publication of many recent reviews on the SM production from in vitro plant cell cultures (Rao and Ravishankar 2000; Wang et al. 2001; Konczak–Islam et al. 2003; Zhang et al. 2009), a number of innovative published reports have occurred in the subject area. Moreover, the studies using different types of reporter genes or selectable markers, viz., β-glucuronidase, green fluorescent protein, red fluorescent protein, or secreted alkaline phosphatase, confirmed the expression of transformed target gene fragments in hairy root cultures. A detailed protocol for *A. rhizogenes* transformation of *G. max* has been reported recently with high stable transformation efficiency after infected cotyledons produced hairy root cultures (Chen et al. 2018). The *GmNAC15* gene (a member of the NAC transcription factor family) overexpression in *G. max* hairy root cultures improved saline tolerance which is a possible method of genetic engineering to improve the abiotic stress tolerance of various important crops (Li et al. 2018). A brief timeline for various research achievements in SM production has been provided in Table 4.2.

4.4.3 *Agrobacterium tumefaciens*-Mediated Transgenic Plants

In medicinal plants, transformation has been reported through *A. rhizogenes*, but those results in the formation of hairy roots; it produces only those chemicals which are synthesized in the roots. Some of the medicinal plants have SM in their shoots, and for that *A. tumefaciens* method is used to cause shooty teratomas for the production of SM in shoots and the expression of SM responsible gene. These hairy root

Table 4.2 Production of secondary metabolites from plant sources, their products, and type of method used for secondary metabolite synthesis

Products	Culture types	Plant species	References
Capsaicin	Callus	*Capsicum annum*	Varindra et al. (2000)
Anthraquinones	Suspension	*Cassia acutifolia*	Nazif et al. (2000)
Gallotannins	Root	*Rhus javanica*	Taniguchi et al. (2000)
Camptothecin	Callus	*Nothapodytes foetida*	Ciddi and Shuler (2000)
Daidzein, retusin, genistein	Callus	*Maackia amurensis*	Fedoreyev et al. (2000)
Formononetin			
Alkaloid	Hairy root	*Atropa belladonna*	Bonhomme et al. (2000)
Protocatechuic aldehyde and caffeic acid	Cell culture	*Capsicum frutescens*	Rao and Ravishankar (2000)
Ginsenoside	Hairy root	*Panax ginseng*	Yu et al. (2000)
Flavonolignan	Root	*Silybum marianum*	Alikaridis et al. (2000)
Ramiflorin	Callus	*Aspidosperma ramiflorum*	Olivira et al. (2001)
Reserpine	Callus	*Rauvolfia serpentina*	Gerasimenko et al. (2001)
Taxol	Suspension	*Taxus* spp.	Jennewein and Croteau (2001) and Wu et al. (2001)
	Cell culture	*Taxus chinensis*	
Withaferin A	Shoot	*Withania somnifera*	Ray and Jha (2001)
Catharanthine	Cell suspension	*Catharanthus roseus*	Zhao et al. (2001)
Anthocyanins	Flower cell culture	*Ajuga reptans*	Terahara et al. (2001)
Terpenoid	Shoot	*Mentha arvensis*	Phatak and Heble (2002)
Plumbagin	Hairy root	*Plumbago zeylanica*	Verma et al. (2002)
Diterpenoids	Suspension	*Torreya nucifera*	Orihara et al. (2002)
Rutin	Callus, shoot culture	*Hemidesmus indicus*	Rao and Ravishankar (2002)
Lehmanin	Callus	*Sophora flavescens*	Kim et al. (2002)
Azadirachtin	Hairy roots	*Azadirachta indica*	Allan et al. (2002)
Anthraquinone	Callus cultures	*Rubia cordifolia*	Bulgakov et al. (2002)
Volatile compounds	Hairy root	*Cichorium intybus*	Bais et al. (2002)
Rosmarinic acid		*Ocimum basilicum*	
Vitexin, isovitexin, orientin/ isoorientin	Callus	*Drosophyllum lusitanicum*	Budzianowski et al. (2002)
Triterpenoid	Suspension	*Ammi majus*	Staniszewska et al. (2003)

(continued)

Table 4.2 (continued)

Products	Culture types	Plant species	References
Hypericin	Multiple shoot	*Hypericum perforatum*	Santarem and Astarita (2003)
Triterpenes, sterols	Suspension	*Hyssopus officinalis*	Skrzypek and Wysokinsku (2003)
Apigenin	Hairy roots	*Saussurea involucrate*	Fan et al. (2003)
Anthocyanins	Callus, cell, and aggregate suspension	*Vaccinium pahalae*, *Glehnia littoralis*	Kahkonen and Heinonen (2003)
Rosmarinic acid and its glucosides	Cell suspension	*Ocimum basilicum*	Petersen and Simmons (2003)
		Anthoceros agrestis	
	Suspension	*Anchusa officinalis*	
	Hairy roots	*Hyssopus officinalis*	
Anthocyanins	Callus and cell suspension	*Ipomoea batatas*	Konczak-Islam et al. (2003)
Crocin	Callus	*Crocus sativus*	Chen et al. (2003)
Saponin	Cell culture	*Panax ginseng*	Hu et al. (2003)
Anthocyanins	Callus	*Hyoscyamus muticus*, *Taraxacum officinale*	Hou (2003)
Plumbagin	Callus	*Plumba gorosea*	Komaraiah et al. (2003)
Alkaloid	Callus suspension	*Catharanthus roseus*	Akcam-Oluk et al. (2003)
Camptothecin	Callus	*Nothapodytes foetida*	Thengane et al. (2003)
Kinobeon A	Cell suspension	*Carthamus tinctorius*	Kanehira et al. (2003)
Anthocyanins	Cell suspensions, callus	*Daucus carota*	Ravindra and Narayan (2003)
Corydaline	Embryo	*Corydalis ambigua*	Hiraoka et al. (2004)
Berberine	Suspension	*Coscinium fenestratum*	Narasimhan and Nair (2004)
Rutin	Callus and suspension	*Fabiana imbricata*	Schmeda-Hirschmann et al. (2004)
Alkaloid	Cell suspension	*Catharanthus roseus*	El-Sayed and Verpoorte (2005)
Flavonoids	Shoot cultures in bioreactor	*Artemisia judaica*	Liu et al. (2004)
Diterpenoids	Surface exudate of the aerial parts	*Salvia wagneriana*	Bisio et al. (2004)
Crocin	Callus	*Crocus sativus*	Ochiai et al. (2004)

(continued)

Table 4.2 (continued)

Products	Culture types	Plant species	References
Anthocyanins	Callus, cell suspension	*Ipomoea batatas*	Terahara et al. (2004)
Taxol	Suspension	*Taxus* spp.	Kim et al. (2004)
Anthraquinones	Callus	*Frangula alnus*, *Rhamnus catharticus*	Kovacevic and Grabisic (2005)
7-Methyljuglone	Shoot culture	*Drosera rotundifolia*	Hohtola et al. (2005)
Rosarin	Callus	*Rhodiola rosea*	
Hypericin	Suspension	*Hypericum perforatum*	
Triterpenes	Callus	*Centella asiatica*	Kiong et al. (2005)
Ginsenosides	Hairy roots	*Panax ginseng*	Choi et al. (2005)
Catechin	Callus	*Rheum ribes*	Farzami and Ghorbant (2005)
Lupeol, rutin	Shoot culture	*Hemidesmus indicus*	Misra et al. (2005)
Kinobeon A	Cell suspension	*Carthamus tinctorius*	Kambayashi et al. (2005)
Phenylethanoid glycosides	Cell suspension	*Cistanche deserticola*	Cheng et al. (2005)
Betalains	Cell suspension, hairy roots	*Beta vulgaris*	Pavlov et al. (2005)
Rosmarinic acid and its glucosides	Suspension	*Anchusa officinalis*	Soobrattee et al. (2005)
	Cell suspension	*Anthoceros agrestis*	
Lithospermic acid B	Hairy roots	*Hyssopus officinalis*	
Piceatannol (a stilbene)	Callus	*Arachis hypogea*	Ovesna and Horvathova-Kozics (2005)
Ginsenosides	Root culture	*Panax ginseng*	Sivakumar et al. (2005)
Withanolides	Hairy roots	*Withania somnifera*	Kumar et al. (2005)
Umbelliferone	Shootlet	*Ammi majus*	Krolicka et al. (2006)
Sennosides	Callus	*Cassia senna*	Shrivastava et al. (2006)
Vincristine	Suspension	*Catharanthus roseus*	Lee-Parson and Rogce (2006)
Gymnemic acid	Callus	*Gymnema sylvestre*	Gopi and Vatsala (2006)
Essential oil	Shoot	*Cymbopogon citratus*	Quiala et al. (2006)
Reserpine	Callus	*Rauvolfia tetraphylla*	Anitha and Kumari (2006)
Anthocyanin	Suspension	*Vitis vinifera*	Qu et al. (2006)

(continued)

Table 4.2 (continued)

Products	Culture types	Plant species	References
Anticancer alkaloid	–	*Nothapodytes nimmoniana*	Padmanabha et al. (2006)
Gymnemic acid	Callus	*Gymnema sylvestre*	Devi et al. (2006)
Cynarin, chlorogenic acid	Callus	*Cynara cardunculus*	Trajtemberg et al. (2006)
Rutin	Hairy roots	*Fagopyrum esculentum*	Hinneburg et al. (2006)
Rosmarinic acid	Callus, cell suspension	*Lavandula officinalis*	Kovacheva et al. (2006)
	Hairy roots	*Salvia officinalis*	Grzegorczyk et al. (2006)
Abietane diterpenoids	Cell suspension	*Torreya nucifera*	Lee et al. (2006)
Baicalin, wogonoside	Hairy roots, cell suspension	*Scutellaria baicalensis*	Huang et al. (2006)
Capsaicin	Callus	*Capsicum annum*	Umamaheswari and Lalitha (2007)
Flavone-C-glycosides	UV irradiated callus	*Passiflora quadrangularis*	Antognoni et al. (2007)
Carnosic acid	Callus, shoot culture	*Rosmarinus officinalis*	Wijeratne and Cuppett (2007)
Hypericins	Multiple shoot	*Hypericum perforatum*	Kornfeld et al. (2007)
Rosmarinic acid	Callus	*Satureja hortensis*	Tepe and Sokmen (2007)
Flavonoids	Callus	*Stevia rebaudiana*	Tadhani et al. (2007)
Rutin	Hairy root	*Fagopyrum esculentum*	Lee et al. (2007)
Hyperforin and adhyperforin	Shoot	*Hypericum perforatum*	Karppinen et al. (2007)
Glucoside	Hairy root	*Gentiana macrophylla*	Tiwari et al. (2007)
Asiaticoside	Hairy root	*Centella asiatica*	Kim et al. (2007)
Flavonoid	Callus	*Momordica charantia*	Agarwal and Kamal (2007)
Camptothecin	Shoot culture	*Ophiorrhiza rugosa*	Vineesh et al. (2007)
Saponins	Shoot	*Primulaveris*	Okrslar et al. (2007)
Eleutherosides	Suspension	*Eleutherococcus senticosus*	Shohael et al. (2007)
Resveratrol	Hairy root	*Arachis hypogaea*	Kim et al. (2008)
Artemisinin	Callus	*Artemisia annua*	Baldi and Dixit (2008)
Azadirachtin	Suspension	*Azadirachta indica*	Sujanya et al. (2008)
Xanthone	Multiple shoot	*Gentianella austriaca*	Vinterhalter et al. (2008)

(continued)

Table 4.2 (continued)

Products	Culture types	Plant species	References
Glycyrrhizin	Hairy root	*Glycyrrhiza glabra*	Mehrotra et al. (2008)
Azadirachtin	Suspension	*Azadirachta indica*	Poornasri et al. (2008)
Serpentine	Callus	*Rauvolfia serpentina*	Salma et al. (2008)
Podophyllotoxin	Hairy root	*Linum album*	Baldi et al. (2008)
Corydalin	Callus	*Cordyline terminalis*	Taha et al. (2008)
Berberin	Callus	*Coscinium fenestratum*	Khan et al. (2008)
Cathine	Suspension	*Brucea javanica*	Wagiah et al. (2008)
Tropane	Hairy root	*Brugmansia candida*	Marconi et al. (2008)
Silymarin	Hairy root	*Silybum marianum*	Rahnama et al. (2008)
Quercetin	Callus	*Pluchea lanceolata*	Arya et al. (2008)
Withanolide A	Hairy root	*Withania somnifera*	Murthy et al. (2008)
Catharanthine	Suspension	*Catharanthus roseus*	Ramani and Jayabaskaran (2008)
Deoursin	Hairy root	*Angelica gigas*	Xu et al. (2008)
Flavones	Callus	*Camellia chinensis*	Nikolaeva et al. (2009)
Stilbenes	Suspension	*Cayratia trifolia*	Roat and Ramawat (2009)
Flavonoid	Callus	*Crataegus sinaica*	Maharik et al. (2009)
Myristin	Shoot	*Myristica fragrans*	Indira et al. (2009)
Podophyllotoxin	Shoot and root	*Podophyllum hexandrum*	Li et al. (2009)
Isoflavones	Multiple shoot	*Psoralea corylifolia*	Shinde et al. (2009)
Taxol	Cell culture	*Cladosporium cladosporioides*	Zhang et al. (2009)
Ajmalicine and catharanthine	Cell suspension, hairy roots, and rootless shoot cultures	*Catharanthus roseus*	Vazquez-Flota et al. (2009)
Guggulsterone	Fed batch culture	*Commiphora wightii*	Suthar and Ramawat (2010)
Flavonoid	Suspension	*Ginkgo biloba*	Hao et al. (2010)
Phenols and flavonoids	Hairy root	*Hypericum perforatum*	Cui et al. (2010)
Anthraquinones	Cell culture	*Rubia cordifolia*	Bulgakov et al. (2010)
Rutin	Root cultures	*Fagopyrum esculentum*	Kim et al. (2010)

(continued)

Table 4.2 (continued)

Products	Culture types	Plant species	References
Alizarin and purpurin	Hairy root	*Rubiaakane*	Lee et al. (2010)
Total fatty acid and gamma-linolenic acid	Hyphae	*Umbelopsis isabellina*	Wei et al. (2010)
Atractylodin	Suspension	*Atractylodes lancea*	Tao et al. (2011)
Taxuyunnanine C	Cell	*Taxus chinensis*	Gao et al. (2011)
Flavonoids	Suspension	*Ginkgo biloba*	Hu et al. (2011)
Triterpenoid	Hairy root	*Codonopsis lanceolatae*	Kim et al. (2011)
Alkaloid	Suspension	*Fritillaria cirrhosa*	Wang et al. (2011)
Flavonoids	Hairy root	*Saussurea involucrata*	Qiao et al. (2011)
Bacopa saponins	Callus cultures	*Bacopa monnieri*	Majumdar et al. (2011)
Xanthone	Shoot cultures	*Gentianella bulgarica*	Jankovic et al. (2011)
Flavonoids	Callus cultures	*Hydrocotyle bonariensis*	Masoumian et al. (2011)
Andrographolide	Suspension	*Andrographis paniculata*	Gandi et al. (2012)
Naphthoquinone	Suspension	*Arnebia euchroma*	Baranek et al. (2012)
Coumarins	Suspension	*Angelica archangelica*	Tomas et al. (2012)
Trans-resveratrol	Cell suspension	*Vitis vinifera*	Belchi-Navarro et al. (2012)
Ginsenoside and polysaccharide	Cell suspension	*Panax quinquefolium*	Wang et al. (2012a)
Caffeic acid	Shoot culture	*Echinacea angustifolia*	Cui et al. (2013)
Saponin	Biosynthesis	*Panax ginseng*	Balusamy et al. (2013)
Artemisinin	Biosynthesis	*Artemisia annua*	Paddon et al. (2013)
Saikosaponin	Adventitious root	*Bupleurum chinense*	Sun et al. (2013)
Essential oils (camphor, camphene, α-thujone, germacrene D, 1,8-cineole, and β-caryophyllene)	Shoot tip, leaf, and node	*Artemisia vulgaris*	Sujatha et al. (2013)
20-Hydroxyecdysone	Suspension	*Achyranthes bidentata*	Wang et al. (2013a)
Valerenic acid	Hairy roots	*Valeriana officinalis*	Torkamani et al. (2014)
Vincamine	Hairy roots and cell suspensions	*Catharanthus roseus*	Verma et al. (2014)
Triptolide and wilforine	Hairy roots	*Tripterygium wilfordii*	Zhu et al. (2014a)

(continued)

Table 4.2 (continued)

Products	Culture types	Plant species	References
Dihydroartemisinic acid glycosides	Suspension	*Artemisia annua*	Zhu et al. (2014b)
Oleanolic acid and ursolic acid	Cell suspension cultures	*Salvia officinalis*, *S. virgata*, and *S. fruticosa*	Haas et al. (2014)
ß-Carboline alkaloids	Hairy roots	*Tribulus terrestris*	Sharifi et al. (2014)
Artemisinin	Hairy roots	*Artemisia annua*	Patra and Srivastava (2014)
Diosgenin	Hairy roots	*Helicteres isora*	Kumar et al. (2014)
Hyoscyamine	Hairy roots	*Anisodus acutangulus*	Cao et al. (2014)
Terpenoid	Cell culture	*Taxus chinensis*	Zhou et al.(2015a)
Alkaloids	Cell culture	*Catharanthus roseus*	Van Moerkercke et al. (2015)
Triterpenoids	Leaf explants	*Centella asiatica*	Singh et al. (2015)
Trans-resveratrol	Cell suspension cultures	*Vitis vinifera*	Almagro et al. (2015)
Steviol glycosides and phenolics	Shoot cultures	*Stevia rebaudiana*	Alvarez-Robles et al. (2016)
Tryptophan decarboxylase and strictosidine synthase	Hairy roots	*Vinca minor*	Verma et al. (2015)
Solasodine	Hairy roots	*Solanum trilobatum*	Shilpha et al. (2015)
Tanshinone	Hairy roots	*Salvia miltiorrhiza*	Hao et al. (2015)
Phenylpropanoid	Hairy roots	*Withania somnifera*	Sil et al. (2015)
Taxadiene	Leaf discs	*Artemisia annua*	Li et al. (2015b)
Xanthones	Roots, hairy roots, and cell suspension cultures	*Hypericum species*	Zubricka et al. (2015)
Rosmarinic acid	Hairy roots	*Salvia wagneriana*	Ruffoni et al. (2016)
Alkaloids	Hairy roots	*Catharanthus roseus*	Sun and Peebles (2016)
Rutin and quercetin	Hairy roots	*Fagopyrum tataricum*	Huang et al. (2016)
Glucosinolates and phenolic compounds	Hairy roots	*Brassica rapa*	Chung et al. (2016)
Diterpene tanshinone	Hairy roots	*Salvia miltiorrhiza*	Shi et al. (2016)
Rosmarinic acid and salvianolic acid B	Hairy roots	*Dracocephalum forrestii*	Weremczuk-Jeżyna et al. (2016)
Bacoside A	Hairy roots	*Bacopa monnieri*	Largia et al. (2016)
Artemisinin	Hairy roots	*Artemisia pallens*	Pala et al. (2016)
Lignan (polyphenol)	Hairy roots and Callus	*Linum usitatissimum*	Gabr et al. (2016)

(continued)

Table 4.2 (continued)

Products	Culture types	Plant species	References
Astragalosides	Hairy roots	*Astragalus membranaceus*	Gai et al. (2016); Jiao et al. (2016)
Abietane diterpenes	Hairy roots	*Salvia sclarea*	Vaccaro et al. (2017)
Anthraquinone	Hairy roots	*Rubia tinctorum*	Perassolo et al. (2017)
Iridoid and phenylethanoid glycoside	Hairy roots	*Rehmannia glutinosa*	Piatczak et al. (2015)
Camptothecin	Hairy roots	*Ophiorrhiza mungos*	Deepthi and Satheeshkumar (2017)
Flavonoids	Hairy roots	*Lactuca serriola*	El-Esawi et al. (2017)
Hypericin, rutin, pseudohypericin, hyperforin, quercetin, emodin, quercitrin, and hyperoside	Hairy roots	*Hypericum tomentosum* and *H. tetrapterum*	Nigutova et al. (2017)
Resveratrol	Hairy roots	*Vitis vinifera*	Hosseini et al. (2017)
Flavonoid	Hairy roots	*Isatis tinctoria*	Jiao et al. (2018a, c)
Polyphenolic compounds	Hairy roots	*Salvia viridis*	Grzegorczyk-Karolak et al. (2018)
Antioxidants and flavonoids	Hairy roots	*Raphanus sativus*	Balasubramanian et al. (2018)
Tropane alkaloids	Hairy roots	*Przewalskia tangutica*	Lei et al. (2018)
Polyphenolic and flavonoid compounds	Hairy roots	*Althaea officinalis*	Tavassoli and Afshar (2018)
Flavonoids, saponins, hydroxycinnamic acids, pectins, and protopectins	Hairy roots	*Nitraria schoberi*	Zheleznichenko et al. (2018)
Saponins	Cell suspension cultures	*Kalopanax septemlobus*	Lee et al. (2018)
Lactoferricin and lactoferrampin	Hairy roots	*Nicotiana tabacum*	Chahardoli et al. (2018)
Tanshinones	Hairy roots	*Salvia miltiorrhiza*	Xing et al. (2018)
Steroidal glycoalkaloids (α-solanine and α-chaconine)	Hairy roots	*Solanum tuberosum*	Nakayasu et al. (2018)
Wedelolactone and other phenolics and flavonoids	Hairy roots	*Sphagneticola calendulacea*	Kundu et al. (2018)
Phenolic acids (rosmarinic acid and lithospermic acid B)	Hairy roots	*Salvia miltiorrhiza*	Zhou et al. (2018)
Camptothecin	Shoot cultures	*Ophiorrhiza mungos*	Krishnan et al. (2018)

cultures can be retained as organ cultures for long durations, and following shoot regenerations can be acquired without any cytological aberrations (Giri and Narasu 2000). *A. tumefaciens*-mediated transformation helps to improve the production of SM in shoot culture as well as cell suspension culture. For example, *A. tumefaciens* transformed *Mentha citrata* shoot cultures produce terpenes (Spencer et al. 1990), and *Coleus forskohlii* transformed cell suspension cultures increase the production of forskolin (Mukherjee et al. 2000). *A. tumefaciens*-mediated genetic transformation system was optimized in *Aloe barbadensis* (He et al. 2007) and *A. annua* (Elfahmi and Chahyadi 2014). An effective antimalarial drug "artemisinin" content was increased up to 38% in *A. annua* plants by overexpressing two novel genes cytochrome P450 monooxygenase and cytochrome P450 reductase through *A. tumefaciens*-mediated genetic transformation (Shen et al. 2012). Activation tagging is a powerful method for producing gain-of-function mutants in various plants, and a high-throughput hairy root-activation tagging technique was reported in transformed hairy roots of *Arabidopsis thaliana*, *Solanum tuberosum*, and *Nicotiana tabacum* (Seki et al. 2005). In this system the vector should be hosted into *A. tumefaciens*, but not into *A. rhizogenes*, and the binary vector T-DNA comprising *rol* gene cluster will be consequently integrated into the plant genome through typical *A. tumefaciens*-mediated transformation method, resulting in the induction of transformed roots and activation tagging of the plant genes therein (Seki et al. 2005). *A. tumefaciens* transformation method was optimized for some more medicinal plant species, i.e., *Panax quinquefolius* (Chen and Punja 2002), *Trigonella foenum-graecum* (Khawar et al. 2004), *Salvia miltiorrhiza* (Yan and Wang 2007), *Linum usitatissimum* (Szopa et al. 2009), *Kalanchoe pinnata* (Jung et al. 2009), *Catharanthus roseus* (Srivastava et al. 2009; Wang et al. 2012b), *C. roseus* (Verma and Mathur 2011), *A. annua* (Li et al. 2015a; Xu et al. 2017), and *Echinacea pallida* (Wang et al. 2017a). The main aim of plant genetic transformation is to multiply the quantity of naturally synthesizing SM and the production of biopharmaceuticals (Bandurska et al. 2016).

4.5 Influence of Fungal Growth as Source of Novel Secondary Metabolite Synthesis

The detection of novel SM is gradually attaining the importance in advanced biotechnology field. Highest frequency and number of resistances beside conventional antibiotics extremely requires novel compounds to stabilize accumulative plant, animal, and human mortality rates. Furthermore, evolution of plant pathogens has to be defined to diminish the yield losses. Another serious question is the post-harvest assembly of harmful mycotoxins in plants. Fungi produce SM, and these natural compounds are low molecular weight that, distinct primary metabolites, remains replaceable for survival of the organism. SM synthesis and fungal development are related processes; therefore, the molecular regulators of growth might be appropriate to determine innovative bio-active mycological compounds or to assist as objectives to regulate fungal growth and development or SM production (Gerke and Braus 2014). Several SM possess biological accomplishments that can range from

favorable to harmful, and some examples of useful SM include anticancer compound taxol, antibacterial agents such as penicillin, antifungal agent caspofungin, immunosuppressive medicine ciclosporin, and importantly cholesterol-dropping drug such as lovastatin. Above 50% of the newly approved drugs between 1981 and 2014 were of SM derivation underlining the high significance of revisions in biotechnology arena (Newman and Cragg 2016).

4.6 Recent Advancements for the Quantitative and Qualitative Analysis of Plant

The improvement of powerful new omics approaches, comprising next-generation sequencing, has been even impelling opportunities for TPCC (Ochoa-Villarreal et al. 2016). However, insufficient genome information of plants contributing procedures that lead to their biosynthesis confines the facility to increase their production by in vitro and in vivo methods (Weeks and Chang 2011). In this regard, the promising transcripts involved in the biosynthesis of therapeutic metabolites in *Swertia japonica* were reported with transcriptome assembly (Rai et al. 2016). Genome sequence information with the understanding of transcriptome expression analysis and accumulating phytochemicals through different explants provide a wide-ranging knowledge of various ongoing metabolic processes, which may provide a support to formulate strategies for improved biosynthesis of active compounds (Rai et al. 2016; Rai and Saito 2016). Recent progressions in the next-generation sequencing analysis with decreased experimental charges and improvement of computational facilities to accomplish the de novo transcriptome analysis, annotation, and subsequent studies have developed the arena of phytochemistry and natural medicine especially in non-model plants with no existing genomic information (Saito 2013; Muranaka and Saito 2013; Rai et al. 2016). Next-generation sequencing technology revolution has fortified the natural bio-active compound research and produced some exciting next-generation sequencing-based SM gene cluster discovery assignments. The genes encoding characteristic SM biosynthetic enzymes in *Camellia sinensis* were typically identified through next-generation sequencing (Li et al. 2015a, b). In this framework, promising new approaches are evolving that hold significant potential for forthcoming applications.

4.7 Conclusion and Future Perspectives

Genome mining approaches consent to the utilization of the information in available genome sequences for the improvement of innovative natural compounds. The cooperation between genome mining approaches and the estimated profusion of SM in plants is a hopeful path to determine the novel natural products as a resource of pharmaceutically important drugs. In order to progress the quantity of active SM in medicinal plants, few important features could be conceded including the

overexpression of functional genes, site-directed mutagenesis of the respective enzymes, gene silencing, synthesizing SM by grouping with chemical synthesis approach, and application of novel elicitors. In the existing state of aggregate resistances in contradiction of established drugs, drastic yield losses, antibiotics, and human mortality rates due to insects, pathogens, and other diseases, the new bioactive SM needs to be identified, for which the enormous medicinal plant species is well suitable. It is equally essential that the molecular mechanisms of virulence, toxin invention, and the control of the biosynthetic pathways will be more clarified.

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5 An Insight into Biotechnological Approaches Used for the Improvement of Secondary Metabolites from the Medicinal Aquatic Plant, Water Hyssop (*Bacopa monnieri* L.)

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Abstract

Water has a significant role on human civilizations by providing food and water along with plants grown on the banks or within the water bodies. Aquatic plants or semiaquatic plants are the group of plants that love to live in or near water bodies, and some of these plants are economically important due to their usage as food or medicinal plants. Water hyssop or brahmi (*Bacopa monnieri* L.) is one of the important semiaquatic/aquatic plants that has been used for medicinal purposes since ancient time in Indian subcontinent. The plant contains several secondary metabolites like bacosides which are used as memory enhancer tonic commercially. Brahmi-based registered drugs are available in India and other countries as memory enhancer tonic and for other diseases like Alzheimer's disease, anxiety, asthma, stomach ulcers, and respiratory ailments and for curing chronic diseases like cancer. Bacopa is facing the threat of extinction from wild as it is not a cultivated plant and propagation through seed is limited due to low availability of and viability of seeds. On the other hand, extensive works on the propagation of this important medicinal plant has been reported to develop in vitro protocols for its conservation and plant propagation for secondary metabolite production. Different in vitro techniques like cell suspension culture, callus culture, and organogenesis have been reported with the objective of producing or enhancing bacoside. Furthermore, application of other biotechnological approaches like *Agrobacterium*-mediated genetic transformation studies, use of mutagens, and in vitro polyploidization have also been reported. Thus, the aim of this chapter is to highlight the application of different biotechnological approaches used for the production, conservation, and secondary metabolite production of *B. monnieri*.

Keywords

Aquatic · Biotechnology · Bacosides · In vitro · Micropropagation

5.1 Introduction

Water hyssop (*Bacopa monnieri*; family, Scrophulariaceae; genus, *Bacopa*) or brahmi (local name in India) is a semiaquatic herb and commonly grows in wetlands, damp, and marshy areas of warmer regions of the world (Al-Snafi 2013; Behera et al. 2016). There are more than 100 aquatic species found in the genus

Bacopa across the globe (Russo and Borrelli 2005). It is native to India and Australia (Aguiar and Borowski 2013) and grown in East Asian countries like Arabian Peninsula, China, Sri Lanka, Nepal, Taiwan, and Vietnam and Florida (Khare 2003; Daniel 2005; Lansdown et al. 2013). The plant is found at 4400 ft altitude and can be cultivated easily depending on the availability of water (Bone 1996). In India, it ranked second among plants, based on uses as medicinal purposes along with advances in research and development and commercial value (Jain et al. 2013). *B. monnieri* is a small semiaquatic creeping, succulent herb having 10–30-cm-long stem and simple leaf and white or blue flowers with 5 mm fruit (capsule). The macroscopic studies revealed 5 mm cylindrical roots and cylindrical and glabrous stem with prominent nodes and internodes. Leaves are simple, sessile, glabrous, opposite, and obovate-oblong to spatulate in shape and generally long (0.6–2.5 cm) and wide (3–8 mm). The flowers are pale blue/pinkish white in color with five corollas, four stamens, two celled anthers, ovary with two chambers, and multiple ovules. Seeds are very small, irregular, and oblong in shape (Jain et al. 2016). Flowers and fruits are produced during summer (Bone 1996).

Uses of *B. monnieri* are known in the complementary and alternative medicines (CAM) since ancient times (Kean et al. 2017). It is an important constituent of Ayurvedic system of medicine and mainly as a nerve tonic for curing various neurological and neuropsychiatric diseases. The ancient Ayurvedic treatises like *Charaka Samhita* since the sixth century AD had mentioned the *Bacopa* formulations, against the mental conditions like anxiety, cognition, and diuretic, and an energizer for heart and nervous system. In the modern era, *Bacopa* is used for commercial mental tonic due to their bio-active compounds like alkaloids, bacosides, saponins, and sterols. The important phytochemicals of *Bacopa* are bacosides which are triterpenoid saponins of dammarane types (Sivaramakrishna et al. 2005) with 12 known analogs of bacoside family (Garai et al. 2009). Other saponins in *Bacopa* are novel bacopasides I–XII (Garai et al. 1996; Chakravarty et al. 2001, 2003) or alkaloids like brahmine, herpestine, nicotine, apigenin, cucurbitacins, D-mannitol, hersaponin, monnierasides I–III, or plantainoside (Kawai and Shibata 1978; Deepak et al. 2005; Bhandari et al. 2007; Kregel and Zhang 2007; Valko et al. 2007; Chakravarty et al. 2008; Phrompittayarat et al. 2007). Other chemical constituents contain glycoside, flavonoids, phytochemicals, amino acids, and esters (Behera et al. 2016). Among these, bacoside A is the most prominent saponin, and most of the research related to brahmi is based on bacoside A.

Based on containing highly important and bio-active metabolic compounds, they are used for curing illness and disorders like Alzheimer's disease (Chaudhari et al. 2017), anti-amnesic activities (Saraf et al. 2008, 2010; Anand et al. 2010), antianxiety and antidepressant activities (Shader and Greenblatt 1995; Bhattacharya and Ghosal 1998), anti-arthritic activities (Viji et al. 2010; Vijayan et al. 2010), Antiepileptic (Dar and Channa 1999), antihyperglycemic activity (Ghosh et al. 2011), anti-inflammatory (Channa et al. 2006), antimicrobial effect (Joshi et al. 2013), antioxidant and adaptogenic properties (Bhakuni et al. 1969; Tripathi et al. 1996; Rao et al. 2000; Chowdhuri et al. 2002; Govindarajan et al. 2005), cardioprotective activities (Mohanty et al. 2010), central nervous system (Rao et al. 2000), DNA damage in humans and astrocytes (Elangovan et al. 1995; Kar et al. 2002),

DNA replication in cancer cell lines (Channa et al. 2006), endocrine effects (Singh and Singh 1980), free radical scavenging effects (Yadav et al. 1989; Sivaranjan and Balachandran 1994), gastrointestinal effects (Sairam et al. 2001; Sumathy et al. 2002; Goel and Sairam 2002; Dharmani and Palit 2006), hepatoprotective (Sumathi and Nongbri 2008; Sumathi and Devaraj 2009), memoy enhancer (Abhang 1993), promoting hair growth (Jain et al. 2012, 2014; Jain 2016), sedative and tranquilizing properties (Bhakuni et al. 1969), stimulatory effect on thyroid function (Jain et al. 1994; Kar et al. 2002), withdrawal effects of morphine (Kar et al. 2002) or healings of wound (Sharath et al. (2010).

The application of combined traditional and modern biotechnological approaches accomplishes the genetic improvement of economic crops. *Bacopa* is mainly used as medicinal plant, and all efforts to conserve or its improvement to date are based solely on its bio-active bacoside A. Biotechnological techniques allow to develop desired traits in short time with elite characteristics. In recent years especially in the last decade or from the beginning of this millennium, *Bacopa* has gained attention of researchers, and studies related to its conservation and genetic improvement using different biotechnological and molecular biology like assessment of genetic diversity, artificial-induced mutation, application of in vitro plant tissue culture techniques for conservation, regeneration and bacoside production, synthetic seed production, use of nanoparticles (NPs) for callus induction or for biosynthesis of NPs, role of microbes for bacoside production, phytoremediation potential of *Bacopa*, genetic transformation studies in *Bacopa*, and genomics and transcriptomics of *Bacopa* have been reported. Thus, the aim of this chapter is to highlight the application of different biotechnological approaches used for the production, conservation, and secondary metabolite production of *B. monnieri*.

5.2 Genetic Diversity in *B. monnieri*

Exploitation of biodiversity of plants for improving the quantitative and qualitative characteristics with wide adaptation in different geographical regions is the basic of breeding programs in the modern scientific era. Rapid increase in human population demands to manipulate local cultivars with modern breeding programs to meet the demand of nutrition and other plant-based medicinal compounds used for the health of human beings. Contrarily, the erosion of genetic material due to overexploitation, use of elite cultivars, and lack of local genetic material bring such plants to endangered category. It is therefore the right demand to save these landraces with novel genes by applying modern biotechnological techniques for their conservation for future. In recent years, characterization of phenotype and genotype of different crops/species and modern techniques like gene mapping and sequencing enable researchers to exploit the functional genomics of desired species (Baloch et al. 2017). Molecular marker technique is used to measure direct genetic among species/genotypes/cultivars on the basis of morphological characteristics or geographical distribution. In *Bacopa*, different molecular markers have been reported to determine genetic diversity, and it is interesting to note that all works related to

genetic diversity to date are based from India, where this plant is found in different parts and used commercially. Researchers collected plants from different geographical regions of India and used different molecular markers to find genetic diversity.

RAPD markers are widely used techniques for assessing the genetic diversity of *Bacopa* compared to other techniques employed (Karthikeyan et al. 2011; Kumar et al. 2013a, b; Srivastava et al. 2016; Anu et al. 2017). The first study of RAPD markers was reported by Darokar et al. (2001). They collected 25 accessions of *Bacopa* mainly from India and from Malaysia and tested with 40 RAPD primers. Out of these, 29 primers generated single or multiple polymorphic bands ranged from 2 to 8 per primer. The similarity of matrices was found between 0.8 and 1.0, which showed the medium polymorphism level. Thereafter, Karthikeyan et al. (2011) applied 10 RAPD markers on 25 of *Bacopa* accessions of different geographical regions of India and compared with in vitro shoot propagation. They got the band size ranged 200–870 bp with 113 amplified bands. Out of 113 bands, only 14 were polymorphic with range of 0–30.77%. A maximum number of 18 amplified bands were generated by OPD 08, but polymorphism was recorded only 1%. Cluster analysis indicated the two subgroups using similar coefficient. Similarly, Kumar et al. (2013a) collected eight accessions from Tamil Nadu region of India and applied six different OPL markers. They identified a total of 30 loci with 50% each of polymorphic and nonpolymorphic loci. The polymorphism ranged 0–83.33% as OPT-18 primers failed to generate any polymorphism (%). Contrarily, maximum polymorphism (%) was achieved when OPL-05 primers were applied.

Bacopa plants (18 accessions) collected from southern part of India were subjected to a total of 20 RAPD primers, out of which only 7 generated single or multiple bands. A total of 490 bands were recorded, and 328 and 162 were polymorphic and nonpolymorphic, respectively. Maximum genetic diversity (%) was recorded from OPT-1 and OPT-18 which was 69.2%, while minimum genetic diversity (%) was obtained from OPL-6 (17.8%). The similarity indices among all accession ranged from 0.8 to 1.0 (Kumar et al. 2013b). Anu et al. (2017) used 15 accessions (Western Ghats, South India), and 22 primers responded to all accession with a total of 197 bands with average polymorphic bands of 8.50. Sixteen out of 22 primers were 100% polymorphic with total of 187 polymorphic bands.

RAPD markers were also used for assessing the variation among in vitro regenerated plants in different studies. Ceasar et al. (2010) assessed the in vitro regenerated plantlets with five different RAPD markers, and all plants were monomorphic in nature and similar to mother plants. In vitro regenerated shoots of *Bacopa* were subjected to ten RAPD markers for assessing genetic fidelity. A total of 58 bands were amplified and only 8 were found polymorphic. The average polymorphism was recorded 13.19% (Pathak et al. 2013). Recently, Sharma et al. (2017a) achieved plant regeneration frequency of 0–20% from cryopreserved shoot tips and compared it with control plants. Their results revealed insignificant variation among shoots from both control and cryopreserved shoot tips using ten RAPD markers and HPLC (high-performance liquid chromatography) to quantify bacoside A contents. Their results revealed the genetic stability of in vitro regenerated shoots after cryopreservation treatment.

RAPD markers are also applied for assessing the genetic stability of *Bacopa* plants encapsulated with alginate. Randomly selected 19 plants after regeneration followed by regrowth of alginate-encapsulated uninodal cuttings were subjected to RAPD markers. A total of 334 bands were amplified with 72 (21.5%) polymorphic bands. The genetic distance of micropropagated plants ranged from 0.00 to 0.92, while encapsulated synthetic seeds showed 0.67–0.92 (Ramesh et al. 2011a). Muthiah et al. (2013) applied 20 ISSR (inter simple sequence repeats) and 25 RAPD primers to in vitro grown plantlets regenerated from encapsulated shoot tips for 6 months at 4 °C. A total of 130 bands with 125 monomorphic bands from ISSR primers were generated, whereas 25 RAPD primers generated 125 bands with 94% monomorphism. Their results from both studies revealed the use of more than one molecular marker for assessing genetic variability of *Bacopa* collected from nature or regenerated under in vitro conditions.

Two different markers RAPD and ISSR were used for amplification of 15 accessions collected from Central Indian States. RAPD markers generated a total of 197 bands with 8.95 bands per primer, and 187 bands were polymorphic, whereas 25 ISSR markers produced a total of 280 bands having 270 polymorphic bands with 10.8 bands/primer. The polymorphic information content (PIC) ranged 0.363 - 0.908 (RAPD) and 0.419 - 0.836 (ISSR). Whereas, similarity index ranged 0.16 - 0.95 (RAPD), 0.18-0.98 (ISSR) and 0.179 - 0.945 for ISSR and RAPD markers (Tripathi et al. 2012). Yadav et al. (2012) collected five different accessions of *Bacopa* from Central and southern and northern region of India. A total of 50 primers were tested, and only 14 produced a total of 515 DNA amplicon. On the basis of sequence of RAPD amplicon, they developed SCAR (sequence-characterized amplified region) primers and obtained single band (406 bp) of *Bacopa* in all five accessions. They concluded that RAPD and SCAR markers can be used for identification of fresh *Bacopa* plants. In the next step, they collected brahmi-based drugs from market, based on *B. monnieri*, and from other plants but sold as brahmi. Application of SCAR markers to these drugs revealed positive results to *Bacopa*-based drug samples, and drug samples from other plants were negative. They concluded that SCAR marker can be useful for the identification of *Bacopa* in fresh and in dry form.

There is a single report about the use of ISSR or amplified fragment length polymorphism (AFLP) markers for assessing the genetic diversity among *Bacopa* plants propagated under in vitro conditions or accession collected from nature (Krishna et al. 2013). They applied 15 ISSR markers to in vitro regenerated shoots of *Bacopa* up to 10 passages and achieved 57 bands with 56 monomorphic and 1 polymorphic band. The dendrogram analysis revealed the no or zero genetic inconsistency of plants cultured on standard or reduced culture conditions, whereas plants cultured on medium enriched with NAA (1-naphthalene acetic acid) and IBA (indolebutyric acid) revealed the minor variation. Impact of ecogeographical region on the quantification of bacoside A to check the chemodiversity was investigated on 75 accession of *B. monnieri*. Results revealed the clear impact of chemodiversity as bacoside A contents varied with region (Srivastava et al. 2016). They subjected 36 AFLP

markers to 9 different samples of each location, and after initial screening, 2 best primer pairs were selected for final fingerprint generation from high- and low-yielding accessions of *B. monnieri*. They recorded 16 bands and 9 were found polymorphic with 56.25% polymorphism. There was no record of specific clustering using principal coordinate analysis (PCoA) or dendrogram.

5.3 Mutation Breeding of *B. monnieri*

The presence of important medicinal metabolites in *Bacopa* and their use as commercial drug create a new window for researchers to improve its characteristics. Researchers developed and are still developing the new protocol of in vitro regeneration with an objective to regenerate plants and to get higher concentrations of bacosides. But there is still a large gap, and researchers are trying to exploit the potential of *Bacopa* to develop new traits which can be grown in field conditions with superior agronomic and medicinal properties. Application of mutation breeding is an important and commonly practiced technique to create genetic variation among existing plant gene pool (Toker et al. 2007) which may help in selection process in a given environment (Yadav et al. 2007). As a result, there is a possibility of gaining large number of alleles (Chopra 2005) with a recessive or segregated (3:1) traits, and these traits must be controlled up to or beyond second generation (Micke and Donini 1993). In plant tissue culture, two types of mutagens, physical and chemical, are used for induced mutation. Limited studies highlight the use of physical mutagen like γ–rays (Varghese and Sathyanarayana 2007; Naik et al. 2012) or chemical mutagens like ethyl methanesulfonate (EMS) (Vajpaye et al. 2006; Naik et al. 2012), methyl methanesulfonate (MMS) (Vajpaye et al. 2006), or colchicine (Escandón et al. 2006; Kharde et al. 2017).

5.3.1 The Use of Physical Mutagens

In vitro nodal segments and leaf-induced calli (1 mg/l 2 4-dichlorophenoxyacetic acid (2,4-D)) of *Bacopa* were treated with γ-rays at the rate of 2.5 Gy/min; 0, 30, 40, 50, 60, 80, 90, and 100 Gy radiation treatments were used for nodal segment explant and 0, 30, 40, 50, 60, and 80 Gy for leaf-induced calli of two cultivars. Treatments of γ–rays induced morphological variability in both plants. They obtained 3.03% w/w bacoside A contents from nodal segment explant of Pragyashakthi cv. compared to 2.60% w/w (calli) and 1.60% w/w (control), whereas bacoside A contents were recorded as 2.61% w/w (calli), 1.75% w/w (control), and 1.17% w/w (nodal segment) from Calcutta Local cultivar (Varghese and Sathyanarayana 2007). Leaf explants of *Bacopa* were treated with 10, 20, 40, and 80 gray (Gy) for 0, 0.5, 1.0, 1.5, 2.0, and 2.5 hours (h) followed by culture on 2.0% sugar- and 2.0 mg/L KIN-containing medium. Eighty-four percent mortality rate of explant at 80 Gy and induced mutation at 10, 20, and 40 Gy (γ-rays) were reported, and five lines were produced which yielded more bacoside A content compared to control (Naik et al. 2012).

5.3.2 The Use of Chemical Mutagens

The plants of *Bacopa* were treated for 2 h with different concentrations of mutagens (0.001–5 mM EMS and 0.01–500 μM MMS) in order to evaluate the ecogenotoxicity by using comet assay to assess DNA damage. Acellular/in vitro exposed isolated nuclei or whole plants were exposed to these mutagens. The results indicated the dose-dependent DNA damage to both mutagens, and this damage was higher in root nuclei compared to leaf nuclei to both mutagens (Vajpaye et al. 2006). A study by Varghese and Sathyanarayana (2007) revealed the exposure of nodal segments and leaf-induced calli explants to 0.5% EMS for 0, 0.5, 1.0, 1.5, 2.0, and 2.5 h for two different cultivars (Pragyashakthi, Calcutta Local cultivar). They reported decreased bacoside A content with increase in exposure time to EM, whereas no increase in bacoside A content was recorded from leaf explant treated with EMS irrespective of exposure time.

Colchicine is another chemical mutagen applied for induced mutation or somaclonal variation in *Bacopa* plant. Colchicine treatment of nodal segments with 0.001% concentration for 24 or 48 h resulted in increased flower size (Escandón et al. 2006). They inoculated the explants on medium having 0.25 mg/l BAP (6-benzylaminopurine) and obtained two different plants (tetraploid) from control plants with difference in size and color of flower and leaf. Kharde et al. (2017) treated leaf explants with 0.1% and 0.2% colchicine for 1, 2, 3, 4, and 5 h and cultured on 1.1 μM IBA and 0.30 μM IBA. They observed changes or variations like leaf shape, number, and arrangements and enhanced bacoside contents which were higher when treated with 0.2% colchicine for 5 h, whereas treatment with 0.1% colchicine for 2 h yielded twofold bacoside contents that were recorded at 0.72%.

5.4 In Vitro Plant Tissue Culture of *B. monnieri*

B. monnieri is medicinal aquatic plant. It contains bio-active compounds, which have been used as medicine and attribute pharmacological activities (Ganjewala and Srivastava 2011). Zhou et al. (2009) reported at least 70 chemical constituents mainly saponins (Chillara et al. 2005), and bacoside A is the main saponin which attributes biological activities (Deepak and Amit 2004; Peng et al. 2010). *Bacopa* is a native plant of India which shows the narrow genetic diversity. The plant is used as memory enhancer (Charles et al. 2011), and a commercial drug is also available. The plant was reported threatened to extinction due to its wild collection and high demand (Tanvir et al. 2010; Tiwari and Singh 2010). Due to these factors, there is a need to develop strategies to conserve plant and also propagate to meet the demand of bacoside A. There are two ways to meet the objective: (a) the use of traditional vegetative propagation or seeds or (b) the application of in vitro plant tissue culture techniques.

Plant cell and tissue culture techniques include callus culture, cell suspension cultures, somatic embryogenesis, or organogenesis (Aasim et al. 2014) for the production of elite plants. These techniques can be used for isolation of economically

important bio-active compounds. The results on different plants/crops show more advantageous for secondary metabolite isolation through in vitro culture compared to plant/seeds taken from field conditions. Furthermore, consistency, controlled conditions, and elite nature of cells/callus/plants taken from in vitro culture make it superior for metabolite production (Talukdar 2014). Furthermore, it is possible to alter the metabolite concentration with the aid of adding different chemicals/enzymes/organic compounds in the culture medium or controlled change in culture conditions like lights, temperature, etc. However, it is also significant to understand the variations in metabolite production or medicinal pathway (Al-Habori and Raman 2002). The in vitro techniques have two parts: (a) in vitro cell/callus/cell suspension and protoplast culture and (b) organogenesis or somatic embryogenesis based in vitro regeneration.

5.4.1 In Vitro Cell Suspension Culture/Callus Culture for Bacoside Production

In recent years, researchers reported work related to in vitro cell/callus/cell suspension of *Bacopa* with main focus on phytochemical production of bacoside. Cell suspension culture from callus is the most widely used technique used for secondary metabolites synthesis (Talukdar 2014). It also provides the facility to investigate the efficacy of variable organic and inorganic chemicals or biotic elicitors (Parale and Nikam 2009) or variable growth conditions on cell growth subsequently followed by secondary metabolite production of economic medicinal plants. Rahman et al. (2002) achieved friable green calli on leaf explant (0.5 mg/l KIN, 1 mg/l NAA, 1 mg/l casein hydrolysate, 30 g/l sucrose) and shifted to liquid medium with the same concentrations in complete darkness. They achieved bacoside A contents at the rate of 1 g/100 g dry cells. Leaf explants of *Bacopa* were used for callus induction by culturing it on 1 μM 2,4-D + 5 μM NAA-containing medium. The medium was also enriched with 0–125 μM glycine or 0–200 μM of phenylalanine, α-ketoglutaric acid, ferulic acid, or pyruvic acid singly. Application of 100 M pyruvic acid significantly enhanced the bacoside A from callus culture (Parale et al. 2010).

Successful callus induction from leaf explant using different combinations of BAP, IAA (Indole-3-acetic acid), KIN (1: 0.05: 0.05 or 1.5: 0.05: 0.05), and 2, 4-D: BAP (1:0.5; 1.5:0.5), was reported by Mendhulkar et al. (2011). They generated the cell suspension by shifting 1 g callus to liquid medium containing 1: 0.5 (2, 4-D: BAP). They treated the 21-day-old cell suspension with 0.2%, 0.6%, and 1.0% DMSO for 3 and 6 h and obtained maximum bacoside contents (4.6 ± 0.03 μg/mg) from suspension culture treated with 1% DMSO for 3 h. Bansal et al. (2014) optimized the KNO_3, KH_2PO_4, glucose, and inoculum density for the growth of cell suspension and bacoside A contents, whereas application of RSM (response surface methodology), 5.67% glucose, 0.313% KNO_3, and 0.29% KH_2PO_4 with inoculum density (0.66%) was optimized and revealed twofold biomass yield and 1.7-fold bacoside A.

Besides of use of callus derived cell suspension culture for Bacoside A production, callus culture using different explants, growth medium or adding different

chemicals have been used also for Bacoside A synthesis. Showkat et al. (2010) induced callus using 0.5 mg/L 2,4-D from leaf explants. Bacoside and fingerprint profile of in vitro regenerated shoots using HPLC or HPLTC from callus revealed the similar phytochemical profile to that of mother plant or plants obtained from markets. Monica et al. (2013) obtained callus of *Bacopa* leaf by culturing it on medium with 0.5 mg/l 2,4 after 20 days and transferred it to a liquid medium similar to Rahman et al. (2002) containing 0.5 mg/l KIN, 1 mg/l NAA, 1 mg/l casein hydrolysate, and 30 g/l sucrose for 20 more days in darkness. They achieved 166% more saponin contents from cell suspension culture compared to plants taken from nature. Talukdar (2014) induced maximum callus culture from leaf and nodal segment explant and achieved highest callus weight on medium containing 0.2 mg/l NAA (24.67 g) or 2.0 mg/l 2,4-D (35.69 g) after 8 weeks of culture. They reported total bacoside content of 1.53% compared to 1.02% from field-grown *Bacopa* using HPLC. Recently, Hegazi et al. (2017a) reported the collection of *B. monnieri* plants from the Eastern Mediterranean coastal region of Egypt (North Sinai). They successful induced callus from leaf explant from medium enriched with 9 μM 2,4-D and 2.3 μM KIN. They also reported the effects of mevalonic acid (precursor) and chitosan and methyl jasmonate (elicitors) and got more biomass with 100 mg/L chitosan and highest bacoside A contents when 10 mM mevalonic acid was used. They also checked the efficacy of 100 mg/l chitosan (elicitors) and obtained 30.76-fold bacoside A contents compared to control plant.

5.4.2 In Vitro Regeneration/Organogenesis of *B. monnieri*

In recent years, large numbers of research work on in vitro regeneration of *Bacopa* have been published especially after in this millennium. The main objective in these studies was to develop or modify the existing protocols for the conservation of *Bacopa* as plant is considered as endangered in the literature. The demand of plant is increasing immensely and researchers are developing new protocols. This section presents the insight as regards in vitro regeneration techniques about growth medium, explants, plant growth regulators (PGRs), rooting, and acclimatization used by researchers. The information given in this section is based on the literature used and analyzed.

In vitro morphogenesis, shoot growth, and rooting vary with nutritional requirement of tissue used and plant type. The basic objective of adding basal medium is to meet the demand of macro- and micronutrients and vitamins, and their requirement also varies with the explant, tissue, or plant type (Saad and Elshahed 2012). The studies on plant tissue culture of *Bacopa* revealed the use of mainly MS medium at different concentrations like full MS (Gurnani et al. 2012; Asha et al. 2013; Jain et al. 2013; Kaur et al. 2013; Koul et al. 2014; Mohanta and Sahoo 2014; Subashri and Pillai 2014; Rency et al. 2016; Wangdi and Sarethy 2016; Haque et al. 2017; Srivastava et al. 2017; Zote et al. 2018), with some reports of using 0.5 MS (Haque et al. 2017) or one half MS (Jain et al. 2014), whereas B5 medium has also been reported in some studies (Mohapatra and Rath 2005; Monica et al. 2013; Koul et al. 2014).

The presence of reducing carbon or nonreducing carbon sources in plant tissue culture media is an important factor for providing energy and carbon source for photosynthesis or maintaining cell's osmotic potential (Sumaryono et al. 2012) in the culture media, which in turn controls the morphogenetic potential (Yaseen et al. 2013). However, it depends mainly on concentration and type of carbon source and technique used for regeneration, callus induction, germination or rotting, etc. Most widely and recommended carbon source in tissue culture are sucrose, fructose, or glucose, but the most preferable carbon source is sucrose due to its effects and cost (Sumaryono et al. 2012). In vitro regeneration studies on *Bacopa* revealed the use of sucrose at different concentrations like 3% (Showkat et al. 2010; Vijayakumar et al. 2010; Asha et al. 2013; Kumari et al. 2014; Pandiyan and Selvaraj 2012; Begum and Mathur 2014; Behera et al. 2015; Nagarajan et al. 2015; Nandhini et al. 2015; Rency et al. 2016; Karataş et al. 2013, 2016, 2018; Narwal 2016; Wangdi and Sarethy 2016; Hegazi et al. 2017a, b; Srivastava et al. 2017; Ranjan and Kumar 2018) or reduced sucrose at the rate of 2.0% (Escandón et al. 2006; Kaur et al. 2013; Jain et al. 2014; Naik et al. 2014; Ranjan et al. 2018) in the culture medium for regeneration and rooting.

In vitro tissue culture of economic plants depends on culture medium composition like gelling agents which makes medium viscous (Jain 2006). There are several commercial gelling agents for plant tissue culture, but agar is the most preferable gelling agent compared to others like gelrite or phytagel or plant-based gums (Babbar et al. 2005). For *Bacopa* regeneration, solid medium gelled with agar or other gelling agents was preferred, but some studies also revealed the use of culture medium with reduced or no gelling agent (liquid medium) based on the need of the experiment. Agar at different concentrations has been successfully employed for in vitro regeneration with concentration of 0.65% (Showkat et al. 2010; Karataş et al. 2013, 2016, 2018, Karataş and Aasim 2014), 0.7% (Escandón et al. 2006; Kaur et al. 2013, Mohanta and Sahoo 2014; Behera et al. 2015; Mishra et al. 2015), 0.75% (Kaur et al. 2013), or 0.8% (Tiwari et al. 2001; Mohapatra and Rath 2005; Joshi et al. 2010; Prabha et al. 2010; Parale et al. 2010; Rout et al. 2011; Gurnani et al. 2012; Pandiyan and Selvaraj 2012; Rao et al. 2012; Asha et al. 2013; Begum and Mathur 2014; Kumari et al. 2014; Naik et al. 2014; Nagarajan et al. 2015; Narwal 2016; Rency et al. 2016; Wangdi and Sarethy 2016; Kashyap et al. 2017; Srivastava et al. 2017; Ranjan et al. 2018), whereas some studies revealed the use of phytagel (Hegazi 2016; Hegazi et al. 2017a, b) and gelrite (Nandhini et al. 2015) in the culture medium. A study by Yusuf et al. (2011) highlighted the comparison of different concentrations of isabgol (1.0%, 3.0%, and 5.0%) with agar (0.7, 1.0, or 1.5%) for in vitro regeneration of *Bacopa*.

Surface sterilization of plant seed or plant parts is the most important step toward plant tissue culture techniques which include the removal or minimizing the exogenous or in some cases endogenous microbial contamination (Buckley and Reed 1994). Micropropagation of aquatic plants usually involves the use of vegetative parts, directly subjected to surface sterilization without any substantial damage to explants during sterilization (Aasim et al. 2013). Selection of proper sterilizing agent and exposure time (Mihaljević et al. 2013) are of utmost importance and depend on

physical or morphological characteristics of plant part like tissue's hardness/softness (Srivastava et al. 2010). Like other aquatic plants, *Bacopa* is propagated through explants taken from vegetative parts and exposed to different sterilizing agents with different times of exposure. Studies on in vitro regeneration of *Bacopa* revealed the use of $HgCl_2$ as major sterilizing agent at different concentrations like 0.01% (Showkat et al. 2010; Vijayakumar et al. 2010; Mohan et al. 2011; Jain et al. 2013; Kumari et al. 2014; Subashri and Pillai 2014), 0.05% (Zote et al. 2018), 0.2% (Koul et al. 2014), and 1% (Mohapatra and Rath 2005) with different exposure times. Thereafter, NaOCl is second most used sterilizing agent but at low concentrations like 0.5% (Soundararajan and Karrunakaran 2011) or 1% (Koul et al. 2014; Zote et al. 2018) and 2% (Naik et al. 2014; Umesh et al. 2014; Hegazi et al. 2017a, b).

Besides that, other detergents or antiseptic chemicals have also been reported for sterilization of *Bacopa*. It includes the use of Labolene detergent (Rout et al. 2011; Asha et al. 2013; Begum and Mathur 2014; Mohanta and Sahoo 2014; Nandhini et al. 2015); Teepol, a multipurpose detergent (Mohapatra and Rath 2005; Gurnani et al. 2012; Rao et al. 2012; Behera et al. 2015; Nagarajan et al. 2015; Kashyap et al. 2017; Ranjan et al. 2018); Savlon, antiseptic detergent (Vijayakumar et al. 2010; Mohan et al. 2011; Pandiyan and Selvaraj 2012; Ranjan and Kumar 2018); and Rankleen (Prabha et al. 2010) and Cetrimide, an antiseptic (Soundararajan and Karrunakaran 2011; Mishra et al. 2015; Srivastava et al. 2017). Sterilization process also involved the use of additive chemicals to enhance the sterilization efficiency. The chemicals used for sterilization with other major sterilizing agents are Tween (Escandón et al. 2006; Sharath et al. 2007; Yusuf et al. 2011; Kaur et al. 2013; Koul et al. 2014; Narwal 2016; Haque et al. 2017), alcohol (Showkat et al. 2010; Jain et al. 2014; Mohanta and Sahoo 2014; Subashri and Pillai 2014; Umesh et al. 2014; Nagarajan et al. 2015; Narwal 2016; Rency et al. 2016; Wangdi and Sarethy 2016; Kashyap et al. 2017; Srivastava et al. 2017; Ranjan et al. 2018), Bavistin fungicide (Kaur et al. 2013; Mohanta and Sahoo 2014; Haque et al. 2017), streptomycin + Bavistin (Mohan et al. 2011; Vijayakumar et al. 2010; Ranjan et al. 2018), and Bavistin + neomycin (Showkat et al. 2010).

The selection of proper explant is an important part of plant tissue culture protocol as it results in the development of adventitious or axillary shoots under in vitro conditions. Besides that, the presence or absence of meristematic cells in the explant also controls the regeneration process as organogenesis and somatic embryogenesis along with other factors like plant growth regulators, culture conditions, etc. For explant selection, different factors like explant age and size, plant quality, genotype, and objective of study (callus induction, somatic embryogenesis, organogenesis) must be taken in account (Smith 2012). For *Bacopa* micropropagation, different explants used can be classified as (a) explants regenerated adventitious shoots or (b) explants regenerated axillary shoots.

For adventitious shoot regeneration, leaf explant is the most widely used explant (Tiwari et al. 2001; Joshi et al. 2010; Parale et al. 2010; Rout et al. 2011; Vijayakumar et al. 2010; Yusuf et al. 2011; Rao et al. 2012; Jain et al. 2013; Koul et al. 2014, 2015; Naik et al. 2014; Umesh et al. 2014; Ayyappadas and Renugadevi 2015; Behera et al. 2015; Nandhini et al. 2015; Haque et al. 2017; Mehta 2017; Ranjan et al. 2018; Srivastava et al. 2017; Zote et al. 2018) followed by internode (Tiwari

et al. 2001; Mohan et al. 2011; Yusuf et al. 2011; Rao et al. 2012; Kaur et al. 2013; Naik et al. 2014; Ayyappadas and Renugadevi 2015; Behera et al. 2015; Kashyap et al. 2017; Mehta 2017; Srivastava et al. 2017) and root explants (Vijayakumar et al. 2010).

Contrarily, different explants are also used for axillary shoot regeneration or callus induction like shoot apex/shoot meristem (Pandiyan and Selvaraj 2012; Jain et al. 2013; Kaur et al. 2013; Subashri and Pillai 2014; Ayyappadas and Renugadevi 2015; Hegazi 2016; Łojewski et al. 2016; Hegazi et al. 2017a, b), nodal segment from different parts of plants (Tiwari et al. 2001; Escandón et al. 2006; Prabha et al. 2010; Showkat et al. 2010; Vijayakumar et al. 2010; Yusuf et al. 2011; Gurnani et al. 2012; Pandiyan and Selvaraj 2012; Asha et al. 2013; Jain et al. 2013, 2014; Kaur et al. 2013; Kumari et al. 2014; Mohanta and Sahoo 2014; Naik et al. 2014; Subashri and Pillai 2014; Umesh et al. 2014; Ayyappadas and Renugadevi 2015; Behera et al. 2015; Mishra et al. 2015; Nagarajan et al. 2015; Narwal 2016; Wangdi and Sarethy 2016; Hegazi et al. 2017a, b; Kashyap et al. 2017; Mehta 2017; Srivastava et al. 2017; Ranjan et al. 2018), apical buds (Narwal 2016), and stem (Vijayakumar et al. 2010; Karataş et al. 2016; Zote et al. 2018).

The provision of PGR in the culture medium along with other factors like explant, basal medium, culture conditions, etc. controls the in vitro callogenesis and organogenesis. These PGRs in the culture medium are used at different concentrations based on the objective of the study. Cytokinins and auxins are used generally for in vitro regeneration. Cytokinins are used either singly or in combination with auxins, whereas auxins alone are used mainly for callus induction followed by organogenesis by transferring the calli to the medium enriched with cytokinins or auxins + cytokinins.

Bacopa is not a recalcitrant in nature and responds well enough to PGRs in the culture medium irrespective of explant even without meristematic regions like leaf or internodes. Different studies on *Bacopa* revealed the use of different cytokinins alone at variable concentrations for different explants. BAP is the most accepted and preferred PGR used for in vitro regeneration (Mohapatra and Rath 2005; Joshi et al. 2010; Prabha et al. 2010; Yusuf et al. 2011; Rao et al. 2012; Asha et al. 2013; Kaur et al. 2013; Jain et al. 2014; Kumari et al. 2014; Behera et al. 2015; Mishra et al. 2015; Nagarajan et al. 2015; Karataş et al. 2016; Srivastava et al. 2017; Haque et al. 2017). Other cytokinins used alone are KIN (Kumari et al. 2014; Naik et al. 2014; Wangdi and Sarethy 2016) and TDZ (Tiwari et al. 2001). A study by Begum and Mathur (2014) reported the use of BAP + KIN combination for in vitro regeneration of *Bacopa*, whereas Subashri and Pillai (2014) optimized different cytokinins (1.0 mg/l each of BAP and TDZ, 4.92 mg/l 2ip) for the regeneration of *Bacopa* in vitro. On the other hand, combination of cytokinin and auxins is also optimized for maximum shoot induction of *Bacopa*. These combinations include BAP + IAA (Gurnani et al. 2012; Narwal 2016; Ranjan and Kumar 2018), BAP + NAA (Rout et al. 2011; Jain et al. 2013; Rency et al. 2016; Ranjan et al. 2018), BAP + IBA (Zote et al. 2018), and KIN + IBA (Mehta 2017), whereas a combination of BAP + KIN + NAA has also been reported (Vijayakumar et al. 2010; Pandiyan and Selvaraj 2012; Ayyappadas and Renugadevi 2015). There are very few studies which reflected the use of TDZ alone or in combination with auxins. Karataş and Aasim

(2014) reported the multiple shoot buds on TDZ, but these buds generated shoots when transferred to MS medium without PGRs. It is also interested to note that *Bacopa* can be propagated without any PGR in the culture medium (Koul et al. 2014) or shoot induction can be achieved by adding IAA or NAA in the culture medium (Mohanta and Sahoo 2014). In conclusion, all explants used for *Bacopa* regeneration respond well to PGRs irrespective of PGR type or concentration. It is also concluded that BAP solely and the combination of KIN and auxins (IAA, NAA, IBA) are most suitable for regeneration.

PGRs are generally used for callus or shoot induction in vitro. Researchers always tried nontraditional organic or inorganic chemicals or biological extracts for enhancing or inducing in vitro regeneration of economic plants. Being an economic plant, *Bacopa* is one of the plants subjected to different chemicals for exploiting the in vitro regeneration potential. Pothiaraj et al. (2016) used seaweed liquid extracts (SLEs) isolated from *Gracilaria edulis* and *Sargassum wightii* and compared with PGRs for *B. monnieri*. Application of 30% (*S. Wightii*) and 40% (*G. Edulis*) liquid extracts significantly enhanced the shoot and root proliferation with increased survivability of in vitro propagated plants. Kashyap et al. (2017) applied humin (a residue taken from acid-base treatment of vermicompost) alone or along with micronutrients, vitamins, or 3.0 mg/l BAP + 1 mg/l IAA in culture media and cultured nodal segment explants for shoot induction. They achieved higher shoot induction, leaf induction, plantlet weight, and survival rate on medium containing humins compared to humins with other supplements.

The rooting of in vitro regenerated shoots is linkage step between transfer of regenerated shoots/plantlets to external field conditions. Rooting followed by adaptation is an important part of successful plant tissue culture protocol. There are studies which skipped the rooting stage due to direct rooting of shoots (plantlets) in the culture medium due to the presence of auxins (Gurnani et al. 2012; Pandiyan and Selvaraj 2012) or even medium containing only KIN (Naik et al. 2014). Other studies even revealed the use of MSO (MS without any PGRs) for rooting and achieved high percentage of rooting (Asha et al. 2013; Mohanta and Sahoo 2014; Subashri and Pillai 2014; Ranjan and Kumar 2018). On the other hand, IBA was used most frequently auxin for rhizogenesis of Bacopa (Tiwari et al. 2001; Joshi et al. 2010; Rao et al. 2012; Kaur et al. 2013; Jain et al. 2013, 2014; Kumari et al. 2014; Behera et al. 2015; Karataş et al. 2016; Srivastava et al. 2017; Zote et al. 2018) followed by IAA (Rout et al. 2011; Narwal 2016; Rency et al. 2016). In all these studies, rooting response was high up to 100%, and these results reflected the easiness of rooting stage. Multiple shoot inductions with callogenesis during rooting medium containing IBA from the cut end of shoots were reported by Karataş et al. (2013). It shows that even auxins alone can also be used for direct plantlet regeneration in short time with longer shoots.

After rooting, the next stage is the adaptation/acclimatization of plantlets to external conditions. *Bacopa* is a semiaquatic plant which can survive in water and also in soil with high moisture. Soil as substrate for transferring plantlets for acclimatization has been reported in almost all of the studies. However, adaptation of in vitro regenerated plantlets in aquariums containing water was reported by Karataş et al. (2013). Furthermore, they also checked the plant growth in aquariums with different pH levels (4–10) and reported maximum plant growth at pH 8.0.

5.4.3 In Vitro Regeneration of *B. monnieri* for Bacoside and Other Metabolites

Although callus culture or cell suspension culture is the most accepted in vitro technique for isolation of secondary metabolites, in vitro regenerated shoots through organogenesis are also a good source of these metabolite isolations. The use of organogenesis for bacoside A and other secondary metabolites production is available. Praveen et al. (2009) regenerated *Bacopa* shoots in semisolid and liquid medium and gained more shoots from liquid medium. Analysis of bacoside A contents revealed more contents compared to semisolid medium, whereas more shoots were recorded from medium containing 2 mg/l KIN. Parale et al. (2010) used leaf explants for shoot induction in liquid medium containing 5 μM BAP. They also used organic supplements in the culture medium and noted enhanced bacoside A contents with 100 μM pyruvic acid. The bacoside contents were higher than control (4-fold) or naturally grown plantlets (1.2-fold). Sharma et al. (2013) applied methyl jasmonate to 1-month-old shoots and cultured it in liquid medium. They obtained maximum bacoside contents (1.8-fold higher than control) after 1 week.

Umesh et al. (2014) obtained plantlets by direct organogenesis (plantlets from leaf explant ın medium enriched with 2 mg/l KIN. However, bacoside contents varied with PGRs, and the highest concentration of bacopasides I and II was recorded on a medium with 1 mg/l BAP + 0.5 mg/l IAA or 2 mg/l KIN, respectively. Nandhini et al. (2015) achieved maximum shoot buds (162.33 ± 21.385) with 0.2 mg/L BAP. They checked the secondary metabolite contents of in vitro regenerated plantlets and found lower flavonoid contents compared to control plants, whereas minor differences in phenol and saponin contents were recorded when compared with control plants. Łojewski et al. (2016) used Mg and other metal-enriched media for shoot induction and Bacosides A contents. They achieved highest bacosides (37.3 mg/g dry weight) from cultures enriched with 1.0 mg/l BAP + 0.2 mg/l NAA + 0.25 g/l serine + 0.1 g/l Mg or 1.0 mg/l BAP + 0.2 mg/l NAA+0.5 g/l serine +0.5 g/l Mg. Hegazi et al. (2017b) obtained maximum shoot multiplication after six subculture (2.45 μM IBA+2.3 μM KIN). They also used precursor (mevalonic acid) and elicitors (chitosan and methyl jasmonate) for biomass and bacoside A production. One hundred micrometer methyl jasmonate enhanced the biomass, while 10 mM mevalonic acid resulted in 8.26-fold more bacoside A accumulation in shoots.

5.5 Regulation of Bacoside Biosynthesis by Beneficial Microbes

The application of biotic elicitors or chemical precursors/elicitors has also been used for increasing callus biomass and bacoside A production. Parale and Nikam (2009) inoculated callus derived from a liquid medium (5 μM NAA and 1 μM 2,4-D) with different strains of fungus used as elicitors. Only inoculation with *Saccharomyces cerevisiae* enhanced the bacoside contents up to 20%, whereas other biotic elicitors resulted in decreased bacoside contents from callus culture. Inoculation of plant beneficial microbes like *Chitiniphilus* sp. MTN22 and

Streptomyces sp. MTN14 with Bacopa plant significantly enhanced resistance against nematode and also up-regulation of Bacoside biosynthetic genes. The genes in the pathway of bacoside biosynthesis were 3-hydroxy-3-methylglutaryl coenzyme A reductase, mevalonate diphosphate decarboxylase, and squalene synthase. Further, the elicitation due to microbes enhanced the bacoside production significantly than the control treatments.

5.6 Cryopreservation of *B. monnieri*

Preservation of plant material for short time of few days to mid or long term upto few months is an important technique in germplasm conservation. These techniques are slow growth storage (in vitro techniques) or cryopreservation using liquid nitrogen (Ozudogru et al. 2010) or combination of different techniques for conservation. Slow growth conservation under in vitro conditions is based on slowing down the growth process of plant tissue without affecting its viability and regrowth under ambient conditions. The two most used techniques for short- to midterm preservation are encapsulation (up to 6 months) and vitrification (up to 12 months) for *Bacopa*. Sharma et al. (2011) used vitrification technique for cryopreservation of shoot tip explants. Their results revealed the significant increase in survival and regeneration frequency of cryopreserved explants when precultured with sucrose at 25 °C. Sharma et al. (2016) optimized the single-step protocol for regeneration, establishment, and medium-term conservation of *Bacopa*. Shoots were preserved for 12 months with relatively high survival rate and confirmed the genetic stability using molecular markers. In another study, Sharma et al. (2017a) cryopreserved the shoot tips of four different accessions with vitrification. They achieved 0–20% regeneration frequency from these cryopreserved explants. Comparison of these plants with non-vitrified plants using RAPD analysis or HPLC for bacosides revealed the genetic and biochemical stability.

5.7 Encapsulation (Synthetic Seed Production) of *B. monnieri*

Synthetic seed technology (SST) deals with the explant encapsulation regenerated in vitro/in vivo by applying alginate (Bukhari et al. 2014). It provides an alternative system for multiplication, storage, short-term preservation, and transportation of elite cloned traits (Gantait et al. 2015a). However, factors like explants, encapsulating agent, and matrix are significant for successful establishment of SST especially in medicinal plants. In recent years, SST has also been employed for *Bacopa* plants taken from in vitro regenerated plantlets. The first study was reported by Bansal and Pandey (2011), and they successfully regenerated the alginate-encapsulated shoot tip explants after storage. Similarly, shoot tip explants encapsulated with calcium alginate beads were stored at 24 ± 2 and 4 °C for 6 months and recorded 100% viability and regrowth of stored encapsulated shoot tips (Hegazi 2016), whereas

shoot tips encapsulated with sodium alginate were also regenerated on medium fortified with cytokinins and auxins (Rency et al. 2016).

Besides shoot tip explant, nodal segment explant was also used for the encapsulation of *Bacopa* using different alginating agents. Sharma et al. (2012) assessed the encapsulated nodal segments of *Bacopa* and obtained 86.67% plantlet conversion after 6–8 weeks of storage. They also checked the efficacy of sodium alginate and $CaCl_2$ on regeneration ability of encapsulated nodal segments. Nodal segments and shoot tips were encapsulated (3% sodium alginate, 80 mM NaCl) and stored at 4, 8, and 24 °C for 1 month. Thereafter, they were regenerated on medium having 0.44 μM BAP + 0.53 μM NAA, whereas storage for 6 months revealed the 100% regeneration from synthetic seeds derived from shoot tip at 4 °C (Muthiah et al. 2013). Gantait et al. (2015b) successfully encapsulated the nodal segment explants and obtained uniform beads when 2.5% sodium alginate + 75 mM NaCl was used and successfully obtained plantlets on 0.5 MS semisolid medium. These results clearly highlight the efficient use of SST for the conservation of *Bacopa*.

5.8 Phytoremediation Potential of *B. monnieri*

Bacopa is collected from nature as wild plant which is found in wet and marshy areas. These areas are generally polluted with industrial or pesticidal contaminants (Hussain et al. 2011) which pollute the water. The heavy metals contained in water are absorbed by *Bacopa* plants. Accumulation of Al, As, Cd, Cr, Cu, Fe, Hg, Mn, Ni, Pb, and Zn elements in *Bacopa* plants collected from nature was reported by Hussain et al. (2011). Similarly, Abdussalam et al. (2011) reported the bioaccumulation of Hg and Cd in *Bacopa* plants. Higher concentrations of Cd, Pb, Cu, and Zn (above threshold level) in *Bacopa* samples tested and reported inappropriate for human consumptions as herbal medicines by Srikanth Lavu et al. (2013). Mishra et al. (2016) collected the brahmi-based drugs from markets [Brahmi Ghrita (BG), Brahmi vati (BV), Saraswat Churna (SC)] and checked the heavy metal and pesticide residues in these samples. Their results highlighted the presence of heavy metals (Cd, Cr, Ni, Pb) or some pesticides like oxamyl, hexachlorocyclohexanes (α-HCH, β-HCH, and γ-HCH), dichlorodiphenyldichloroethylene, and dichlorodiphenyltrichloroethane. However, their concentration was below toxicity level. Keeping in view, collection of plant samples for herbal preparation is significant, and samples collected from the wild must be screened prior to making herbal medicines. Application of biotechnological techniques such as in vitro regeneration using plant tissue culture can be used to alleviate such types of risks.

5.9 Genetic Transformation Studies in *B. monnieri*

Advancement in genetic engineering techniques in recent years helps the researchers to incorporate elite genes of interest in plants in order to get traits with desired agronomic characteristics (Karami 2008). The development of reliable and

repeatable in vitro regeneration protocol is prerequisite for successful genetic transformation. Besides that, other factors like genetic transformation technique used, type of genotype/cultivar and explant, PGR type and concentration, proper use of selection medium, *Agrobacterium* strains and cell density, etc. are also important to increase transformation efficiency. Furthermore, application of histochemical and molecular biology techniques (PCR, RT-PCR, hybridization) and bioassay is also vital for the confirmation of insertion, integration, and expression of genes in transgenes of different progenies. Genetic transformation of medicinal plants in recent years is gaining popularity in order to increase the economically important secondary metabolites.

Bacopa is also an important medicinal aquatic plant because it contains bacoside A which is a commercial brahmi-based drug used as brain tonic. Several techniques are in use for genetic transformation in plants, but interestingly, only *Agrobacterium tumefacien*s (Nisha et al. 2003; Ramesh et al. 2011b; Aggarwal et al. 2013; Yadav et al. 2014; Kumari et al. 2015; Paul et al. 2015; Croom et al. 2016; Sharma et al. 2017b)- and *Agrobacterium rhizogenes* (Majumdar et al. 2011; Bansal et al. 2014; Paul et al. 2015; Largia et al. 2016)-mediated genetic transformation have been reported for *Bacopa* to date. *Agrobacterium*-mediated genetic transformation is now most widely used genetic transformation technique for both monocots and dicots (Karami 2008). The advantages of *Agrobacterium*-mediated transformation are stable DNA integration into genome but with low copy numbers (Shou et al. 2004) and stable transgene expression in progeny (Hu et al. 2003).

5.9.1 *A. tumefaciens*-Mediated Genetic Transformation in *B. monnieri*

The first report on *Agrobacterium*-mediated genetic transformation of *Bacopa* was reported by Nisha et al. (2003). After that, the number of other studies by different researchers were published in current decade (Ramesh et al. 2011b; Aggarwal et al. 2013; Yadav et al. 2014; Kumari et al. 2015; Paul et al. 2015; Croom et al. 2016; Sharma et al. 2017b). In these studies, researchers used different *Agrobacterium* strains with plasmid-containing genes, selection medium, explant type, and different techniques for confirmation of transgenes. Leaf explant was the most preferable explant for genetic transformation studies (Aggarwal et al. 2013; Yadav et al. 2014; Kumari et al. 2015; Paul et al. 2015; Sharma et al. 2017b), whereas other explants like node (Ramesh et al. 2011b) and TCL from leaf or stem (Croom et al. 2016) were also used successfully for genetic transformation. For these explants, different types and concentrations of PGRs were used like 1.5 mg/l BA + 0.1 mg/l NAA + 0.1 mg/l GA3 (Nisha et al. 2003) and 1.0 mg/l BAP + 0.1 mg/l NAA + 0.1 mg/l GA3 (Ramesh et al. 2011b). On the other hand, 1–2 mg/l BA and 0–0.2 mg/l IAA (Kumari et al. 2015) and 2.0 mg/l BAP and 2.5 mg/l KIN (Sharma et al. 2017b) were added in the culture medium for putative transgenic shoot induction. Contrarily, Paul et al. (2015) cultured leaf explants on MSO for transgenic shoot induction of *Bacopa*.

Incorporation of specific gene of interest is the basic aim of any genetic transformation study that is driven by specific constitutive or non-constitutive promotors. The genetic transformation studies of *Bacopa* revealed the use of reporter or selectable marker genes driven by constitutive promotor. In these studies, *uid* was the most widely used gene (Nisha et al. 2003; Ramesh et al. 2011b; Aggarwal et al. 2013; Yadav et al. 2014; Kumari et al. 2015; Croom et al. 2016; Sharma et al. 2017b), whereas genes like neomycin phosphotransferase (*nptII*) (Nisha et al. 2003; Aggarwal et al. 2013; Yadav et al. 2014; Paul et al. 2015), hpt (Ramesh et al. 2011b; Kumari et al. 2015) and GFP (Croom et al. 2016), cryptogein gene (Paul et al. 2015), and tryptophan decarboxylase (tdc) or strictosidine synthase (str) (Sharma et al. 2017b) were also reported. It was also interested to note that CAMV 35S was the most used promotor in these studies. NOS promotor was also used for *nptII* gene in some studies (Nisha et al. 2003; Aggarwal et al. 2013). Based on the presence of reporter or selectable marker genes along with explant type, provision of proper selective agent and its concentration are also important for the enhancement of genetic transformation efficiency. The studies reflected the use of single antibiotic at the rate of 50 mg/l hygromycin (Sharma et al. 2017b) or two selective agents (antibiotics) like 15 mg/l kanamycin (kan) and 300 mg/l cefotaxime (cef) (Nisha et al. 2003; Yadav et al. 2014), 10 mg/l hygromycin (hyg) and 250 mg/l cef (Ramesh et al. 2011b), 50 µg/ml kan and 500 µg/ml carbenicillin (Aggarwal et al. 2013), 200 mg/l cef and 10 mg/l hyg (Kumari et al. 2015), or 500 mg/l cef and 100 mg/l kan (Paul et al. 2015).

After successful development of transgenes, the confirmation of gene integration and expression in different progenies is an important factor to obtain transgenic plants or lines. Techniques like GUS (β-glucuronidase) activity, polymerase chain reaction (PCR) analysis, and reverse transcription polymerase chain reaction (RT-PCR) were used by Nisha et al. (2003) and Kumari et al. (2015), whereas Ramesh et al. (2011b) confirmed transgenes by GUS and PCR analysis. Aggarwal et al. (2013) used different techniques like GUS, PCR, and RT-PCR analysis of *nptII* gene for confirmation. Yadav et al. (2014) applied techniques like histochemical GUS analysis, PCR analysis of *nptII* and GUS gene, and fluorometric GUS assay for the confirmation of transgenes, whereas RT-PCR analysis of GFP transcript and GUS analysis of putative transgenes were reported by Croom et al. (2016). Sharma et al. (2017b) used series of techniques like GUS, PCR, Southern blot hybridization, and RT-PCR. They also used metabolite profiling and quantification by HPLC.

5.9.2 *A. rhizogenes*-Mediated Genetic Transformation in *B. monnieri*

Hairy root (HR) culture is an important technique used for developing adventitious root induction using *Agrobacterium rhizogenes* for obtaining secondary metabolites. The inoculation of explants taken from medicinal plants with *A. rhizogenes* helps to produce bio-active compounds. There are few reports available which highlight the successful use of *A. rhizogenes* for hairy root production to increase

bacoside production. Different *A. rhizogenes* strains were inoculated with explants like leaf (Majumdar et al. 2011; Bansal et al. 2014; Paul et al. 2015; Largia et al. 2016) or internode (Bansal et al. 2014) followed by culture on MS medium without any PGR (MSO). For selection, different antibiotics like 500 mg/L ampicillin (Majumdar et al. 2011; Largia et al. 2016) or 500 mg/l cef and 100 mg/l kan (Paul et al. 2015) were added in the selection medium. After genetic transformation, putative transgenes (HR) were confirmed by PCR and RT-PCR of rol AB or rol A, TR, and ags genes (Majumdar et al. 2011). Paul et al. (2015) used PCR analysis for the detection of the rol genes (*rolA*, *rolB*, *rolC*, *rolD*) and TR DNA (*aux1*, *aux2*, *ags*, *mas1*, *mas2*) and also used semi-qRT-PCR technique. They also checked the bacoside contents of transgenes by HPLC, whereas Largia et al. (2016) confirmed the transgenes with PCR for rol A gene, Southern blot hybridization, and elicitation of transformed plants with chitosan and also performed HPLC analysis to confirm the bacoside contents.

5.10 Application of Nanoparticles (NPs) in *B. monnieri*

Application of nanoparticle (NP) in vitro studies is gaining popularity among researchers in recent years. These NPs are in use for various purposes like antimicrobial activities (Klaine et al. 2008) or toxicological studies (Krishnaraj et al. 2012). These studies are majorly on microorganism or model organism. In recent years, NPs are also applied on plants for different objectives like germination or plant growth (Monica and Cremonini 2009). Furthermore, these NPs have clear impact on biological and pharmacological activities of some plants (Gandhare et al. 2016). In vitro plant regeneration techniques provide an alternative and efficient way of using NPs for different plant species of economic importance. Krishnaraj et al. (2012) exposed the Bacopa seeds to AgNPs and $AgNO_3$ at different concentrations (10 ppb, 100 ppb, 10 ppm and 100 ppm) or cultured the Bacopa seedlings in hydroponic system containing 10 ppm AgNPs, 10 ppm $AgNO_3$ and control without any NPs. Results revealed no effects of AgNPs, while $AgNO_3$ hindered the seed germination with 45% at 10 ppm and zero at 100 ppm. Scanning electron microscopy (SEM) studies revealed the no severe toxic effects on plant morphological characteristics subjected to AgNPs. A couple of studies revealed the use of two different NPs in the culture medium at the rate of 16×10^{10}, 16×10^{5}, and 16×10^{3}. In first study, silver nanoparticles (Kalsaitkar et al. 2014) and, in second study, copper nanoparticles (Gandhare et al. 2016) were applied for callus induction. In both studies, callus were induced at first and then transferred to medium with respective NPs resulting in almost similar results for both NPs. Minimum to medium callus growth was recorded on medium containing 16×0^{5} and 16×10^{3} AgNPs or CuNPs. Callus color was changed from green to light brown (16×10^{5}) or dark brown (16×10^{5}) but with no change in color when cultured on medium with 16×10^{3} AgNPs or CuNPs, whereas complete callus inhibition was recorded at higher concentration of AgNPs or CuNPs. Besides using NPs on plant growth or callus induction, *B. monnieri* have been reported for

biosynthesis of different NPs like gold (Babu et al. 2013; Bommavaram et al. 2013; Bindhu and Umadevi 2014) or platinum (Nellore et al. 2013).

5.11 Transcriptomics and Genomics Resources of *B. monnieri*

B. monnieri is a diploid plant species with chromosome number $2n = 64$. Only up to recent its transcriptomic and genomics studies have been studied. Comparative transcriptomic studies revealed high-quality reads of 22.48 million and 22.0 million in shoot and root samples, respectively. Overall, 26,412 and 18,500 genes were annotated in root and shoot samples, respectively. Lastly, the 43 transcripts related to secondary metabolism were selected after mapping to 133 KEGG pathways (Jeena et al. 2017). The bacoside biosynthesis-related transcripts such as CYP450 monooxygenases, GTs, and β-amyrin synthase were in excess in root tissues; however, their expression was dominating in shoot tissues indicating the site of biosynthesis (Jeena et al. 2017). The identified genes would be useful for bacosides and other secondary metabolites by metabolic engineering either in homologous or heterologous expression system. Another study of de novo assembly of transcriptome of the plant revealed 10,556 simple sequence repeat (SSR) out of 8892 transcripts (Prabhudas and Natarajan 2017).

5.12 Conclusion and Future Prospects

B. monnieri is widely distributed in different geographic regions, but genetic variability studies are very limited. Similarly, use of physical and chemical mutagens resulted in increased Bacoside A contents. Due to its high bacoside A contents, the plant is widely collected from field conditions, and studies revealed the presence of certain heavy metals and pesticidal residues collected from the wild. Therefore, screening of these plants to heavy metals prior to use for making herbal medicines is important. Furthermore, the plant is also considered as threatened endangered plant by some researchers. This problem can be overcome by employing plant tissue culture techniques for its conservation and mass production to enhance bacoside A contents by using callus or cell suspension culture or directly from regenerated shoots. Other important advancements in recent years are the encapsulation technique to make synthetic seeds, the use of biotic elicitors for bacoside production, and the use of NPs for callus induction and also biosynthesis of NPs. Studies in *Bacopa* revealed the *A. tumefaciens*- and *A. rhizogenes*-mediated genetic transformation. However, in these studies, reporter or marker genes were used, and there is a need to incorporate genes related to bacoside A contents. During the last two decades, *Bacopa* is the most important aquatic plant due to its commercial value, but one major area in which the plant needs more research work is the functional genomics, genome sequencing, gene expression, and plant omics. Application of biological tools like QTL or MAS for identifying the potential genes to exploit the full potential of *Bacopa* plants.

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Prospects for the Use of Plant Cell Culture as Alternatives to Produce Secondary Metabolites

6

Hera Nadeem and Faheem Ahmad

Contents

Abstract

Plants have proven to be a beneficial means for uncovering new products having therapeutic interest in the drug augmentation. Human beings uses plant-produced secondary metabolites since from the prehistoric times. Due to high usage of secondary metabolites in diverse marketing sectors, such as pharmaceutical, food, and chemical industries, the demand for the most relevant and accepted method to separate these metabolites from plants is huge. Different extraction techniques

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have been used to obtain secondary metabolites, and many of these techniques are built on the extracting strength of solvents and the application of mixing and/or heat. In addition to traditional methods, several new methods have been established, but till now none of them are considered as a standard method for elicitation of secondary metabolites. In the late 1960s, plant cell culture technologies were found as a promising tool for both investigating and designing plant secondary metabolites. With the help of cell cultures, phytochemicals are not only produced in adequate quantity, but also discard the existence of intrusive compounds that develops in the field-grown plants. This technology serves advantageous over classical methods. Many approaches have been used to amplify the yield of secondary metabolite manufacture by cultured plant cells. Among these approaches are selecting a plant with immense biosynthetic capacity, acquiring efficacious cell line for growth and production of the concerned metabolite, manipulating culture environment, elicitation, metabolic engineering, and organ culture. Mass cultivation of plant cells is done with the help of different bioreactors. Application of cell culture provides various benefits including the synthesis of secondary metabolites, working in controlled conditions as well as autonomous to soil and climate conditions. Elicitor which may be biotic or abiotic is considered as one of the stress agents to obtain increased amount of secondary metabolites from different parts of the plants. Polysaccharides like chitosans are natural elicitors which are benefitted for plant cell's immobilization and permeabilization. A new path has been initiated in current years for secondary metabolite production with the help of elicitors in plant tissue culture. The different criteria that influence the production and accumulation of secondary metabolites include elicitor concentrations, exposure time, cell line, nutrient composition, and age or stage of the culture. In a number of plant cell cultures, elicitors have intensified the production of sesquiterpenoid, phytoalexin, terpenoid indole alkaloids, isoflavonoid, phytoalexins, coumarins, etc. Regardless of these efforts of the past few decades, plant cell cultures have led to very little economic successes for the production of esteemed secondary compounds. Thus, the aim of this chapter is to highlight the prospects of plant cell culture to produce secondary metabolites, and also provides an overview on the important approaches used for the secondary metabolite production and their improvement strategies.

Keywords
Conventional techniques · Elicitation · Bioreactors · Organ culture · Secondary metabolites

6.1 Introduction

The hunt for natural bio-active compounds having promising results for the analysis and prevention of diseases is presently a concern topic for various laboratories and industries. The ability of these bio-active compounds to appropriately combine

with proteins, DNA, and other biological molecules to synthesize a suitable product would be taken advantage for crafting natural product-derived therapeutic agents (Ajikumar et al. 2008). With the advancement of technologies and evolution of advanced methods to enhance the production, detection, separation, and characterization have transformed the screening of natural bio-active compounds, which can be used efficiently for various needs (Van-Lanen and Shen 2006; Wang and Weller 2006). An array of bio-active compounds released from plants as secondary metabolites assist them to enhance their competency to survive and reduce local challenges by approving them to collaborate with their surroundings (Harborne 1993). Plants respond to the attack of pathogens, wounds, insects, and herbivores or to other biotic stresses such as malnutrition (Graham 1991) and abiotic stresses such as low temperature (Zimmerman and Cohill 1991) by stimulating a multitude of defense mechanism including induction of biosynthesis of secondary metabolites. It is very difficult to retrieve a uniform pattern of secondary metabolites in vivo by classical agriculture practices. In a bioreactor, cultivation of plant cells by in vitro which is an industrial alternative offers a precise supply of secondary metabolites with homogenous quality and yield independent of the external factors (Fowler 1985). Many complications have to be faced for acquiring secondary metabolites from plants that include environmental factors, political and labor inconstancy in the producing countries, unbounded variations in the crop quality, inefficiency of authorities to prohibit crop adulteration, and losses in storage and handling. Cell culture technology is a desirable mean for study and synthesis of plant secondary metabolites. The emerging significance of secondary metabolites has appear to be high level of concern for improving cultivation technology with the prospect of increasing their production (Zhong 2001), and researchers are now aimed in altering the production of secondary metabolites by manipulating plant cell culture. Bacteria and fungi, during the past 40 years, have been used particularly in Japan, Germany, and the USA for the production of a vast range of secondary metabolites, the same way they were used for antibiotic or amino acid production (Mulabagal and Tsay 2004).

According to the World Health Organization, most of the organs of medicinal plants contain substances that can be benefited for therapeutic purposes, which are the prototype for chemo-pharmaceutical semi-synthesis. Different parts of plants like leaves, roots, rhizome, stems, flowers, fruits, grains etc. contain biologically active components hence used in control of plant diseases. These plant-derived chemical compounds or bio-active components are responsible for guarding the plant against the microbe infections or infestations by pests (Nweze et al. 2004; Doughari et al. 2009). Plant products can be mainly of two types: (i) primary plant metabolites and (ii) secondary metabolites (Fig. 6.1). Unlike primary metabolites which are directly associated with growth and development, secondary metabolites are not directly involved with the normal growth and development or reproduction of an organism. Though these secondary metabolites are not essential for the plants, they play crucial role in plant defense mechanisms. Secondary metabolites such as alkaloids, steroids, tannins, glycosides, volatile oils, fixed oils, resins, phenols, and flavonoids are found in abundance in

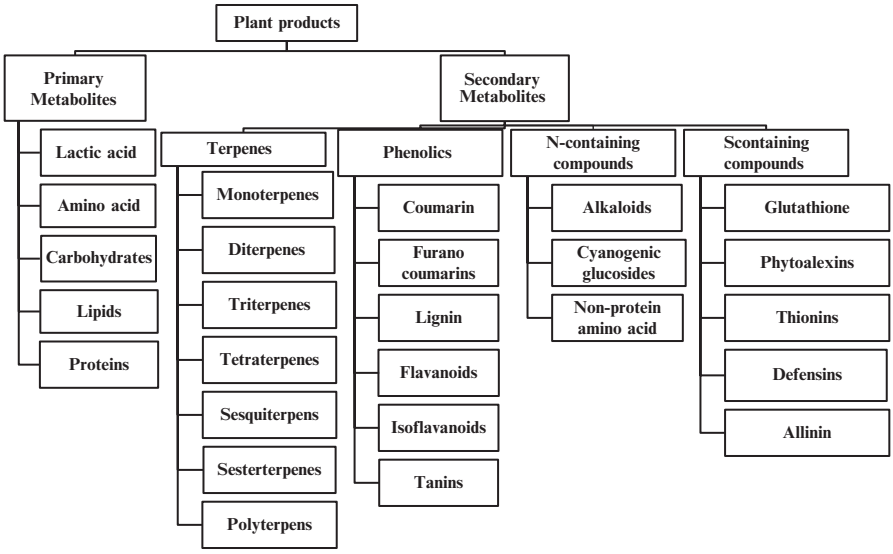

Fig. 6.1 Classification of plant-derived metabolites

various plant parts like leaves, flowers, bark, seeds, fruits, and roots. Phytochemicals obtained from secondary metabolism have been refined for pharmaceuticals, food additives, flavors, and fragrance and for products like latex and tannins. Traditionally, phytochemicals have been derived by distillation from plants thriving in the wild or in plantations. Some callus and cell suspension cultures turn red with time, show lignified tracheid, or emit odor which is a sign of the capability of such cultures to manufacture secondary metabolites. The antioxidant property of bio-active compounds and their beneficial use in processed food as a natural antioxidant have been significantly increased in current years. These natural products either as pure form or standardized contain exceptional chemical diversity, which provides an ideal opportunity for discovery of new drugs (Cosa et al. 2006). More than 80% of the world's population entrust on conventional medicine for their basic healthcare needs, according to the World Health Organization (WHO). Phytochemicals derived from plants are safe and considerably effective alternatives with less unfavorable effect. Almost 20% of recognized plants have been used in pharmaceutical studies; advantageous biological functions such as anticancer, antimicrobial, antioxidant, antidiarrheal, and analgesic and wound-healing property were reported from secondary metabolites (Naczk and Shahidi 2006). Plants containing useful phytochemicals may complement human body needs by acting as natural antioxidants (Suffredini et al. 2004). For example, vitamins A, C, and E and phenolic compounds such as flavonoid, tannin, and lignin present in plants all perform as antioxidants (Boots et al. 2008). By delaying or inhibiting oxidation generated by reactive oxygen species (ROS), antioxidant controls and reduces the oxidative damage in foods and conclusively increases the shelf life and quality of these foods (Ames et al.

1993). Beta-carotene, ascorbic acid, and many phenolic compounds also play a vital role in delaying aging, lowering inflammation, and inhibiting certain cancers (Duthie et al. 1996). Secondary metabolites are mainly classified into five types depending upon their biosynthetic origin:

(i) Polyketides – produced by the acetate-mevalonate pathway
(ii) Isoprenoids – produced via mevalonate pathway
(iii) Alkaloids – synthesized from various amino acids
(iv) Phenylpropanoids – produced from amino acids
(v) Flavonoids – produced by a combination of (i) and (iv)

Studies on callus and cell culture had been done extensively for the production of secondary plant metabolites by late 1950s. The main prospect of implementing such type of technique is to synthesize secondary metabolites from the by-product of cultured cell or tissue which can be used for commercial purposes like pharmaceuticals and cosmetics, hormones, enzymes, proteins, antigens, food additives, and natural pesticides (Terrier et al. 2007). Plant biotechnology provides an excellent opportunity to manipulate cells, tissues, organs, or whole organisms by culturing them in vitro and then getting the required compounds (Rao and Ravishankar 2002). By using different biotechnological approaches, these biologically active metabolites can be developed from callus cultures, cell suspension cultures, and/or organ cultures. From various studies it was found that secondary metabolites are in great amount in differentiated plant tissue, so to harvest these metabolites for the intention to synthesize medically important compounds, various efforts are incorporated to cultivate the entire plant in *in vitro* conditions (Biondi et al. 2002). The organ culture has much more benefit over the conventional culture of undifferentiated cells as they are more reliable for secondary metabolite production (Rao and Ravishankar 2002). Under stress, secondary metabolite biosynthesis in plant cells can be persuaded by elicitors or precursors and/or by utilization of both. Precursors are chemical stress factors that are key substrates, intermediate products, or enzymes of secondary metabolite biosynthesis pathways. Despite, if not used at the correct stage and/or right concentration, they may have toxic or inhibitory effects on the plant cells (Gueven and Knorr 2011). Elicitors are biotic or abiotic chemicals such as heavy metals, pesticides, and detergents or physical factors such as cold shock, UV, and high pressure that induce enzymatic activity against stress (Rao and Ravishankar 2002) triggering accumulation of secondary metabolites (Zhang et al. 2002). General elicitors generate secondary metabolism in a variety of different plants, whereas specific elicitors trigger secondary metabolism in a specific plant. The magnitude of elicitation depends on the effective dose which differs depending on the plant species. Escalation of secondary metabolite production is a delicate process that relies on the dosage of environmental stress besides its stage of application during agriculture. Independent of external factors, bioreactors support a controlled supply of secondary metabolites with consistent quality and yield through in vitro plant cells cultivation (Fowler 1985). During the last five decades, secondary metabolite production employing plant cell cultures has been a scientific

challenge due to insignificant cell yield, moderate growth, and genetic fluctuation of productive cell lines which makes the process inconsistent. Most of the scientific studies on feasibility of the plant cell cultures have been directed (Memelink et al. 2001; Zhong 2001; Verpoorte and Memelink 2002; Sumner et al. 2003). Thus, the aim of this chapter is to highlight the prospects of plant cell culture to produce secondary metabolites and also provide an overview on the important approaches used for the secondary metabolite production and their improvement strategies.

6.1.1 Biotechnology Engineering Coupled with Biochemistry Led to Better Yield of Secondary Metabolites

The involvement of interdisciplinary approaches like biochemistry and biotechnological techniques had managed to get a notable improvement in secondary metabolite production (Cusido et al. 2014; Dias et al. 2016). One of the best examples where biotechnology in conjugation with biochemistry led to the significant growth in production of secondary metabolites is hairy root culture. In this methodology the plant part is selected to infect with *Agrobacterium rhizogenes* favoring higher genetic constancy and growth, and therefore bio-active compounds released to the medium can conveniently be separated and purified to get higher yields (Anand 2010). Hence the higher yield of these bio-active compounds can efficiently be used for various applications in food and pharmaceutical industries.

6.1.2 Importance of Secondary Metabolites

The applications of plant secondary metabolites are tremendous. They may be utilized as therapeutic compounds because of their antimicrobial, anti-inflammatory, and anticancer properties. For example, vincristine (an alkaloid obtained from *Catharanthus roseus*) is an anticancer compound, diosgenin (a saponin obtained from *Dioscorea* species) is used as contraceptive, and menthol (a monoterpene obtained from oil of peppermint) is used in toothpaste. They may be used for their colors and fragrances in food and cosmetic industries and as pesticides and insecticide.

6.1.2.1 Benefits of Plant Tissue Culture Over Traditional Agricultural Practices

As the *in vitro* produced plants are independent to different external factors like geographical and seasonal variations, they provide a continuous and standardized supply of metabolites with homogenous quality and yield as compared to the traditional production. Unique compounds which cannot be easily obtained through parent plants can easily be created through plant tissue culture. An overview on secondary metabolite production by means of plant tissue culture has been shown in Fig. 6.2. Similarly, stereo- and region-specific biotransformation of the plant cells can be done for the manufacturing of bio-active compounds from effective

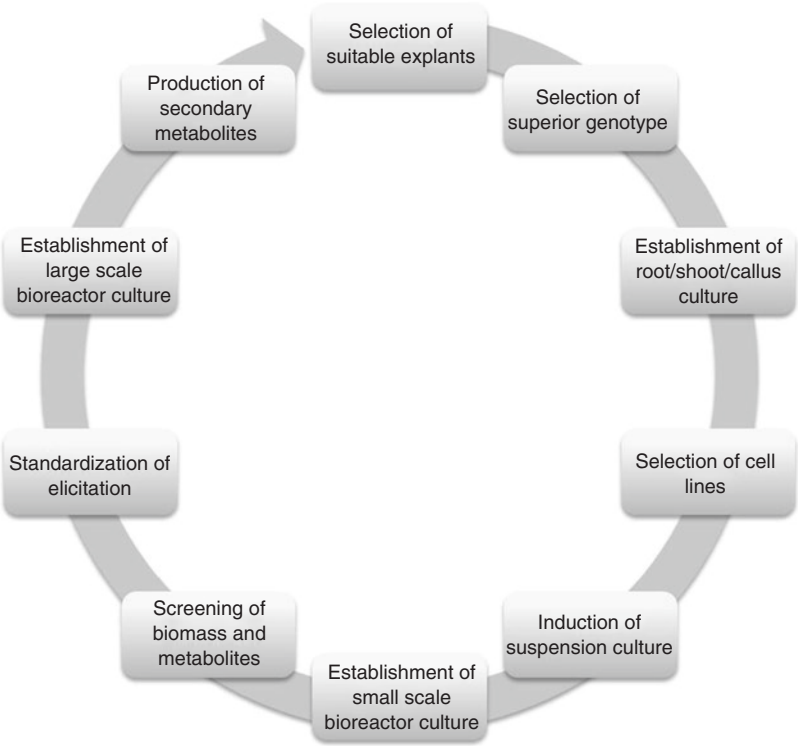

Fig. 6.2 Summary of culture techniques development and production of target secondary metabolites

prototype and is independent to any political intervention. Secondary metabolite biosynthesis is sensitive to aeration because:

(i) Secondary metabolite biosynthesis increased with increase in the diameter of cell aggregates.
(ii) High mass transfer resistance caused by large aggregate size induces secondary metabolite biosynthesis due to lack of mass transfer toward the center of the cell aggregates.
(iii) Cell aggregate size causes diffusion resistance hindering diffusion of intracellular substrates.

6.2 Plant Cell Factory-Mediated Secondary Metabolite Production

The yields of secondary metabolites are highly dependent on internal factors like physiological and developmental phase of plants. Different biotechnological methodologies have been experimented and implemented to get improved and enhanced

quantity of secondary metabolites from medicinal plants. Plant tissue culture serves as an efficient substitute system to get desired natural products which are not sufficiently present in nature. Secondary metabolites produced via plant cell culture are much more favoured over the conventional agricultural production because: (i) It is independent of geographical and seasonal variations and various environmental factors; (ii) It offers a defined production system, which ensures the continuous supply of products, uniform quality and yield; (iii) It is possible to produce novel compounds that are not normally found in the parent plant (Rao and Ravishankar 2002). Secondary metabolite production from plant system includes screening of high-yielding cell line, media modification, precursor feeding, elicitation, large-scale cultivation in bioreactor system, hairy root culture, plant cell immobilization, biotransformation, and others (Rao and Ravishankar 2002; Vanishree et al. 2004).

6.2.1 Bioreactor-Mediated Secondary Metabolite Production

In vitro production of secondary metabolites is an interdisciplinary field, which needs joint efforts between various scientists, plant physiologists, cell and molecular biologists, pharmacologists, toxicologists, chemists, and chemical engineers to assess:

(i) Tissue composition and organization
(ii) Flow and mass transfer conditions in the bioreactor
(iii) Kinetics of cell growth and product formation
(iv) Genetic stability of productive cell lines
(v) Control of micro- and macroenvironment in the bioreactor
(vi) Implications of bioreactor design on downstream processing
(vii) Potential for process scale-up

Bioreactor operation can be batch, fed-batch, or continuous. Batch bioreactors are used to regulate optimum production conditions upon scale-up from small-scale fermentations in a flask. If the cell culture is under the impact of limiting nutrient, fed-batch operation is favored. The usual operation mode after optimization studies is the continuous mode or the chemo state which allows continuous supply of the nutrient medium and removal of the products allowing a steady state operation. If secondary metabolite biosynthesis is growth-related, a single-step bioreactor is sufficient. Elseways, stagewise fermentation is proposed where the first bioreactor is used for culture growth and the second one is used for secondary metabolite biosynthesis (Payne et al. 1993). Intracellular products usually require batch or fed-batch operations, while extracellular products allow continuous production schemes.

6.2.1.1 Application of Bioreactors

Bioreactors are one of the main and essential requirements for application of plant tissue culture for secondary metabolite production. Hence, bioreactors are designed according to the cell culture method so that improved quality and quantity of

secondary metabolites can be produced. Plant cell bioreactors are chiefly divided into five types on the basis of their structure:

(i) Mechanical stirring
(ii) Airlifting bioreactors
(iii) Bubbling bioreactors
(iv) Nutrient mist bioreactors
(v) Temporary immersion bioreactors

All the bioreactors mentioned above have some features that they share in common which includes properly blended media and sterile air. In the case of shaking flask, shaking makes a continuous contact of media with air, whereas in bioreactors the media is agitated or supported by air bubbles or blended with air accordingly, and after that it is transferred to cultured cells. Consequently, distribution of air becomes much critical for bioreactors. In bioreactors, there are some sensors designed which regulate the change in pH, temperature, dissolved oxygen, and bubbles generated. Though mechanical bioreactors can create topmost dissolved oxygen, due to their susceptibility to shear forces, they are not generally employed for plant and tissue culture. Both airlift and bubbling bioreactor have many features in common, and they are mostly employed for plant and tissue culture. Especially for hairy root culture, bioreactors are equipped with stainless steel mesh for giving hairy roots the required support. For tissue culture, the nutrient mist and temporary immersion are used as they have common characteristics. These bioreactors consist of two components: one is for media storage and another one is for tissue culture. With the help of atomizer, the mixture of media and sterile air is sprayed in the form of very small droplet on the outer of cultured tissue; this is the mechanism for nutrient mist bioreactors. In temporary immersion bioreactors, the media is moved on the tissue culture part and there it is kept for short period of time and then later on it is pumped back to the storage tank. With the employment of these bioreactors, many potential secondary metabolites were isolated from medicinal plant cell cultures which are of great importance to industrial use.

6.2.2 Elicitors and Elicitation

Formerly, elicitor was used to describe the molecules that generate production of phytoalexins, but now it is conventionalized as a compound that improves the defense mechanism of plant (Hahn 1996; Nurnberger 1999). Elicitors can also be explained as component when added in little quality to cell system, incites the synthesis of certain important compounds. So, elicitation can be elucidated as accelerated and upgraded biosynthesis of compounds resulting from addition of elicitors in small quantity (Radman et al. 2003; Angelova et al. 2006). Among the tremendous usage of elicitors, it is also practiced for releasing the metabolites into the medium (Pitta-Alvarez et al. 2000).

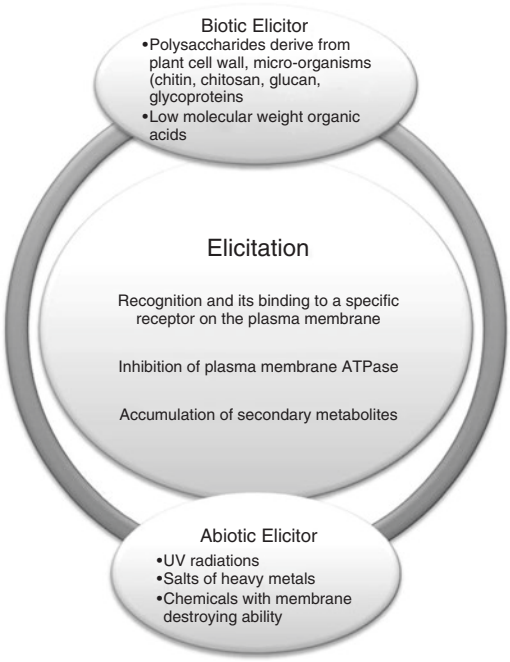

Fig. 6.3 Overview of plant-derived secondary metabolite production via abiotic and biotic elicitors

To protect themselves from the attack of pathogen, plants release secondary metabolites. Elicitors are thus used to stimulate the production of secondary metabolites, and they also lower down the time required to obtain the increased amount of desired compound (Barz et al. 1988; Dicosmo and Tallevi 1985) (Fig. 6.3). Employing varied elicitors, synthesis of many beneficial secondary metabolites was reported (Wang and Zhong 2002a, b; Lee and Shuler 2000). We have summarized some of them in tabular form (Table 6.1).

6.2.2.1 Types of Elicitor

Molecules that trigger protection and stress-generated reaction in plants are collectively termed as elicitors (Radman et al. 2004). Elicitors incorporate both the pathogen-derived compounds and the substance discharged from the plants due to the activity of pathogens. Elicitors can be biotic or abiotic. The biotic elicitors as the name signifies have biological origin obtained either from pathogens or through plants themselves, whereas abiotic elicitors can be physical or chemical component (Kumar and Shekhawat 2009).

Biotic Elicitors

Carbohydrates and proteins come under the category of biotic elicitors. Biotic elicitors include different components of existing organisms like polysaccharide present in plant cell wall, namely, pectin and cellulose, as well as the excerpt of microbes particularly chitin, glucans, and glycoproteins (Nishi 1994; Benhamou 1996; Shirsau et al. 1997. In response to the invasion by pathogens along with the

Table 6.1 Recent reports on the use of biotic and abiotic elicitors in plant cell culture to influence the production of plant-derived secondary metabolites

Plant name	Secondary metabolite	Type of culture	Elicitor	Report
Abrus precatorius	Glycyrrhizin	Cell suspension	Fungi	Karwasara et al. (2010)
Ajuga bracteosa	Phenols and flavonoids	Root suspension	Methyl jasmonate	Saeed et al. (2017)
Ajuga bracteosa	Phenols and flavonoid	Shoot	Thidiazuron	Ali et al. (2018)
Arachis hypogaea	Resveratrol	Hairy root	Sodium acetate	Condori et al. (2010)
Artemisia absinthium	Phenols and flavonoids	Suspension	Gibberellic acid	Ali et al. (2015)
Artemisia annua	Artemisinin	Hairy root	Fungi	Wang et al. (2009)
Artemisia annua	Artemisinin	Cell suspension	Methyl jasmonate	Caretto et al. (2011)
Astragalus membranaceus	Isoflavonoid	Hairy root	Methyl jasmonate	Gai et al. (2016)
Bacopa monnieri	Bacoside A	Shoot	Methyl jasmonate	Sharma et al. (2013)
Cannabis sativa	Tyrosol	Cell suspension	Jasmonic acid	Pec et al. (2010)
Catharanthus roseus	Ajmalicine	Cambial cells	Cyclodextrin	Zhou et al. (2015)
Catharanthus roseus	Lochnericine	Hairy root	Light irradiation	Binder et al. (2009)
Calophyllum inophyllum	Inophyllum	Cell suspension	Fungi	Pawar et al. (2011)
Centella asiatica	Asiaticoside	Hairy root	Methyl jasmonate	Kim et al. (2004)
Datura stramonium	Hyoscyamine	Hairy root	Jasmonic acid	Amdoun et al. (2010)
Eleutherococcus koreanum	Eleutherosides B and E	Adventitious root	Salicylic acid	Lee et al. (2015)
Eruca sativa	Glucosinolate	Hairy root	Salicylic acid and ephephon	Kastell et al. (2018)
Glycine max	Isoflavonoid	Cell suspension	Cold shock	Gueven and Knorr (2011)
Gymnema sylvestre	Gymnemic acid	Cell suspension	Methyl jasmonate	Chodisetti et al. (2015)
Hypericum perforatum	Hypericin	Cell suspension	Salicylic acid	Gadzovska et al. (2013)
Hypericum perforatum	Hypericin	Cell suspension	Ozone exposure	Xu et al. (2011)
Isatis tinctoria	Flavonoid	Hairy root	*Aspergillus niger*	Jiao et al. (2018)
Lachenalia spp.	Caffeic and ferulic acid	Shoot	White, blue-red light	Bach et al. (2018)
Melissa officinalis	Hydroxycinnamic acid	Suspension	Cobalt chloride	Urdova et al. (2015)

(continued)

Table 6.1 (continued)

Plant name	Secondary metabolite	Type of culture	Elicitor	Report
Oldenlandia umbellata	Anthraquinones, alizaril	Adventitious root	Pectin, yeast extract, xylan	Krishnan and Siril (2018)
Panax ginseng	Ginsenosides	Hairy root	Methyl jasmonate	Corchete and Bru (2013)
Panax ginseng	Phenols and flavonoid	Root suspension	Salicylic acid	Ali et al. (2007)
Plumbago indica	Plumbagin	Hairy root	Jasmonate	Gangopadhayay et al. (2011)
Portulaca oleracea	Dopamine	Hairy root	Salicylic acid	Ahmadi et al. (2013)
Pueraria candollei	Isoflavonoid and genistein	Hairy root	*Agrobacterium* and yeast	Udomsuk et al. (2011)
Pueraria mirifica	Isoflavonoids	Hairy root	Chitosan	Korsangruang et al. (2010)
Podophyllum hexandrum	Podophyllotoxin	Cell	Methyl jasmonate	Hazra et al. (2017)
Polygonum multiflorum	Phenolic compound	Adventitious root	Yeast extract and chitosan	Ho et al. (2018)
Rhodiola imbricata	Phenol and flavonoid	Callus culture	Light	Kapoor et al. (2018)
Salvia miltiorrhiza	Tashinones	Hairy root	Methyl jasmonate	Hao et al. (2015)
Salvia sclarea	Aethiopinone	Hairy root	Methyl jasmonate	Kuzma et al. (2009)
Salvia miltiorrhiza	Tanshinone	Hairy root	Hyperosmotic stress	Shi et al. (2007)
Salvia miltiorrhiza	Phenolic acid	Cell suspension	Salicylic acid	Dong et al. (2010)
Satureja khuzistanica	Rosmarinic acid	Cell suspension	Methyl jasmonate	Khojasteh et al. (2016)
Scutellaria lateriflora	Baicalein and scutellarin	Hairy root	Light and Cyclodextrin	Marsh et al. (2014)
Stephania venosa	Dicentrine	Cell suspension	Salicylic acid and chitosan	Kitisripanya et al. (2013)
Silybum marianum	Silymarin	Cell	Cyclodextrin	Almagro et al. (2011)
Stevia rebaudiana	Phenols and flavonoids	Callus	Light	Ahmad et al. (2016)
Taxus spp.	Taxane	Cell	Cyclodextrins	Sabater-Jara et al. (2014)
Taxus baccata	Phenolic content	Cell suspension	Squalestatin	Jalalpour et al. (2014)
Vitis riparia	Resveratrol	Cell suspension	Cyclodextrin	Zamboni et al. (2006)
Vitis vinifera	Anthocyanins	Cell suspension	Pectin	Cai et al. (2011a)
Vitis vinifera	Anthocyanins	Cell suspension	Ethephon	Cai et al. (2011b)

environmental destruction, plant releases antimicrobial compounds, i.e., phytoalexins, which are actually secondary metabolites. Nowadays in cultured cells, biotic elicitors chiefly the fungal elicitors are consider as a dynamic path for escalating secondary metabolites (Siddiqui et al. 2010).

Abiotic Elicitors

As compare to the biotic elicitors, abiotic elicitors have not been able to gain much attraction in plant cell culture (Angelova et al. 2006). Nonbiological in origin, abiotic elicitors include inorganic salts and various environmental factors chiefly UV rays, heavy metal salts like copper and cadmium ions, as well as pH. In recent times, it was concluded that the tropospheric ozone has the ability to trigger biochemical plant responses that are analogous to the compounds released during fungal attack (Zuccarini 2009). As reported by Schmeller and Wink in 1998, *Taxus* plant is of great importance because of its anticancer properties. Wu et al. (2001) experienced amplification of taxol synthesis when lanthanum was used as an elicitor in *Taxus* spp. cell culture.

6.3 Important Approaches for Production of Secondary Metabolites

6.3.1 Organ Culture-Mediated Secondary Metabolite Production

Many therapeutic compounds and other important constituents are derived from root cultures (Pence 2011; Li et al. 2002). Essential alkaloids like hyoscyamine and scopolamine and important drugs can easily be obtained by using root culture method without many efforts (Fazilatun et al. 2004). Root cultures have far more importance over the conventional higher plant root system, and it is now being explored on a high note, as root system has very slow growth rate and is much more challenging. The requirement for some secondary metabolites is increasing for commercial purpose; to cope with, plant shoot cultures are employed instead of relying on the natural plant produce (Khanam et al. 2000). Different kinds of bioreactors are employed for root and shoot cultures (Kasparova et al. 2009; Kim et al. 2002).

6.3.2 Callus Culture-Mediated Secondary Metabolite Production

Callus is an unspecialized, unorganized, growing, and dividing mass of cells. It is produced when explants are cultured *in vitro* on an appropriate medium, with concentration of both auxin and cytokinin in accurate ratio. Callus cultures are generally categorized into two types: embryogenic or non-embryogenic. In embryogenic type of callus culture, a single cell or a small group of competent cells follow a developmental pathway that leads to reproducible regeneration of non-zygotic

embryos which are capable of producing a complete plant (Ptak et al. 2013). The major application of somatic embryogenesis include clonal propagation of genetically uniform plant material, elimination of viruses, provision of source tissue for genetic transformation, generation of whole plants from single cells called protoplasts, and development of synthetic seed technology. However in non-embryonic callus culture contains more or less similar cluster of dedifferentiated cells are taken for synthesis of secondary metabolite. *Maackia amurensis* has been investigated for secondary metabolites by employing callus culture (Fedoreyev et al. 2004). Biosynthetic totipotency of plant cell is the major objective behind the concept of production of secondary metabolites using cell suspension culture; hence, the genetic composition of each cell in the culture remains the same, and thus a wide range of bio-active compounds can be extracted which are available in entire plant.

6.3.3 Hairy Root Culture-Mediated Secondary Metabolite Production

In a phytohormone-deficient medium, hairy roots grow hastily with immense branching with oblique or horizontal growth (Hu and Du 2006). Hairy roots obtained from *Agrobacterium rhizogenes* have huge application in various commercial areas. Hairy roots have the benefit over others of not failing the genetic and biosynthetic stability; they produce secondary metabolites over subsequent generations (Giri and Narasu 2000). Hairy root cultures have been investigated abundantly in root nodule research. With the help of transformed root cultures, many possibilities of secondary metabolite biosynthesis have been examined (Kuzovkina and Schneider 2006). The substantial interrelationship between secondary metabolite production and morphological differentiation gives more momentum to utilization of cell culture technique for the production of phytochemicals on a commercial scale.

Synthesis of two different bio-active compounds synchronously is achievable through adventitious root co-cultures (Wu et al. 2008). The promising results obtained by implementing hairy root culture, now bioreactors, are incorporated to achieve much more bio-active compounds (Mehrotra et al. 2008). To obtain various valuable alkaloids and alkannins, hairy root cultures of plants, namely, *Lithospermum erythrorhizon*, *Harpagophytum procumbens* (Ludwig-Muller et al. 2008), and adventitious roots of *Panax ginseng* (Jeong et al. 2008) and *Scopolia parviflora* (Min et al. 2007) were examined in different volumes of bubble column bioreactors. Ginsenoside, which is a class of natural product steroid, glycosides, and triterpene saponins can also be synthesized by employing adventitious root culture in combination with stirred tank bioreactors (Jeong et al. 2008). To cope with the increasing demands, improved and modified bioreactors are employed having stainless steel tank plant cell growth in addition to the vessels that were also armed with specialized hangers. Among all mentioned cultures, hairy root culture has gained tremendous popularity due to its distinctive capability to achieve secondary metabolite production on a large scale.

For secondary metabolites that are released as a result of defense responses, their primary role is to protect plants, but because of its therapeutic properties, researchers have focused their attention toward it. Due to seasonal and environmental instabilities along with little knowledge about the biosynthesis and signal transduction pathway of these secondary metabolites, it becomes very challenging for pharmaceutical industries to obtain these bio-active compounds. Plant cell culture provides an excellent medium for sustainable, easily expandable production of secondary metabolites to restrict the hurdles. To boost up the yield, noticeable approaches like manipulating the supplements and bettering the culture environment and elicitation are taken into consideration (Kumar and Sopory 2008).

Secondary metabolites obtained from plants *via in vitro* conditions have been acknowledged with great passion (Stafford 1991; Smith 1996). For a variety of medicinal plants, secondary metabolite production through *in vitro* plant cell suspension culture systems has been reported (Tripathi and Tripathi 2003). Plant cell culture is usually considered as an ideal method for analyzing the biological consequences of secondary metabolites and for generating natural products for biotransformation (Walker et al. 2002). Secondary metabolites obtained from callus, cell, and cell suspension cultures (Pepin et al. 1995; Shibli et al. 1997, 1999) along with plant parts like leaves and flowers are enlisted in Table 6.2. To exhibit accumulation of secondary metabolites in callus and cell suspension culture, various distinct determinants are practiced; the substantial ones are the chemical composition of the media compared to the growth regulators (Nawa et al. 1993), concentration and source of carbon (Decendit and Merillon 1996; Mori and Sakurai 1994), and concentration and source of nitrogen (Mori and Sakurai 1994; Sato et al. 1996). The main significance of cell cultures includes:

(i) It is independent to different environmental factors like soil and climatic condition.
(ii) Antagonistic biological impacts that disturb secondary metabolite production in the nature are excluded like microorganisms and insects.
(iii) Selection of suitable cultivars with the intention of achieving greater supply of secondary metabolites is possible.
(iv) It is cost effective.

6.4 Secondary Metabolites and Its Assimilation in Plant Cell Cultures

In order to obtain high-quality uniform product from cell culture, it is important to develop techniques that are economically feasible (Berlin and Sasse 1985). Collection of increased amount of several products in cultured cells is obtained by precise selection of productive cells and cultural conditions. For achieving higher yield of secondary metabolites for commercial demands, several strategies and efforts have been aimed for accelerating the biosynthetic activity of cultured cells (Dixon 1999; Buitelaar and Tramper 1992). Various methods are now being used to escalate the production of secondary metabolites through plant cell culture including manipulation of nutrient media and elicitation.

Table 6.2 Plant-derived secondary metabolites isolated from plant via different cell culture types

Plant name	Secondary metabolite	Type of culture	Report
Adhatoda vasica	Vasine	Shoot culture	Shalaka and Sandhya (2009)
Agastache rugosa	Rosmarinic acid	Hairy root	Lee et al. (2007)
Aloe vera	Aloe emodin and chrysophanol	Adventitious root	Lee et al. (2013)
Ammi majus	Umbelliferone	Shootlet	Krolicka et al. (2006)
Andrographis paniculata	Andrographolide	Adventitious root	Parveen et al. (2009)
Arachis hypogaea	Resveratrol	Hairy root	Condori et al. (2010)
Artemisia	Artemisinin	Hairy root	Ikram and Simonsen (2017)
Artemisia annua	Drimartol A	Hairy root	Abbott et al. (2010)
Artemisia annua	Artemisinin	Callus	Baldi and Dixit (2008)
Astragalus membranaceus	Saponins and isoflavonoids	Adventitious root	Wu et al. (2011)
Brucea javanica	Cathin	Suspension	Wagiah et al. (2008)
Brugmansia candida	Anisodamine	Hairy root	Cardillo et al. (2010)
Bupleurum chinense	Saikosaponin	Adventitious root	Hao and Guan (2012)
Bupleurum chinense	Saikosaponin	Adventitious root	Kusakari et al. (2012)
Castilleja tenuiflora	Phenylethanoid glycosides	Adventitious root	Gomez-Aguirre et al. (2012)
Catharanthus roseus	Catharanthine	Hairy root	Wang et al. (2010)
Catharanthus roseus	Alkaloids	Hairy root	Li et al. (2011)
Cayratia trifoliata	Stilbenes	Suspension	Roat and Ramawat (2009)
Centella asiatica	Asiaticoside	Adventitious root	Mercy et al. (2012)
Coleus blumei	Rosmarinic acid	Hairy root	Bauer et al. (2009)
Crataegus sinaica	Flavonoid	Callus	Maharik et al. (2009)
Datura stramonium	Hyoscyamine	Hairy root	Amdoun et al. (2010)
Echinacea angustifolia	Caffeic acid derivatives	Adventitious root	Cui et al. (2013)
Echinacea angustifolia	Caffeic acid derivatives	Adventitious root	Murthy et al. (2014c)
Eleutherococcus senticosus	Eleutherosides	Suspension	Shohael et al. (2007)
Eleutherococcus korean	Eleutherosides	Adventitious root	Lee and Paek (2012)
Fagopyrum esculentum	Rutin	Hairy root	Lee et al. (2007)

(continued)

Table 6.2 (continued)

Plant name	Secondary metabolite	Type of culture	Report
Gentiana macrophylla	Gentiopicroside	Hairy root	Zhang et al. (2010)
Gentiana macrophylla	Glucoside	Hairy root	Tiwari et al. (2007)
Gentianella austriaca	Xanthone	Multiple shoot	Vinterhalter et al. (2008)
Glycyrrhiza glabra	Glycyrrhizin	Hairy root	Mehrotra et al. (2008)
Glycyrrhiza uralensis	Flavonoid	Hairy root	Zhang et al. (2009)
Glycyrrhiza uralensis	Glycyrrhizic acid	Adventitious root	Yin et al. (2014)
Gossypium hirsutum	Gossypol	Hairy root	Verma et al. (2009)
Gynochthodes umbellata	Anthraquinone	Callus	Anjusha and Gangaprasad (2017)
Gynura procumbens	Phenylpropanoids	Adventitious root	Saiman et al. (2012)
Hypericum perforatum	Phenolics, flavonoids, chlorogenic acid, and sphingoid base-1-phosphate	Adventitious root	Wu et al. (2014)
Hypericum perforatum	Hypericin	Suspension	Hohtola et al. (2005)
Hypericum perforatum	Hypericins	Multiple shoot	Kornfeld et al. (2007)
Globularia trichosantha	Catalpol, aucubin, and verbascoside	Callus	Colgecen et al. (2018)
Mentha × piperita	Menthol, pulegone	Shoot	Fejer et al. (2018)
Momordica charantia	Flavonoid	Callus	Agarwal and Kamal (2007)
Momordica dioica	Flavonols, hydroxycinnamic acid	Hairy root	Thiruvengadam et al. (2016)
Morinda citrifolia	Anthraquinones	Adventitious root	Baque et al. (2012)
Myristica fragrans	Myristin	Shoot	Indira et al. (2009)
Ophiorrhiza rugosa	Camptothecin	Shoot	Vineesh et al. (2007)
Panax quinquefolium	Ginsenoside	Hairy root	Mathur et al. (2010)
Periploca sepium	Periplocin	Adventitious root	Zhang et al. (2011)
Piper solmsianum	Piperine	Suspension	Balbuena et al. (2009)
Pluchea lanceolata	Quercetin	Callus	Arya et al. (2008)
Plumbago indica	Plumbagin	Hairy root	Gangopadhayay et al. (2011)

(continued)

Table 6.2 (continued)

Plant name	Secondary metabolite	Type of culture	Report
Polygonum multiflorum	Anthraquinones, hydroxybenzoic acids, hydroxycinnamic acids, and flavonols	Hairy root	Thiruvengadam et al. (2014)
Polygonum multiflorum	Anthraquinones, stilbenes, flavonoids, tannins, and phospholipids	Root culture	Thanh-Tam et al. (2017)
Primula veris	Saponins	Shoot	Okrslar et al. (2007)
Psoralea corylifolia	Daidzein	Hairy root	Shinde et al. (2010)
Psoralea corylifolia	Isoflavones	Multiple shoot	Shinde et al. (2009)
Rauvolfia serpentina	Reserpine	Callus	Nurchgani et al. (2008)
Rauvolfia tetraphylla	Reserpine	Callus	Anitha and Kumari (2006)
Rubia akane	Anthraquinone	Hairy root	Park and Lee (2009)
Salvia miltiorrhiza	Tanshinone	Hairy root	Yan et al. (2011)
Salvia officinalis	Flavonoid	Multiple shoot	Grzegorczyk and Wysokinska (2008)
Salvia sclarea	Diterpenoid	Hairy root	Kuzma et al. (2009)
Salvia viridis	Rosmarinic acid and caffeic acid	Hairy root	Grzegorczyk-Karolak et al. (2018)
Silybum marianum	Silymarin	Hairy root	Rahnama et al. (2008)
Spirotropis longifolia	Spirotropin A, spirotropin B, and spirotropaone	Adventitious root	Basset et al. (2012)
Stevia rebaudiana	Steviol-glycosides	Adventitious root	Reis et al. (2011)
Taxus × *media*	Paclitaxel	Hairy root	Syklowska-Baranek et al. (2009)
Tinospora cordifolia	Berberine	Suspension	Ramarao et al. (2008)
Tripterygium wilfordii	Triptolide, alkaloids	Adventitious root	Miao et al. (2014)
Vitis vinifera	Resveratrol	Callus	Kin and Kunter (2009)
Withania somnifera	Withanolides	Adventitious root	Murthy and Praveen (2013)
Withania somnifera	Withanolide A	Hairy root	Murthy et al. (2008)
Withania somnifera	Steroidal lactone	Callus	Mirjalili et al. (2009)
Zataria multiflora	Rosmarinic acid	Callus	Francoise et al. (2007)

6.5 Yield Improvement Strategies

6.5.1 Preliminary Considerations

For the production of secondary metabolites employing plant tissue culture, information of the variety, cultivar, and species of the desired plant along with the complete profile of the bio-active compound present in them must be known (Ananga et al. 2013). Firouzi et al. (2013) destine the consequences of utilizing four ecotypes of *Silybum marianum* on growth method and flavonolignan production in cell culture. Particular ecotypes showed critical variation in the considered parameters. Hence selection of apt explant is an important and essential step for initiating callus culture. Usually, a good and viable explant should be small, healthy, and taken from middle part of the plant and should contain meristematic tissues.

6.5.2 Screening Cell Lines

A complete strategy for production of secondary metabolites from the desired plant cell culture must be planned before moving further. Various factors about the selection of cell line must be taken into consideration which include growth rate, culture stability, and tolerance of the culture (Shuler 1999). The term clonal selection is used for production of a population of cells having the same trait. For economic point of view, growth rate of the culture plays a crucial role. Genetic and epigenetic factors are the reason behind the fluctuation in the culture. Epigenetic factors are resulted from change in the environment and do not conclude in permanent change in cell genome.

6.5.3 Alteration of the Components of the Culture Medium

Plant tissue culture media include some or all of the following components: macronutrients, micronutrients, vitamins, amino acids, carbon source, growth regulators, solidifying agent, and undefined organic supplements (Saad and Elshahed 2012). The most frequently used media are Murashige and Skoog (MS) medium (Murashige and Skoog 1962), Linsmaier and Skoog medium (Linsmaier and Skoog 1965), Gamborg medium (Gamborg et al. 1968), and Nitsch and Nitsch medium (Nitsch and Nitsch 1969). For providing optimum growth to the desired culture and deriving required amount of secondary metabolites, some of the media are usually altered.

6.6 Conclusion and Future Prospects

Due to high concern for low yield and productivity of useful plants, with increase demand of food and health benefit products, plant tissue culture techniques are well accomplished. It was observed that plant tissue culture predicts more efficient and

reliable source for most of secondary metabolite production, but on the other hand, there are only some cell cultures that can produce stable and efficient source of secondary metabolites. There were some achievements in the formulation of important secondary metabolites because of upgrading culture technique, choice of cell line, and model of bioreactor with passing time. There is no ambiguity that the in vitro culture of secondary metabolites from plant cell culture is an interesting technology for obtaining useful product. Plant tissue culture technique is an important approach for the production of those plant species which were at risk though having potential secondary metabolites which can be commercially applied for the preparation of valuable food and medicines in future.

It is the starting point for the production of valuable secondary metabolites from both plant and cell culture; therefore, there is a need to develop more research for large-scale production of compounds for economic and other purposes. Incorporation of molecular biology is the most efficient tool for handling and expression of secondary metabolite production on a large scale. There are some other studies that predict that developing the research in the area of plant tissue culture day by day results in large production of secondary metabolites. Many other examples could be presented with plant cell culture technique as this research area is developing actively to increase the production. A significant shift in the appeal of the cell culture technologies will likely come from a better understanding of the biological mechanisms that operate biosynthetic pathways and the application of this knowledge to engineering economically competitive high-value product yield.

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7 Biotechnological Exercises in the Production of Secondary Metabolites and Its Significance in Healthcare Practices

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Abstract

Plants produces various secondary metabolites that are economically vital. They deliver products for medications in the form of naturally attained stuffs, like fragrances and flavor, pigments and dye, foodstuff or additives, pesticides, and therapeutics. The increasing industrial prominence of secondary metabolites in recent years has resulted in an exceedingly prodigious curiosity in the production of secondary metabolites to meet required demands. Biotechnological exercises in the production of secondary metabolites have a great importance. For certain phytochemicals, bioreactors can be used for the large-scale production. In the case of culture production of secondary metabolites, the right media selection could increase yeild up to 20–30 times, but for several other bio-active compounds, which are in very small amounts, cell culture techniques are not feasible. Elicitation by phytoalexins has led to raised production of secondary metabolites. The approach of root/shoot hairy cultures is another technique, but is hindered at scaling-up stages. On the other hand, metabolic engineering is a technique, which could aid in the enhancement of certain secondary metabolites. This methodology can fabricate secondary metabolites in cell cultures or in the plants itself. Thus, metabolic engineering gives the impression of provocative approach to enhance the metabolites produced by the cell. Therefore, current technologies will benefit to encompass and improve the sustained utility of the higher plants as rekindling sources of phytochemicals, particularly compounds related to therapeutic importance. The present chapter summarizes the different biotechnological exercises associated with the fabrication of secondary metabolites. Moreover, it also discusses the various biotechnological techniques for the production of specific and valued secondary metabolites used in the healthcare practices.

Keywords

Cell suspension culture · Gene duplication technology · Phytochemicals · Micropropagation · Secondary metabolites

7.1 Introduction

Plants have the capability to synthesize diverse secondary metabolites, which are made up of organic molecules with unique carbon arrangements. Secondary metabolites are not required for cells to survive, but it plays a vital character in an interface of the cells with its ambiances, confirming the constant existence of the organism to its biomes (Ncube and Staden 2015). Normally the secondary metabolites are of low molecular weight, and its production is specific to cell, tissue, and organ. These compounds frequently change among germplasm from a similar population of plants in reverence to their quantity and forms (Matsuura et al. 2018). Secondary metabolites look after plants against biotic and abiotic stresses, viz.,

microorganisms, nematodes, insects, and animals and temperature, moisture, shading, injury, and heavy metals, respectively. Due to the excessive commercial importance, they are extensively used as chemical such as for medications, flavors, essence, insect repellent, and dyes. Most of the significant therapeutic biomolecules are alkaloids which are biosynthesized mainly from amino acids. However plant secondary metabolites can be chemically categorized into different types, i.e., phenolics (Wuyts et al. 2006; Iqbal et al. 2017), terpenes (Singh and Sharma 2015), compounds containing nitrogen (Ejaz et al. 2017), and compounds containing sulfur (Kang and Kim 2007). Due to the occurrence of varied diversity and multifaceted performance of secondary metabolites, it is assumed to be of enormous significance. Secondary metabolites hold various therapeutic properties, and thus it is of immense importance to mankind for health benefits (Forbey et al. 2009).

Subsequently secondary metabolites are bio-active compounds which are substantial for the stability of the organisms. Several secondary metabolites meddle with pharmacological assets, which mark them attention-grabbing for various biopharma and agri-biotechnological utilizations (Rai et al. 2009). The outcome of secondary metabolites as by-products from organism in response to the external stimuli (biotic and abiotic) is another cause. Thus, the yield of bio-active products is a natural, biochemical, and bioenzymatic process that happens in all organisms during metabolism process (Dias et al. 2012). The system of metabolites, functional with enzymatic reaction throughout the course of metabolic process, is known as metabolome. According to Moghe and Last (2015), a metabolome comprises of all the manacles of responses, relating to enzymes and its substrates in the metabolic process and finishing up in the materialization of the metabolites (primary and secondary). In the process when pyruvate enters the mitochondria to undergo in tricarboxylic acid cycle, it gets converted into acetyl CoA. The acetyl CoA on further metabolism produces secondary metabolites required for the cell. On the other hand, the mitochondria undergoing tricarboxylic acid cycle synthesize macromolecules or primary metabolites required for the survival of the cell. The systematic synthesis of metabolite (primary and secondary) is shown in Fig. 7.1. Various secondary metabolites are produced during this process, essential for the development of organism. Thus, the usage of compounds like perfumes, caffeine, ephedrine, nicotine, essential oils, piperine, capsaicin, and strychnine and hallucinogen compounds like tetrahydrocannabinol, heroin, cocaine, morphine, and natural dyes is formed as secondary metabolite by various organisms. Therefore, for biotechnologists, it's a huge task to elucidate techniques to outgrow these bio-active compounds in better quality and in abundant amount. The principal and customary mode to excerpt the phytochemicals is to cultivate the individual plant in glasshouses or in the field. In this circumstance, cell culture or tissue/organ culture is imperative methods of in vitro micropropagation to extract specific phytochemicals.

As an alternative methodology, biotechnologist could isolate and express the genes responsible for the formation of particular secondary metabolite of therapeutic significance in a particular biosynthetic pathway. If this technique will be successful, then recombinant DNA technology on bacteria or yeasts could flourish, which may produce valuable plant secondary metabolites for therapeutic use

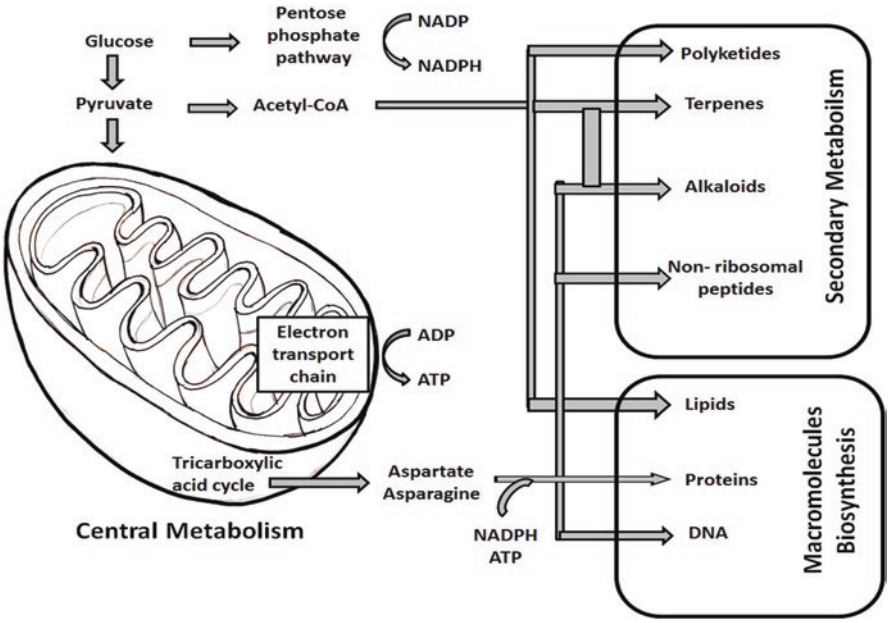

Fig. 7.1 Metabolomic biosynthesis of primary and secondary metabolites

(Pandey et al. 2014). The biosynthesis of antibiotics by means of genes coding enzymes approach, at present, is of inordinate attainment. In recent years, microorganisms have been exploited, and techniques have been developed to express the genes responsible for alkaloid biosynthesis for its overproduction (Pickens et al. 2011). Eventually, it is conceivable to yield desired alkaloids from cells of yeast or bacteria recombinant DNA technology. Although if particular secondary metabolite of plant or microorganism causes prevention to the growth of pests or pathogens, thus genetic transformation of vulnerable plants may perhaps be added prospective for its utilization. For almost two decades, researchers in the field of biotechnology have attempted to yield beneficial secondary metabolite in cell or tissue/organ cultures. However cell cultures which are undifferentiated have generally botched to form such beneficial compounds judiciously, as distinguished tissue/organ cultures like root cultures linger on as lively as whole plant (Ochoa-Villarreal et al. 2016).

The basic benefit of present techniques is to make available of uninterrupted, consistent source of plant-originated phytochemicals and possibly will be amplified on large-scale production culture of cells or tissue/organ for its extraction. The technique holds prominent advantage for meticulous fabrication of numerous, useful secondary metabolites upon application. The present output and production of secondary metabolite cannot achieve the commercial goal of phytochemicals bioprocessed for the fabrication of most the secondary metabolites of therapeutic importance. In the direction to expand the boundaries, recent progressions and new

prospects in plant organ or cell/tissue culture-based procedures are being critically studied and experimented. In such experimentations, novel approaches to develop the preferred secondary metabolites should be taken into consideration for better future. One of the foremost glitches that come across in this arena of research is the absence of elementary information of biosynthesis pathways and mode of action accountable for the fabrication of secondary metabolites. Moreover, it also discusses the various biotechnological techniques for the production of specific and valued secondary metabolites used in the healthcare practices.

7.2 Recent Advancements in Secondary Metabolite Production

Plant cell or tissue/organ cultures embrace prodigious potential for meticulous propagation of innumerable, beneficial secondary metabolites upon requirement and demand. Outcomes associated to cell cultures achieving the fabrication of specific therapeutic phytochemicals at a level parallel or better than that of whole plant have been enhanced in the last few decades (Vijaya et al. 2010; Ncube and Staden 2015). In the course to accomplish amplified yields, appropriate to utilize commercially, attempts have been made to find the biosynthetic actions of cell cultures, accomplished by proper rectification of conditions for growing of culture, choosing high-producing strains with precursor nourishment, biotransformation, and immobilization procedures (DiCosmo and Misawa 1995; Ncube and Staden 2015). Organ culture technique (transgenic root hair cultures) has transfigured the character of secondary metabolite in plant tissue culture. The method is inimitable in their biosynthetic and genetic permanency, quick in growth, and efficiently sustainable. By improvising the approach, an extensive variety of biochemical compounds has been manufactured (Giri and Narasu 2000). Recent progresses in cell or tissue/organ culture technique, associated with enrichment by genetic engineering, have developed biomolecules of pharmaceutical and nutraceutical significance and other additional valued constituents (Hansen and Wright 1999). Furthermore, improvements in the enzyme technology, molecular biology, microbiology, biochemistry, and fermentation technology enhanced the cell culture techniques that have developed a feasible source of secondary metabolites of therapeutic and agricultural importance (Abdin 2007; Barbulova et al. 2014).

Genome alteration technique resulted in comparatively producing huge amounts of expected biosynthetic compounds provided by plants on treatment with genome engineered viruses. The transgenic plants could produce biomolecules in limited quantity, without any additional interference with other biosynthetic pathways (Abdin and Kamaluddin 2006). However, for the large-scale production of secondary metabolites, tissue culture technique is established, which is an attractive methodology beside outmoded approaches for the production of secondary metabolites, as it deals with meticulous resource of phytochemicals that are not dependent on the availability of whole plants (Sajc et al. 2000). Some of the metabolites of nutraceutical and pharmaceutical significances are summarized in tabular form (Table 7.1).

Table 7.1 Bio-active compounds of nutraceutical and pharmaceutical significance

Class	Known metabolites numbers	Examples	References
Alkaloids	21,000	Quinine, cocaine, psilocin, reserpine, caffeine, nicotine, morphine, atropine, berberine, ephedrine, vincristine, galantamine, vincamine, quinidine	Wink (2010) and Kennedy and Wightman (2011)
Non-protein amino acids (NPAAs)	700	Azatyrosine, canavanine	Wink (2010)
Amines	100	Methylamine, dimethylamine, trimethylamine, aziridine, piperidine	Iqbal et al. (2014a, b)
Cyanogenic glycosides	60	Amygdalin, dhurrin, linamarin, lotaustralin, prunasin	Wink (2010)
Glucosinolates	100	Sinigrin, glucotropaeolin, gluconasturtiin, glucoraphanin	Wink (2010)
Alkamides	150	*N*-Isobutyl-2*E*-decenamide and *N*-isobutyl-decanamide	Molina-Torres et al. (2004)
Lectins, peptides and polypeptides	2000	Concanavalin A	Reeke et al. (1975)
Terpenes	>15,000	Azadirachtin, artemisinin, tetrahydrocannabinol	Tiwari and Rana (2015)
Steroids and saponins	NA	Cycloartenol	Babiychuk et al. (2008)
Phenylpropanoids, lignins, coumarins and lignans	2000	Resveratrol	Kennedy and Wightman (2011)
Polyacetylenes, fatty acids and waxes	1500	Oleic acids	Minto and Blacklock (2008)
Polyketides	750	Aflatoxin B1, geldanamycin, erythromycin	Crawford and Townsend (2010)
Carbohydrates and organic acids	200	Formic acid, lactic acid, citric acid	Nakui et al. (2009)

7.3 Current Biotechnological Exercises Employed in the Production of Secondary Metabolites from Higher Plants

Cell or tissue/organ cultures of plant can be done consistently within sterilized surroundings from portions of the plant parts like roots, stems, leaves, and meristems for proliferation and extraction of valuable secondary metabolites. Enrichment in secondary metabolite fabrication could be done by strain development approaches, as variety of cell lines, and media optimizations can be used (Jan et al. 2017). At present the sturdy and mounting requirement in marketplace for natural and non-convectional products has progressed. Considering the in vitro plant-originated

compounds as promising industrial unit for secondary metabolite products has generated the path for innovative exploration for secondary phyto-product countenance (Karuppusamy 2009). There are many different benefits of fabricating valued secondary phyto-metabolites by cell/tissue culture, than by in vivo technique in the plant. It can be summarized as under:

- Production of phytochemical can be more consistent, natural, and more foreseeable.
- Extraction of the phytochemical can be quick and effective, when compared with isolation from complete plant.
- Phytochemicals generated in vitro can directly be equivalent to the phytochemicals produced by complete plant.
- Inquisitive phytochemicals can be evaded in cell cultures, which cannot be done in the plants grown infield.
- Cell/tissue cultures can harvest definite and customary phytochemicals in bulk quantities.
- Cell/tissue cultures are a prospective exemplary to investigate and testify elicitation.
- Radiolabeling in cell/tissue cultures could be done, so that the stored secondary metabolites, when delivered as feedstuff to experimental models (animals), can be outlined during metabolic process.

Various research on enhancement of secondary metabolites production, has increased now a days for producing an extensive variety of valued secondary phytochemicals in callus or suspension cultures, however further research is required to establish technique for organ cultures of well-known plant as well (Davioud et al. 1989). This condition repeatedly arises when the phytochemical of importance is only formed in specific plant tissues/organ of the parental plant. A crucial case in point is in *Panax ginseng*. As in this plant, in vitro saponin and additional valuable metabolites are particularly formed in its organ (root) culture. Likewise, medicinal plant *Hypericum perforatum*, which holds the hyperforins and hypericins (in foliar glands), has not established the capability to accrue phytochemicals in undistinguished cells (Smetanska 2008). Therefore approaches need to be developed for fabrication of secondary metabolites on large scale. The rigorous accomplishments have been focused on generating natural remedies or chemo-protecting compounds (secondary metabolites) obtained from plant cell or tissue/organ culture by the following subsequent approaches.

7.3.1 Therapeutically Significant Secondary Metabolite Production by Plant Tissue Cultures Technique

With the advancements in the field of research and development, the technique like tissue culture for fabrication of phytochemicals has embellished beyond its potentials (Vijaya et al. 2010). The chief edges of a cell culture methodology over the standard cultivation process of complete plants may be illustrious as under:

- Production of valuable phytochemicals under controlled environment without the impact of conditions of soil or fluctuations in climatic.
- Microorganisms and pests free cells cultures.
- Reproduction of cells from any plant could simply enrich particular metabolites.
- Reduction of labor expenses and increased production, as automated regulation of cell growth and balanced parameter of metabolite progressions, could be done.
- Callus cultures could be the source of organic constituents which can be easily extractable.

Some of the secondary metabolite productions in culture medium are cathinone alkaloids by suspension culture (Anderson et al. 1987), allicin by callus culture (Malpathak and David 1986), caffeine by callus culture (Waller et al. 1983), anthraquinones by suspension culture (Dornenburg and Knorr 1999), ginkgolide A by suspension culture (Carrier et al. 1991), L-DOPA by suspension culture (Wichers et al. 1993), etc.

7.3.2 Secondary Metabolite Production by Organ Cultures Technique

Organ culture is a technique where rapid propagation can be done by cutting small sections of the plant organ. Small slicing of *Fritillaria unibracteata* can swiftly propagate the bulb by organ culture process. The sliced bulbs were grown in MS media, supplemented with 4.44 mole indole-3-butyric acid (IBA) and 5.71 mole indole-3-acetic acid (IAA). The cultivated bulbs were collected after 50 days of culture period. The growth rate was enhanced by 30–50 times, which was higher than that of normal conditions. The magnitude of secondary metabolites was elicited like alkaloids and other valuable phytochemicals in the cultured bulbs than in the normal growing bulb (Gao et al. 2004). Micropropagation of shoot development on the MS medium, supplemented with 1-naphthaleneacetic (0.1 mg/l) and thidiazuron (0.1 mg/l) on *Frangula alnus*, was attained, and the production of secondary metabolite, anthraquinone, was maximum in the shoots than in the plant grown under normal condition (Namdeo 2007).

7.3.3 Secondary Metabolite Improvement by Addition of Precursors

The treatment of plant cells or tissue/organ with factors like biotic and/or abiotic has an expedient approach to enhance secondary metabolite fabrication grown under media culture (Karuppusamy 2009). The utmost commonly used precursors in prior studies were yeast extract, fungus, polysaccharides, methyl jasmonate, and chitosan. One of the most recognized elicitor methyl jasmonate is an established

indicator compound and is the most effectual elicitor for Taxol fabrication in *T. chinensis* Roxb (Wink et al. 2008). Gonsenoside, a secondary metabolite found in *P. ginseng*, can be illicitly produced in the supplemented media in Meyer cell/organ culture (Yagi et al. 1983; Xu et al. 2008; Yamanaka et al. 1996).

Biosynthesis of hyperforin and adhyperforin by shoot culture process of *H. perforatum* when treated with amino acids was reported (Kim et al. 2004). Upon supplementation of shoot cultures by amino acids, valine conformed to side chain of hyperforin and isoleucine conformed to side chain of adhyperforin, separately. Nourishing the shoot cultures with amino acid like isoleucine (2 mM) prompted three- to sevenfolds of elicitation in the production of hyperforin (Kim et al. 2004). It was reported that, when amino acids (leucine) were treated in callus and cell suspension cultures of *Centella asiatica*, the triterpene production was increased. The method was found to be quite impressive for the elicitation of asiaticoside. In the callus culture, multifarious increase of asiaticoside was observed by this approach (Karppinen et al. 2007).

7.3.4 In Vitro Elicitation Technique

In vitro treatment of cells or tissue/organ by microbial, physical, or chemical elements which causes morphological and physiological changes is called "elicitation," and the compounds are so-called elicitors. An elicitation is a method of tempting or enhancing the fabrication of secondary metabolites by cells or tissue/organ culture to make sure of their existence, perseverance, and effectiveness (Karuppusamy 2009; Kiong et al. 2005). In a study, abiotic elicitor was applied in the hairy roots of *P. ginseng* to improve growth and to elicit ginseng saponin biosynthesis. However in the study, elicitor treatments were performed to inhibit the development of the root hairs, but at the same time it was enhancing the content of ginseng saponin biosynthesis (Jeong and Park 2006). Elicitor treatment of benzo (1,2,3)-thiadiazole-7-carbothionic acid S-methyl ester and autoclaved lysate of cell suspension of *E. sakazaki* was done for the fabrication of secondary metabolites in callus culture, cell suspension culture, and hairy roots of *Ammi majus*. The study showed noteworthy outcomes (Staniszewska et al. 2003). The investigation based on GC and GC-MS estimation of methanolic and chloroform excerpts exhibited greater accretion of umbelliferone in the treated (elicited) tissues (Staniszewska et al. 2003). In a study on *Rubia akane* cell culture, chitosan (polysaccharide) was used as a biotic elicitor. The results were prompting the multifarious upsurge of anthraquinone fabrication (Jin et al. 1999).

7.3.5 Enhancement of Secondary Metabolites by Cultures of Hairy Root

Secondary metabolite synthesis in plant roots, based on inoculation by *Agrobacterium rhizogenes* with hairy root system, has become common in the past few years

(Palazon et al. 1997; Karuppusamy 2009). However, in the absence of lateral root branching, physical factor like geotropism and factor like genetic stability could affect the growth of the root hair, ultimately affecting the production of secondary metabolite by hairy root culture technique. Hairy roots ascending for the formation of secondary metabolites by the treatment of plant material by *A. rhizogenes* are analogous to those normally produced by parent roots of whole plant, with parallel or greater yields (Sevón and Oksman-Caldentey 2002). The unperturbed genetic stability and sudden advancement in normal media that are deficient in hormones mark them particularly appropriate for biochemical analysis, which was not easy to undertake in root hair cultures of plant. The hairy roots of the plant are firstly sterilized and then interact with the parts of the plant by infecting it with *A. rhizogenes*. For the period of the interaction course, *A. rhizogenes* transmits the portion of DNA (T-DNA) situated in the root-persuading plasmid to plant cells, and the confined genes in the region are expressed in the identical manner as the normal endogenous genes of the plant cells. Some strain of *A. rhizogenes* (like A4) possess T-DNA divided into two segments (i.e., TL-DNA and TR-DNA). Thus, both are assimilated independently into the plant's genome (Jouanin 1984).

7.3.6 Production of Secondary Metabolite by Genetic Transformation in Hairy Root Culture

Genetically transformed roots deliver a favorable substitute for biotechnological utilization of the plant cells (Pandey et al. 2014). *A. rhizogenes* intervened with the transformation of plant's genome, which could be utilized in the way similar to well-established technique, engaging *A. tumefaciens*. The transformation of *A. rhizogenes* enables the growth of plantlets that have been renewed and also to yield transgenic cultures of hairy root of the plant (Karuppusamy 2009). The exclusion of the limiting sequences, not any of the supplementary T-DNA sequences are obligatory for the transmission. The leftover T-DNA could be switched by the external DNA (sequences are firmly hereditary in a Mendelian fashion) and inserted into the cells by which the regeneration of the whole plants can be achieved (Zambryski et al. 1989). Transformations by *A. rhizogenes* possess advantage of being capable to relocate any external gene of prominence, situated in transformed clone of binary vector of hairy root. In a study, the gene of interest with respect to enhancement of secondary metabolism was introduced into hairy roots. In the process 6-hydroxylase gene of *Hyoscyamus muticus* was incorporated in hyoscyamine-rich *Atropa belladonna* by the help of *A. rhizogenes*. An amplified quantity of enzymatic activity with five times more concentration of scopolamine was observed in engineered roots (Hashimoto et al. 1993).

7.3.7 In Vitro Secondary Metabolite Production by Endophytes

There are three origins of supports on the formation of secondary metabolites produced by plants. There is disagreement that plants and endophytic microorganism

are coevolved with similar pathways to form these naturally occurring products. One more assumption states that primeval horizontal gene transmission is attained among plants and endophytic microorganism. The last suggestion is that moreover plants or endophytic fungus yield particular secondary metabolites, thus relocating them to the other symbiotic organism (Karuppusamy 2009; Jennewein et al. 2001). Studies based on radiolabeling of biosynthetic pathways by means of precursor-like amino acids show that fungal endophyte and plants have analogous but different metabolic paths for the fabrication of secondary metabolites (Zhang et al. 2009). It is still under investigation that whether the phytochemicals produced by the plants are naturally produced or it is the result of a mutualistic association of beneficial organisms with the plant. However studies reveal that the blend of influencing factors of plants and fungal endophyte upsurges the secondary metabolite accumulation in both the organisms (Li et al. 2009; Engels et al. 2008). However, the symbiotic relationship among plants and fungal endophytes and the effects on each other in the course of production of substantial bio-active compounds (therapeutically significant) could be processed. This could deliver the background for upcoming natural product fabricated by the process of genetic engineering and metabolic engineering (Komaraiah et al. 2003).

7.3.8 Secondary Metabolites Scaling-Up by the Use of Bioreactors

In this technique bioreactors are used for large-scale production of secondary metabolites. This can be achieved thru scaling-up, by the use of bioreactors, for large-scale modification of plant cells. The process would lead to the formation of exclusive bio-active phytochemicals in a vigorous process. During the process, the plant cells in liquescent suspension provide a distinctive combination of physicochemical environs that is essential for bioreactor's large-scale progression (Ruffoni et al. 2010; Gupta et al. 2014). The fabrication of secondary metabolites using bioreactor from cell culture of Sandalwood and Periwinkle was done by Valluri (2009). In the study, the activity of phenylalanine ammonia lyase was inhibited by the use of trans-cinnamic acid; as a result substantial upsurge in the formation of alkaloid from the cell culture of periwinkle was observed. When cells were exposed to mannitol-induced osmotic stress, it yields noticeable enhancement in the production of total alkaloid. Biotic and abiotic stresses induce additive stimulation in alkaloid accumulation. However, no secondary metabolites (essential oils) are identified, in the form of phenolics from sandalwood cell cultures manufactured in the bioreactor (Valluri 2009).

7.3.9 Secondary Metabolite Immobilization and Accumulation

Advancements in immobilization techniques and scaling-up methodologies provide significant upsurge in numerous plant cell/tissue culture applications, for the formation of bio-active compounds with prominent and additional importance. Compounds

derived from plants with anticancer, chemotherapeutic, or antioxidative properties use Taxol and rosmarinic acid in place of therapeutic agent. Cell cultures of *Plumbago rosea* were immobilized in calcium alginate. It was then cultured in MS media containing 10 mM calcium chloride for the formation of plumbagin, an essential therapeutic compound. Investigations were performed to elucidate the influence of immobilization on improved deposition of secondary metabolite (plumbagin). Calcium alginate immobilization improved the formation of plumbagin by one- to threefold increase, as compared to control (Vanisree and Tsay 2004).

7.3.10 Secondary Metabolite Production by Metabolic Engineering

Metabolic engineering encompasses the objective and focuses modification of metabolic pathways occurs in an organism. It would deliver improved knowledge and usage of various cellular pathways for supramolecular assembly, transduction of energy, and alteration of chemical (Lessard 1996). This method implements on plants which will allow endogenous pathways (biochemical pathways) to be influenced and could result in the development of transgenic crops. Thus in the process, the synthesis of natural products by the plants is altered to deliver valuable biomolecules of therapeutic significance (Kinney 1998). As in numerous studies, fabrication of secondary metabolites is excessively low to be used commercially; therefore metabolic engineering can offer several approaches to:

- Better output, like by increasing the number of cells employed for producing secondary metabolites.
- Overexpression of genes could increase the carbon flux by making use of biosynthetic pathway.
- Categorize for rate regulation of enzyme or hindering feedback and competitive inhibition mechanism.
- Reduction in catabolism.

A number of genes coding biosynthetic pathways of alkaloids such as nicotine, berberine, and scopolamine were engineered and executed. Cloned gene expression of two enzymes, viz., putrescine *N*-methyltransferase and (*S*)-scoulerine 9-*O*-methyltransferase, in *A. belladonna* and *N. sylvestris* (transgenic plants), respectively, in cell culture of *C. japonica* and *E. californica,* respectively, was performed. The results reveal that putrescine *N*-methyltransferase was overexpressed and amplified the content of nicotine in *N. sylvestris* (Sato et al. 2001). Metabolic engineering by yeast is another technique for the fabrication of valued secondary metabolites. Thus by exploiting yeast, the cloning of genes from different plant species and microorganisms can be done easily. It can be done for the production of the following:

- Flavonoid production using yeast (Yan et al. 2005)
- Terpenoid production using yeast:
  - Monoterpenoids production using yeast (Oswald et al. 2007)
  - Sesquiterpenes production using yeast (Ro et al. 2007)
  - Carotenoids production using yeast (Gunel et al. 2006)
- Alkaloid production (plant-origin) by using yeast (Geerlings et al. 2001)

7.4 Secondary Metabolites of Pharmacological Significance

Exploration in the arena of tissue culture (plant) technique has led to the formation of various phytochemicals of therapeutic significance, which are beneficial for human health. New-fangled developments in the production of therapeutic compounds by cell culture technique hassled to extensive assortment of medications such as phenolics, alkaloids, saponins, terpenoids, steroids, amino acids, flavonoids, etc. (Abdin and Kamaluddin 2006; Jordon and Wilson 1995). Efficacious efforts to yield some of these valued medications in comparatively bulky amounts by cell cultures are Taxol (paclitaxel) (Cragg et al. 1993; Fett-Neto et al. 1994; Suffness 1995), diosgenin (Tal et al. 1983; Zenk et al. 1978), L-3, 4-dihydroxyphenylalanine or L-DOPA (Daxenbichler et al. 1971; Brain and Lockwood 1976), capsaicin (Holden et al. 1988; Ravishankar et al. 2003; Sanatombi and Sharma 2007), camptothecin (Sakato and Misawa 1974; Thengane et al. 2003), morphine and codeine (Furuya et al. 1972; Yoshikawa and Furuya 1985), and berberine (Hara et al. 1991; Vanisree et al. 2004). Some of the structures of the secondary metabolites are given in Fig. 7.2.

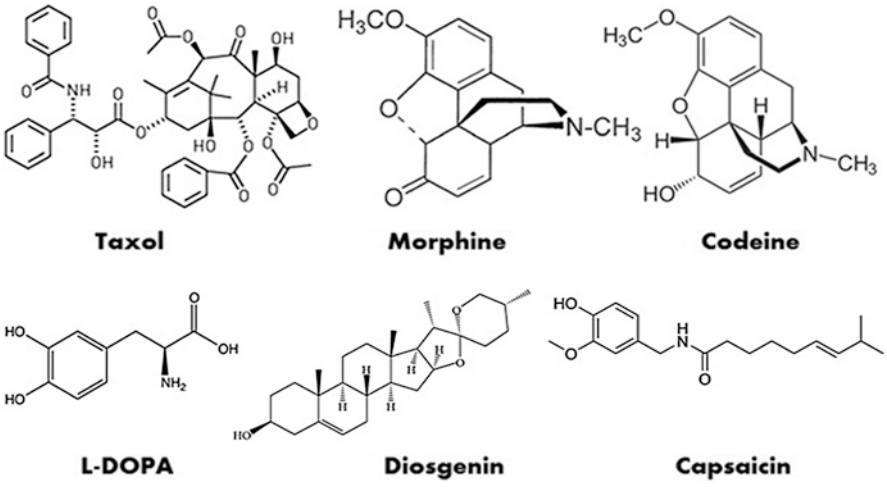

Fig. 7.2 Structure of secondary metabolites of therapeutic importance

7.5 Secondary Metabolites Significant in Healthcare

Certain secondary metabolites possess explicit effectiveness in healthcare which is superfluous to conventional vitamin utilities, for instance, the importance of carotenoids such as lutein and zeaxanthin are required for muscular strength (Roberts et al. 2009; Bai et al. 2011). Presently, there is considerable attention paid in potentially long-standing nutritional assistance by amplified usage of wide range of plants derived secondary metabolites. The extraction process of secondary metabolite and its application are summarized in Fig. 7.3. Several in vitro and in vivo investigations like epidemiological study, minor animal trials, and nutritional interference trials have delivered indications that secondary metabolite consumptions cause cancer reduction, reduced level of cardiovascular diseases, several metabolic disorder, and several neuron disintegration syndromes like Alzheimer's or Parkinson's disease (Crozier et al. 2009; Miller and Snyder 2012). Despite the validity of all the outcomes of the investigations, the probable mechanism pathways for health-related benefits are still unexplained. Several previous investigations reveal evident worth of secondary metabolite intake, which were due to their antioxidant properties (Iqbal et al. 2014a, b; Tripathi et al. 2016). On the other hand, the correlation between antioxidant activities to the findings monitored in in vitro explorations on animal/human trials marks qualm on the antioxidant postulate (Rastogi et al. 2018). However, phytochemicals like phenolics; carotenoids; glucosinolates; vitamins B, C, and E; folates; isothiocyanates; glutathione; and lycopene have been reported to possess substantial antioxidant ability. Nevertheless, various investigations currently focus on other activities other than antioxidant properties, as there are several

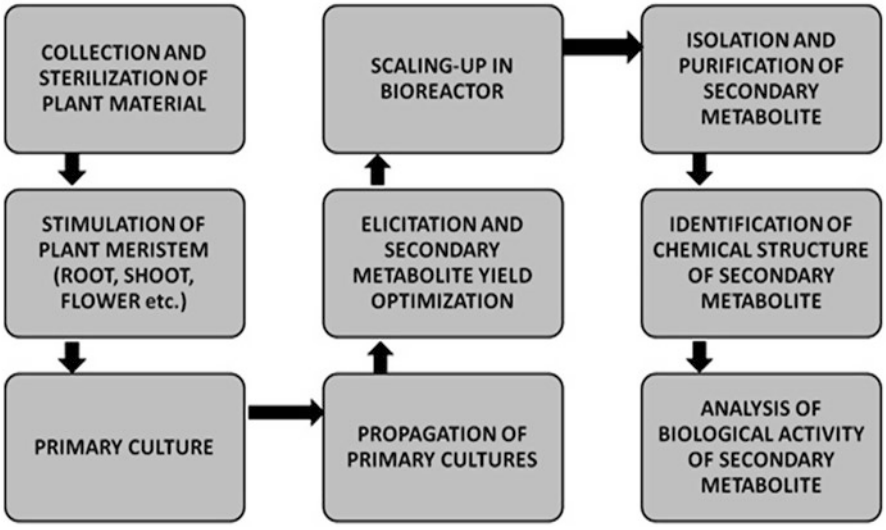

Fig. 7.3 Systematic representation of secondary metabolite production and its biological application

other types of metabolites which possess distinct property. Trials based on human involvement, genetically modified prototypes, and animal models possessing isogenic lines have been investigated, but there is still a shortage of well-organized investigations, precisely analyzing the acclaimed health benefits of secondary metabolites. The benefits and drawbacks of various bases of validation for the effects on health and action mechanism of secondary metabolites of plants have been extravagantly reviewed by Traka and Mithen (2011). While going through the literature, irrespective of the mechanistic action of secondary metabolite, it is ostensible that various investigators contemplate their views, indicating that several plant secondary metabolites actually do have noteworthy benefits associated to health.

An additional barrier in outlining an appropriate dosage of a particular secondary metabolite in food is microbiota of the human, which interacts in the metabolic action and absorption of compounds. An equivalent quantity of secondary metabolite could affect differently, which may cause diverse levels of reaction in different individuals. This deviation in effect is associated to persons' genetic character, age, fitness level, and medications. On the other hand, an auxiliary difficulty is that for several secondary metabolites to be taken into account its bioavailability. The particular secondary metabolite can consecutively influence the tissues with its various effects; further, it could be responsible for the metabolic transformation of the tissues as well. Therefore, it requires information of the absorption, metabolic action, position, and, eventually, defecation of respective bio-active compound, based on the trials on human/animal models to investigate these factors.

As we know, fruits and vegetables are the source of innumerable sets of secondary metabolites taken by our body. These phytochemicals are significant to understand how every compound is absorbed, and, therefore, its bioavailability can be elucidated. Although, most of the studies emphasize on bioavailability of plant-derived secondary metabolites in human/animal tissues. However the actual effect might be subsidiary, as it is intermediated by means of modifications by gut's microbiota which may support to elucidate the inconsistency among the comparatively low grade of apparent bioavailability and its definite nutritional advantages (Duynhoven et al. 2011; Manach et al. 2009). Moreover, the existence of particular enterotypes is more persistently linked with nutrition than ethnicity or topography. The association of microbiota enterotype might affect both the individual's possibility of chronic ailment and also have an impact on the way bio-active compounds are absorbed. It is probably that particular enterotypes will be affected by continuing specific dietary habit, but also short nutritive mediation of merely for a day could modify microbiome association (Wu et al. 2011). Though the multifarious interrelationship with microbiota are usually mutualistic, it could turn out to be pathological, for instance, in the situation of inflammatory bowel disease (Fava and Danese 2011). Minor population of bifidobacteria and greater occurrence of gastrointestinal pathogenic bacteria like *E. coli*, *Campylobacter*, *Helicobacter*, and *Salmonella* are frequently related to chronic immune diseases. Food can also affect the peril aspects, such as metabolic syndrome, which are generally related with fast food and junk dietaries, and could cause metabolic syndromes like obesity and diabetes (Fava et al. 2013).

Extensive effect of flavonoids, in human-involved investigations, on cardiac risk disease associated with high and low levels of flavonoid intakes over a period of 18 weeks revealed an upsurge in potentially valuable bacterial groups for the high flavonoid intake, for example, as in *Bifidobacterium* (Chong et al. 2013). Polyphenolics are another category of secondary metabolites. Polyphenols are mostly well-examined biomolecule, and they are permitted through metabolic action and are abdominally absorbed (McGhie and Walton 2007). Relating to the diet, polyphenols are mainly delivered by fruits, vegetables, grains, and beverages like tea, coffee, wine, and beer (Grosso et al. 2014). Anthocyanin is a class of phytosecondary metabolite. Its constancy of structure is determined by the type of sugar component associated. The anthocyanins are derived compounds like gallic acid, protocatechuic acid, syringic acid, and aglycones, which are revealed to be biotransformed by microflora (Forester and Waterhouse 2010). The bacterial-reliant metabolic action of anthocyanins can then, in turn, modify abdominal bacterial inhabitant like *Lactobacillus* and *Bifidobacterium*, recommending an optimistic association among bacterial condition and phytochemical intake (Hidalgo et al. 2012). As earlier reviewed, studies associated to human subjects have established the absorption of secondary metabolites derived from plant, but elucidating the consequence of the method and the metabolic outcome still faces methodological challenges. The approaches like using bioinformatical tools (next-generation sequencing or metagenomics) and animal/human model studies would improve our knowledge of the secondary metabolite interaction with tissue and its metabolism. It would elucidate the pathways of metabolism of secondary metabolite and the development of future nutritional food with enhanced level of secondary metabolites which could be obtained naturally.

7.6 Conclusions and Future Prospects

Secondary metabolites produced by the plants support them to contest and stay alive in extreme environmental conditions. Various biotechnological methodologies are employed for fabrication and enhancement of secondary metabolites by genetic engineering process and plant tissue culture techniques. Genomic knowledge by metabolic engineering for fabrication of secondary metabolites derived from plants is presently well innovated. Thus, metabolic engineering and biotechnological exercises can be applied as a substitute for the production of naturally active, economically valuable, and pharmaceutically important secondary metabolite. Developments in bio-techniques, mainly the technique of plant cell cultures, could deliver new worth for therapeutically and economically bio-active compounds. The main benefit of the in vitro cell cultures comprises the fabrication of secondary metabolites, cultivated in controlled environmental conditions, thus enabling us to extract important and particular phytochemicals in elicited quantities. The practice of genetic engineering is another emerging tool which can regulate the pathways for the fabrication of therapeutically significant secondary metabolites. Knowledge of biosynthesis of

desired phytochemicals obtained from plants and its cultures are still in its preliminary stages; therefore accordingly approaches are required to advance the information based on molecular and cellular level. The advancement in new-fangled methods of molecular biology to yield cultures by transgenic and to understand the effect of the expression and regulation of biosynthetic pathways is possibly to be a noteworthy step in the direction of making cell culture technique more relevant to produce commercially important secondary metabolites. These new techniques will contribute to spread and improve the sustained efficacy of higher plants **as** non-conventional sources of compounds, specifically therapeutic compounds. It is anticipated in this field that prolonged and escalated efforts will lead to contribute efficacious biotechnological fabrication of secondary metabolites. It is further to be explained the effect of particular secondary metabolite associated with health benefits, which could be useful for neutraceutical and pharmaceutical industries. This in turn could permit the elucidation of innovative bio-active compounds and support to fix objectives for the improvement of nutritionally efficient foodstuffs for health benefits.

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8 Salient Biotechnological Interventions in Saffron (*Crocus sativus* L.): A Major Source of Bio-active Apocarotenoids

Maryam Vahedi, Roghaye Karimi, Jitendriya Panigrahi, and Saikat Gantait

Contents

Abstract

Crocus sativus (L.) is considered to be one of the high-value spices cultivated around the globe, and hence is under scanner of the genomic approaches that have been used to study the identification, expression, and regulation of the key

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genes involved in its flower development and apocarotenoid biosynthesis. *C. sativus* flower contains in excess of 150 compounds of aromatic and vaporescent. It produces remarkable amounts of apocarotenoids, such as crocin, picrocrocin, and safranal, that exhibit a wide range of anticancer, neuroprotective, anti-inflammatory, and cardioprotective activities. These apocarotenoids displaying such a wide range of pharmacological activities are of huge interest to culinary and pharmaceutical industries. Advances in biotechnological interventions, like genomic technologies, functional genomics, and transcriptomics studies, have revealed the expression of genes and/or structure, function, evolution, mapping, and editing of genes encoding apocarotenoid biosynthesis and enabled *C. sativus* genetic improvements in an efficient way through molecular breeding programs. The application of genomic tools and techniques has encouraged *C. sativus* breeders to adopt precision breeding approaches. The present chapter attempts to traverse across the recent developments in genetics and genomics-based researches conducted in *C. sativus* to perceive the biosynthetic pathways of its major secondary metabolites.

Keywords
Crocetin · Genetic transformation · Picrocrocin · Safranal · Transcriptomics

8.1 Introduction

The dried stigma of *Crocus sativus* (L.) i.e., "Saffron" is taken into account as the utmost valuable flavoring herb in the world. It is an autumn-flowering perennial plant with underground storage organ (in the form of corm) (Fernández 2004). The major saffron-producing countries are Iran, India, Greece, Morocco, and Spain, wherein Iran ranks first in terms of the worldwide production (Molina et al. 2005; Carmona et al. 2006). Saffron cultivation earns priority mainly for its red stigmas (style branches) that hold the unique flavoring and coloring attributes (Melnyk et al. 2010). Saffron flower comprises of over 150 aroma and volatile compounds. Significant amounts of apocarotenoids like crocin, picrocrocin, and safranal are produced by saffron stigma. These apocarotenoids exhibit an array of anticancer, neuroprotective, anti-inflammatory, and cardioprotective activities (Baba et al. 2015a).

Naturally, saffron, a renowned member of Iridaceae is a non-fertile herb owing to the triploid nature (2n = 3x = 24) of its genome (Harpke et al. 2013). Its triploid condition is attributed to an irregular meiosis process and allows vegetative propagation via corms that limits its genetic base and hinders its genetic enhancement (Renau-Morata et al. 2012). The data on nuclear DNA content and other karyological features in the genus *Crocus* present a large and complex genome of about 10 Gb (for *C. sativus*) (Busconi et al. 2015). Such genome size and ploidy level eventually restrict saffron breeding and genetic improvement. The recent advances in genomic approaches provide new techniques that allowed investigation of the evolutionary

origins of saffron. A number of research studies were carried out on the molecular basis of *Crocus*, but the genetic origin of *C. sativus* is not clear yet. Phylogenetic analysis based on chloroplast, ribosomal, and nuclear single copy genes sequence could not find the origin of saffron (Petersen et al. 2008; Harpke et al. 2013). Despite these intensive studies, the questions on the ancestor species and the allied evolutionary processes still remain unresolved. The whole genome sequencing of the *Crocus* provides a powerful tool to reveal diversity, relationships between species, and the origin of saffron, but till today it persists as a challenging problem for cultivated saffron carrying the intricate genome of considerable size. However, a number of sequencing projects are still under way mainly funded by the European Commission Brussels, Belgium. Therefore, it is expected that the whole genomic sequences of diploids and polyploid species of the genus *Crocus* would provide a fundamental knowledge for understanding the evolution and domestication of saffron.

In addition, not much is understood regarding the synthesis and accumulation of apocarotenoid compounds in the course of stigma growth and development. Numerous researches were carried out to study the transcriptome sequence data for the identification of structural and functional organization of the saffron genome. The same was also used for putative gene identification and networks that are involved in the production of biologically active plant compounds. Expressed sequence tags (ESTs) provide information about the genes expressed in a specific tissue or organ. However, limited EST collections from saffron corms (Álvarez-Ortí et al. 2004a) and mature stigmas (D'Agostino et al. 2007) are available till date. Recently, next-generation transcriptome sequencing efforts were performed for the stigma and flower tissues by Baba et al. (2015a) and Jain et al. (2016) to elucidate the molecular basis of apocarotenoid biosynthesis and its accumulation. A number of MADS-box and MYB-transcription factors that are involved in the flower development were cloned, and their expression were characterized (Tsaftaris et al. 2007; Gómez-Gómez et al. 2012). The surge in expression frequencies of apocarotenogenic genes suggested that the apocarotenoid accumulation might be regulated by gene expression during the stigma and tepals development (Ahrazem et al. 2015). Bioinformatics approaches offer the essential techniques for the identification of responsible genes and pathways of medicinal plants; in addition such approaches analyze the bulk amount of information, generated from high-throughput techniques (Sharma and Sarkar 2012). Bioinformatics studies can contribute in all stages of genotyping experiments in saffron such as structural genomics, comparative genomics, transcriptomics, proteomics, phylogenetic analysis, and system biology (Husaini et al. 2009). In recent years, the development of genomic tools and techniques, such as ESTs, genome and transcriptome sequencing, and bioinformatics, facilitated the research on genetic enhancement of saffron. This chapter provides an outline of the recent developments in genomics- and transcriptomics-based researches of saffron and also summarizes these omics approaches to identify molecular mechanisms of apocarotenoid biosynthesis.

8.2 Saffron Apocarotenoids and Their Use

Phytochemical studies on saffron have shown the presence of more than 150 constituents in its stigmas including crocin, crocetin, safranal, picrocrocin, etc. (Fig. 8.1) (Tarantilis et al. 1995; Escribano et al. 1996; Lozano et al. 2000; Bathaie and Mousavi 2010). Amid all the components of saffron extract, crocetin is the prime contributor toward key pharmacological functions (Abe and Saito 2000). Few of the other such constituents, such as volatile agents like safranal, are considered for significant contributor of pharmacological activities of saffron, whereas the other important constituents are the bitter-tasted (picrocrocin) and dye-yielding (like crocetin and its glycosides, crocin) active principles (Rios et al. 1996). The significant uses of saffron apocarotenoids have been described briefly in Fig. 8.2. In the conventional medication system, saffron stigma is employed as an antiedermatogenic medication. Several studies conducted have supported the pharmacological protective properties of crocins and crocetins and established their antioxidant features (Ahmad et al. 2005; Shen and Qian 2006; Xiang et al. 2006). According to Xiang

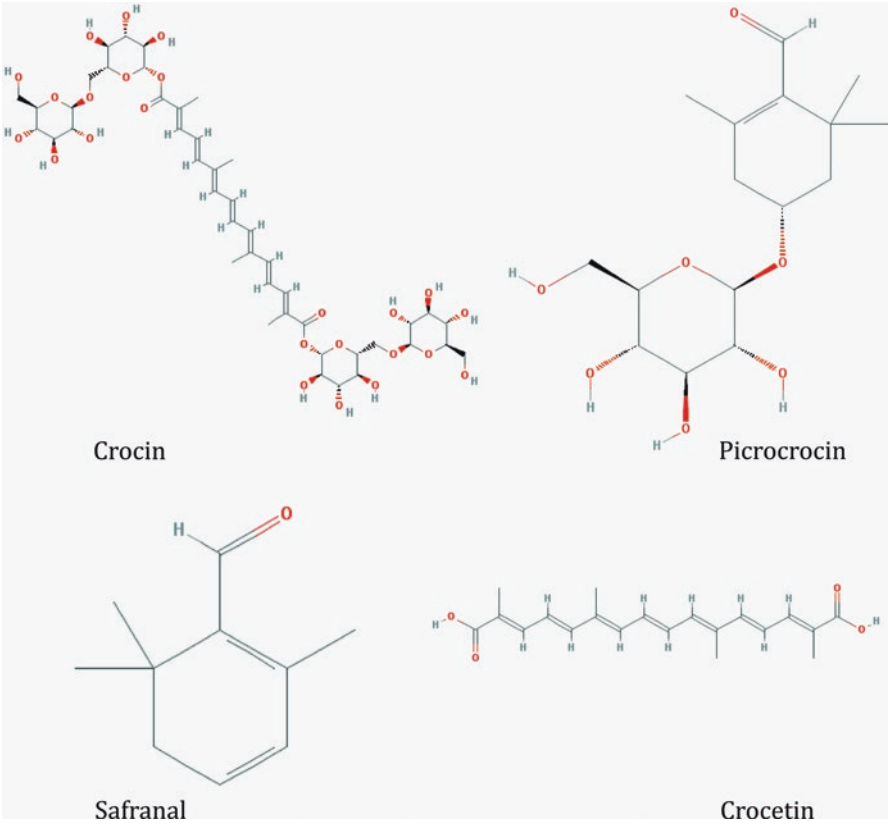

Fig. 8.1 Structures of some key saffron apocarotenoids

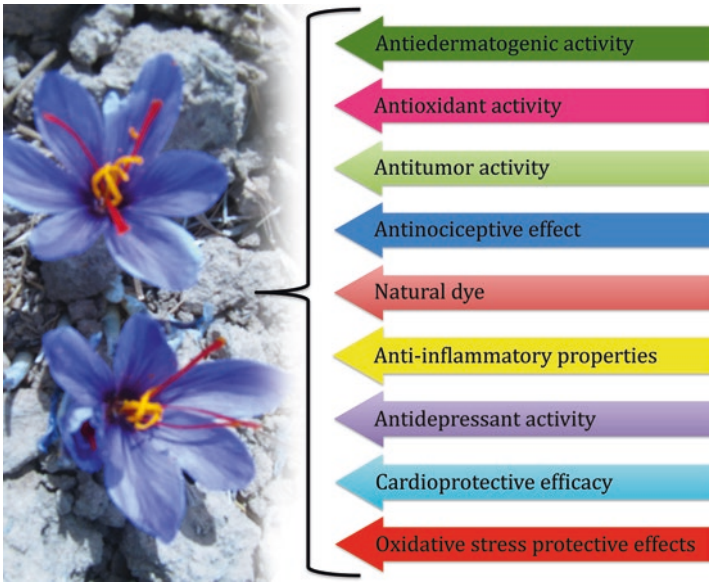

Fig. 8.2 Pharmacological activities and other uses of saffron apocarotenoids

et al. (2006), crocetin involved in the inhibition of leukocyte adherence to vascular endothelial cells might be the consequence of vascular barriers. Besides, their study also revealed that crocetin could safeguard myocardium cell mitochondria. In similar ways, crocetin has significantly reduced the lipid peroxidation content (LPO), superoxide dismutase (SOD) activity, and GSH-Px in cardiac hypertrophy and considerably enhanced the myocardial, pathological, and histological transformations stimulated by norepinephrine (Shen and Qian 2006). The neuroprotection study was conducted in a hemi-Parkinson rat model by treating crocetin. It was revealed that the levels of dopamine and glutathione content were unaltered, but the thiobarbituric acid content was declined in crocetin-treated groups (Ahmad et al. 2005). An in vitro study involving the treatment of crocetin, crocin, and picrocrocin over human cancer cells revealed that crocin is the more promising saffron product and can be considered with antitumor activities (Escribano et al. 1996). Coupled with other plant parts of saffron, the aqueous and ethanolic extracts of stigma and petal have also been shown to possess an antinociceptive potential accompanied by chronic or/and acute anti-inflammatory properties in mice (Hosseinzadeh and Younesi 2002). The ethanolic and aqueous extracts of *C. sativus* containing crocin and safranal have been evaluated for their antidepressant potential on mice imposed with swimming experiment. This study revealed that the immobility time was shortened by the influence of both crocin and safranal. Likewise, safranal helped in inhibiting the uptake of serotonin and crocin via dopamine and norepinephrine (Hosseinzadeh et al. 2004). Similarly, Magesh et al. (2006) evaluated the mechanism of crocetin, the saffron plant derivative in hindering the tumor cell proliferation. They treated crocetin to lung cancer-bearing mice during pre- and post-initiation

periods and discovered that crocetin effectively reverted back all the pathological changes perceived in cancerous animals. In addition to this, crocetin was proved to scavenge free radical and played a significant role in cellular function. The chemopreventive and genotoxin-promoted oxidative stress protective effects of saffron were also documented in Swiss albino mice (Premkumar et al. 2003). They have treated the dehydrated stigmas of saffron on cyclophosphamide, mitomycin-C, urethane, and cisplatin and studied the stimulated modifications in lipid peroxidation and antioxidant activities in Swiss albino mice. The significant results were obtained with respect to a simultaneous escalation in the enzymatic (CAT, SOD, GPx, and GST) and also nonenzymatic antioxidants, for instance, diminished glutathione in saffron-pretreated albino mice in contrast to the only genotoxin-treated subjects. Crocin, i.e., digentiobiosylcrocetin, has been shown to have glycoside pigments (Tarantalis et al. 1995). These pigments might possibly be the additional product for the artificial food dye tartrazine (Timberlake and Henry 1986). Dufresne et al. (1999) described their report on the conversion of crocetin to several glycosyl esters with the help of cell-free suspension culture when fed with the encapsulated substrate. Nevertheless, the attributes of glucosyl transferase, associated with crocin synthesis, were studied and depicted that the enzyme actively participated in crocin synthesis by creating ester bonds involving the glucose moiety of UDP-glucose and the carboxyl groups of crocetin (Côté et al. 2000). Notably, crocin also has the cardioprotective efficacy, and this claim was supported by Goyal et al. (2010). Their investigation on the induction of crocin derived from *C. sativus* in isoproterenol (ISO)-stimulated cardiotoxicity based on hemodynamic, histopathological, ultrastructural, and antioxidant factors. Crocin (20 mg/kg/day) administered in ISO-treated rats and significant modulation was observed in hemodynamic and antioxidant derangements. In addition, ultrastructural and histopathological assessments pronounced the cardioprotective potential of crocin. In another literature, the significant effect of vitamin E, safranal, and crocin could preclude the dangerous effect of the diazinon-induced rise and intensification of few specific biomarkers in rats (Hariri et al. 2010). The significant events that were created by diazinon on rats, such as the decrease in total protein and albumin concentrations, and the elevation in the levels of direct 8-iso-prostaglandin $F_{2\alpha}$, TNF-α, and soluble protein-100 β (S100 β) were notably annulled with the application of vitamin E, safranal, and crocin. Likewise, Joukar et al. (2010) studied on the effect of saffron consumption on rat's heart with isoproterenol-induced myocardial injury based on biochemical and histopathological findings. The saffron plus ISO group significantly decreased serum levels of heart troponin I, and also reduced the intensity of tissue destruction of the heart.

Saffron tablet was referred without much clinical importance based on some biochemical parameters and hematological changes (Mahamadpour et al. 2013). A safety evaluation of saffron tablets in healthy volunteers had shown a certain change in hematological and biochemical parameters. However, these changes were not in abnormal values and not so critically important (Modaghegh et al. 2008). Saffron (200 mg tablets) was observed to show a positive response on a sexual function by enhancing duration of some erectile events in patients with erectile dysfunction

after ingesting for a period of 10 days (Hosseinzadeh 2009). One of the reports proposed that crocins may play a critical role in controlling the obsessive-compulsive disorder, a type of psychiatric disorder. It showed a functional interaction among crocin and the serotonergic system (Georgiadou et al. 2012). Several such clinical trials were executed on saffron to evaluate its pharmacological activities (Moshiri et al. 2014; Bhandari 2015). Rajaei et al. (2013) confirmed the hypoglycemic and antioxidative properties of crocin wherein a dose of 60 mg/kg was observed to substantially decline the blood glucose level in diabetic animals. In addition, crocin was reported to suppress the proliferation of K-562 human chronic myelogenous leukemia cells expressing Bcr-Abl protein tyrosine kinase activity (Geromichalos et al. 2014). Crocin supplements were found to be beneficial that enhanced the serum cholesteryl ester transfer protein in patients with metabolic syndrome (Javandoost et al. 2017). As reviewed by Moradzadeh et al. (2018), crocetin has the ability to inhibit cancer cell proliferation via preventing nucleic acid synthesis, improving antioxidative system, as well as stimulating apoptosis and differentiation pathways. A number of preclinical researches have exhibited that dietary intake of some carotenoids has potent antitumor effects both in vitro and in vivo, suggesting their potential preventive and/or therapeutic roles in several tissues (Bolhassani et al. 2014). Pitsikas (2015) critically reviewed the advancements in research on the influence of crocin to deal with memory disorders and explain its benefit over currently commonly used cognitive enhancers. However, these reports seldom considered the clinical safely issue emerged from usage of saffron tablets. On the contrary, Mahamadpour et al. (2013) evaluated the clinical safety of crocin and reported a comparatively safe and standard profile for crocin (in form of tablet) intake at a dose of 20 mg/day for 30 days for healthy human volunteers.

8.3 Biosynthesis of Saffron Apocarotenoids

Structurally, crocins are trans-crocetin di-(β-D-gentibiosyl) ester, crocetins are natural carotenoid dicarboxylic acid, and picrocrocin is the degradative product of zeaxanthin carotenoid, whereas safranal is an aromatic aldehyde. A simultaneous study of crocin, picrocrocin, and safranal showed the interconvertible products along with HTCC (4-hydroxy-2, 6, 6-trimethyl-1-cyclohexene-1-carboxaldehyde), wherein picrocin was converted to HTCC with the involvement of β-glocusidase. Consequently, HTCC was transformed to safranal when exposed to acid treatments. Likewise, picrocrocin, when treated with alkali or acid, was hydrolyzed to safranal (Himeno and Sano 1987). The saffron apocarotenoid being volatile and crocetin glycosides being water-soluble, they are collected in vacuoles to result in pigmentation (Rosati et al. 2009). The particular color of saffron is produced owing to the existence of crocins that possess glycoside carotenoid structure (Bolhassani et al. 2014). The biosynthetic pathway was first proposed by Rubio-Moraga et al. (2004). The biosynthesis of apocarotenoids was derived from a putative precursor zeaxanthin to generate cyclic carotenoid volatile organic compounds (picrocrocin and safranal) and crocetin that is ultimately glycosylated to crocin (Fig. 8.3). The

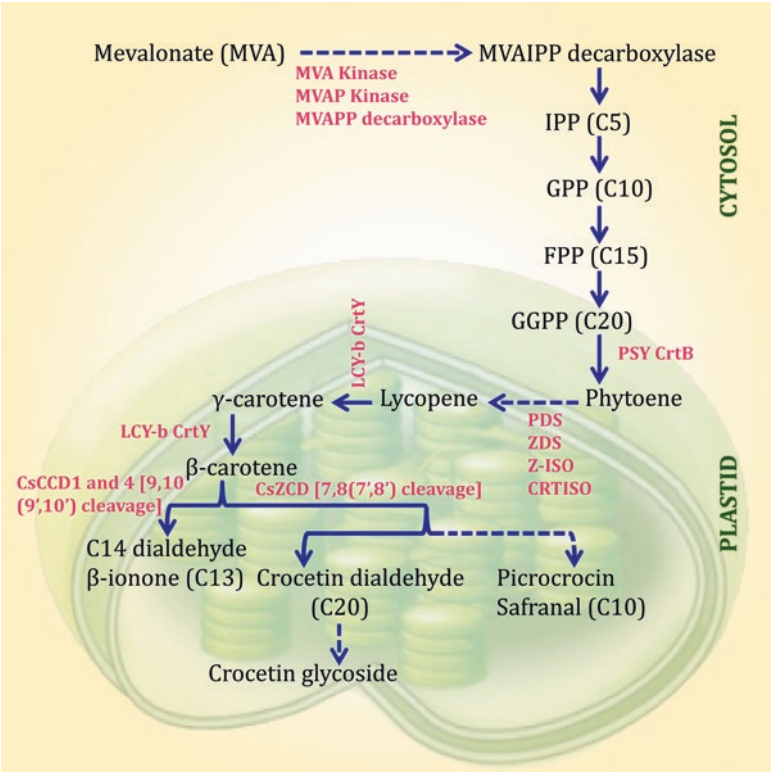

Fig. 8.3 Elementary pathways for synthesis of saffron apocarotenoids. The enzymes involved in these pathways are in red color. The steps that involve multiple enzymes are specified with dashed arrows. (Adapted and redrawn from Rosati et al. 2009)

key enzymes engaged in these biosynthetic phases were identified and assessed. The *Crocus* carotenoid-specific cleavage dioxygenases (CCDs) enzymes characterized up to now, analogous to CCD1 and CCD4 enzymes, are differentially expressed in floral organs, and only CCD4s comprise predictable transit peptides for plastoglobule localization (Rubio-Moraga et al. 2008). The CCD1-like generic CsCCDs contain 9,10 (9′,10′) cleavage action on several carotenoid substrates (Bouvier et al. 2003; Rubio-Moraga et al. 2008). The precursor (zeaxanthin) was cleaved by the enzyme *Crocus* 7, 8 (7′, 8′)-zeaxanthin dioxygenase (CsZCD). This leads to the biogenesis, color, and odor of crocetin and picrocrocin (Namin et al. 2009). CsZCD enzyme finds 98–100% structural similarity with CCD4-like CsCCD4a and CsCCD4b proteins (Rubio-Moraga et al. 2008). CsZCD is shorter than CsCCD4 enzymes, which transport the plastid transit peptide. The glucosylation of hydroxyl-β-cyclociral subsequently produced picrocrocin. Moreover, 7, 8–7′, 8′ oxidoreductase catalyzed crocetin dialdehyde into crocetin; at the same time, safranal was the modified form of safranal. However, advanced exploration is indispensible to

explain the *Crocus* CCD4/ZCD in terms of its precise number, protein configuration, and enzymatic action (Rosati et al. 2009).

8.4 Omics Approaches

The latest advancement in molecular genetics has accelerated the identification of apocarotenoid biosynthesis pathway. The sequences of MADS-box genes expressed in saffron flower have been cloned to appraise the molecular events governing the growth and development of flower in the associated wild progenitor species of *Crocus* as well as cultivated saffron. Comparative structural and phylogenetic analysis of these proteins may be helpful to solve the origin of the cultivated triploid *C. sativus* (Tsaftaris et al. 2007). The genetic origin or ancestral species of saffron is not yet clearly disclosed (Petersen et al. 2008; Harpke et al. 2013). The current advances in genome sequencing technology of diploids and polyploid species of the series *Crocus* may clarify some important details of the genus *Crocus.* Chloroplast genome sequencing of seven *Crocus* species was performed using illumina platform, which provided genetic information on the phylogeny, species identification, and population genetics of this valuable spice. A new and simple technique of genome walking relying on the initiation of circular genomic DNA fragments (cgDNA) and rolling circle amplification of the circular genomic DNA has been reported to isolate the promoter regions of numerous genes from *C. sativus* (Tsaftaris et al. 2010). Apart from the biodiversity array in medicinal plants, DNA barcoding can also be applicable for the identification/authentication of such commercially important plants in the postgenomic era (Gantait et al. 2014; Mishra et al. 2016). Three DNA barcodes such as *trnH-psbA* intergenic spacer (trnH-psbA), a sizable subset of ribulose-bisphosphate carboxylase (rbcL-a), and nuclear internal transcribed spacer 2 (ITS2) were employed to discriminate saffron from its adulterants by sequences diversity assessment (Huang et al. 2015). The barcoding melting curve analysis approach (Bar-MCA) that utilizes the unanimous chloroplast plant DNA barcoding zone *trnH-psbA* could be a faster method to authenticate saffron and detect its adulterants (Jiang et al. 2014). The sequence of the plastid genes *matK* and *rbcL* was applied to construct the new marker for saffron and the adulterant species reorganization (Soffritti et al. 2016). A practical standard operating procedure (SOP) has been introduced by Zhao et al. (2016) for the authentication of saffron. For SOP, loop-mediated isothermal amplification (LAMP) method is used, and it requires four to six different primers that are projected depending on the nucleotide sequence of the internal transcribed spacer 2 (ITS2) nuclear ribosomal DNA of *C. sativus* to distinguish saffron from its adulterants. A sequence characterized amplified region (SCAR) technique also can be applied for the validation of a wide variety of dried food products containing saffron (Torelli et al. 2014). SCAR markers as a quick, profound, and inexpensive screening approach were developed for identifying dehydrated commercial saffron stigmas often mixed with seven common bulking components (saffron adulterants) (*A. montana*, *B. orellana*, *C. officinalis*, *C. tinctorius*, *C. vernus*, *C. longa*, and

Hemerocallis sp.). SCAR markers are effective in identifying these adulterants substantiately (Marieschi et al. 2012). This approach facilitated the recognition of even a very low quantity of each adulterant. Apart from the sequence-related amplified polymorphism (SRAP), the new SCAR combined with ITS maker-based multiplex PCR analysis developed for the rapid identification of substitutes in saffron at molecular level (Babaei et al. 2014a, b). This approach could identify the occurrence of any anticipated plant material and adulterant materials in a single sample. DNA fingerprints are the barcode-like patterns that can be used for authentication of herbal medicines (Ganie et al. 2015).

The transcriptome is the study of all RNA transcripts in one cell at the specific developmental stage that focuses on the gene expression. The *C. sativus* transcriptome provides insights for understanding the molecular basis of flavor and color biogenesis. The first genomic characterization of a mature saffron stigmas has revealed the occurrence of 6603 high-quality ESTs across the Saffron Genes database (http://www.saffrongenes.org) that categorized into 1893 clusters, each related to a differently expressed gene, and interpreted. Homology analysis by blastX showed the high expression level of some transcripts contigs (TCs) (D'Agostino et al. 2007). A computational analysis was used to identify miRNAs, and their targets using this EST library from mature saffron stigmas, two putative miRNAs (miR414 and miR837-5p), and co-expressed genes including transcription factors and protein kinase which may play roles in apocarotenoid biosynthetic pathways have been characterized (Zinati et al. 2016). Three novel miRNAs, csa-miR1, csa-miR2, and csa-miR3, were forecasted by computational approaches. These objects ensure a function in biotic and abiotic stress resistance, senescence, as well as growth and development of plant. Furthermore, certain objects are engaged in mRNA transfer, translation, and posttranslational amendments (Guleria et al. 2012).

Some of the genes and enzymes that are involved in the abovementioned steps are studied and characterized. Likewise, the *Crocus* carotenoid cleavage dioxygenase gene (*CsCCD*) was also cloned (Bouvier et al. 2003). According to Rubio-Moraga et al. (2008), the *Crocus* CCDs, characterized till date, are analogous to CCD1 and CCD4 enzymes and are differentially expressed in flower organs, and CCD4s solely carry predicted transit peptides for plastid localization. The CCD1-like generic *CsCCD*s possess 9,10 (9′,10′) cleavage activity on various carotenoid substrates (Bouvier et al. 2003). CCD4-like CsCCD4a and CsCCD4b proteins (Rubio-Moraga et al. 2008) are 98–100% comparable to the CsZCD enzyme, earlier reported to cleave zeaxanthin at the 7,8(7′,8′) positions, resulting in synthesis of crocetin dialdehyde (Bouvier et al. 2003). CsCCD4 enzymes are longer than CsZCD and contain a plastid transit peptide. They perform a 9,10(9′,10′) cleavage and are also able to cleave zeaxanthin, although the expected apocarotenoids could not be detected by neither LC nor GC (Rubio-Moraga et al. 2008). However, advanced experimentations are necessary to explain the enzymatic activity, protein structure, and a precise number of *Crocus* CCD4/ZCD enzymes. Furthermore, apocarotenoid volatiles and water-soluble crocetin glycosides are collected in vacuoles to express pigmentation. As reviewed by Rosati et al. (2009), an UDP-glucose crocetin 8-8′-glycosyltransferase enzyme was purified from cell suspensions and

characterized (Côté et al. 2000), and the product of the stigma-expressed *UGTCs2* gene was shown to glucosylate crocetin aglycones and glycosides in vitro (Rubio-Moraga et al. 2004). Although the recent efforts have been focused on the identification of genes that are involved in the apocarotenoid biosynthesis, there are certain genes absent in the entire apocarotenoid biosynthetic pathway. Several enzymes that were identified to catalyze apocarotenoid biosynthesis pathway are the product of the crucial genes, such as PSY, LCY, CCD, BCH, and ZCD, which control the biosynthesis of apocarotenoids during the course of multiple phases of stigma development (Gómez-Gómez et al. 2010; Mir et al. 2015a). The molecular functions of two *Crocus* carotenoid cleavage dioxygenases, namely, CsCCD and *CsZCD*, have been detected by Bouvier et al. (2003). *CsZCD* precisely catalyzes the synthesis of crocetin dialdehyde from zeaxanthin, and *CsZCD* is responsible for the pigment and aroma synthesis in saffron. The expression patterns of *CsPSY*, *CsPDS*, *CsLYCb*, and *CsBCH* genes were investigated throughout the growth of stigma. By the modification of immature yellow to completely matured red stigmas, an accumulation of zeaxanthin was detected, supplementing with the expression of *CsPSY*, phytoene desaturase, and *CsLYCb*, besides the substantial collection of *CsBCH* and *CsZCD* transcripts (Castillo et al. 2005). The garnering of apocarotenoids and expression framework of apocarotenoid biosynthesis genes were researched on focusing on three particular phases of stigma growth (yellow, orange, and scarlet). Reverse transcription (RT)-PCR analysis revealed a distinct association amid apocarotenoid gene expression and apocarotenoid content throughout developmental period (IqbaLMzr et al. 2013). Maximum apocarotenoid biosynthesis and highest levels of *CsZCD* gene expression occurred during the fully developed scarlet stage of stigma development (Mir et al. 2012). CCD2 was identified during the first steps of stigma development using the 454-based transcriptome sequencing. The expression of *CsCCD2* was correlated with the accumulation of crocin since it catalyzes the first step in crocetin biosynthesis (Frusciante et al. 2014). The model of crocin accumulation and the expression of apocarotenoid-related genes were investigated to find the agents affecting the garnering of such bio-active compounds and to recognize the main stages of their biosynthetic pathway. The results showed that the expression of the carotenogenic genes *PSY*, *ZDS-V*, *BCH*, and *LCY-II* was associated with the accumulation of crocins and increases the transcript levels of *CCD2* genes during stigma and tepal development (Ahrazem et al. 2015). Four CCD genes, namely, *CsCCD1a*, *CsCCD1b*, *CsCCD4a*, and *CsCCD4b*, were identified from *C. sativus.* The four CCDs are divided into two phylogenetically dioxygenase categories with the same enzymatic activity even though their expression and localization were different (Rubio et al. 2008). In a study on the expression of three isoforms of CCD4 gene (*CsCCD4a*, *CsCCD4b*, and *CsCCD4c*) in response to different stresses, the results indicated that *CsCCD4a* and *CsCCD4b* showed enhanced expression in response to dehydration, salinity, and methylviologen, but *CsCCD4c* did not show any change in expression (Baba et al. 2015a). Functional characterization of *Cs*BGlu12, a β-glucosidase from *C. sativus*, has shown its role in abiotic stress through reactive oxygen species (ROS) scavenging (Baba et al. 2017). The association between expression of *CstNCED* and the endogenous ABA quantity was

studied in corms and stigma; the results showed the participation of *CstNCED* in the modulation of ABA-associated activities, for example, corm dormancy and flower senescence of saffron (Ahrazem et al. 2011). *CCD7* and *CCD8* genes that control the branching of shoots through apical dominance were required for strigolactones (SL) biosynthesis and were first isolated by Rubio-Moraga et al. (2014a, b). The expression patterns of two lycopene-b-cyclase genes, *CstLcyB1* and *CstLcyB2a*, were explored in multiple saffron tissues; *CstLcyB1* was substantially expressed in stigma and leaf tissue, and at lesser levels in tepals, contrastingly, *CstLcyB2a* was characterized only in the stigma tissue (Ahrazem et al. 2010). The spatial and temporal expression array of *CsGT45* was investigated by RT-PCR during stigma development. The results showed that *CsGT45* expression is developmentally controlled. The *CsGT45* expression level in the yellow and orange phases was low, but enhanced since the red phase, and touched its ultimate state during anthesis. *CsGT45* is an effective enzyme that performs a major responsibility in the synthesis of flavonoid glucosides in the stigma of saffron (Rubio-Moraga et al. 2009).

Three distinctive homologous *CsAP1* genes, viz., *CsAP1a*, *CsAP1b*, and *CsAPc*, are the originally described MADS-box genes that were characterized from leaves and flowers of saffron. The expression pattern genes showed that the transcripts of each of these genes exist in leaves, together with the flowers of *C. sativus* (Tsaftaris et al. 2004). The expression of a family of five PISTILLATA/GLOBOSA-like (PI/GLO-like) MADS-box genes have been studied in the saffron flower, recognized to produce heterodimers for stamens and petals (Kalivas et al. 2007). SEP3-like cDNAs, transcribed from three genes, were isolated and their expression configurations and prospective protein interactions with other saffron MADS-box proteins investigated (Tsaftaris et al. 2011). The isolated *MYB* gene from *C. sativus* when expressed displayed an enhanced expression in the red stigmas of saffron, but a comparatively reduced expression was detected in tepals, alongside no transcripts identified in anthers and leaves (Gómez-Gómez et al. 2012). The first analysis of a comparative expression analysis of floral homeotic genes in relation with senescence was performed at different stages of flower development, identifying the pathway can make last longer flowering of saffron by activation of particular key genes (Wafai et al. 2015). Later, Ashraf et al. (2015) reported the modulatory role of *CsULT1* in biosynthesis of *Crocus* apocarotenoid for the first time; it suggested a potential function in controlling the biosynthetic pathway of crocin. Differentially expressed genes, early inducible proteins (ELIP) and SOUL heme-binding proteins, engaged in the response of saffron stigmas against light, were recognized in saffron stigma (Ahrazem et al. 2016).

There is lack of study about gene expression pattern in the corm of saffron. At a stage characterized by storage accumulation and corm growth, a remarkable amount of sequences with similarity to genes related to cell growth, protein synthesis, folding and degradation, transcription factors, and proteins related to the formation and maintenance of cell wall and other cellular structures were identified (Alvarez-Orti et al. 2004a). The expression profile of the key storage protein, mannose-binding lectin, of saffron corm was greater throughout summer season before sprouting and then declined immediately after sprouting of corm (Álvarez-Ortí et al. 2004b).

The first study on transcriptome sequencing of saffron stigma and flower tissues was carried out using illumina platform that generated 64,604,402 flower and 51,350,714 stigma reads, and 64,438 de novo assembled sequences were categorized into 32,204 unigenes comprising of 9853 clusters and 22,351 singletons. The database provides a basis to identify the regulatory pathway of *C. sativus* flower development and biosynthesis of apocarotenoids (Baba et al. 2015c). Furthermore, differential gene expression (DGE) in saffron stigma against the rest of the flower indicated that biosynthesis of carotenoids and their subsequent degradation into apocarotenoids occur mainly in stigma. Eighty-one zinc-finger genes were detected in stigma divided into eight subfamilies (Malik and Asharaf 2017). Expression patterns indicated a probable role for *CsSAP09* in apocarotenoid metabolism regulation that found to be highly expressed in stigma at anthesis stage corroborating with the accumulation pattern of apocarotenoids. From 206 million high-quality paired-end studies, following the standardization of de novo transcriptome organization, as many as 105,269 distinctive transcripts were attained. Functional annotation helped the discovery of genes involved in flavor and color biogenesis in spice; 54% of *C. sativus* transcripts could effectively be interpreted with the aid of public databases (Jain et al. 2016). Comprehensive databases in the Yet Another Tool Suite for analyzing RNA-seq derived transcriptome (YeATS) suite from the NCBI and Ensembl databases were established to accelerate the characterization of the saffron metagenome from the transcriptome obtained by Jain et al. (2016). Soybean mosaic virus was detected to be abundantly expressed in all five tissues analyzed; several putative pathogen bacterial and fungal genera transcripts were identified according to the factors based on the homology comparison (Chakraborty 2016).

8.5 Genetic Modifications

Genetic modifications with the aid of biotechnological tools and techniques could be a source for bringing variations in saffron. In fact, genetically transformed saffron could be evolved as a source of new and desirable traits with high economic value and wider adaptability. Such an avenue of research could only be taken up when there are established in vitro protocols for direct and/or indirect regeneration of saffron. As reviewed by Gantait and Vahedi (2015), there are an ample number of in vitro protocols reported by several researchers, and these can pave the way forward for genetic engineering in saffron. The other aspect for genetic modification in saffron is the identification of desirable genes and their regulatory behavior that can fulfill the demand of the breeder or consumer (Mir et al. 2015b). Since recent past, genetic modification through *Agrobacterium*-mediated gene transfer technology attained significant progress in the regulated genetic enhancement of traits in demand for several other plants in Iridaceae family where this technology has emerged out to be the key approach in modern molecular breeding. Several research achievements have been reported on gene manipulations and modifications, for instance, genes responsible for abiotic stress tolerance and insect resistance, regulation of genes involved in the biosynthetic pathways of secondary metabolites, etc.

However, such reports on the genetic modification in saffron are scanty until now. Instead, there are multiple attempts that have been reported on genetic information related to synthesis of aroma compounds during the development of saffron stigma. Naturally, the young stigma has almost no odor, but at pre-anthesis, the aromatic compound β-ionone turns out to be the volatile norisoprenoid in the stigma. Rubio-Moraga et al. (2008) isolated four CCD genes (namely, *CsCCD1a*, *CsCCD1b*, *CsCCD4a*, and *CsCCD4b*) from saffron. Subsequently, they observed the expression pattern wherein *CsCCD1a* displayed an incessant expression and *CsCCD1b* was expressed exclusively in stigma tissue; however, during the stigma development, only *CsCCD4a* and *CsCCD4b* expressed harmoniously with the maximum levels of carotene and ionone release. Similarly, Ahrazem et al. (2010) isolated and analyzed the *CCD4* genomic DNA regions in saffron. They also recognized multiple alleles, such as *CsCCD4a* (that includes or excludes an intron) and *CsCCD4b* (that includes an atypical intron). In addition, they confirmed the occurrence of individual gain or loss based on the relationship of the locations of *CCD4* introns within the coding region with *CCD4* genes from other plant species. *CCD4a* promoter sequence was found appropriate to initiative GUS expression in the saffron flower specifically in pollen. This was a functional characterization of *CCD4a* promoter, was carried out via stable transformation of *Arabidopsis* plants with a 1400 bp DNA fragment (P-*CsCCD4a*) integrated to the β-glucuronidase (*GUS*) reporter gene. Following the isolation of *CCD4* genes (*CsCCD4a* and *CsCCD4b*) from the saffron stigma tissue and the establishment of their relation to the synthesis of some distinct volatile compounds to attract the pollinators, Rubio-Moraga et al. (2014a, b) confirmed other *CCD4* individuals that are linked with carotenoid-derived volatile synthesis during stigma growth. They observed the expression of *CsCCD4c* confined within the saffron stigma tissue, and it was found to be associated with the synthesis of megastigma-4,6,8-triene.

Additionally, upregulation of *CsCCD4c* was induced following any external injury or environmental stress that eventually suggests that the apocarotenoid product of this gene is involved during adapting with abiotic stress. Lately, Baba et al. (2015b) studied the substrate specificity of three isoforms of *CsCCD4* based on their molecular modeling and docking analysis. High substrate specificity for β-carotene was exhibited by all the three isoforms. Furthermore, they have exposed the three *CsCCD4* isoforms to variable stresses and analyzed their expression pattern, which confirmed that *CsCCD4a* and *CsCCD4b* showed amplified expression toward water stress, salinity stress, and methylviologen. Such finding supports the earlier observation of Rubio-Moraga et al. (2014a, b) on the function of *CsCCD4* isoforms facilitating the defense response of plants against environmental stress. An overexpression of *CsCCD4b* in genetically transformed *Arabidopsis* confirmed this attribute of *CsCCD4* isoforms. The transgenic *Arabidopsis* displayed comparatively long roots and more lateral roots in comparison to wild type/non-transformed plants. Additionally, the genetically transformed *Arabidopsis* exhibited increased performance of reactive oxygen species metabolizing enzymes signifying that *CsCCD4b* generated β-ionone and β-cyclocitral which could function as stress signals and intervene in the rearrangement of stress-responsive genes that eventually results in plant defense.

8.6 Conclusions and Future Prospects

There is a huge prospect, opportunities, as well as bottlenecks in the concept, implementation, and biotechnological improvement of saffron. The ever-increasing scientific progress and information updates offer remarkable innovative prospective to explain genetic relationships, genomic evolution, and biotechnological improvement of saffron and to use these scientific techniques and database for the persistent advancement of this important medicinal and aromatic plant. Nevertheless, at the same time, if we are unable to utilize these information and technologies at an optimal level, it could be obvious that we ascertain the safeguarding of crucial germplasms; increase storage, manipulation, and access to enormously accumulating genomic data; and establish upgraded functional genomic technologies for phenotyping and genetic management of saffron.

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Recent Advances in Extraction, Characterization, and Potential Use of Citral

9

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Contents

Abstract

Citral is the main product of the lemongrass. Citral is found in various oils extracted from different plant species including *Lemon myrtle*, *Listea citrata*, etc. Citral, the most significant natural occurring metabolite, having strong

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lemon smell and essence, is commonly applied as additives in diets, drinks, and maquillages with high purchaser acceptance. Citral is found in two forms, E-isomer and Z-isomer. E-isomer is generally identified as geraniol or citral A, while Z-isomers as neral or citral B. Citral is an aroma compound and generally used in perfumery. Citral is naturally unstable and vitiates in both water and watery solutions due to catalyzation and oxidation of acidic part of citral which decreases the natural flavor of citral and makes off-flavors. Citral has strong sensitizing effect that is why it can be used only with anti-sensitizing agent. Some reports indicated the allergic effect of citral on people. Extensive work has been done to explore the various aspects related to citral such as extraction from different species, synthesis of synthetic drugs, production, characterization, etc. There are several reports related to antimicrobial, antibacterial, antifungal properties of citral content. The present chapter summarizes the work on citral content in various aspects carried out in the last decades in relation to their traditional and modern uses as culinary, medicinal, and cosmetic and also deals with quality issues and toxicity.

Keywords
Citral · Lemongrass · Geraniol · Secondary metabolites

9.1 Introduction

Various plant parts including secondary metabolites of medicinal plants are used as vernacular medicine in traditional treatment of diseases and ailments. Increment in the number of cases in opportunistic diseases related to side effect of synthetic drugs makes a pressure to increase the efforts to search for biological optional drugs with little side effect. Therefore, struggles are focused to elucidate plausible sources such as plants containing therapeutic agents (Patil et al. 2010). Newly and advanced techniques of extraction helped in better investigation of natural occurring compound of plant origin with more exactitude leading to separation of biomolecule from crude combinations of secondary metabolites (Wang and Weller 2006). Several ethnoplants have medicinal properties, and lemongrass is one of these plants. Lemongrass is recurrent grass broadly dispersed globally and most especially in countries with humid atmosphere (Francisco et al. 2011). The commercially significant grasses of lemongrass family are C4 tropical plants. Leaves of lemongrass contain major portion of all secondary metabolites of the plant that includes up to 1.5% (d.wt) aromatics and essential oils with high smell of lemon, yellow or amber in color (Adejuwon and Esther 2007). Most of the essential oils obtained from aromatic and medicinal plant species are useful in production of perfumes, soaps, toiletries, syrups, and sauces. Some of these oils are used in traditional and folk medicine for various medical purposes. Inquiries about the assessment of the biological actions of essential and aromatic oils of medicinal plants have discovered the

therapeutic properties like antifungal, insecticidal, antiviral, and antibacterial. Essential oil mainly used in aromatherapy to treat serious skin diseases like superficial mycoses showed significant antimicrobial effect on skin pathogens (Tajidin 2012). Citral is the major component of lemongrass oil (LO) (Tajidin 2012), a natural combined form of geranial (a-citral) and neral (b-citral) (Pengelly 2004). Both geranial and neral are aldehydes and isomers to each other. The high citral content of lemongrass oil has made it significant for numerous therapeutic combinations. Citral, also known as 3,7-dimethyl-2,6-octadienal, is a monoterpene chemical that can be obtained from citrus fruits, herbs, plants, and grasses (Negrelle and Gomes 2007). Citral has been used as a natural preservative to foodstuff, maquillages, and drinks due to the presence of strong flavor and lemon aroma (Maswal and Dar 2013). The citral molecule can be move freely across the membranes due to its small size and hydrophobic nature. Citral has strong electrophile property due to the activity of α, β-unsaturated carbonyl and can be readily digested by mammal cells (Esterbauer et al. 1975; Diliberto et al. 1990).

Citral has antifungal, bactericidal, deodorizer, insecticidal, linctus, weak diuretics, stimulating, spasmolytic, and craving properties. Citral also has the mild effect on inflammation. Citral exhibited bacteriostatic effect and was found significant against *Staphylococcus aureus* with minimum inhibitory concentration (MIC) values at the range of 75–150 μg/ ml. Citral has no effect on cell wall but can degrade cell membrane by affecting its potential, obstructing efflux pump. Citral can reduce the load of staphylococcal infections in liver tissues and spleen according to dose-dependent manner which further decrease when combined dose of citral and norfloxacin is used. Citral has no effect on mortality or morbidity at the dose of 500 mg/kg body weight and can elongate effect of norfloxacin in the case of the post-antibiotic effect. Green and Berenbaum (1994) advised that being a volatile molecule, it can be worked as natural repellent and protect the plant from insects and other predators by repelling. They found citral as toxic agent for cabbage loopers (*Trichoplusia ni*), and they also observed that ultraviolet light can increase citral toxicity. Devi et al. (2011) reported calcium antagonist role of citral. The existing chapter recapitulates the work on citral content in various aspects carried out in the last decades in relation to their traditional and modern uses as culinary, medicinal, and cosmetic and also deals with quality issues and toxicity.

9.2 Source of Citral

Citral, chemically known as 3,7-dimethyl-2,6-octadienal, is an aromatic bio-active molecule present in the extracted essential oils obtained from lemongrass, citrus fruits, verbena (*Verbena officinalis*), and ginger. Citral is made up of monoterpenes (isomeric mixture of geranial and neral) and myrcene being found (Dudai et al. 2005). Zeng et al. (2015) proved that extract of ginger, obtained from steam distillation, has 30–40% citral.

9.3 Extraction and Characterization of Citral

Supercritical extraction of essential oil with the help of CO_2 is most reliable technique in which dried powder of leaves is used. As compound with high molecular mass is started to extract at higher solvent density, yellowish semisolid mass extracted in place of yellow essential oil. At 90 bar and 50 °C, the optimum extraction citral yield is obtained from CO_2 supercritical extraction. At above conditions, citral yield was 68% of the essential oil. The citral content was higher in hydrodistilled essential oil than that of supercritical extracted oil.

Schaneberg and Khan (2002) developed method to quantify the bio-active markers like neral, geraniol, geranial, citronellal, limonene, and β-myrcene, based on gas chromatography with flame ionization. They compared processes for the extraction of oils from *C. citratus*. These procedures were steam distillation, supercritical fluid extraction, and accelerated and simple solvent extraction.

Mei et al. (2010) determined the stability of citral in emulsions (oil-water) with octadecane in both liquid and solid phase at pH 3.0. The results of Schaneberg and Khan (2002) experiment showed faster degradation of citral in anionic sodium dodecyl sulfate stabilized in comparison with nonionic polyoxyethylene lauryl ether-stabilized emulsions.

Rapid degradation of citral was noted when octadecane crystallized in emulsions like nonionic polyoxyethylene lauryl ether and anionic sodium dodecyl sulfate. The solid and liquid phase of octadecane also affects the citral partitioning. In liquid phase of octadecane, 18–25% partitioning of the total citral was noted while 41–53% in solid phase. They suggested that increment in the rate of citral degradation is due to an increment in citral partitioning outside of emulsion (oil-water) droplets. These results emphasized the use of technology able to decrease citral partitioning and contact to acidic phases to enhance stability of citral in emulsions. Tian et al. (2018) prepared nanoparticles using solid lipid loaded with citral (citral SLNs) by a method of homogenization (high-pressure); the lipid known as glyceryl monostearate (GMS) is used and a mixture of 1:1 (weight ratio) of Span 80 and Tween 80 as the surfactant. The GC data indicated that citral stability increase and 67% of the total citral stayed in the suspensions of citral-SLN while only 12% in control. They concluded that covering of citral with solid lipid can increase citral stability in acidic phases.

Citral, a key molecule of lemongrass essential oil, can be isolated by using steam distillation (Rao et al. 2015). The analysis of results and conditions explained that the time of distillation and volume were 98.21 min and 0.0 53 µl, respectively. The citral yield was 85.1416% at optimum conditions. The 83.8% yield of citral was noted in revised and confirmation experiment. The data of refractive index, flash point, density, and specific gravity of isolated product were 1.488, 91 °C, 0.89031 g/cm^3, and 0.8904 which were similar to data of above properties of standard citral.

9.4 Biological Properties of Citral

9.4.1 Anti-inflammatory Properties

There are several severe health issues in the world; inflammation is one of these issues.

The main causes of inflammation of tissue include physical stress and chemical inducers like lipopolysaccharides. The discharge of proinflammatory facilitators like prostaglandin E2 (PGE2) and nitric oxide (NO) by incubated lipopolysaccharides with macrophages can cause inflammation. The fluctuation in nuclear factor kappa-B cells (NF-κB), tumor necrosis factor-TNF-α, interleukins, reactive oxygen species (ROS), and cytokines are other factors that can induce inflammation. Several investigators reported that isolated citral has the strong property of anti-inflammation, while solvent extracts of lemongrass and polyphenol-rich extractants showed low to mild anti-inflammatory activities. The secondary metabolites of lemongrass including citral have anti-inflammatory effect on paw edema and peritonitis induced by carrageenan in model rat. Paw edema was reduced by using citral, and peritonitis was reduced due to mitigation of leukocyte conversion to peritoneal cavity. Generally, citral is dose reliant in decreasing protein expression, both alpha and gamma peroxisome proliferator-activated receptor, COX-2 mRNA in human macrophage (U937) induced by LPS (Katsukawa et al. 2010). Alpha and gamma peroxisome proliferator-activated receptor is cluster of nuclear receptor proteins that have important role to control the metabolism, differentiation, and cell development by acting as transcription factor (Kulinsky 2007). Citral also reduced the production IL-10, IL-6, and IL1-β resultant in the inhibition of cytokine in both LPS introduced peritoneal macrophage and animal as well as in control (Sforcin et al. 2009; Bachiega and Sforcin 2011). Treatment with citral oil in mice with lung injury induced by LPS inhibited IL-1β, TNF-α, and IL-6 levels both in vivo and in vitro, demonstrating that the citral can inhibit a possible inflammatory response (Shen et al. 2015).

It was also demonstrated that the alcoholic extract of lemongrass, which has, as major compound citral, reduced the generation of TNF-α in bronchoalveolar macrophages stimulated with LPS, enhancing the anti-inflammatory property of citral and indicating that modulation of the COX-2 and TNF-α genes can be one of the processes involved in such activity (Tiwari et al. 2010). Citral inhibited the phosphorylation interaction with inhibitory proteins kB (IkB), blocking translocation of the p50 and p65 subunits of NF-kB and leading to a low expression of inducing enzyme nitric oxide synthetase (iNOS) (Lee et al. 2008).

9.4.2 Antioxidant Properties

Free radicals, superoxide anion, and hydrogen peroxide are the major reactive oxygen species (ROSs) formed by the reaction of oxidation in tissue, cell, and organ systems of human (Heo et al. 2003). ROSs are very reactive and can damage various

cell components and biomolecules such as DNA, structure and nature of proteins, cellular lipids, and cell membranes (Devasagayam et al. 2004). Furthermore, ROSs can induce health problems like muscle destruction, rheumatoid arthritis, and atherosclerosis. The body has antioxidants which are able to fight ROSs and can provide protection against oxidation effect of free radicals (Finkel 1998; Thannickal and Fanburg 2000). DPPH scavenging test showed the antioxidant potential of lemongrass oil. The data available in literature shows that extracts of both leaves and stalk have antioxidant potential which were dose reliant (Mirghani et al. 2012). Bouzenna et al. (2017) examined the antioxidant effect of citral and its possible protecting effects against toxicity induced by aspirin in in vitro condition. Ferric reducing antioxidant power (FRAP), β carotene/linoleic, and 1,1diphenyl-2-picrylhydrazyl (DPPH) are used generally to find out the antioxidant potential of various molecules including citral. Citral showed FRAP with effective concentration (EC 50) 125 ± 28.86 μg/ml and inhibits the oxidation of linoleic acid as well as moderates the DPPH. The combined dose of aspirin and citral reduced cell death induced by aspirin. Citral controlled the activities of superoxide dismutase (SOD) and glutathione. Citral also prevents the activation of MAPKs. The data obtained from above study suggested that citral can provide the protection to IEC-6 cells from oxidative stress, induced by aspirin. Oxidative stress is helpful to search new molecules from available natural substances with antioxidant potential. The antioxidant potential of pure citral is similar to the antioxidant potential of ascorbic acid. A high treatment dose of citral (50 mg/kg body weight) did not show any mutagenic effect on mice. High dose citral showed no harmful effect on direct ingestion (Rabbani et al. 2005). Citral has antioxidant potential, and it can serve as antioxidant defense to protect the plant from ROSs or free radicals.

9.4.3 Antibacterial Properties

Gupta et al. (2017) studied the combined action of norfloxacin and citral against *Staphylococcus aureus* (SA) and drug-resistant strains. Espina et al. (2017) studied the sterilizer power of carvacrol (500–2000 μl/l) or citral against mature biofilms of *L. monocytogenes* (EGD-e), *S. aureus* (SC-01), and *E. coli* (MG1655). Carvacrol at the rate of 1000 ppm reduced sessile cells (reduced 5 log cycles) making mature biofilms of all three studied species. The results of above study showed the potential of the citral capable to eradicate the biofilm of foodborne pathogens. In recent times, various plant materials were tested for antibacterial activity, and results are very promising which make positive approach to discover new biomolecules with antibacterial potential. The antibacterial potential of different extracts of lemongrass including essential oil has also been examined by various workers (Grace et al. 1984). α-citral and β-citral also known as geranial and neral, respectively, are among the main aromatic compound of lemongrass oil. Both α- and β-citral showed antibacterial activity against both gram-positive and gram-negative bacteria. Another component of lemongrass oil, myrcene, has no direct effect on bacteria individually but improves the activity when used in combination with other components (Grace

et al. 1984). Use of the essential oil citral in the local therapy of infectious diseases triggered by *S. aureus* showed positive action. However, the existence of a harmful action or interference in the physiology and/or structures of bacterial cells of this natural product and its bioavailability when used in living beings are not well understood. The joint influence of the air pouch model, essential oil citral, and *S. aureus*, since the citral appears to be a potential therapeutic agent for treating local infections triggered by *S. aureus*. In conclusion, the treatment with essential oil citral in the infection triggered by *S. aureus* led to a reduction of some features of acute inflammation, including the number of monocytes. The TNF-α cytokine has proved to be a more sensitive biomarker, in ELISA and RT-qPCR array. By reducing TNF-α concentration, EOC promoted the reduction of transcription of genes related to proinflammatory cytokines. The action of the EOC seems to have a better response in a period of 4 h; thus, this suggests that the EOC can act as a modulator of the immune system by decreasing cellular migration and the production of proinflammatory cytokines following infection with *S. aureus*.

9.4.4 Anti-obesity and Antihypertensive Properties

Aqueous extract of citratus at a dose of 500 mg/kg/day reduced hypoglycemic index significantly in the presence of counter-regulators like glucan, cortisol, and catecholamine. It was noted that hypolipidemic effect reduced in blood stream with low lipid density level. The extracts of lemongrass including essential oil relaxed various tissues like rat mesentery, rat aortic rings, and rabbit ileum (Bastos et al. 2010; Devi et al. 2011, 2012). For example, citral produced a dose-reliant vasorelaxation in phenylephrine aortic rings (pre-constricted) of male SHRs or WKRs (Devi et al. 2012). Similarly, intravenous administration of citronellol (acyclic monoterpenoid) created a hypotensive response in Wistar rats. Factors like indomethacin, hexamethonium, and atropine have no effect on such type of hypotensive response (Bastos et al. 2010). Citronellol used endothelium-independent process to prompt relaxation to superior mesenteric artery of rat. The potassium channels dependent on tetraethylammonium has no relation with arteries without endothelium. Calcium channels operated by voltage inhibited Ca^{2+} influx to activate citronellol and regulate intracellular Ca^{2+} stores (caffeine gated) and IP3 (Bastos et al. 2010).

Citral was found to be a moderate inhibitor of mammalian alpha-amylase, with an IC50 of 120 μM and caused also a decrease of alpha-amylase levels in vivo (Najafian et al. 2011). Moderate lowering of postprandial glucose, alongside with normalization of blood lipid profile, was observed in diabetic rats upon treatment with the compound. Water intake and urine volume of diabetic rats are also showing a remarkable decrease with the use of 16 mg/kg of citral, which is in accordance with its effect on blood glucose, and interesting in terms of the therapeutic benefits that it could have on these discomforting consequences of diabetes in patients. Citral was also found to be able to promote weight loss and to decrease food intake. On the basis of above findings, Najafian et al. (2011) proposed citral as a possible antihyperlipidemic agent in diabetes and potential therapeutic in obesity.

9.4.5 Antinociceptive Properties

Lemongrass oil containing citral is used in experiments on three nociception models of mice to find out the antinociceptive properties. In hot plate test, intraperitoneal supply of essential oil increased the response to stimuli in mice, while induction by acetic acid exhibited that oral and intraperitoneal supply of essential oil inhibits the contraction in the abdomen in a dose-reliant manner. In another test with formalin, supply of essential oil through IP inhibited licking time in both (first and second) phases of experiment (Viana et al. 2000). They observed the role of opioid receptors in the action of antinociceptive as antagonist naloxone obstructed function of essential oil found in the extract. The investigators of the same group pronounced that differences in reports published previously might be due to chemotypes used in experiments.

Quintans-Junior et al. (2011) reported antinociceptive potential of citral isolated from lemongrass. They used acetic acid writhing and formalin-induced nociception to study the antinociceptive properties of citral. Conclusively, citral is able to exhibit antinociceptive property by inhibiting nociception and writhing.

9.4.6 Anti-fungi Properties

Citral showed antifungal activity by damaging cell wall and membrane of spore of *Aspergillus flavus*. Inside the cell, citral interacts with DNA and their mitochondrial processes and also aggregates protein-like molecule that leads further damage of the cell. All these events inside the cell lead disorder in metabolic reaction which diminished the germination ability of the spore (Luo et al. 2004). The three fungi known as *F. subglutinans*, *C. gloeosporiodes*, and *C. musae*, responsible for postharvest diseases of fruits, are affected by citral as it can alter the morphology of fungal hyphae (Garcia et al. 2008). The antifungal activity of citral is also reported for *Penicillium digitatum*, a postharvest pathogen of lemon fruit (Ben-Yehoshua et al. 1995).

Agar dilution method is used to determine the minimum lethal concentration (MLC) and minimum inhibitory concentration (MIC) of citral oil against different isolates of four dermatophytes (*M. gypseum*, *E. floccosum*, *T. rubrum*, and *T. mentagrophytes*). The data of MLC and MIC indicated that citral has mild effect on all isolates of dermatophytes than that of essential oil. *M. gypseum* was the most resistant which is followed by *T. rubrum*. The results of above study proved the antifungal activity of citral and lemongrass oil, and both can be used as fungicides. The hole diffusion assay was followed in vitro condition to study the effectiveness of cream with four different doses of oil of lemongrass. The cream containing 2.5% oil of lemongrass showed minimum concentration to control the fungal infection hence used to make antifungal cream for further clinical study (Wannissorn et al. 1996).

Desai and Parikh (2012) used a hydrotropic combined solution of sodium cumene sulfonate and sodium salicylate to extract the citral content from leaves of lemongrass (*C. flexuosus*). Plant material, temperature, solid loading, and hydrotrope concentration can affect directly the yield of citral. Taguchi method gave highest

extraction in which both hydrotropes registered highest citral yield with conditions as 5% solid loading, temperature of 30 °C, and size of 0.25 mm of pieces of leaves. Lower performance of sodium cumene was noted for extraction than that of sodium salicylate. Extraction mechanism can be understood by microscopic analysis of leaves that provide insight of leaves. The efficiency of hydrotropes for extraction was checked from the kinetic study. The organic solvent can be avoided in the extraction of citral with help of hydrotropes under hydrotropic extraction. Hydrotropic extraction technique can be used to extract different biomolecules and oils from plants as this technique is very simple and environment friendly.

In another observation by OuYang et al. (2018), they noted that citral prevents the growth of *P. digitatum* by accumulation of ROS as a result of damage in cell membrane and oxidative phosphorylation.

9.4.7 Anticancer Properties

The anticancer property of citral was exposed when a report published to claim that caspase 3 activity induced by citral in the HL60 and U937 cell lines in 2005. The potential of citral to treat the cancer has not been completely explained, but citral is among the natural compound of plant origin that gave some promising results against several human cancer cells like HL60, ovarian cancer cells, U937, etc. (Liu et al. 2012). Another positive observation regarding citral is that it showed very little or negligible cytotoxic effect on normal epithelial cells but showed sufficient toxicity against breast cancer cell line and indicated cancer-specific effect of citral (Patel et al. 2015). In vitro condition, citral can induce the cell death in the cells of leukemia and breast cancer (Dudai et al. 2005; Xia et al. 2013). Maruoka et al. (2018) observed that citral alone or combined dose with chemotherapeutic agents can suppress proliferation of lung cancer cell by inhibiting Src/Stat3 activities. Naz et al. (2018) studied the potential of citral and mode of its action to inhibit the activity of microtubule affinity-regulating kinase 4 (MARK4).

Citral can bind the active site effectively and stabilize the complex with several interactions. The above observation is made by docking studies. They noted the strong stability in binding of citral with MARK4. The similar findings were obtained from fluorescence binding studies which also indicate that citral inhibits enzyme activity of MARK4 that measured through kinase inhibition assay. Citral-treated cells of MCF-7 showed inhibition in growth as these cells are arrest in cell cycle phase (G2/M phase) and citral-induced apoptosis. Citral treatment decreased synthesis of prostaglandin E2 within 48 h. The above study established the fact that citral can be used to treat the cancer by MARK4 inhibition (Chaouki et al. 2009). Dubey et al. (1997) noticed that citral has anticancer potential. Citral showed the cytotoxic effect on mouse leukemia cells (P388) at IC50 value (7.1 μg/mL). Micronucleus antimutagenic assay was used to study antimutagenic effect on mutagens like nickel metal ($NiCl_2$), mitomycin C, and cyclophosphamide. High dose of citral was used to check the mutagenic potential, and the result showed that no significant change in micronucleus frequencies of erythrocytes that proved citral is

nonmutagenic. Moreover, this study proposes that citral reduces nuclear injury prompted by the clastogens by utilizing antioxidant potential (Rabbani et al. 2005). White et al. (2017) examined effect of citral on immortalized rhabdomyosarcoma (RMS) cells and found significant death rate in cancer cells at and above the dose of 150 μM citral, and significant changes were noted in morphology of mitochondria of the cell incubated with 10 μM citral.

9.5 Conclusions and Future Prospects

Citral is one of secondary metabolites of lemongrass and has lemon-like aroma. Due to its aromatic nature, it involved to provide the fragrance to several formulations and take part in the formation of consumer products as flavor gradient. The citral molecule is unstable and lost its properties like flavor over time in watery solutions because of the oxidative reactions. The use of citral in food industry is a big challenge due to its unstable nature. Another challenge is to develop the delivery system of citral content for food industry. Colloidal systems are generally used to encapsulate and in delivery technique of citrate. There is need to focus the technical problems like stabilization of citral, use of cofactors, instability of citral under various environmental stresses, and the preparation of citral-based nanoparticle, etc. All above technical problems related with stability of citral and development of new techniques to use citral in the protection of various pathogenic diseases including cancer should be addressed as future prospects to develop particular formulation to treat particular disease without any side effect.

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Hairy Root Cultures as an Alternative Source for the Production of High-Value Secondary Metabolites

10

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Abstract

Hairy roots are rapidly growing, highly differentiated transformed root cultures induced by *Agrobacterium rhizogenes* infection usually at the infected site of the representative medicinal plant. Hairy roots have the ability to rapidly multiply in the culture medium devoid of any hormones. Unlike other plant cell cultures, hairy root cultures are genetically and biochemically stable and produce a variety of secondary metabolites. In the past three decades, researchers across the world have successfully initiated and cultured hairy roots in vitro for a large number of medicinal plants. Hairy root technology is becoming a promising source for the production of pharmaceutically and industrially important secondary metabolites. This is due to the characteristics of hairy roots, such as rapid growth, the lack of geotropism, extensive lateral branching, and, more importantly, genetic stability. This chapter explores the applications of secondary metabolites in drug formulation, cosmetic preparation, food processing, and the study of plant metabolic pathways. It also briefs about the recent advancements in the area of hairy root culture involving other biotechnological approaches like metabolic engineering or genetic engineering, elicitation, metabolic trapping, and phytoremediation. This chapter certainly benefits the researchers to further explore on the applications of hairy root culturing technology to produce desired plant secondary metabolites on a large scale.

Keywords

Agrobacterium rhizogenes · Genetic engineering · Hairy roots · Medicinal plants · Secondary metabolites

10.1 Introduction

Medicinal plants produce a variety of biologically active compounds, i.e., secondary metabolites which play a vital role in plant self-defense mechanisms. Especially, roots play major roles in plants, including anchoring plants to the soil, uptake of minerals and water from the soil, storage of nutrients in perennial plants, and defending themselves from other plants or microbes present in the soil by producing a wide variety of chemical compounds, popularly known as secondary metabolites. These secreted metabolites not only provide protection to plants from biotic and abiotic stresses like pathogens, insects, and other environmental stresses but also useful in improving human's and other animal's health (Tian 2015). These compounds are produced in trace amounts during the secondary metabolism, but not essentially necessary for plant growth and development. Plant-based compounds, including alkaloids, flavonoids, saponins, terpenes, anthraquinones, and anthocyanins, are the essential source for the preparation of drugs, food additives, dyes, oils, resins, and agricultural chemicals (Kim et al. 2002; Zhou et al. 2011; Bharati and Bansal 2014). Obtaining the chemical compounds directly from the wild- or field-grown plants is not promising as the yield obtainable is being very low and has limited availability in their habitat. Moreover, it may lead to the

destruction of the natural habitat due to over exploitation of these plants. The artificial synthesis of chemical compounds also has several disadvantages including high cost of production, the difficulties in the synthesis, unavailability of the optimized methods for the compound synthesis, and characterization. These problems can be overcome by the using the biotechnological approaches such as plant tissue culture, transgenic medicinal plants, etc. to enhance the synthesis of valuable phytochemicals from medicinal plants (Zhou et al. 2011). In this regard, the hairy root technology is widely preferred by biotechnologists for the large-scale production of diverse secondary metabolites from various medicinal plant resources (Veena and Taylor 2007).

Hairy roots are the by-products from the *Agrobacterium rhizogenes* (gram negative, soil bacterium)-infected sites, commonly known as hairy root disease or syndrome. This soil bacterium transfers its T-DNA segment from Ri (root-inducing) plasmid into the host plant genome. The T-DNA region contains a set of genes encoding for the specific enzymes, which control the biosynthesis of natural auxins and cytokinins. The new changes, i.e., insertion of new genes, cause hormonal imbalance in the host plant and induce the formation of proliferating roots (hairy roots) from the wounded sites infected with *A. rhizogenes* (Guillon 2006). Hairy roots are characterized by the abnormal multiplication on the phytohormone free medium by retaining genetic stability. Hairy roots have several unique properties including fast growth rate, able to accumulate vast variety of chemical compounds, no requirement of exogenous hormone in the medium, and genetic and biochemical stability (Giri and Narasu 2000). The schematic representation of hairy root induction and its application is shown in Fig. 10.1. Nowadays, many research groups are paying attention toward in vitro culturing of hairy roots for producing wide varieties of root-oriented plant secondary metabolites. Recent advancements have provided a better understanding about the molecular mechanisms involved in the T-DNA transfer and their integration into the host plant genome. This has paved a new way for producing plant secondary metabolites through employing metabolic engineering strategies. Also, hairy roots have shown the capability of absorbing some of the threatening recalcitrant pollutants and thus can be used to clean the environment (phytoremediation). In this chapter, detailed information about hairy roots and their applications in the production of valuable plant secondary metabolites are discussed. Further, more recent advances in the field of hairy root culture technology are highlighted.

10.2 Production of Secondary Metabolites Through Hairy Root Cultures

From several decades to now, worldwide population is still depending on plants and plant-derived products for their daily needs. Even today, around 80% of the human population depends on plants as a traditional medicine to cure several diseases (Ekor 2014; Swamy et al. 2016). Terrestrial plants are the greatest source for several chemical compounds with wide-ranging pharmaceutical applications. As these compounds occur in trace amounts in plants, they generally do not meet the huge demand in the pharmaceutical industry. Hence, this has raised a curiosity among researchers to make use of biotechnological approaches to commercially produce

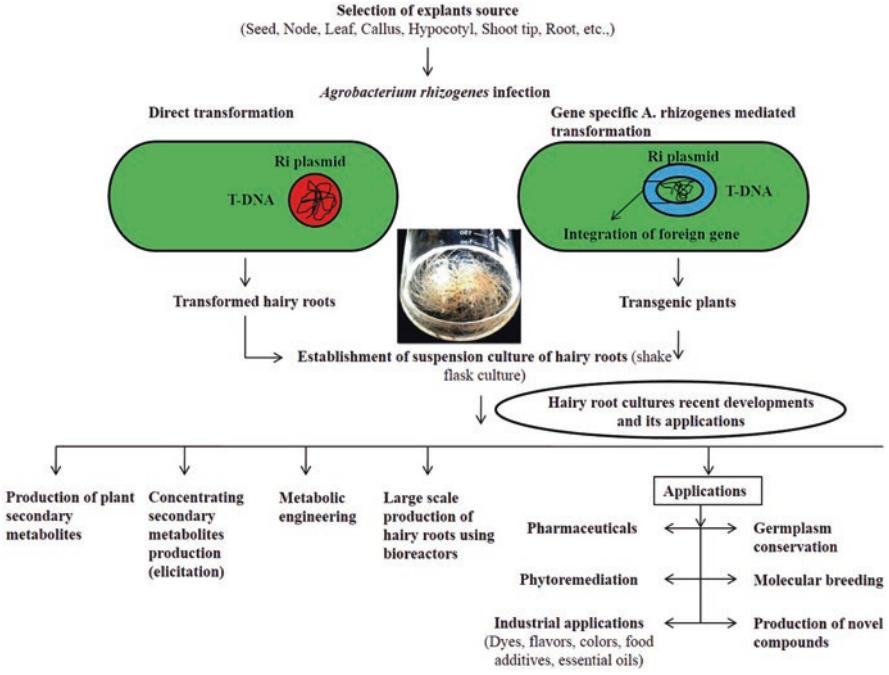

Fig. 10.1 The schematic representation of hairy root induction and its application

these valuable compounds using plant sources (Verpoorte et al. 1999). In search of this is the hairy root culture technology, an alternative approach which offers the production of secondary metabolites in a large scale. Moreover, hairy roots have the unique characteristics of fast growth, and also levels of secondary metabolites produced are equal to or superior than the parent plants (Roychowdhury et al. 2013). The genetic and biosynthetic stability of hairy roots is another advantage for the production of valuable secondary metabolites. In addition to that, transformed hairy roots can be proficient to regenerate into entire viable plants and also preserve their genetic stability throughout and further successive subculturing and plant regeneration (Giri and Narasu 2000). There are several important secondary metabolites produced through hairy root cultures in many medicinal plant species which are endangered and pharmaceutically important. The list of few important secondary metabolites produced through hairy root cultures from various medicinal plants has been described in Table 10.1. In the recent era, hairy root cultures are not only used for secondary metabolite production but also widely used as model systems for studying plant physiology and metabolism, regulation of metabolic pathways, and identification of key genes for production and regulation of particular metabolite (Shanks and Morgan 1999; Sharma et al. 2013; Tian 2015). For example, the roots of *Panax ginseng* plants were rich in ginsenosides, saponin which possesses immunomodulatory, adaptogenic, and antiaging properties. The hairy roots of *P. ginseng* produce twofold increased concentration of ginsenosides than the wild-type roots

Table 10.1 Establishment of hairy root cultures for plant secondary metabolite production

Plant species	Secondary metabolite	Biological properties	References
Artemisia annua	Artemisinin	Antimalarial	Weathers et al. (2005)
Beta vulgaris	Betalains	Antioxidant, colorant	Pavlov and Bley (2006)
Bixa orellana	Stigmasterol	Antimalarial	Zhai et al. (2014)
Chlorophytum borivilianum	Stigmasterol and hecogenin	Antioxidant	Bathoju et al. (2017)
Clitoria ternatea	Taraxerol	Anticancer	Swain et al. (2012)
Datura innoxia	Scopolamine and hyoscyamine	Anticholinergic	Dechaux and Boitel-Conti (2005)
Echinacea sps.	Alkamides	Anti-inflammatory, immune-stimulatory	Romero et al. (2009)
Eschscholzia californica	Benzylisoquinoline	Antimicrobial, anticancer	Vázquez-Flota et al. (2017)
Fragaria x ananassa cv. Reikou	Polyphenols (proanthocyanidins, flavonoids, hydrolyzable tannin)	Antioxidant, anticancer	Motomori et al. (1995)
Gingko biloba	Ginkgolide	Against cardiovascular and aging diseases	Ayadi and Tremouillaux-Guiller (2003)
Hyoscyamus niger	Tropane alkaloids	Anticholinergic	Jaziri et al. (1988)
Isatis tinctoria	Flavonoids	Antioxidant	Gai et al. (2015)
Linum flavum	Aryltetralin lignans Lignans coniferin	Anticancer	Renouard et al. (2018) and Lin et al. (2003)
Linum usitatissimum	Lignan	Anticancer	Gabr et al. (2016)
Nasturtium officinale	Glucosinolates (gluconasturtiin, glucotropaeolin)	Anticancer, antifungal, antibacterial, antinematode, anti-insect	Wielanek et al. (2009)
Ophiorrhiza pumila	Camptothecin	Antitumor	Saito et al. (2001)
Papaver somniferum	Morphine Sanguinarine Codeine	Sedative, analgesic	Le Flem-Bonhomme et al. (2004)
Polygonum multiflorum Thunb	Anthraquinones	Antifungal, anti-inflammatory, antimicrobial	Thiruvengadam et al. (2014)
Rauvolfia micrantha	Ajmalicine Ajmaline	Antihypertensive	Sudha et al. (2003)

(continued)

Table 10.1 (continued)

Plant species	Secondary metabolite	Biological properties	References
Rauwolfia serpentina	Terpenoid indole alkaloids (reserpine, ajmalicine, ajmaline, serpentine, yohimbine)	Hypertension, high blood pressure, mental illness	Mehrotra et al. (2015)
Solanum chrysotrichum	Saponin	Antifungal	Caspeta et al. (2005)
Stevia rebaudiana	Stevioside glycosides	Antioxidant, anti-inflammatory, antihypertensive	Kumari and Chandra (2017)
Taxus brevifolia	Taxol	Anticancer	Huang et al. (1997)
Valeriana wallichii	Iridoids (valepotriates)	Sedative, spasmolytic	Banerjee et al. (1998)
Withania somnifera	Steroidal lactones (withanolide A)	Anticancer	Murthy et al. (2008)

(Yoshikawa and Furuya 1987). In addition to that, *P. quinquefolium* is another important *Panax* species, and its hairy roots produced 0.2 g g^{-1} dry weight of ginsenoside content within 10 weeks of hairy root culture (Mathur et al. 2010). The hybrid plant was made between *P. ginseng* and *P. quinquefolium* which was more dynamic in ginsenoside production than the parental plant. The hairy roots (8-week-old) derived from the hybrid plant containing equivalent amounts of ginsenosides present in the field-grown parental plant roots revealed the biosynthetic potential of hairy roots maintained in the parent plants (Washida et al. 1998; Tian 2015).

10.3 Role of Bioreactors in Large-Scale Production of Secondary Metabolites

Scaling-up process of commercially important secondary metabolites through bioreactor at the industrial level is the next step after establishing in vitro hairy root cultures (Giri and Narasu 2000; Bourgaud et al. 2001). Bioreactors work as a chemical factory and offer a big hope for the large-scale production of high-quality biologically active compounds from medicinal and aromatic plants cells/tissues. This process is also known as molecular farming (Shanks and Morgan 1999). Large-scale production of secondary metabolites using bioreactor is not an easy process, because designing of the bioreactor and optimization of culture conditions are very difficult. The successful cultivation of hairy roots in bioreactor depends on several requirements, including growth characteristics, morphology, nutrient uptake and availability, oxygen supply, composition of the medium, inoculum concentration, and distribution which can facilitate the growth of inoculum (Giri and Narasu 2000; Roychowdhury et al. 2013; Ho et al. 2017). Also, the productivity in bioreactors depends on several physical and chemical parameters like light, temperature, pH, water, substrate availability, impeller designs, composition of gases, choice of hairy root clone, removal of toxic by-products, reactor operation, etc. (Roychowdhury

et al. 2013; Sharma and Shahzad 2013). There are several types of bioreactor designs that have been reported for hairy root culturing. Generally, three major types of bioreactors are used for hairy root cultivation, namely, liquid-phase reactors, gas-phase reactors, and hybrid reactors (a combination of both liquid-phase and gas-phase reactors) (Srivastava and Srivastava 2007). Liquid-phase reactors are commonly known as submerged reactors, in which roots remain submerged in the culture medium and air is passed or bubbled on culture medium to supply oxygen. The best examples for liquid-phase reactors are air lift, stirred tank, bubble column, liquid-impelled loop, and submerged connective flow reactors. In gas-phase bioreactors, hairy roots were occasionally exposed to air, nutrient liquid, and other gaseous mixtures in the bioreactors. In these reactors, nutrients are provided as either in the form of either spraying liquid nutrients onto the roots or roots getting nutrients in the form of droplets, which significantly depends on the varying sizes. Trickle bed, liquid-dispersed, droplet phase, and nutrient mist reactors are some examples for the gas-phase reactors. In hybrid reactors, hairy roots were first exposed to liquid phase and then grown in a gas phase (Roychowdhury et al. 2013). Bioreactor culture systems are mainly used in the industrial application, and they have several advantages, such as requiring very small amount of the inoculum, controlled environmental conditions, increased working volumes, and standardized growth parameters, viz., pH, light, temperature, nutrient media composition, etc. for inducing metabolite production effectively. In addition, easy separation of the target compounds, reproducible yield of the end product, and simpler and quicker harvesting of the cells are some of the other advantages of using bioreactors (Sharma and Shahzad 2013). Some examples for the production of secondary metabolites through the use of bioreactors are mentioned in Table 10.2. For example, artemisinin and its derivatives are high efficient drugs used for the treatment of *Plasmodium falciparum* (both chloroquine-sensitive and chloroquine-resistant strains) which is the causative agent of cerebral malaria. Traditionally, it is obtained from the plant source *Artemisia annua* which contains low concentrations of artemisinin. Patra and Srivastava (2016) reported that large-scale artemisinin production by *A. annua* hairy roots in nutrient mist bioreactor.

10.4 Advances in Metabolic Engineering of Hairy Roots

A new promising technology known as metabolic engineering or genetic engineering was evolved in the early 1990s (Bourgaud et al. 2001). Metabolic engineering in plants involves the alteration of metabolic pathways to increase the flux toward desired secondary metabolites or to attain better understanding of metabolic pathways and use of cellular pathways for chemical transformation, energy transduction, and supramolecular assembly (Chandra and Chandra 2011; Hussain et al. 2012). In other words, metabolic engineering is the alteration or improvement of the cellular activities involving transport and enzymatic and regulatory functions of the cell by using rDNA technology (Bourgaud et al. 2001; Hussain et al. 2012). It is one of the fastest-growing applications for the production of industrially important

Table 10.2 Examples of some important plant secondary metabolites produced through bioreactors

Plant species	Secondary metabolite	Bioreactor type	References
Artemisia annua	Artemisinin	Mist and bubble column reactor; gas- and liquid-phase bioreactors	Kim et al. (2001) and Patra and Srivastava (2016)
Astragalus membranaceus	Astragaloside IV and polysaccharide	Air lift bioreactor	Du et al. (2003)
Artemisia annua	Terpenoids	Mist and bubble column reactor	Souret et al. (2003)
Atropa belladonna	Tropane alkaloids	Stirred bioreactors	Lee et al. (1999)
Atropa belladonna	Tropane alkaloids, atropine	Bubble column bioreactor	Kwok and Doran (1995)
Beta vulgaris	Betalains, peroxidase	Bubble column reactor	Rudrappa et al. (2004, 2005)
Catharanthus roseus	Ajmalicine	Bubble column and rotating drum bioreactor	Thakore et al. (2017)
Datura stramonium	Hyoscyamine	Isolated impeller stirred tank reactor	Hilton and Rhodes (1990)
Eleutherococcus koreanum	Saponins	Air lift bioreactor	Lee et al. (2015a, b)
Genista tinctoria	Phytoestrogens	Prototype basket-bubble bioreactor	Luczkiewicz and Kokotkiewicz (2005)
Hypericum perforatum	Hypericin	Balloon-type bubble bioreactor	Cui et al. (2010)
Hyoscyamus muticus	Tropane alkaloids	Trickle bed bioreactor	Flores and Curtis (1992)
Nicotiana rustica	Nicotine	Air-sparged vessel stirred tank	Rhodes et al. (1987)
Panax ginseng	Ginsenosides	Air bubble bioreactor	Murthy et al. (2017)
Panax ginseng	Saponins	Air lift bioreactor	Yoshikawa and Furuya (1987)
Panax ginseng	Ginsenosides	Wave bioreactor	Palazon et al. (2003)
Polygonum multiflorum Thunb	Anthraquinones, stilbenes, flavonoids, tannins,	Air lift bioreactor	Lee et al. (2015a, b)
Stizolobium hassjoo	Levodopa	Mesh hindrance mist trickling bioreactor	Sung and Huang (2006)
Trigonella foenumgraceum	Diosgenin	Air lift bioreactor	Rodriguez-Mendiola et al. (1991)

bio-active compounds from various plant sources. The main aims of this technique are (1) overproduction of a desired compound which is normally produced in less quantity or increased metabolite production by transferring the pathways to another plant or microorganisms, (2) reducing the production of unwanted compounds, and (3) production of a new compound that is usually produced in nature but not present in the host plant (Verpoorte and Memelink 2002; Capell and Christou 2004; Chandra

and Chandra 2011). This can be achieved by conquering the rate-limiting steps or by jamming competitive pathways and blocking of catabolism successfully.

Now, multistep metabolic engineering is possible, which overtakes single-step engineering, and it is the best way to produce secondary metabolites in transgenic plants (Capell and Christou 2004). The main advantage of this method is that it is convenient and cost-effectively produces industrially important secondary metabolites continuously (Hussain et al. 2012). Also, this technique is used as a tool for improving crop plants that are resistant to various diseases, plants producing allelopathic compounds to control the weeds, pest-resistant plants to improve the importance of ornamentals and fruits, and enhanced pollination by modifying scent profiles (Chandra and Chandra 2011). Another advantage is the production of valuable secondary metabolites under controlled environment which is free from climate and soil conditions (Hussain et al. 2012). Engineering or structural design of secondary metabolite pathways is quite difficult in plants, because it requires a detailed knowledge of the whole biosynthetic pathways and a detailed perception of its regulatory mechanisms. But, such information is not explored in many medicinal plants known to have vast variety of bio-active metabolites (Oksman-Caldentey and Inze 2004). Recent advances in metabolic engineering have open a new way for the production of secondary metabolites in higher quantities. However, the success of this approach depends on the metabolic pathway elucidation and metabolite pathway mapping and identifying specific restraining enzyme activities. This process can be further improved by using an appropriate genetic transformation procedure. So far, most of the biosynthetic pathway strategies developed for producing secondary metabolites were through various ways which include isolating and expressing of the respective genes in more efficient organisms, construction of promoters to enhance the expression of a target gene, or antisense and co-suppression techniques for knockdown of particular plants for the desired traits (Bourgaud et al. 2001). For example, engineering of the flavonoid pathway in *Saussurea involucrata* by a transgenic approach increased the production of apigenin. The gene responsible for apigenin production in *S. medusa* was found to be chalcone isomerase (*chi*) gene. A complete cDNA sequence of *chi* gene construct was prepared under the control of the cauliflower mosaic virus (CaMV) 35S promoter. The *chi* gene was introduced into the *S. involucrata* genome by *A. rhizogenes*-mediated transformation which resulted in the establishment of transgenic hairy root lines. The enzyme chalcone isomerase converts naringenin chalcone into naringenin, which is the precursor of apigenin. After 5 weeks of incubation, C46 hairy root line accumulated 32.1 mg/l of apigenin with total flavonoids at 647.8 mg/l. The accumulation of apigenin and flavonoid content was found to be 12 and 4 times, respectively, which is superior when compared to the wild-type hairy roots. The enhanced enzyme productivity was obtained due to the superior activity of chalcone isomerase (Li et al. 2006). In addition to that, hairy root metabolic engineering has been widely used to enhance the production of pharmaceutically important secondary metabolites and also the production of certain recombinant proteins. For example, solasodine glycoside harmfully controls its own biosynthesis. A recombinant gene construct, i.e., anti-solamargine (As)-scFv gene, contains single-chain fragment variable (scFv)

antibody region derived from hybridoma cell lines. Transformed hariy root cultures with anti-solamargine (As)-scFv gene controls and enhances the solasodine glycoside concentration up to 2.3-fold more in the transgenic *S. khasianum* than wild-type hairy roots (Putalun et al. 2003). Metabolic engineering of the hairy roots is also used to make the de novo synthesis of secondary metabolites by introducing the specific genes that encode related enzymatic process in other organisms. The transfer of three genes from *Ralstonia eutropha* bacterium into the genome of sugar beet hairy roots directed the accumulation of poly(3-hydroxybutyrate) (Menzel et al. 2003). Recently, Hidalgo et al. (2017) reported the metabolism of tobacco hairy root for the production of stilbenes. In this study, in order to achieve the holistic response in the phenylpropanoid metabolic pathway and also direct the upregulation of multiple metabolic process, transformed tobacco hairy root (HR) cultures carrying the gene stilbene synthase (STS) derived from *Vitis vinifera* and *Arabidopsis thaliana* transcription factor (TF) AtMYB12 were established. In addition to that, the normal flux was arrested through the incorporation of an artificial microRNA responsible for chalcone synthase (amiRNA CHS); otherwise there will be a heavy competition with STS enzyme for precursors. The transgenic tobacco hairy roots were capable to synthesize the target compound, stilbenes.

10.5 Enhancement of Secondary Metabolites Through Elicitation

Elicitation is an efficient and promising method for increasing the production of secondary metabolites using an elicitor which is a substance that when introduced into a living cell system in ideal/little concentrations improves the biosynthesis of secondary metabolites. The mechanism involved in this process is that the addition of elicitors (both biotic and abiotic) into the plant system attacks the plant cell wall and triggers the production of plant-defensive secondary metabolites (Namdeo 2007; Bensaddek et al. 2008).

In general, the plant cells recognize the elicitor compounds through various signaling molecules and interact or bind with specific receptors present on the plasma membrane. These interactions later generate signals and activate genes that are responsible for the defense reactions including systemic acquired responses (SAR) and induced systemic resistance (ISR). This stimulates the biosynthesis of pathogenesis-related (PR) proteins or defense secondary metabolites, and these finally lead to the production of secondary metabolites (Zhao et al. 2005). The mechanism involved in the production of secondary metabolites through elicitors was showed in Fig. 10.2. Elicitors are broadly divided into two types, viz., biotic and abiotic; mostly abiotic elicitors are inorganic salts (minerals) and physical and chemical factors such as pH, temperature, UV light, heavy metal salts (Cu and Cd ions), etc., while biotic elicitors are polysaccharides derived from plant cell wall and microorganisms (pectin, cellulose, chitin, and glucans), glycoproteins (G-protein or intracellular proteins), pathogenic fungi and bacteria, plant hormones (methyl jasmonate and salicylic acid), etc. (Donenburg and Knorr 1995; Bourgaud et al. 2001;

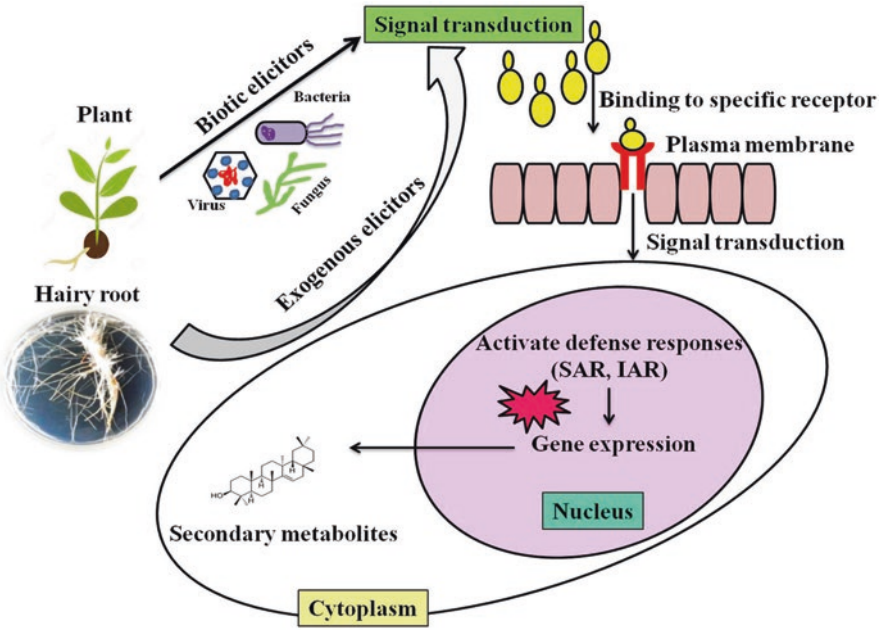

Fig. 10.2 The mechanism of elicitors in secondary metabolite production

Namdeo 2007; Ramirez-Estrada et al. 2016). In addition to that, new types of elicitors have been recently introduced and successfully used in few plant cell cultures. These new elicitors include voliticin, caeliferins, and inceptins. These compounds are derived from plants and insects (which are mostly found in oral secretions of insects). Recently, it was found that they act as an elicitor by activating jasmonates and lead to the production of secondary metabolites, mainly the volatile compounds (Ramirez-Estrada et al. 2016). However, improved production of the metabolites from plant cell cultures through elicitation depends on several parameters, such as selection of suitable elicitor, concentration of elicitor, duration of elicitor treatment, age of the explants, cell line, nutrient composition of the media, growth regulation, etc. (Namdeo 2007). Elicitation method for the plant cell culture system has shown a positive result in secondary metabolite production. However, the study about how plant cells or tissues and their metabolic pathways respond to both abiotic and biotic elicitors is a key route to design the new strategies to enhance the industrially important bio-active compounds in a large scale. For example, a few important bio-active compounds produced through elicitation with biotic and abiotic elicitors are Taxol (Veersham et al. 1995), phytoalexins (Kuroyanagi et al. 1998), saponins (Wu and Lin 2002), tropane alkaloids (Lee et al. 1998), etc. Different types of elicitors used for the production of valuable metabolites are listed in Table 10.3. For example, Largia et al. (2016) reported that the transformed hairy roots plants of *Bacopa monnieri* elicited with 10 mg/L chitosan for 2 weeks enhanced the accumulation of bacoside A (5.83%) content, which is a five- and fourfold increase when compared

Table 10.3 Production of plant secondary metabolites by using different elicitors

Plant species	Secondary metabolite	Elicitors	References
Ammi majus	Coumarine, furocoumarine	BION® *Enterobacter sakazakii*	Staniszewska et al. (2003)
Arachis hypogaea	*Trans*-resveratrol	Sodium acetate	Medina-Bolivar et al. (2007)
Arachis hypogaea	Resveratrol, piceatannol, arachidin-1, and arachidin-3	MeJA and cyclodextrn	Yang et al. (2015)
Astragalus membranaceus	Calycosin and formononetin	*Aspergillus niger*	Jiao et al. (2017)
Artemisia annua	Artemisinin	Chitosan	Putalun et al. (2007)
Azadirachta indica	Azadirachtin	Salicylic acid, jasmonic acid	Satdive et al. (2007)
Catharanthus roseus	Alkaloids (indole)	*Penicillium* sp.	Rijhwani and Shanks (1998)
Centella asiatica	Asiaticoside	Methyl jasmonate	Kim et al. (2007)
Datura metel	Atropine	AgNO3, nanosilver, *Bacillus cereus*, *Staphylococcus aureus*	Shakeran et al. (2015)
Hyoscyamus muticus	Sesquiterpenes	*Rhizoctonia solani*	Singh (1995)
Hyoscyamus niger	Polyamines and tropane alkaloids	Methyl jasmonate	Zhang et al. (2007)
Linum album	Lignan	Coniferaldehyde and methylenedioxycinnamic acid	Ahmadian Chashmi et al. (2016)
Oxalis tuberose	Harmaline, harmine	*Phytophthora cinnamomi*	Bais et al. (2003)
Lotus corniculatus	Isoflavonoids	Glutathione	Robbins et al. (1991)
Papaver orientale	Morphinan alkaloids	MeJA and salicylic acid	Hashemi and Naghavi (2016)
Panax ginseng	Ginseng saponin	Selenium, NiSO4, NaCl	Jeong and Park (2006)
Pharbitis nil	Umbelliferone, scopoletin, skimmin	CuSO4, MeJA	Yaoya et al. (2004)
Salvia miltiorrhiza	Tanshinone	Sorbitol	Shi et al. (2006)
Scopolia parviflora	Scopolamine	*Pseudomonas aeruginosa*, *Bacillus cereus*, *Staphylococcus aureus*	Jung et al. (2003a, b)
Solanum tuberosum	Sesquiterpene, lypooxygenase	*Rhizoctonia bataticola*, B cyclodextrin, MeJA	Komaraiah et al. (2003)
Tagetes patula	Thiophene	*Furasium conglutanis*, *Aspergillus niger*	Mukundan and Hjortso (1990) and Buitelaar et al. (1993)

to wild plants and unelicited transformed plants. Similarly, Shilpha et al. (2016) reported that *Solanum trilobatum* hairy roots (ST-09 clone) elicited for 2 weeks with 4 μM for methyl jasmonate enhanced the solasodine content, which is 1.9- and 6.5-fold higher than unelicited hairy roots and wild roots.

10.6 Biotransformation

Biotransformation is the process in which a substance is transformed from one chemical to another, and it is catalyzed by the effective enzyme structures of biological systems. Plant cell or organ cultures have the capability to convert exogenously added organic compounds into functional analogs (Banerjee et al. 2012; Roychowdhury et al. 2013). This type of protocols has been done by using plant cell/organ cultures which have generated the libraries of analog compounds with limited structural modifications, and it also ensures the sustainable use of the resource under defined culture conditions free from seasonal variations and pathological constraints. The resulted compounds will have the important characteristic potency of a parent molecule and can also attain a superior selectivity, safety, and physicochemical properties with lower toxicity. This can be more appropriate to be used for newer therapeutic applications. The biotransformation method is very useful for the discovery of novel phytochemicals having therapeutic and commercial advantages. Also, this method is attaining more attention toward the green chemistry, because of the reduced usage of hazardous chemicals in the process of chemical modifications. The major reactions involved in biotransformation methods include oxidation, reduction, glycosylation, esterification, methylation, isomerization, and hydroxylation. Hairy root cultures have various advantages as biocatalysts over cell suspension cultures, because of their genetic and biochemical stability, multi-enzyme biosynthetic potential comparable to the parent plant, and cost-effectiveness. Therefore, hairy root cultures also act as an experimental model system in biotransformation studies (Giri et al. 2001; Banerjee et al. 2012). Biotransformation studies were reported in *Ri*-transformed root cultures of several plant species for producing valuable secondary metabolites and are briefly described by Banerjee et al. (2012). For example, the biotransformation ability of *Atropa belladonna* hairy root cultures has been explored by using three carbonyl substrates such as 3,4,5-trimethoxybenzaldehyde, 3,4,5-trimethoxy-acetophenone, and 3,4,5-trimethoxy-benzoic acid. Among the three substrates used, 3,4,5-trimethoxybenzaldehyde and 3,4,5-trimethoxy-acetophenone were biotransformed, but, 3,4,5-trimethoxy-benzoic was not biotransformed. The 3,4,5-trimethoxybenzaldehyde was biotransformed by oxidation and reduction of substrate into 3,4,5-trimethoxy-benzoic acid and 3,4,5-trimethoxy benzyl alcohol, respectively (Srivastava et al. 2012). Overall, the biotransformation using hairy root cultures has got potential to generate new products or to generate already known products very efficiently. The list of reactions involved in biotransformation of hairy roots for metabolites production are shown in Table 10.4.

Table 10.4 Biotransformation of hairy roots for plant secondary metabolite production

Plant species	Types of reaction	Product	References
Anethum graveolens	Acetylation, reduction	Menthyl acetate linalool, α -terpineol, citronellol	Faria et al. (2009)
Anisodus tanguticus	Oxidation	Androst-4-ene-3,17-dione 6 α-hydroxy androst-4-ene-3	Liu et al. (2004)
Astragalus membranaceus	Deglycosylation	Calycosin Formononetin	Jiao et al. (2017)
Atropa belladonna	Reduction	Scopolamine	Subroto et al. (1996)
Brassica napus	Reduction, glycosylation	6-(1(S)-hydroxyethyl)-2,2-dimethyl-2,3-dihydro-4H-chromen-4-one	Orden et al. (2006)
Brugmansia candida	Glucosylation	4-Hydroxyphenyl β-D-glucopyranoside (arbutin)	Casas et al. (1998)
Coleus furskohlii	Glycosylation	Methyl β-D-glucopyranosides, methyl β-D-ribo-hex-3-ulopyranosides	Li et al. (2003)
Cyanotis arachnoidea	Reduction	Deoxyartemisinin	Zhou et al. (1998) and Ligang et al. (1998)
Daucus carota	Reduction	(S)-1-phenyl ethanol)	Caron et al. (2005)
Lobelia sessilifolia	Glucosylation	Protocatechuic acid 3-O-β-D-glucopyranoside	Ishimaru et al. (1996)
Lobelia sessilifolia	Glucosylation	(+)-catechin 7-O-β-D-glucopyranoside	Yamanaka et al. (1995)
		Protocatechuic acid, protocatechuic acid 3-O-β-D-glucopyranoside	
		(–)-epicatechin 7-O-β-D-glucopyranoside	
		(–)-epiafzelechin 7-O-β-D-glucopyranoside	
Levisticum officinale	Isomerization	Linalool, nerol	Nunes et al. (2009)
Panax ginseng	Esterification	Digitoxigenin stearate	Kawaguchi et al. (1990)
		Digitoxigenin palmitate	
		Digitoxigenin myristate	
		Digitoxigenin laurate	
Panax ginseng	Glycosylation	(RS)-2-phenylpropionyl β-D-glucopyranoside	Yoshikawa et al. (1993)
		(2RS)-2-0-(2-phenylpropionyl) D-glucose	
		(2RS)-2-phenylpropionyl) 6-0-β-D-xylopyranosyl β-D-glycopyranoside	

(continued)

Table 10.4 (continued)

Plant species	Types of reaction	Product	References
		Myoinositol ester of (R)-2-phenylpropionic acid	
Panax ginseng	Glycosylation	30-O-[β-D-glucopyranosyl (1→2) β-D-glucopyranosyl]	Asada et al. (1993)
		18 β-Glycyrrhetinic acid	
		30-O-[β-D-glucopyranosyl] 18 β-glycyrrhetinic acid	
		3-O-[β-D-glucopyranosyl -(1→2) β-D- glucopyranosyl] 18 β-glycyrrhetinic acid	
		3-0-[β-D-glucopyranosyl-(1→2)-β-D-glucopyranosyl] -30-0-(β-D-glucopyranosyl) 18 β-glycyrrhetinic acid	
Panax ginseng	Glycosylation	p-carboxyphenyl β-D-glucopyranoside	Chen et al. (2008)
		p-hydroxybenzoic acid	
		β-D-glucopyranosyl ester	
		m-carboxyphenyl β-D-glucopyranoside	
Pharbatis nil	Glucosylation	Skimmin	Kanho et al. (2004, 2005)
		4-Methylskimmin	
		Scopoline	
		3,4,8-Tri methylskimmin	
		Scopolin, aesculin, eichoriin, vanillin-4-O-β-glucopyranoside	
		Vanillyl alcohol-4-O-β-D-glucopyranoside	
Physalis ixocarpa	Glucosylation	Arbutin	Bergier et al. (2008)
Plantago lanceolata	Glucosylation	(E)-p-coumaroyl-1-O-β-D-glucopyranoside	Fons et al. (1999)
Polygonum multiflorum	Glycosylation	3-oxo-eremophila 1,7(11)-dien-12,8-olide	Yan et al. (2008)
		3-oxo-8-hydroxy-eremophila 1,7(11)-dien-12,8-olide	
Polygonum multiflorum	Glucosylation	4-Hydroxybenzene derivatives: 1-4-benzendiol	Yan et al. (2007)
		4-Hydroxybenzaldehyde	
		4-Hydroxybenzyl alcohol	
		4-Hydroxybenzoic acid	
Polygonum multiflorum	Glucosylation	5-Methyl-2-(1-methylethyl) phenyl-β-D-glucopyranoside	Dong et al. (2009)

10.7 Hairy Root Applications in Environmental Protection (Phytoremediation)

Environmental pollution is a universal problem that adversely affects both the developed and developing countries. The major reason for environmental pollution is due to human activities and natural hazards. Contaminants are usually classified into two types: organic and inorganic. Due to the human activities including oil spills, agriculture wastage, military explosives, fuel production, and wood treatment, organic contaminants are released into the environment. Some of important organic pollutants such as trichloroethylene (TCE), atrazine, trinitrotoluene, polycyclic aromatic hydrocarbons, benzene, toluene, polychlorinated biphenyls (PCBs), polycyclic aromatic hydrocarbons, and methyl tert-butyl ether contaminating the soil and water are a challenge to the world. Generally, inorganic contaminants are originated from either human activities or natural processes. The most dangerous inorganic contaminants include heavy metals such as copper, zinc, manganese, lead, molybdenum, mercury, and nickel which are released into the environment by natural and human activities causing a health threat to humans and livestock (Suza et al. 2008). The removal of these contaminants from the environment is not an easy task, and decontamination is a very expensive process. Phytoremediation, as an emerging alternative technology, is highly appreciated in recent times for its effectiveness in cleaning up of the contaminated environment. Phytoremediation is defined as the ability of plants to uptake contaminants from the polluted environment (soil, water, or air) and convert the toxic chemical molecules to harmless forms enzymatically (Roychowdhury et al. 2013; Guillon et al. 2006). The key advantage of phytoremediation technique is that it is about ten times less expensive than conventional environmental cleanup methods, and it is a safe method. Generally, plants act as natural soil stabilizers, reduce the amount of contaminants, and maintain the surroundings free from pollutants. Phytoremediation is better than bioremediation methods that uses microbes in terms of easy monitoring. This is because, in phytoremediation, the plants' condition is visible, and the presence of pollutants in plant tissues can be easily tested (Doty 2008). The major phytoremediation strategies involved in the removal of contaminants include phytoextraction, phytostabilization, and rhizofiltration of organic and inorganic pollutants (Gonzalez et al. 2006). In this regard, hairy root technology also plays an important role in the process of phytoremediation. Some of the advantages offered by hairy roots for this purpose include fast growth and high branching of hairy roots allowing increase absorption of contaminants, high biochemical and genetic stability, easy maintenance, scaling-up in bioreactors being easy, and provision of a huge surface area of contact with the contaminants. Moreover, hairy roots contain essential enzymes and metal chelating agents to detoxify the harmful compounds (Gonzalez et al. 2006; Roychowdhury et al. 2013). In recent years, hairy roots are serving as a potential tool to decontaminate the environment and are being highly appreciated by environmental biologists for its effectiveness. A wide variety of environmental pollutants that can be removed by hairy roots derived from different plant species are shown in Table 10.5. However, it is required to completely understand the enzymatic machineries involved in the

Table 10.5 Phytoremediation of environmental pollutants by hairy root cultures

Plant species	Pollutant	Reference
Solanum nigrum	PCBs (polychlorinated biphenyls) and zinc	Macková et al. (1997a, b) and Subroto et al. (2007)
Thlaspi caerulescens	Cadmium	Nedelkoska and Doran (2000) and Boominathan and Doran (2003)
Alyssum sp. *A. bertolinii*, *A. tenium*, and *A. troodi*	Nickel	Nedelkoska and Doran (2001) and Suresh et al. (2005)
Catharanthus roseus	RDX (hexahydro-1,3-5-trinitro-1,3-5-triazine) and HMX (oxtahydro-1,3,5,7-tetranitro-1,3,5,7-tetrazocine)	Bhadra et al. (2001)
Daucus carota	Phenol and chloroderivatives	De Araujo et al. (2002)
A. bertolonii and *Thlaspi caerulescens*	Nickel, and cadmium	Boominathan and Doran (2002)
Atropa belladonna	TCE (trichloroethylene)	Banerjee et al. (2002)
Brassica napus	2,4-Dichlorophenol, Phenol	Agostini et al. (2003) and Coniglio et al. (2008)
B. juncea and *Chenopodium amaranticolor*	Uranium	Eapen et al. (2003)
B. juncea and *Cichorium intybus*	DDT (Dichloro-diphenyl-trichloroethane)	Suresh et al. (2005)
Helianthus annuus	Tetracycline and oxytetracycline	Gujarathi et al. (2005)
Lycopersicon esculentum	Phenols	Wevar-Oller et al. (2005)
Daucus carota, *Ipomoea batata*, and *Solanum aviculare*	Guaiacol, catechol, phenol, 2-chlorophenol, and 2,6-dichlorophenol	De Araujo et al. (2004, 2006)
Brassica juncea	Phenol	Singh et al. (2006)
Lycopersicon esculentum	Phenol	Wevar-Oller et al. (2005) and González et al. (2006)
Alyssum murale	Nickel	Vinterhalter et al. (2008)
Solanum lycopersicon	Phenol	Wevar-Oller et al. (2005) and González et al. (2006)
Nicotiana tabacum	Phenol, 2,4-DCP	Alderete et al. (2009) and Talano et al. (2010)
Armoracia rusticana	Uranium	Soudek et al. (2011)

bioconversion of toxic contaminants to nontoxic complexes and also the mechanisms involved in the hyperaccumulation and metal tolerance (Roychowdhury et al. 2013). In the future, the application of genetic engineering to insert specific detoxifying genes in hairy roots enhances their capacity to effectively clean up the contaminant.

10.8 Germplasm Conservation

Germplasm conservation is one of the prominent techniques to preserve/restore the plant biodiversity, because most of the plants do not produce viable seeds and propagate vegetatively, while some plants produce recalcitrant seeds, and the storage of seeds is affected by pests or other pathogens. So, the conservation of wild, rare, and endangered medicinal plant species for future use has become a big problem, and more efforts are initiated in this direction. Biotechnological tools such as plant tissue culture micropropagation and cryopreservation have certainly benefited in protecting plant germplasms including vegetatively propagated plant species, genetic resources of recalcitrant seeds, rare and endangered plant species, cell lines with special attributes, genetically transformed plant material, and clones obtained from elite genotypes (Engelmann 2011). Based on the storage duration, in vitro conservation methods are classified into three types, namely, short-, medium-, and long-term storage. Among them, cryopreservation is the most efficient technique for long-term conservation of the germplasm of a valuable plant, because of its cost-effectiveness and safety. Three types of cryopreservation methods are highly employed for the biodiversity conservation. They include freeze-induced dehydration, encapsulation-dehydration, and encapsulation-vitrification (Shibli et al. 2006). Hairy root cultures can be used for the germplasm conservation, because hairy root cultures are significantly a good resource for the production of several secondary metabolites and, in recent times, they are obtained in many medicinal plants for commercial applications. Hence, conserving such hairy roots will be more useful for future applications. However, there are only very few reports available on the conservation of hairy roots of medicinal plants. Hairy roots in the form of artificial seeds are a reliable delivery system for the clonal propagation of elite plants with genetic uniformity, high yield, and low production cost. Cryopreservation method for root tips was first developed by Benson and Hamill (1991) from hairy root cultures of *Beta vulgaris*, and the same technique was implemented in *Nicotiana rustica*. Yoshimatsu et al. (1996) reported the cryopreservation of *Panax ginseng* hairy roots. In addition to that, cryopreservation of hairy roots was reported in some more medicinal plants like *Artemisia annua* (Teoh et al. 1996), *Armoracia rusticana* (horseradish) (Phunchindawan et al. 1997; Hirata et al. 1998), *Atropa belladonna* (Touno et al. 2006), *Eruca sativa*, *Astragalus membranaceus* and *Gentiana macrophylla* (Xue et al. 2008), *Maesa lanceolata* and *Medicago truncatula* (Lambert et al. 2009), and *Rubia akane* (nakai) (Kim et al. 2010, 2012; Salma et al. 2014).

10.9 Omics Approaches in Secondary Metabolite Production

The omics approaches, namely, genomics, transcriptomics, proteomics, and metabolomics, have been majorly utilized in hairy root-based secondary metabolite production. As transcriptomic tools the microarrays and expressed sequence tags (EST) were useful in measuring the gene expression studies in large scale. Expression of target genes in a plant cell can be modified through various methods such as

precursor feeding, elicitor treatment, overexpression or silencing of transgenes, etc. Generation of cDNA microarrays and EST database provides the information about the changes at mRNA level and also briefs the functions of genes and its regulation in secondary metabolism of hairy root cultures. Transcriptome analysis of hairy root cultures has been done in several plants including *P. ginseng* (ginsenoside), *C. roseus* (indole alkaloids), *Medicago truncatula* (anthocyanin), *S. miltiorrhiza* (tanshinones), etc. (Jung et al. 2003a, b; Murataa et al. 2006; Pang et al. 2008; Gao et al. 2009; Wang et al. 2010). In studying the tanshinone biosynthesis, *S. miltiorrhiza* hairy root cultures were used as a model system. The combined analysis of metabolite profiling and cDNA-AFLP identified the candidate genes which are potentially involved in the biosynthetic pathway (Yang et al. 2012). Proteomics is an important, powerful, and under-explored omics technology for the secondary metabolite elucidation in hairy root cultures. Proteomic approach for hairy root cultures has been initiated in *P. ginseng* and opium poppy (Kim et al. 2003; Zulak et al. 2009). Metabolomics is an emerging approach which is highly useful in secondary metabolite production (Yang et al. 2012). The systems biology approaches with a combination of omics approaches will offer a great opportunity for high-throughput secondary metabolite elucidation in various plant species.

10.10 Conclusions and Future Prospects

In the modern era, humankind is facing the problem of high demand for several potent plant secondary metabolites possessing many bio-pharmacological activities. Previously, in vitro dedifferentiated plant tissue cultures were used for obtaining plant metabolites. As the years passed, cell suspension and adventitious root cultures were widely adopted for the same. However, to elucidate such metabolites, there is a need to develop an efficient and reliable, fast-growing in vitro tissue culture model to overcome the problem of wild plant availability. In this regard, hairy root cultures offer a great value to the continuous production of several precious secondary metabolites, because of their unique characteristics discussed above. Since the emergence of hairy root technology, a lot of improvements have been made day by day especially the use of bioreactors, application of elicitation strategy, and biotransformations. Overall, hairy root technology has shown its wide utility in many medicinal plants. Moreover, the production of plant secondary metabolites in the hairy root culture system has delivered very encouraging findings, for example, illuminating the sites of biosynthesis or rate-regulating stages, precursor's requirements, role of regulatory genes, transcription factors, and putative metabolite intermediates relating to secondary metabolite biosynthesis. Also, it offers the possibility of recognizing a suitable gene candidate required for metabolic engineering of specific plant traits and to improve their secondary metabolite secretion. However, more efforts are to be encouraged to better understand the biosynthetic pathways and regulatory cascades involved in secondary metabolite synthesis. Therefore, it is crucial to make use of genetic engineering approaches in order to fully realize the biosynthetic prospective of hairy roots. Plant biotechnologists are required to work

closely with bioengineers to overcome the challenges faced during the scaling-up of hairy root cultures in bioreactors. In the future, research efforts should be encouraged toward making use of hairy root culture technology for producing high-value secondary metabolites commercially from many unexplored medicinal plant species.

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Plant Cell Culture as Alternatives to Produce Secondary Metabolites

11

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Abstract

Plants are often regarded as "natural chemical factories" and produce variety of biomolecules for their metabolism and survival. They are mainly represented as primary metabolites and secondary metabolites. Primary metabolites are essentially required for the plant growth and its survival, whereas secondary metabolites are chemical compounds which are not directly associated with their growth, survival, development, and reproduction but are involved in various defense mechanisms and environmental adaptations. These compounds have been used throughout the history as medicines, flavors, fragrances, and pigmented colors. In recent years, these natural products have started dominating the food, healthcare, cosmetics, and pharmaceutical industries and are considered economically important and high-valued marketed products. The production of these phytochemicals is generally dependent on the plant species, geographical locations, climatic conditions, and edaphic factors. The main constraint in their large-scale production is quality and quantity control during their synthesis. In this chapter, the choice of plant tissue culture as an alternative way for the production and extraction of secondary metabolites is discussed. The expression of the synthetic pathway for their mass production can be enhanced and altered by selecting proper cell lines, manipulating media components, addition of precursors and elicitors, biotransformation, permeabilization, and immobilization. A combinatorial research in the field of plant tissue culture, metabolite synthesis pathway, and downstream processing can exploit the potentiality of these natural factories for large-scale production of secondary metabolites.

Keywords

Natural factories · Secondary metabolites · Plant tissue culture · Biotransformation and immobilization

11.1 Introduction

Plants are the most successful form of life existing on this planet. They cannot run away when attacked by pathogens, predators, or herbivores without any immune system to fight against any infections. Interestingly, plants have survived for millions of years on the earth. There are thousands of structurally distinct molecules which have evolved during time course to provide defense against bacteria, fungi, virus, and herbivores (Schäfer and Wink 2009). Secondary metabolites are the diverse chemicals and apparently lack in the primary functions of plant but have specific negative impacts on other organisms and pathogens which lead to a hypothesis that these metabolites are evolved for their protective values (Delgoda and Murray 2017). Many secondary metabolites are toxic in nature which repels herbivores and pathogens and therefore plays an important role in defense mechanisms. There are several pathogens that cause numerous diseases in humans including

inflammation, flu, diarrhea, dysentery, and other disorders. In earlier time, humans completely rely on natural drugs to treat bacterial and fungal diseases. Those drugs were extracted from natural sources like plants and their parts. Medicinal systems was developed thousands of years ago and completely relied on natural or herbal medicines (Wink 2015). The records of herbal medicines are found in Ayurveda, traditional Chinese medicine and European medicines (Markus 2012). Modern medical systems are completely based on antibiotics and synthetic drugs, but these medicines soon begin to develop drug resistance and thus show negative impact on human health. For the improvement of modern molecular medicinal systems, proteomic and genomic analysis is extensively used for the identification of more new targets involved in the human diseases. These targets are used for drug testing which inhibits them. In this context, secondary metabolites library have been created, and these molecules can either be used directly or modified synthetically in modern medicinal system. There are mainly three categories of secondary metabolites terpenes, phenols, and nitrogen-containing compounds which are used as a source for natural colors, flavors, pesticides, insecticides, cosmetics, fragrances, medicines, and therapeutics (Chiang and Abdullah 2007). As a result, these metabolites are commercially and economically more important than primary metabolites, since the synthesis of these metabolites generally depends on the plant species, geographical locations, climatic conditions, and edaphic factors making their extraction and purification more difficult (Tyler and Russo 2015). Plant cell culture can provide an alternative route over traditional cultivation methods and chemical synthesis methods for the secondary metabolite production. These approaches have been investigated by many scientists to provide a new, stable, and promising biosynthetic platform for desired natural compounds (Bhatia et al. 2015). There are different methods used for plant tissue culture like cell suspension, callus culture, shoot culture, and hairy root cultures. Organ cultures often show metabolite characteristics similar to parent plant, but sometimes the accumulation of desired products is very low (Kolewe et al. 2008). Hairy root culture which is obtained by the transformation of *Agrobacterium rhizogenes* is genetically very stable and enhances the secondary metabolite production (Giri and Narasu 2000). But the major limitations in the commercial usage of this method are the cultivation of hairy roots under controlled bioreactors systems (Giri and Narasu 2000). This technique offers an alternative and potent method for the production of high-valued natural marketed products such as artemisinin (Baldi and Dixit 2008), resveratrol (Cai et al. 2012), paclitaxel (Li et al. 2009), and ajmalicine (Ten Hoopen et al. 2002). This chapter provides a comprehensive review on the current status of secondary metabolite production by classical and nonclassical approaches of plant tissue culture. It is mainly focused on the secondary metabolite synthesis pathway, better cell line section, precursor feeding, and other classical approaches adopted for their enhanced production. In addition, technological advancements like biotransformation, cell immobilization, cell culture, and organ cultures are employed for the improved commercial production of phytochemicals. Some important metabolites produced by different in vitro tissue culture methods are listed in Table 11.1.

Table 11.1 Some important secondary metabolites produced by different plant tissue culture techniques

Plant species	Secondary metabolite	Mode of action	Culture condition	Culture type	References
Catharanthus roseus	Catharanthine	Nicotinic receptor inhibitor and anticancer property	MS + 2,4-D + UV-B radiation	Suspension	Ramani and Jayabaskaran (2008)
Catharanthus roseus	Vincristine	Anticancer agent	MS + 2,4-D + GA3	Shoot	Lee-Parsons and Royce (2006)
Ammi majus	Umbelliferone	Anti-inflammatory and sunscreen agent	MS + BAP	Shoot	Królicka et al. (2006)
Lithospermum erythrorhizon	Shikonin	Antioxidant treatment of capillary bleeding	MS + 2,4-D + kinetin	Hairy root	Fukui et al. (1998)
Rauvolfia serpentina	Reserpine	Antipsychotic and antihypertensive	MS + IAA + Cu_2^+	Callus	Nurcahyani et al. (2008)
Silybum marianum	Silymarin	Hepatoprotective	MS + IAA + GA3	Hairy root	Rahnama et al. (2008)
Vitis vinifera	Resveratrol	Cardiac and anticancer agent	MS + IAA + GA3 +UV	Callus	Keskin et al. (2009)
Pluchea lanceolate	Quercetin	Antioxidant	MS + NAA + BAP	Callus	Arya et al. (2008)
Plumba gorosea	Plumbagin	Antimicrobial, anti-inflammatory	MS + $CaCl_2$	Callus	Komaraiah et al. (2003)
Glycyrrhiza glabra	Glycyrrhizin	Peptic ulcer treatment	MS + 2,4-D + GA3	Hairy root	Gopi and Vatsala (2006)
Gymnema sylvestre	Gymnemic acid	Antidiabetic	MS + 2,4-D + IAA	Callus	Mehrotra et al. (2008)
Eleutherococcus senticosus	Eleutherosides	Antidiabetic effects	MS + 2,4-D	Suspension	Shohael et al. (2007)
Rheum ribes	Catechin	Antioxidant	MS + IBA + BA	Callus	Sepehr and Ghorbanli (2005)
Azadirachta indica	Azadirachtin	Insecticidal	MS + 2,4-D	Suspension	Sujanya et al. (2008)
Centella asiatica	Asiaticoside	Wound healing	MS + 2,4-D	Hairy root	Kim et al. (2007)
Cassia acutifolia	Anthraquinones	Purgative	MS + 2,4-D + kinetin	Suspension	Nazif et al. (2000)

11.2 Biomolecules of Plants

Metabolism is the sum of all the reactions taking place in an organism. There are many organic molecules and compounds which are synthesized by an organism via enzyme-regulated reactions known as metabolic pathways (Fig. 11.2). These synthesized organic molecules are called as metabolites. Based on their involvement in the growth and development of plants, these biomolecules are categorized into primary metabolites and secondary metabolites.

Primary metabolites are the organic compounds which are directly associated with its growth, development, and reproduction. These metabolites generally perform various physiological functions which is essentially necessary for the survival of plants (Muranaka and Saito 2010). These mainly comprise of various types of organic molecules like, carbohydrates, proteins, lipids, and nucleic acids. These metabolites are formed during the exponential growth phase, trophophase. Basic primary metabolites are starch, sucrose, cellulose, ethanol, certain amino acids, DNA, and RNA.

Secondary metabolites are the molecules which are indirectly associated with the basic function of plants such as growth and reproduction. Though this class of metabolites is not associated with the direct survival of plant, it plays a significant role in adaptation and defense mechanisms (Sato and Matsui 2012). These metabolites activate only in a particular growth stage, stress conditions, nutrient limitations, or microorganism attack. These metabolites are produced only in the stationary phase of growth, i.e., idiophase. These bio-active molecules are structurally and chemically extremely diverse in nature. Many secondary metabolites are toxic in nature which repels herbivores and pathogens and therefore plays an important role in defense mechanisms (Zhou et al. 2015).

11.2.1 Categorization of Secondary Metabolites

These compounds are broadly classified into three main categories on the basis of their chemical structures, elemental composition, solubility, and synthesis pathway (Kabera et al. 2014). Some of the examples of different categories of secondary metabolites are depicted in Fig. 11.1.

11.2.1.1 Terpenes

Terpenes or terpenoids represents the largest category of secondary metabolites and entirely composed of carbon and nitrogen atoms. They are derived either from acetyl CoA or from other intermediates in glycolysis pathway and are generally insoluble in water. These are further classified on the presence of number of C5 isoprenoid units in their molecular skeleton which is shown in Table 11.2 (Devika and Koilpillai 2012).

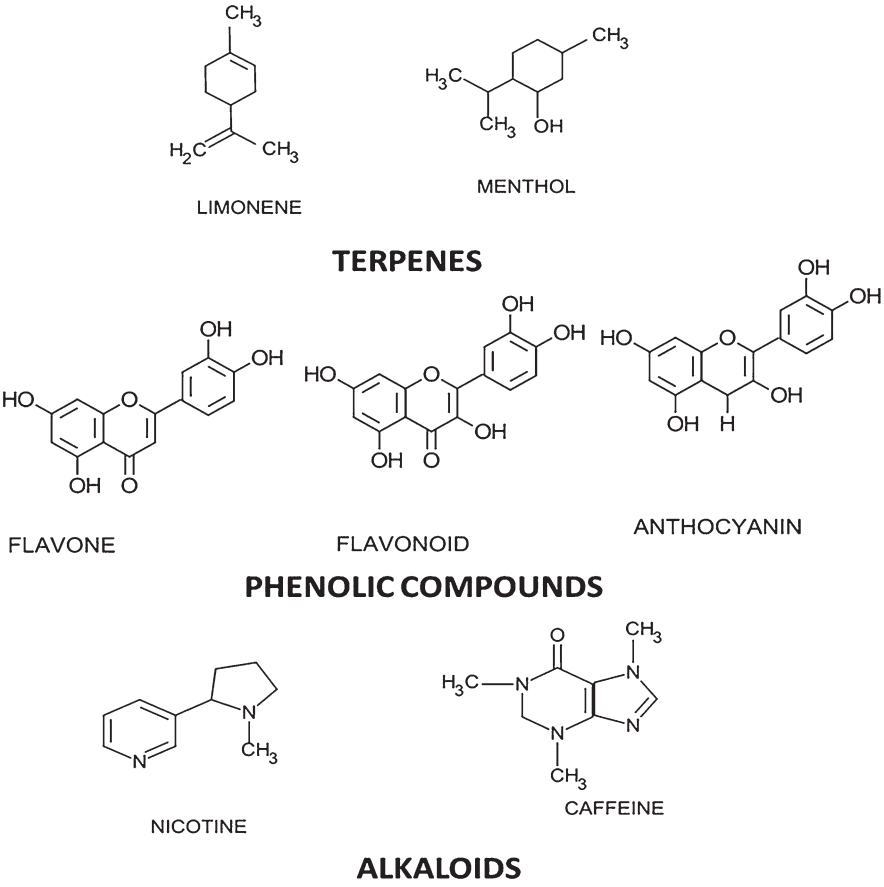

Fig. 11.1 The chemical structures of some important secondary metabolites

Table 11.2 Different classes of terpenes

Class of terpene	Number of carbon atoms	Number of isoprenoid units	Type of secondary metabolite
Hemiterpenes	5	1	Isoprene, prenol, isovaleric acid
Monoterpenes	10	2	Limonene, menthol, geraniol, terpineol
Sesquiterpenes	15	3	Abscisic acid, farnesol, humulene
Diterpenes	20	4	Taxol, cafestol, phytol
Triterpenes	30	6	Stigmasterol, lanosterol
Tetraterpenoids	40	8	Beta-carotene, lycopene
Polyterpenes	>100	>1000	Rubber, latex

Table 11.3 List of different phenolic compounds

Secondary metabolites	Source	Medicinal properties
Salicylic acid	*Willow*	Antifungal
Tannins	Fir or oak	Antimicrobial, astringent
Estradiol	Soybean	Estrogen hormone
Isoflavonoid	Legumes	Insecticidal
Capsaicin	Pepper and chillies	Pungent taste
Propofol	–	Anesthetic
Thymol	*Thymus vulgaris*	Antiseptic
Eugenol	Cloves	Essential oil

11.2.1.2 Phenolic Compounds

The next category in the secondary metabolites is the compounds which contain a phenol group and therefore known as phenolic compounds. Phenolic compounds contain a functional hydroxyl group which is attached to aromatic rings (Forkmann and Martens 2001). The wide chemical diversity of these compounds plays some very important role in plants like in defense mechanism against pathogens and herbivores, mechanical support by providing strength, pollination by attracting pollinators and fruits dispersers, absorption of harmful UV radiations, and growth inhibition of other competing nearby plants (Winkel-Shirley 2001). There are many phenolic compounds which show strong medicinal properties and illustrated in Table 11.3 (Devika and Koilpillai 2012).

11.2.1.3 Nitrogen-Containing Compounds

Nitrogen-containing compounds are the secondary metabolite which contains nitrogen in their structure. Alkaloids are the largest class of nitrogen compounds and synthesized by common amino acids (like tryptophan and tyrosine) and also from terpenes synthesis pathway. Generally, these compounds are toxic in nature and taste bitter and accumulate in plant parts like fruits or leaves which can be easily ingested by herbivores (Bird et al. 2003). Atropine is an alkaloid synthesized by *Atropa belladonna* plant which is used as an anesthetic drug. Indole alkaloids such as vincristine, which is produced by *Catharanthus roseus*, found to have anticancerous properties (Zhu et al. 2014). Another alkaloid, quinine which is extracted from the *Cinchona* bark, is highly poisonous for malarial parasite and has been used as an antimalarial drug in most of the tropical regions of the world (Kacprzak 2013). The second class of nitrogen containing compounds is plant glycosides or glucosinolates. These compounds are generally found in Brassicaceae family plants and are also involved in defense mechanisms (Holst and Fenwick 2003).

11.2.2 Secondary Metabolite Synthesis Pathway

The biosynthetic pathway for the secondary metabolite production is derived from various precursors involved in the primary metabolism which is shown in Fig. 11.2.

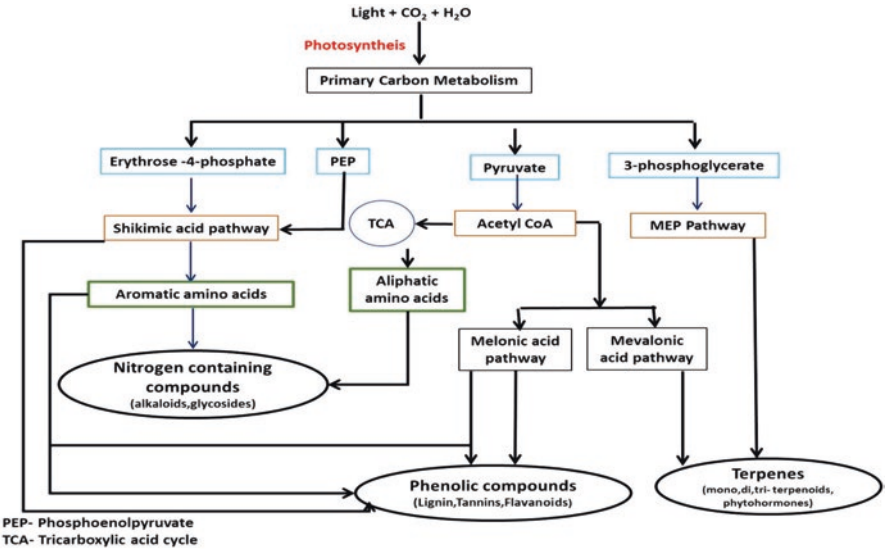

Fig. 11.2 The secondary metabolite production is highly regulated with the primary metabolite biosynthesis pathway

These pathways are very complex in nature which has many junction nodes in which an intermediate simultaneously acts as a precursor for another part of the same pathway. The major precursors are derived from carbohydrate, protein, and lipid metabolism. The final end products are derived from three main classes of compounds. Aromatic compounds, such as phenols and flavonoids, are synthesized by aromatic amino acids (Mijts and Schmidt-Dannert 2003). Acetyl CoA is a main component formed by glycolysis pathway and also via the β-oxidation of fatty acids, used in TCA cycle for the synthesis of aliphatic amino acids which works as a precursor for the synthesis of alkaloids and glycosides (Parsaeimehr et al. 2011). In addition, acetyl CoA is also involved in the synthesis of terpenes.

11.3 Plant Tissue Culture and Its Advantages Over Conventional Agricultural Techniques

Plants synthesize important secondary molecules via various primary intermediates. These molecules are produced naturally by different plant species but have many disadvantages like:

(a) Production of these molecules is completely dependent on seasonal conditions.
(b) Synthesis and production is unpredictable.
(c) Low yield of desired molecules.

(d) Problem of contamination with the other metabolites.
(e) Extraction and purification of desired molecules is very difficult.
(f) Production depends on edaphic and geographical factors.

Several strategies have been adopted to increase the metabolite production to meet market demands. There are mainly two approaches, classical and nonclassical approach, to achieve the goal (Bhatia and Bera 2015). Classical approach involves selection of high-yielding cell lines and media. Classical approach has been used from decades to enhance the in vitro secondary metabolite production. Recent development in the field of tissue culture led to base of nonclassical approach. This approach includes addition of precursors and elicitors, genetic manipulation, cell immobilization, and biotransformation.

Plant tissue culture can be defined as in vitro alterations and manipulations in plant cells and tissues. Plant tissue culture relies totally on totipotency phenomena, i.e., a single plant cell, which has the ability to regenerate into a whole plant. It was first started by Haberlandt in 1902. Due to advancement in the area of plant tissue culture, it has become an alternative approach for the secondary metabolite production. This technology has various applications and thus bloomed beyond expectations. The main advantages of this technique over the conventional method of cultivation are as follows (Hussain et al. 2012):

(a) Production of these compounds is simpler, reliable, and more predictable.
(b) Production of these compounds is under controlled conditions.
(c) It maintains the homogeneity of the culture (Xu et al. 2011).
(d) Synthesis and production is independent of geographical and edaphic factors.
(e) Easy and efficient extraction of molecules.
(f) High yield and improved productivity of desired compounds.
(g) Cultured cells are free from microbial contamination.
(h) Automated and controlled production will reduce the labor costs.
(i) Production of rare, novel, and economically important molecules has become easy (Yadav et al. 2012; Zhu et al. 2011).

Despite of several advantages of this alternative approach, plant tissue cultures have various constraints and limitations (Smetanska 2008). These include:

(a) Plant tissue culture is an expensive technology and requires heavy maintenance costs.
(b) This technique reduces the genetic diversity.
(c) This requires highly trained and skilled workers.
(d) High chances of contamination.
(e) It is not suitable for some plant species where components in growth media can kill explants.

11.4 Strategies for Enhanced Secondary Metabolite Production by Plant Tissue Cultures

11.4.1 Selection of High-Yielding Cell Lines

The selection of desired cell lines is very important for the identification of high-yield and fast-growing cultures. Natural metabolites are species specific and have crucial accumulation (Murthy et al. 2014). This selection process is achieved by various chemical-based approaches like identification of high-yielding cell lines by using exogenous intermediates. The high-yielding cell lines of *Lavandula vera* were obtained for the production of rosmarinic acid by adding phenylalanine substrate (phenylalanine ammonia-lyase, PAL) into the culture medium. It was found that the cell lines with high levels of PAL activity show enhanced levels of rosmarinic acid (Georgiev et al. 2006). Similarly, high-yielding cells lines of *Mentha arvensis* were screened for the enhanced menthol production at large scale (Dhawan et al. 2003a).

The major limitation of this classical approach is that the production ability of the desired product decreases with increase in subculturing of cell lines (Wilson and Roberts 2012; Georgiev et al. 2009). This reduced or complete loss of desired product may be due to genetic instability. During subculturing of cell lines, the chromosomal rearrangement results into genetic modifications like insertion and deletion which leads to polyploidy and aneuploidy (Ochoa-Villarreal et al. 2016). Genetic modifications have been seen after 1 year, in case of *Taxus media* subcultures which result in low taxol production (Baebler et al. 2005). To overcome these problems, selectable markers have been used in plant cells combined with other sorting methods to maintain high performance in culture medium during the course of metabolite production (Raven et al. 2015).

11.4.2 Manipulation in Media Components

Plant cell culture medium requires simple sugars as carbon source; amino acids as nitrogen source; nitrogen, phosphate, and potassium as macro elements; and auxins and cytokinin as phytohormones which are required for the metabolite production (Holland et al. 2013). These all culture components along with the culture conditions like light, temperature, and pH of the media effects the production of secondary metabolites in cell culture (Murthy et al. 2014). Modifications in the culture medium include alterations in carbohydrate and nitrogen ratios, elimination of certain phytohormones, low level of phosphates, and enhancement in the sucrose levels (Bhojwani and Dantu 2013). Therefore, the classical approaches are very important for the production of desired natural products and can increase the yield by 20- to 30-folds (Verpoorte et al. 2002).

Phytohormones or plant growth regulators play a crucial role in the accumulation and production of secondary metabolites. Each plant species requires specific phytohormone at specific level for the induction and growth of callus and also for the production of metabolites (Vasilev et al. 2013). Increased levels of auxins stimulate

cell dedifferentiation, cell division, and callus growth and therefore, the concentration of auxins in cell culture medium for metabolite production is tightly regulated. 2,4-D, IAA, and NAA are commonly used auxins in culture medium. IAA has been shown for the enhanced production of shikonin and anthraquinones in the suspension culture of *Lithospermum erythrorhizon* and *Morinda citrifolia*, respectively (Tabata 2006; Zhong 2001). Media manipulation is also used for the production of M12 antibody in hairy root cultures of tobacco (Hakkinen et al. 2014) and BY-2 antibody in cell suspension of tobacco cultures (Vasilev et al. 2013).

11.4.3 Addition of Precursors

Precursors are the compounds which can be converted into secondary metabolites by living systems when added endogenously or exogenously. These compounds are basically the intermediate compounds or the compounds used in the beginning of the biosynthetic route for secondary metabolite production. Therefore, feeding of precursor in the cell culture medium is an obvious approach for the enhanced production of secondary metabolites. Addition of geraniol in the cell culture of *Catharanthus roseus* plant led to the accumulation of citronellol and nerol (Lee and Shuler 2000). Likewise, addition of phenylalanine in the suspension culture of *Salvia officinalis* stimulates the rosmarinic acid production with decreasing the synthesis period in culture medium (Kim et al. 2004).

11.4.4 Addition of Elicitors

Plants generally synthesize secondary metabolites under environmental stress or as a part of defense mechanism against pathogens, microorganisms, insects, or herbivorous predators. Elicitors are the compounds which increases the specific metabolite production when added in very less quantity in the culture medium (Murthy et al. 2014). Depending on their origin, these can be differentiated into two categories biotic elicitors and abiotic elicitors. Biotic elicitors are the biological components present in any microbe and can trigger the stress condition in plant cell. These include microbial cell wall components, polysaccharides like chitin, various enzymes, and glycoproteins (Roberts et al. 2003; Zhao et al. 2001). For example, addition of a signaling molecule methyl-jasmonate (MeJA) in the culture medium of *Taxus cuspidata* drastically enhances the production of paclitaxel drug (Lee et al. 2010). Also, the addition of MeJA with cyclodextrin in the grapevine cultures shows a synergistic effect on the resveratrol production (Lijavetzky et al. 2008). Abiotic elicitors are non-biological substances, and these include physical components like ultraviolet irradiation, mechanical wounding, high salt content, high or low osmolarity, high pressure, extreme temperatures, and chemical components like heavy metals and inorganic salts (Pauwels et al. 2009; Luo and He 2004).

11.4.5 Immobilization and Its Applications

The term immobilization refers to a technique in which catalytically active enzymes are physically confined or localized in a certain defined region of space with retention of their catalytic activities, and which can be used repeatedly and continuously (Mohamad et al. 2015). This technique is used for the production of carbohydrates, amino acids, and other therapeutic products by entrapping enzymes on different kinds of gels like agarose or calcium alginate gels (Brena et al. 2013). However, calcium alginate gels are the most preferred matrix for entrapment method because of their simple and nontoxic nature which has been employed for the production of various metabolites like paclitaxel (Bentebibel et al. 2005), vanillin, ajmalicine, and capsaicin (Rao and Ravishankar 2002). Cell cultures of *Plumbago rosea* were immobilized in calcium alginate and then cultured in MS media containing 10 mM $CaCl_2$ for the enhanced production of plumbagin (Mulabagal et al. 2004). This technique provides high biomass concentrations in bioreactors to protect cells which prevents shear stress and enhances product accumulation by increasing the biomass production (Murthy et al. 2014). Immobilization technique has been used for the enhancement of commercial production of human granulocyte-macrophage colony-stimulating factor (GM-CSF) in tobacco cells by around 50-fold (Bodeutsch et al. 2001). An immobilized plant cell provides potential benefits in the research and development of plant tissue cultures but has limitations with the nutrient mass transfer in a bioreactor.

11.4.6 Biotransformation and Its Applications

Biotransformation is a technique which involves the chemical modifications of organic compounds by living biological systems or cell cultures. This technique has three major advantages: (a) formation of pure chiral products, (b) transformed cells that can perform regiospecific modifications, and (c) synthesis of novel products (Rao and Ravishankar 2000). The biotransformation of cinnamyl alcohol to rosavins enhances the production of glycosides by 80–95% in the root culture of *Rhodiola kirilowii* (Grech-Baran et al. 2014). In another study, a novel indole alkaloid, 3-hydroxy-4-imino-catharanthine, was identified in the suspension cultures of *Catharanthus roseus* by biotransformation of catharanthine (He et al. 2015). The biotransformation of cinobufagin in the suspension cultures of *Catharanthus roseus* was reported to synthesize a novel compound, 1b-hydroxyl desacetylcinobufagin which shows cytotoxic activities against HL-60 cell lines (Ye et al. 2003). In the suspension cultures of *Saussurea involucrata*, bufadienolide molecules are transformed into new 11 metabolites (Zhang et al. 2013). In another study, three novel compounds have been reported by biotransformation of 21-O-acetyldeoxycorticosterone molecule in the Digitalis lanata suspension culture (Agrawal et al. 2012). Therefore, biotransformation is an alternative way for the semi-synthesis of new novel products which can reduce the energy, cost of manufacture, and undesired by-products.

11.4.7 Membrane Permeabilization and Its Applications

Plant cells are semipermeable in nature which results in the blockage of synthesized metabolites inside the vacuole only. Permeabilization facilitates the release of products without influencing the biosynthesis capacity and cell viability of plant tissue culture. Permeabilization can be achieved by using UV radiations, electric pulses, high pressure, thermal application, and sonication. In the suspension culture of *Taxus chinensis*, the addition of hexadecane, decanol, and dibutyl phthalate in low concentrations leads to the release of paclitaxel into the medium (Wang et al. 2001). XAD-7 resin has been used for the enhanced production of paclitaxel, serpentine, anthraquinones, ajmalicine, and plumbagin (Malik et al. 2013). These processes can improve the culture productivity, product recovery, and mitigating toxicity which result in the low downstream processing cost (Ochoa-Villarreal et al. 2016).

11.5 Cell, Tissue, and Organ Cultures for the Secondary Metabolite Production

11.5.1 Cell Suspension Cultures

For the commercial production of secondary metabolites, cell suspensions are cultured by directly inoculating callus into the liquid media. These inoculated flasks kept for continuous agitation in horizontal shakers are later transferred to bioreactors columns (Bourgaud et al. 2001). During the past decades, secondary metabolite production by cell suspension cultures has been attracted much industrial and academic interest because it can solve the issues related to product quality due to environmental influence (Rao and Ravishankar 2002; Yamamoto et al. 2002). In the suspension cultures of *Vitis vinifera*, the production of anthocyanin accumulation is increased after addition of jasmonic acid or light irradiation. After a week, maximum anthocyanin produced was 13.8 CV (color value)/g FCW (fresh cell weight) (Zhao et al. 2010).

11.5.2 Callus Cultures

Callus is a growing mass of undifferentiated cells which is derived from plant parenchyma cells and extensively used in tissue culture research. Callus culture can be defined as the callus cells culture into the medium containing different phytohormones in an appropriate quantity. The origin of callus cultures can be embryogenic or non-embryogenic in nature. The callus cultures of *Maackia amurensis* plant were analyzed for different isoflavonoids, and the maximal yield of isoflavones was found to be 20.8 mg/g (DCW) which is four times higher than the naturally synthesized by plant (Fedoreev et al. 2004). The callus cultures of *Sericostoma pauciflorum was* reported to synthesize bio-active molecules like *β-sitosterol and caffeic acid*, which are used in anticancer and antidiabetic drugs (Jain et al. 2012). In the callus cultures

of *Sophora flavescens*, addition of polysaccharides for the production of flavanones (sophoraflavanone G and lehmanin) was studied, and it was found that the production of these flavanones was enhanced by fivefold with addition of 2 mg/ml yeast extract (Yamamoto et al. 2002). The quantification and distribution of taxol in explants and callus culture of different *Taxus* sp. like *Taxus baccata*, *Taxus canadensis*, *Taxus brevifolia*, and *Taxus cuspidate* was studied. It was found that the taxol production is highest in *T. canadensis* culture as compared to other species.

11.5.3 Organ Cultures

There are mainly two organs, shoot and root, which are used as organ cultures. Shoot cultures are generally used to overcome the dependency of commercial products on the natural plant products (Khanam et al. 2000) and also to bring in vitro somaclonal variations for the selection of high-yielding clones (Dhawan et al. 2003b). In a study, seeding explants are used for shoot organogenesis for the production of aucubin and verbascoside and is over bacoside molecules and shown to be highly and regenerative in nature even after 4 years (Piątczak et al. 2015). Root cultures are a good source for the production of valuable medicinal secondary metabolites (Pence 2011; Li et al. 2002). Root cultures are used for the production of important medicinal alkaloid compounds such as hyoscyamine and scopolamine (Fazilatun et al. 2005). Though there are several bioreactors which are used for the organ cultures (Kašparová et al. 2009) (Kim et al. 2002), the main problem is the scale-up at the commercial level due to their poor sensitivity to shear stress (Kaimoyo et al. 2008) and high maintenance and equipment cost.

11.5.4 Hairy Root Cultures

Hairy roots show rapid and plagiotropic growth and are highly branched in nature which can be easily subcultured on a phytohormone-free synthetic medium (Hu and Du 2006). These cultures are derived when the *Agrobacterium rhizogenes bacteria* successfully transformed into plant cell. Transformed cultures have been used for artificial seed production. These cultures can produces metabolites without losing their genetic stability even after many successive generations (Giri and Narasu 2000). On the other hand, adventitious root cultures can simultaneously produce two different secondary metabolites (Wu et al. 2008). Hairy root cultures of *Lithospermum erythrorhizon* and *Harpagophytum procumbens* (Ludwig-Müller et al. 2008) were studied for the production of shikonin and harpagoside, respectively, whereas *Panax ginseng* (Jeong et al. 2008) and *Scopolia parviflora* (Min et al. 2007) cultures were used for the production of ginsenosides and alkaloids, respectively. Transfection of *Agrobacterium rhizogenes* with cucurbits produces the hairy root culture which is characterized by a geotropism, and lateral branching shows to produce high levels of secondary metabolites (Rekha and Thiruvengadam

2017). Thus, the commercial production of secondary metabolites can be greatly improved by above discussed tissue culture techniques.

11.6 Large-Scale Production of Secondary Metabolites Using Plant Tissue Culture

With the research and technological advancements in the area of plant tissue, culture has resulted in the commercial production of many important pharmaceutical secondary metabolites like alkaloids, flavonoids, and steroids (Yamamoto et al. 2000b). For example, the industrial-scale production of shikonin by cell cultures of *Lithospermum erythrorhizon* is mainly based on the classical approach. This process yields shikonin with a rate of 60 mg/g/week which is around 1000-fold higher than the natural production by plant roots (Yamamoto et al. 2000a).

11.6.1 Taxol

Taxol is an alkaloid found in the bark of the *Taxus* tree which has anticancerous properties and is one of most promising natural anticancer drugs available in the market (Cusido et al. 2014). In order to achieve the maximum yield of taxol, several manipulations are used in the culture medium. The production of taxol in the callus culture of different *Taxus* sp. like *Taxus baccata*, *Taxus canadensis*, *Taxus brevifolia*, and *Taxus cuspidate* was studied. It was found that the taxol production is highest in *T. canadensis* culture as compared to other species. Taxol is also produced by the suspension culture of *Taxus baccata* and further optimized for its large-scale production (Malik et al. 2011).

11.6.2 L-DOPA

L-dopa is also known as levodopa or L-3,4-dihydroxyphenylalanine. It is known as a precursor for the production of betalain and melanin alkaloids in plants such as *Baptisia*, *Lupinus*, *Mucuna*, and *Vinca faba (Giray Kurt* et al. 2009). L- DOPA also acts as a precursor for the neurotransmitters in animals. The deficiency of these neurotransmitters leads to a progressive disabling disorder called as Parkinson's disease. Hence, L-DOPA is being used as a potent drug against this disease, and, therefore, its demand in market is increased drastically in last few years. The production of L-DOPA was carried out by classical approach in different *Mucuna* sp. Suspension cultures of *M. pruriens* and *M. prurita* by using methyl jasmonate and chitin as elicitors and L-tyrosine as precursor in the culture medium enhance the production of L-DOPA. The efficiency of L-DOPA production was found to be higher in *M. pruriens* culture as compared to *M. prurita* culture (Raghavendra et al. 2012).

11.6.3 Morphine and Codeine

Morphine and codeine are produced by *Papaver somniferum plant. This alkaloid shows potential analgesic properties and commercially used in most of the pain killer medicines.* The callus cultures of *P. somniferum* were reported to produce morphine and codeine at threefold yield (Li Siah and Doran 1991). Stepwise culture methods are used using genetically engineered strains for the production of thebaine with a 300-fold increased yield (Nakagawa et al. 2016).

11.6.4 Berberine

Berberine is an alkaloid which is found in the roots of *Coptis japonica* and cortex of *Phellodendron amurense* plants. It shows an antibacterial activity against various Gram-negative and Gram-positive bacteria. A suspension culture of *C. japonica* is *used to produce berberine at large-scale production* (Mulabagal and Tsay 2004).

11.7 Conclusions and Future Prospects

In last few decades, there is a drastic increase in the market of natural products, and the trend will continue in the future. Since the availability of medicinally important secondary metabolites is very limited and people start to use natural products, it is important to search for alternative ways for the commercial production of therapeutic metabolites. It is widely recognized that cultured plant cells can be potentially used as a source of valuable natural bio-active compounds but unfortunately only a few cell cultures are commercially used as stable source for the secondary metabolite production. The major advantage of cell culture technique is to provide a controlled and stable environment for the synthesis of secondary metabolites which is independent of environmental factors which can affect their synthesis and yield. In last few decades, many strategies, such as high-yielding cell line selection, media manipulation, elicitor and precursor addition, cell immobilization, and biotransformation, have been investigated for the best optimized conditions at large-scale production. In order to achieve this, it is very important to introduce new molecular biology and genetic tools to produce transgenic cultures which can affect the expression and regulation of biosynthetic pathways and overcome the dependency on artificial products. In recent years, the usage of plant cell culture systems is increased rapidly perhaps due to an improved understanding of the bio-active biosynthetic pathway in economically important crops. Advancement in the tissue culture field also provides a new means for the cost-effective and commercial production of exotic and rare plants and the compounds they produce. The combined efforts by interdisciplinary researchers of various fields like tissue culture, molecular biology, biochemistry, and downstream processing can exploit the potential of plant cells for the large-scale production of secondary metabolites. These classical and nonclassical approaches will enhance and extend usefulness of plants for the secondary metabolite production.

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12 Metabolic Engineering Strategies for Enhancing the Production of Bio-active Compounds from Medicinal Plants

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Abstract

Bio-active compounds, isolated from medicinal plants play a vital role in modern medicine as some of them have become very potent drugs. Although these compounds are produced and accumulated in different parts of the plants, there are many bottlenecks in getting plant-based bio-active compounds. In particular, phytocompounds are limited to a particular species/genus and are produced only under specific conditions, such as pathogen attack, stage of growth and development, etc. In this regard, metabolic engineering is very promising as it offers the possibilities of overcoming the dearth of desired plant compounds by using various strategies that include increased flux of precursors, blocking of competitive pathway by using the intermediate compounds, introducing new metabolic pathways, overcoming rate-limiting steps, and the overexpression of regulatory genes or transcription factors for inducing the biosynthetic pathways. The metabolic engineering approach has been exploited in transforming plants as "cell factories" for producing various bio-active compounds. Due to the tremendous biological potential of these compounds, the understanding of their synthesis, accumulation, and manipulation in different parts of the plant along with their regulation is very crucial. By utilizing different genomics and metabolomics tools, the production of various bio-active compounds has been enhanced. This chapter provides the present-day knowledge on the production of some plant-derived bio-active compounds, such as polyphenols, alkaloids, terpenes, and saponins. Further, various biotechnological approaches for increasing the accumulation of bio-active compounds through metabolic pathways engineering in plants are discussed in this chapter.

Keywords

Medicinal plants · Bio-active compounds · Metabolism · Metabolic engineering · Next-generation sequencing · miRNA profiling · High-throughput technologies · Genomics

12.1 Introduction

The term "bio-active" is a combination of two words, i.e., "bio" and "active," which corresponds to life and full of energy, respectively (Bernard and Dromard 2011). Scientifically, a bio-active compound is a substance that possesses

biological activities (Cammack et al. 2006). Since olden times, medicinal plants have been considered as the richest bio-resource of important drugs, which have been widely utilized for the therapeutic uses (Akthar et al. 2014; Swamy and Sinniah 2015; Swamy et al. 2016). In the modern society, the usage of herbal products as an alternative to synthetic medicines has increased dramatically (Choudhary et al. 2013; Dey and De 2015; Swamy and Sinniah 2016). Presently, plant-derived folk medicinal systems are continuing to play a vital role in the healthcare industry, and more than 80% of the global populaces depend on the plant-derived medications for the treatment of their primary health issues (Sudipta et al. 2014; Swamy et al. 2015). The growing interest of consumers toward phytocompounds is due to the fact that they are clinically safe and cause no or negligible side effects while treating many common diseases and chronic illnesses (Arumugam et al. 2016; Ahmed et al. 2018). In nature, plant-derived bio-active metabolites have a significant role in various physiological processes of plants, such as cellular respiration, pollination, color development, photosynthesis, nutrient assimilation, solute transport, and protein synthesis (Dhar et al. 2015).

Since the chemical synthesis method used for the production of drug has some constrains, the demand for plant-derived pharmaceutical compounds in recent years has been increased (Vogt 2010). Owing to various pharmacological activities of plant bio-active compounds and their extensive use in the pharmaceutical industry for commercial herbal preparations has led to the gradual depletion and extinction of medicinal plant resources from the natural populations due to their ruthless collection which causes the destruction of habitat. Moreover, the supply of phytocompounds is limited due to the fact that they occur in very low quantities in nature, and hence large amounts of plant materials are required for extracting the drug. Since the synthesis of a target bio-active phytocompound depends on various factors, such as plant's adjustment to variations in temperature, light conditions, and stress under the pathogen infections (Lucchesini et al. 2009), the procedures for their extraction from plant sources should be standardized for the commercialized production. On the other hand, field cultivation practices are time-consuming, quite expensive, and labor intensive and leave the material vulnerable to natural calamities. Therefore, viable alternative/methodologies, such as plant cell and tissue culture, biotic/abiotic elicitation, and genetic or metabolic engineering, need to be developed for germplasm conservation and for the manipulation of existing pathways for the enhancement and accumulation of medicinal compounds of commercial importance in plant or its parts.

Heterologous biosynthesis of target natural product in host organisms such as microbes or different plant species is another alternative approach for increasing the yield of required plant-derived bio-active compound. Being environmentally friendly in comparison with chemical synthesis process (Marienhagen and Bott 2013), it is one of the acceptable approaches for enhancing the production of desired bio-active compound, but there are certain limitations. Since the biosynthetic pathways of many valued plant compounds and their related genes are not yet fully elucidated (Wang et al. 2011; Miralpeix et al. 2013), there is a need to characterize such fundamental target pathways in medicinal herbs. The chapter summarizes different classes/subclasses of bio-active compounds available from natural sources,

along with the current information on various applications of recent biotechnological tools for the enhancement of these compounds in plants. The literature cited in the text on scientific developments latest technical progresses and research trends have evidently proved that bio-active compounds/metabolites are the most significant resources for new formulations of drugs in the future world.

12.2 Classification of Bio-active Compounds

Plants are considered as the most important resource of bio-active metabolites, which are classified either into primary or secondary metabolites depending on their functional roles (Wu and Chappell 2008; Talreja 2011). Some of the primary metabolites include sugars, nucleic acids, amino acids, fatty acids, and few other compounds that are needed by the plants for their growth and development. In contrast, secondary metabolites have no direct role in the plant's growth and development but provide additional benefits to the plants. Secondary metabolites perform diverse functions throughout the plant's life cycle including chemical communications between plants and their surrounding vicinities (Balandrin et al. 1985). On the basis of biosynthetic origin, secondary metabolites have been divided into three main groups: (i) phenolics (e.g., phenolic acids, coumarins, stilbenes, flavonoids, tannins, and lignin), (ii) terpenes (e.g., plant volatiles, cardiac glycosides, sterols, and carotenoids), and (iii) nitrogen-containing compounds (e.g., alkaloids and glucosinolates) (Rea et al. 2010; Krzyzanowska et al. 2010).

Terpenoids, the organic chemicals derived from terpenes, represent the largest and highly diverse class of biologically active natural products that are identified with different structures (>50,000) till date. They are chiefly formed in the vegetative tissues, such as leaves, flowers, and roots (Dudareva et al. 2004). Terpenoids range from linear to polycyclic molecules with variable sizes from 5-carbon hemiterpenes with a single isoprene unit to natural rubber, a polymer of isoprene units (Misawa 2011; Vranova et al. 2012). Further, they are classified into sesquiterpenoids (C_{15}), monoterpenoids (C_{10}), diterpenoids (C_{20}), triterpenoids (C_{30}), and tetraterpenoids (C_{40}) depending upon the number of isoprene units (Smanski et al. 2012; Sato 2013).

Terpenoids include both primary and secondary plant metabolites with wide-ranging functional roles, for example, as hormones (e.g., abscisic acid, brassinosteroids, cytokinins, and gibberellins), photosynthetic pigments (e.g., chlorophyll, phytol, and carotenoids), electron carriers (e.g., ubiquinone and plastoquinone), essential constituent of membranes (e.g., phytosterols), and polysaccharide assembly mediators (e.g., polyprenyl phosphates) (McGarvey and Croteau 1995). Terpenoids isolated from plant sources such as ginkgolides, artemisinin, Taxol, and crocetin have been reported to act therapeutically against a variety of diseases (Stromgaard and Nakanishi 2004; Weathers et al. 2006; Lenka et al. 2012; Dhar et al. 2017). In addition, many specific terpenoids serve as attractants for pollination and help in defending plants against herbivores and pathogenic attacks (Gershenzon and Dudareva 2007; Parsaeimehr et al. 2011).

Phenolic compounds are very widespread metabolites in nature and can be described as the compound containing an aromatic ring structure with single or several hydroxyl substituents including the functional derivatives, such as esters, methyl ethers, and glycosides (Parsaeimehr et al. 2011). These compounds have assorted structures, which include both soluble compounds (e.g., flavonoids, phenolic acids, and quinones) and non-soluble compounds (e.g., cell wall bound hydroxycinammic acids, lignins, and tannins) (Krzyzanowska et al. 2010). Phenolic compounds such as stilbenes and flavonoids possess active pharmacological and biological activities. Flavonoids represent important phenolic compounds present in different tissues (fruits, roots, leaves, and tubers) of edible crops as well as in coffee, legumes, tea herbs, and spices. Flavonoids originate from a specific product, i.e., chalcone, which further gets diversified into multiple biosynthetic branches to include anthocyanins, flavonols (quercetin and myricetin), and isoflavones (daidzein and genistein).

Phenolics are considered as the best antioxidants due to the presence of phenol moiety, which is highly reactive in nature. They play a critical role in radical scavenging either by hydrogen atoms or electron donation along with the delocalization of the unpaired electron inside the aromatic ring (Fernandez-Panchon et al. 2008). Phenolics are involved in various kinds of plant physiological actions, such as pigmentation, reproduction, pathogenic resistance, and stresses due to heavy metals and various biotic and abiotic factors (Ferrari 2010; Cheynier et al. 2013).

Nitrogenous compounds, for example, alkaloids and glucosinolates, are pharmacologically active compounds produced by living organisms, and they contain either a single or multiple heterocyclic nitrogen atoms. Alkaloids with >12,000 isolated structures are one among the major nitrogen-containing compounds, where nitrogen is derived from amino acids, like tryptophan, tyrosine, lysine, and aspartate. They are grouped based on their ring structure containing nitrogen (either pyrrolidine or piperidine) and the origin of their biosynthetic pathways (alkamides, amino acids, cyanogenic glycosides, and amines) (Paiva et al. 2010; Khadem and Marles 2012). The presence of different alkaloids protects the plants against major UV radiation, pathogen, and herbivore attack (Parsaeimehr et al. 2011; Bohinc et al. 2012). Glucosinolates represent diverse functional groups (~200 compounds) of sulfur which are derived from glucose (Angelova et al. 2010). They are relatively small but possess nutritional importance. Depending upon the amino acid structure, glucosinolates can be grouped into either aliphatic-, aromatic-, or indole-type compounds (Halkier and Gershenzon 2006). Aliphatic glucosinolates are derived from alanine, leucine, isoleucine, valine, and methionine, while aromatic and indole glucosinolates are derived from phenylalanine, tyrosine, or tryptophan. A large number of glucosinolates have nutritional importance, for example, red radish and broccoli contain glucoiberin and glucoraphenin. Likewise, red and white cabbage, cauliflower, and brussels sprouts possess progoitrin and sinigrin, whereas mustard and horseradish are contained with sinigrin and gluconasturtiin (Ishida et al. 2014). At the time of cell damage/repair cycle, myrosinase enzyme hydrolyzes the glucosinolates in cell vacuole and then cleaves off the glucose unit from its structure. The remaining molecules get rapidly transformed into thiocyanate, isothiocyanate, or

nitrile that work as natural pesticides and provide defense against various pathogens (Paiva et al. 2010; Agerbirk and Olsen 2012).

12.3 Biogenesis of Plant-Derived Bio-active Compounds and Their Applications

The term biogenesis refers to the process of synthesis of bio-active compounds/metabolites from a sequence of various biosynthetic reactions in a pathway (Bruneton 1999). The natural products are assorted on the basis of their biogenesis where the photosynthetic process plays an active role. Many researches have demonstrated the biosynthesis of terpenes via mevalonic pathway, which uses acetyl-CoA as the precursor, while phenolic compounds are made through shikimate or mevalonic pathway. Also, alkaloids are biosynthesized basically from aromatic acids derived from the shikimate pathway or from the aliphatic amino acids derived from tricarboxylic acid pathway (Fig. 12.1).

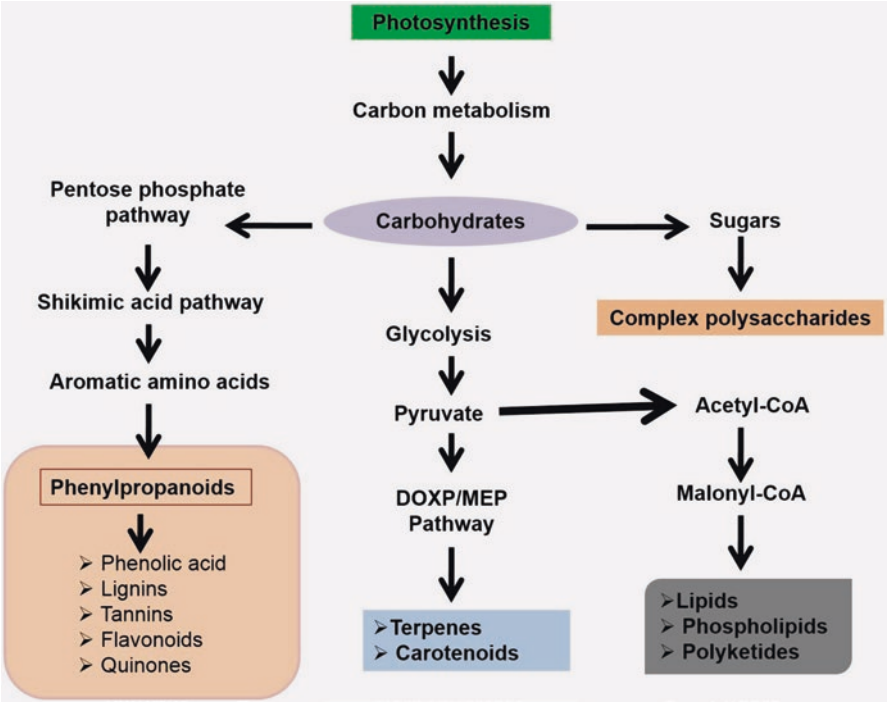

Fig. 12.1 General biosynthetic pathway of secondary metabolites

12.3.1 Biogenesis of Terpenoids

Terpenoids are synthesized from the basic isoprene units, dimethylallyl diphosphate (DMADP) and isopentenyl diphosphate (IDP) derived from two independent pathways. One of the above pathways, i.e., methylerythritol phosphate (MEP) pathway, is confined within the plastids and commences with the synthesis of 1-deoxy-D-xylulose 5-phosphate (DXP) produced from pyruvate (PYR) and D-glyceraldehyde-3-phosphate (GAP) (Banerjee and Sharkey 2014), while the other pathway, i.e., mevalonic acid (MVA) pathway, occurs in cytosolic, endoplasmic reticulum and peroxisomes and consists of six enzymatic steps (Gutensohn et al. 2014). 3-Hydroxy-3-methylglutaryl-CoA reductase (HMGR) is considered to be a rate-limiting enzyme of the pathway which is localized in the peroxisomes (Opitz et al. 2014). The prenyltransferases condense both IDP and DMADP to form intermediate linear terpenoid molecules that act as the precursors for the synthesis of various terpenoids. The condensation of IDP and DMADP molecules mediated by geranyl diphosphate synthase (GDS) results to form C_{10}-compound, i.e., geranyl diphosphate (GDP) within the plastid. One molecule of DMADP and two molecules of IDP are converted into C_{15}-compound farnesyl diphosphate (FDP), which acts as the precursor for sesquiterpenoid synthesis by farnesyl diphosphate synthase (FDS) in the cytosol. Moreover, squalene (the precursor of triterpenoids) is formed by the condensation of two molecules of FDP by squalene synthase (SQS) localized in the ER membrane. Similarly, the biosynthesis of diterpenoids takes place by the condensation of one molecule of DMADP and three molecules of IDP using geranylgeranyl diphosphate synthase (GGDS) in the plastid. Finally, two GGDP molecules lead to the synthesis of a tetraterpenoid phytoene in the plastid (Loto et al. 2012).

12.3.2 Biogenesis of Terpenoid Indole Alkaloids

Terpenoid indole alkaloids, such as quinine, ajmalicine, strychnine, and vincamine, are widely distributed in angiosperm families, namely, Rubiaceae, Loganiaceae, Apocynaceae, and Nyssaceae (Yamamoto et al. 2000). These alkaloids consist of a terpenoid and indole components, which are derived from the secologanin and tryptamine, respectively. Tryptamine is obtained by the decarboxylation of tryptophan catalyzed by the enzyme, tryptophan decarboxylase, while secologanin is produced via the triose phosphate/pyruvate pathway, and its synthesis plays an important regulatory role in terpenoid indole alkaloid biosynthesis. Hydroxylation of geraniol to 10-hydroxygeraniol represents the first committed step in secologanin biosynthesis which ends up with the biosynthesis of secologanin from loganin (Yamamoto et al. 2000). Strictosidine, the precursor for the synthesis of all terpenoid indole alkaloids, is formed by a Pictet-Spengler condensation reaction of tryptamine and secologanin which is catalyzed by strictosidine synthase. The subsequent removal of the strictosidine glucose moiety leads to the production of strictosidine-derived aglycone which is further converted via several unstable intermediates to

dehydrogeissoschizine. This step represents a key branch point from where several diverse terpenoid indole alkaloid pathways are formed (Ziegler and Facchini 2008).

12.3.3 Biogenesis of Phenolic Compounds

Phenolic compounds have a high chemical diversity and are derived from the shikimate, phenylpropanoid, and flavonoid pathways (Krzyzanowska et al. 2010). The phenolic compounds, i.e., phenylpropanoids with a C6-C3 skeleton, are the products of the general phenylpropanoid (phenylalanine/hydroxycinnamate) pathway, where the first step includes the conversion of *L*-phenylalanine to the hydroxycinnamic acids (Wink 1999). Phenylalanine ammonia-lyase is the first enzyme involved in this pathway (Koukol and Conn 1961). It catalyzes the deamination of *L*-phenylalanine to (*E*)-cinnamate, which is further hydroxylated to 4-coumarate by cinnamate 4-hydroxylase enzyme (Gabriac et al. 1991). The 4-coumarate-CoA ligase catalyzes the activation of HCAs (4-coumarate, caffeate, ferulate, and sinapate) to the respective CoA esters (feruloyl-CoA, caffeoyl-CoA, 4-coumaroyl-CoA, and sinapoyl-CoA) (Hamberger and Hahlbrock 2004). The synthesis of caffeate, ferulate, and sinapate takes place by the hydroxylations and methylations at different steps of the pathway. The biosynthetic pathways leading to produce lignin, coumarins, lignans, hydrolyzable tannins (gallotannins and ellagitannins), and flavonoids which are derived from phenylpropanoids are the most elucidated biosynthetic pathways in the plant kingdom (Seigler 1998).

12.3.4 Application of Bio-active Compounds in Human Health

The usage of medicinal plants for producing various bio-active phytocompounds and recombinant molecules of industrial interest has received growing interest over the past two decades. In spite of the abundance and development of synthetic drugs, a percentage of the populace of growing nations still depend upon conventional medicines system for their health care requirements (Lesney 2004). Typically, bio-active compounds are not involved in metabolism of plants; however they play essential roles in the survival of plants by using their capabilities in the protection of the plant toward pathogenic organisms and predatory herbivores (Bernhoft 2010; Ingebrigtsen 2010). Moreover, bio-active compounds may also function as vehicle for the removal of nitrogenous waste products and maintenance of plants life during drought stress (Belonwu et al. 2014; Tadele 2015).

Nutritional flavonoids, such as kaempferol, quercetin, and isorhamnetin, have anti-inflammatory, antihistamine, and antioxidant activities. They inhibit the peroxidation of lipids, exhibit free radicals scavenging activity, and modulate various cellular signaling pathways (Cote et al. 2010). It has been reported that a constant consumption of polyphenol-rich cocoa helps to control blood pressure, increases cerebral blood flow, and boosts the mental health (Kim et al. 2011). Flavonoids shield the oxidation of low-density lipoprotein cholesterol that prevents the

deposition of atherosclerotic plaques in the arterial wall. Saponins also reduce blood cholesterol level, prevent growth of cancer cells, and help in the stimulation of the immunity. Some saponins, including sapotoxin, may be toxic for human beings and induce inflammation in the gastrointestinal tract (Satwadhar et al. 2011).

Alkaloids are known to be pharmacologically active compounds responsible for appetite loss and also act as diuretic (Yadav et al. 2014). The available reports have confirmed the therapeutic applications of alkaloids as local anesthetic and anti-arrhythmia (Kuete 2014), stimulants (e.g., theobromine, caffeine, nicotine, and methylated derivatives of uric acids, such as theacrine, methylliberine, and libertine) (Ramawat et al. 2009), anti-cancer drugs (e.g., camptothecin, vinblastine, vincristine) (Parekh et al. 2009; Ramawat et al. 2009), cholinomimetics (e.g., aceclidine) (Kuete 2014), and antimalarial (e.g., quinine and artemisinin) (Ramawat et al. 2009). The antimicrobial, antiparasitic, antihyperglycemic, antiviral, antiallergenic, anti-inflammatory, and immune modulatory activities of terpenoids have been demonstrated by various research groups (Wagner and Elmadfa 2003; Rabi and Bishayee 2009; Kumar et al. 2011).

12.4 Strategies of Metabolic Engineering Used for the Enhancement of Bio-active Compound

Plants contain a number of metabolic pathways required for the biosynthesis of various biologically active metabolites. Metabolic engineering is an alternative approach for unraveling and improving the valuable metabolites in plants. In plants, many biotechnological techniques have been used for the stimulation of synthesis of bio-active compounds, screening and selection of highest producing cell line, culture media standardization, elicitation, commercial production using bioreactors, cell immobilization, feeding metabolic precursors, and biotransformation (Parsaeimehr et al. 2011) (Fig. 12.2). The latest developments in the area of gene editing techniques have considerably helped in exploiting the capabilities of plant cells to increase the production of bio-active compounds (Woo et al. 2015).

12.4.1 Plant Cell Culture as Factories for the Production of Natural Bio-active Compounds

Only about 10% of the medicinal plants are cultivated, while the remaining 90% are exploited from the wild (Julsing et al. 2007). Harvesting of herbal products from the wild population results in the loss of genetic diversity along with the destruction of plant habitats and ecosystems. Therefore, it is necessary to develop the appropriate strategies/mechanisms for rapid propagation and the improvement of medicinally important plants for enhanced productivity. Together with domestication, the practice of good agricultural practices is the key step toward the standardization and commercial production of quality medicinal plants. Tissue culture techniques have added advantages over conventional methods of propagation, for meeting

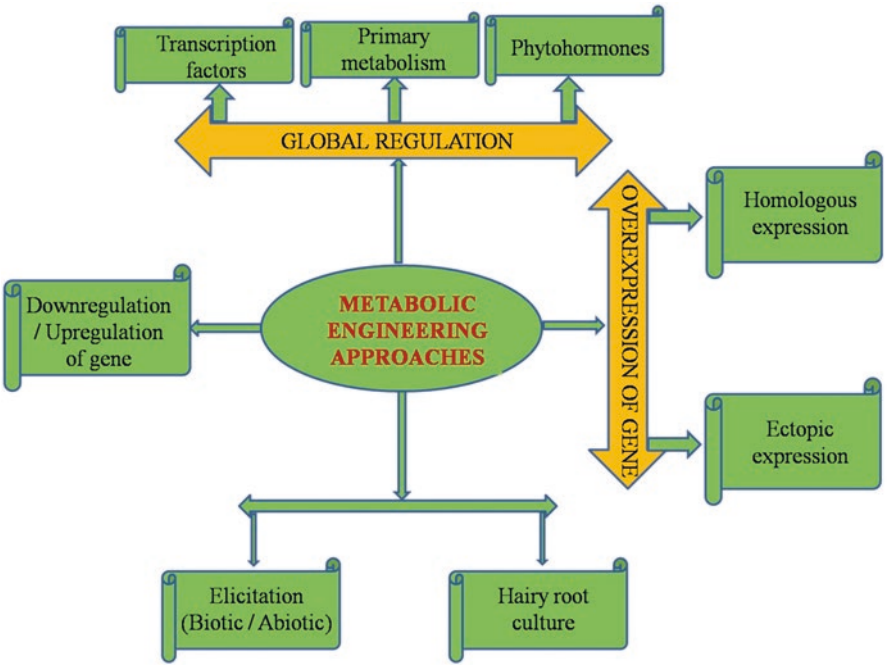

Fig. 12.2 Plant metabolic engineering approaches employed for stimulating bio-active compounds

ever-growing demand of the medicinal plants (Rout et al. 2000). It also acts as an alternative means for the synthesis of medicinally/industrially valued metabolites through the cell and organ culture in various plants (Vanisree et al. 2004; Vongpaseuth and Roberts 2007; Sharma et al. 2015).

Plant cells act as factories for producing chemical compounds that are useful products of biochemical pathways for survival and propagation of the plants (Hadacek 2002). Plant cell culture systems were introduced during the 1960s as a potential tool for yielding biologically active compounds. Since then, various strategies for to enhance the yield of bio-active compounds in cell culture systems have been developed. The cell culture system has emerged as a feasible biotechnological tool and is widely used to culture plant cells in mass scale for obtaining bio-active principles. For example, geraniol, an important monoterpenoid used in flavor and fragrance industries has been produced using cell culture approach (Chen and Viljoen 2010). For the production of other natural products, cell culture has proved to be commercially viable system that can be handled simply (Vasilev et al. 2014).

The plant cell culture is a well-established technology platform to produce plant pharmaceuticals continuously. This approach allows cells to proliferate at higher growth rates under a closed and controlled environment and is independent of environmental or climatic conditions (Niraula et al. 2010; Rahimi et al. 2012). Hence, in vitro culture has been recommended as a process for the production of natural

bio-active compounds for food purposes by the Food and Agriculture Organization of the United Nations (Anand 2010; Roberto and Francesca 2011). Some of the important metabolites produced by plant cell cultures at a higher yield in comparison with parent plants are compiled in Table 12.1.

For the commercialization of natural products, the most critical step includes the scaling-up of the culture production from laboratory to industrial scale. Therefore, it is important to choose a bioreactor that can fulfill the adequate biological requirements and engineering needs of the culture. The scaling-up step from shake flask cultures to bioreactors requires the optimization of basic culture parameters, such as nutrient availability, low-shear environment, and adequate mixing and reducing mass transfer for improved oxygen (Georgiev et al. 2013). Plant cells have been cultured in a variety of bioreactors, namely, air-life bioreactors (ALB), bubble column bioreactors (BCB), standard stirred-tank bioreactors (STR), and recent bioreactor designs (e.g., hollow fiber bioreactors, wave bioreactors, membrane bioreactors, and rotating drum reactors) (Huang and McDonald 2012). Among all, STR is the most widely employed bioreactor due to the fact that it offers easy scaling-up process, oxygen transfer ability, and good fluid mixing and relatively matches all the requirements of the present good manufacture practices. However, the BDB and ALB bioreactors are less expensive, and in some cases, they provide an easy route to scale-up, and their construction is more straightforward as compared to other reactors (Ochoa-Villarreal et al. 2016).

12.4.2 Targeted Production of Natural Compounds to Particular Plant Cell Compartments

Plant cell has a complex intercellular organization, where the flow of metabolites is highly controlled and coordinated among different compartments depending upon the specific requirements of the plants (Wu and Chappell 2008; Hendrawati et al. 2012).

Table 12.1 Comparison of product yield of secondary metabolites in cell culture and parent plant

Plant	Product	Yield (% dry weight)		References
		Cell culture	Plant	
Catharanthus roseus	Ajmalicine	1.0	0.3	Lee and Shuler (2000)
Morinda citrifolia	Anthraquinones	18	2.2	Zenk (1977)
Coptis japonica	Berberine	13	2	Fujita and Tabata (1987)
Vanilla planifolia	Caffeic acid	0.02	0.05	Knorr et al. (1993)
Panax ginseng	Ginsenoside	27	4.5	Matsubara et al. (1989)
Nicotiana tabacum	Nicotine	3.4	2.0	Mantell et al. (1983)
Coleus blumei	Rosmarinic acid	27	3	Petersen and Simmond (2003)
Lithospermum erythrorhizon	Shikonin	20	1.5	Kim and Chang (1990)

Factors contributing to the overall production of complex secondary metabolites include the versatile nature of compartmentalized enzymes and their substrates, precursor requirement, and metabolic intermediates (Gomez-Galera et al. 2007; Kayser and Warzecha 2012). The compartmentalization plays a major regulatory role in the biosynthesis of secondary metabolites as most of the biosynthetic pathways operate in different compartments of the plant cell. For example, GAP/pyruvate pathway occurs in the plastids and thus represents the major source of terpenoids, such as mono- and diterpenoid biosynthesis in plastids (Lichtenthaler et al. 1997). Similarly, terpenoid-type indole alkaloids biosynthesis needs three compartments, namely, plastids for terpenoid moiety and tryptophan, cytosol for the decarboxylation of tryptophan, and vacuole for coupling of tryptamine with secologanin (Verpoorte et al. 2000; Kirakosyan et al. 2009).

Plants also possess several specific and differentiated organs, where various physiological processes/pathways and gene expression vary considerably. Also, the compartmentalization of secondary metabolic biosynthetic paths occur at subcellular levels (Pasquali et al. 2006), and both the temporal and developmental progressions influence or decide on whether and when a gene has to be active or inactive. Intra- and intercellular location of the enzymes is another key factor influencing on the secondary metabolite production. Studies have suggested that the localization of enzymes to cellular compartments has proved to be helpful in proper protein assembly of the alkaloid biosynthetic pathway (Gomez-Galera et al. 2007; Ziegler and Facchini 2008). Targeted expression of genes to a specific compartment/organelle that carries specific precursors has increased the level of target metabolites/compounds. For the targeted gene expression, specific amino acid sequences have been reported that help in retaining the proteins to the specified organelles (Lessard et al. 2001). By using the above tactic, the target genes can be overexpressed either to cytosol or plastids, allowing the transport of common precursors into a proper direction of metabolic flux, which leads to >1000-fold increase in the levels of sesquiterpenes (patchouli alcohol and amorpha-4,10-diene). Likewise, in transgenic tobacco plants, the production of monoterpene limonene was found to be about 10- to 30-fold higher when compared to control plants (Brouwer et al. 2002; Hendrawati et al. 2012).

12.4.3 Upregulation/Downregulation of Biosynthetic Pathway Genes Involved in the Production of Bio-active Compounds

The regulation of expression of various genes constitutes the most vital step in plant developmental processes leading to the production of large number of bio-active metabolites in different compartments of a plant cell. The regulation relies on large number of mechanisms that lead to the differential (increased/decreased) production of gene product, i.e., protein. Gene expression can be manipulated at any step during transcriptional initiation, to RNA processing and finally to the posttranslational modification of protein products (Petrillo et al. 2014) (Fig. 12.3). In a gene regulation network, one gene controls another gene in so many different ways.

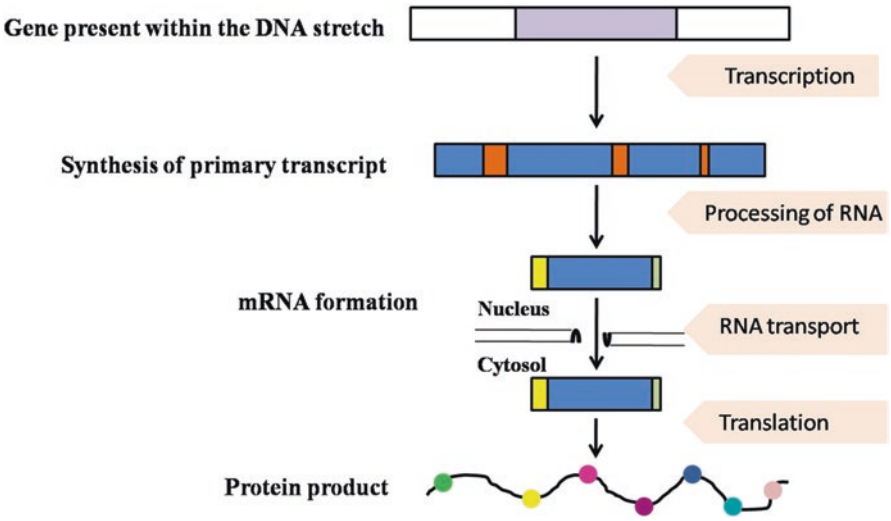

Fig. 12.3 Schematic representation showing that at which stages DNA/mRNA/protein pathway expression can be controlled

Upregulation in plants is triggered by a signal (internal/external), which culminates in increased expression of one or more genes within the cell resulting in specific protein products encoded by those genes. On the other hand, the process of downregulation results in the decreased gene expression and finally decreased synthesis of protein products.

The gene regulation in various kingdoms is carried out by either upregulation or downregulation of key genes involved in a particular pathway like bio-active metabolic pathways in plants. In the latter case, upregulation of various genes is exhibited by many mechanisms such as using strong promoters, modulating transcription factors, inserting highly expressed genes taken from other plant species, etc. so as to increase the production of various bio-active metabolites in different parts of the plant (Dhar et al. 2015). Conversely, downregulation is carried out by knocking down the expression of genes operating in competitive pathway so as to increase the metabolic flux toward the biosynthesis of desired bio-active metabolite in a specific plant species.

Biosynthesis of phenolic compounds starts from phenylalanine which gets converted into cinnamic acid by phenylalanine ammonia-lyase (PAL), a key enzyme involved in the biosynthesis of various plant secondary metabolites. In many plant species, multiple genes encode this PAL enzyme which shows differential expression in specific tissues and under definite controlled conditions. This makes the regulation of *PAL* gene very complex which further hints toward the multifaceted dynamics of signaling mechanisms and biosynthesis of secondary metabolites in plants (De Jong et al. 2015; Cheng et al. 2015).

Jasmonic acid (JA) is a key signaling molecule, which actively participates in the processes of secondary metabolite synthesis, e.g., triggers the emission of volatile

products, like homoterpenes (Nabity et al. 2013). JA has been found to enhance secondary metabolite levels due to activation of genes controlling the synthesis of PAL enzymes (Dar et al. 2015).Upregulation/overexpression of transcription factors (TFs) has led to the increased gene expression and finally the enhanced metabolite in the plant cell (Fits and Memelin 2000; Li et al. 2014; Olivoto et al. 2017). Fits and Memelin (2000) demonstrated that overexpression of genes encoding TFs, i.e., MYB that regulate JA, resulted in higher accumulation of terpenoids in *Arabidopsis*. There are genetic and physiological evidences in favor of *myb* regulation triggered by JA resulting in immediate accumulation of anthocyanins. Also overexpression of TF MYB75 led to the accumulation of anthocyanins in *Arabidopsis* Coronatine-Insensitive 1 (*coi1-1*) mutants in comparison with the wild-type genotype. Van Acker et al. (2014) characterized a key enzyme, cinnamoyl-CoA reductase (CCR), linked to the biosynthesis of lignin. CCR is reported to convert hydroxycinnamoyl-CoA esters to their corresponding aldehydes. The authors demonstrated that down-regulation of *ccr* gene increases production of ethanol after processing of wood. Although downregulation of *ccr* gene resulted in the reduced biomass because of inferior growth rates, the total yield of saccharification suggested that this strategy of downregulating the associated genes could lead to a higher production of biofuel, efficiently.

The *SQLE* gene, coding for squalene epoxidases (SE), catalyzes the rate-limiting step of biosynthesizing triterpenoid saponins and phytosterol. RNA silencing of *pgSQE1* gene derived from *Panax ginseng* led to the downregulation of *pgSQE1*, which resulted in the reduced production of pharmacologically important ginsenosides (Han et al. 2010; Guo et al. 2016). This suggested that overexpression of *pgSQE1* gene possibly enhances the production of ginsenosides in *P. ginseng*. Transgenic *Artemisia annua* (which contains artemisinin, an effective antimalarial drug), expressing an hpRNA construct which targets squalene synthase (SQS), a key enzyme involved in the biosynthetic pathway of sterol, considerably improved artemisinin production (~3 fold) in comparison with control plants (Zhang et al. 2009; Guo et al. 2016).

In transgenic *Papaver somniferum* (Opium poppy) plants, the downregulation of codeinone reductase (COD) by using RNA silencing approach resulted in the increased yield of nonnarcotic alkaloid, reticuline (a key compound derived from isoquinoline alkaloid biosynthetic pathway), at the expense of the downstream products, including oripavine, morphine, thebaine, and codeine (Allen et al. 2004; Guo et al. 2016). Similar studies were conducted by Kempe et al. (2009), where the downregulation of salutaridinol 7-O-acetyltransferase (*salAT*) gene in *P. somniferum* effectively improved the yield of pharmaceutical products, salutaridine and salutaridinol.

Cassava (*Manihot esculenta*) represents one of the main staple foods in tropical nations but remains less utilized due to toxic compounds (cyanogenic glucosides) occurring in its tuber. In the transgenic cassava, the downregulation of CYP79D1 and CYP79D2 genes coding for cytochrome P450 enzymes that are responsible for catalyzing the first committed reaction in the biosynthesis of lotaustralin and linamarin significantly decreased cyanogenic glucoside production up to 90% levels in

its tuber (Siritunga and Sayre 2003; Guo et al. 2016). In plants, the activity of different genes of flavonoid biosynthetic pathway leads to the establishment of different colored petals and fruits. Flavonol synthase (FLS) is one of the key enzymes which lead to the production of flavonols. Overexpression of the gene encoding FLS in crabapple resulted in significant increase in flavonol content using transient expression systems (Tian et al. 2015). Thus, upregulation or downregulation of key genes leading to the biosynthesis of bio-active compounds could not only help in characterizing genes but also serve as a potential tool in the future for increasing the metabolic flux toward the production of important bio-active compounds in different parts of the plant.

Watanabe et al. (2017) elucidated the development of different carotenoids in the petals of Japanese morning glory, *Ipomoea nil*, which only accumulates the trace amounts of carotenoids. The downregulation of the key enzyme 9-cis-epoxycarotenoid dioxygenase (NCED) of the abscisic acid biosynthetic pathway by using RNA silencing method has led to the increased accretion of upstream metabolites, mainly the β-carotene and lycopene (Guo et al. 2016). Also, RNAi technology showed its utility in enhancing β-carotene and lutein contents in potato by downregulating the expression of β-carotene hydroxylase (BCH) that converts β-carotene to zeaxanthin (Van Eck et al. 2007; Guo et al. 2016).

12.5 Current and Emerging Trends Involved in Metabolic Engineering of Bio-active Compounds

With the advent of genome sequencing, gene editing technologies, and bioinformatics tools, the genes encoding various enzymes involved in specific metabolic pathways have become easy to discover, characterize, and manipulate so as to enhance the production of bio-active compounds (Olivoto et al. 2017). This strategy has become successful for the isolation and enhancement of high-value plant-derived bio-active compounds by either reconstituting the plant pathways in heterologous hosts or in native producer in order to confer new properties to the selected plant species (Tatsis and O'Connor 2016). Thus a huge potential for manipulating the metabolic pathways to produce and enhance the concentration of bio-active metabolites exists. This can be harnessed with large number of metabolic as well as genetic engineering approaches which have been highlighted below.

12.5.1 High-Throughput Sequencing/Genome-Wide Association Study (GWAS)

In the recent decade, the advent of high-throughput or next-generation sequencing technologies (NGS) has provided opportunities to revolutionize the discovery of new metabolites in plants, including biosynthetic pattern of genes, their regulation, regulatory networks, etc. through genome-enabled technologies. The approach employs a simple hypothesis of classical forward genetics, by which the novel

genes (DNA sequencing) and their expression patterns (RNA-Seq) are initially recognized through the NGS technologies which are further used to match the new proteins and metabolites via routine metabolomics techniques and proteogenomics (or metabologenomics). Various genes involved in the production of novel metabolites which affect the plant nutrients, taste, and tolerance to biotic and abiotic stresses, etc. have been found through NGS technologies. Nowadays, up to 80% of the sequencing of DNA extracted from plants, actively producing bio-active metabolites, has been carried out by Illumina® technology (read length 120–300 kb). However, some new high-throughput technologies such as PacBio® with longer sequencing reads (up to 15 kb) have emerged in order to facilitate the DNA sequencing with more specificity, sensitivity, and reliability (less error rate). Various analyses such as SNP/Indel detection, phylogenetic analysis, structural variation, network analysis, transcriptome analysis, and physical cluster identification can be carried out using freely available web-based and Linux-based bioinformatics tools such as Galaxy platform (https://usegalaxy.org/).

With the use of NGS methods, millions of SNPs can be discovered in a genome, in which many of them belong to the secondary metabolic pathways. Availability of both the phenotypic data and SNP mapping in a genome enable a user to discover the association at a genome-wide scale. NGS has led to the improvement of natural biomedicines and improvised the selection process of cultivars having good agronomic traits for producing increased levels of desired metabolites of pharmaceutical importance (Unamba et al. 2015; Hao and Xiao 2015). Till date, whole-genome sequencing data of 183 terrestrial plants have been released and submitted in the Genome Sequence DataBase (GSDB) of National Center for Biotechnology Information (NCBI), out of which, 58 have been completed and submitted in 2016 alone which are being used primarily for medicinal purposes. Thus NGS technology has provided the essential information related to the plant origin, evolution, heritable traits, physiological and developmental schematics, metabolic potential, and epigenomic regulation (Rai et al. 2017).

12.5.2 Role of miRNAs in Biosynthesis of Bio-active Compounds and Their Accumulation

Gene coding for the functional proteins is modulated by various types of regulatory factors, including microRNAs (miRNAs), transcription factors, etc. miRNAs represent small endogenous RNAs of about 20–24-nucleotide length, which act as an important regulator during posttranscriptional modifications via translational inhibition/repression or degradation of their target mRNAs through complementary base pairing (Zhang and Wang 2015). Recently, miRNAs are becoming new tool for crop improvement and protection through genetic engineering (Djami-Tchatchou et al. 2017). Several studies have showed that plant miRNAs prefer to target transcription factors, which are involved in numerous growth and developmental processes (Zhang 2015; Samad et al. 2017). Thus, alteration in the expression levels of miRNAs leads to the significant changes in plant growth and developmental

processes which ultimately effect the bio-active compounds/metabolites production (Rubio-Somoza and Weigel 2011; Kamthan et al. 2015).

The traditional method of engineering for targeting the protein-coding transcripts to enhance bio-active compounds is limited to a small number of genes. However, miRNAs are considered as the negative gene regulators, and they might target numerous genes at a time in a complex gene network. Therefore, miRNAs represent a new target for enhancing the yield or metabolite production directly (Zhang and Wang 2016). Recent studies have shown that the manipulation of a single miRNA gene can expressively improve both crop yield and metabolite production under different conditions like stress and cold/hot temperatures (Zhang and Wang 2015).

Due to the biological significance of plant derived bio-active metabolites such as carotenoids, flavonoids, and alkaloids etc. it is important to understand the regulatory mechanism involved in the activation of key genes responsible for their enhanced production. Presently, significant efforts have been made in characterizing the role of miRNAs regulating the synthesis of bio-active compounds in medicinal herbs. Gou et al. (2011) reported the role of miR156 during anthocyanin regulation in the stem tissues of *Arabidopsis* by targeting SPL9 protein. Higher expression of miRNA156 leads to the reduction of SPL activity, which subsequently enhances flavanone 3-hydroxylase, dihydroflavonol 4-reductase, and other anthocyanin biosynthetic gene expression, and leads to the production of higher levels of anthocyanin. Zhang et al. (2015) reported the involvement of miR8154 and miR5298b in the regulation of phenylpropanoid and flavonoid biosynthesis. Computational identification of several miRNAs (miR172i, miR1438, miR829.1, miR1873, and miR5532) was found to target important phenylpropanoid and flavonoid biosynthetic pathway genes (Biswas et al. 2016). In several other studies, miRNAs were seen to enhance the terpenoid biosynthesis in plants. For example, miRNA156 targets the transcription factor SPL9 and binds directly to the promoter of terpene synthase 21 (TPS21) gene and controls its expression to regulate the biosynthesis of sesquiterpenoid in *Arabidopsis* and *Pogostemon cablin* (Patchouli) (Yu et al. 2015). Similarly, miR4995 targets the gene coding for 3-deoxy-7-phosphoheptulonate synthase involved in the biosynthesis of picroside in *Picrorhiza kurroa* a medicinal herb (Vashisht et al. 2015). Likewise, in *Stevia rebaudiana* plant, 11 miRNAs validated by Saifi et al. (2015) reported their involvement in the biosynthesis of steviol glycoside. Further, the authors also have established a correlation between the steviol glycoside contents and the expression levels of their target mRNAs. In a similar way, researchers used NGS approaches to map and validate several other miRNAs that are involved in the biosynthetic pathway of sesquiterpene in *X. strumarium* (Gupta et al. 2017). Lately, bioinformatics techniques have been exploited to recognize the miRNAs that take part in the biosyntheis of terpenoid in *Podophyllum hexandrum* (Biswas et al. 2016) and *Mentha* spp. (Singh et al. 2016).

12.5.3 Gene Editing Using CRISPR/Cas9

Three main genome editing tools include TALEN (transcription activator-like effector nuclease), ZFN (zinc finger nuclease), and the CRISPR (clustered regularly interspaced short palindromic repeats)/CRISPR/Cas9 (CRISPR-associated protein 9) systems (Ma et al. 2015; Petolino 2015; Zhang et al. 2017). But the implementation of TALEN and ZFN tools is very laborious because of the complications in protein design, their synthesis, and validation. However, the above problem was overcome with the discovery of a CRISPR/Cas9 system, the most emerging and adopted tool because of its simplicity, flexibility, design, high efficiency, and low cost (Ma et al. 2015). This system has an endonuclease-type activity which breaks double strand at the specific genome sites (Fig. 12.4). Such breaks are further restored by the error-prone nonhomologous end joining (NHEJ) pathway, which often leads to loss of gene function due to bases deletion, insertion, or substitution event (Wang et al. 2017; Ma et al. 2015). Some of the genome editing approaches applied in plants are given in Table 12.2.

In *Salvia miltiorrhiza*, which is known for its antiarrhythmic and vasorelaxation effects, CRISPR/Cas9 system has been employed to knock out the committed gene *SmCPS1* coding for diterpene synthase involved in the biosynthesis of tanshinone using *Agrobacterium rhizogenes*-mediated transformation (Li et al. 2017). The diterpene synthase is the key enzyme that utilizes geranylgeranyl diphosphate (GGPP) as its substrate for producing tanshinones (Narula and Arora 2017). Likewise, GGPP acts as a precursor during the biosynthesis of taxol by blocking the metabolic flux of forming GGPP to tanshinone and switching it to biosynthesize

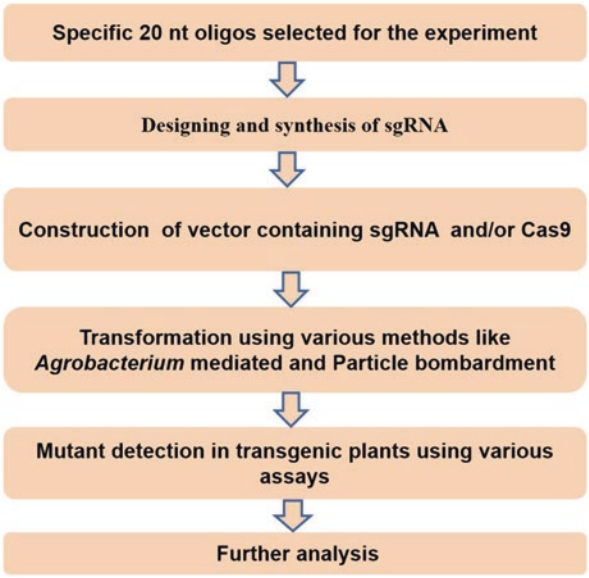

Fig. 12.4 Flow diagram of CRISPR/Cas9 genome editing of target genes

Table 12.2 Genome editing approaches applied in plants

Plant	Target genes	Editing techniques	References
Tomato	Regions in the viral genome	CRISPR/Cas9	Ali et al. (2015)
Maize	ALS	CRISPR/Cas9	Svitashev et al. (2015)
Rice	ALS	TALEN	Li et al. (2016)
Rice	GW2, GW5, and TGW6	CRISPR/Cas9	Xu et al. (2016)
Maize	Waxy	CRISPR/Cas9	Chilcoat et al. (2017)
Rice	SBEI and SBEIIb	CRISPR/Cas9	Sun et al. (2017)
Soya bean	*Bar*, *GmFEI1*, *GmFEI2*	CRISPR/Cas9	Jacobs et al. (2015)
Red sage	SmCPS1	CRISPR/Cas9	Li et al. (2017)
Red sage	SmCPS1 and SmKSL1	CRISPR/Cas9	Bai et al. (2018)
Barrel clover	*GUS*	CRISPR/Cas9	Michno et al. (2015)

Taxol. CRISPR/Cas9 system generated 3 homozygous and 8 chimeric mutants from the 26 independent transgenic hairy root lines of *Salvia*. Metabolomics analysis also proved the above results evidenced by the zero accumulation of tanshinone in homozygous mutants and a declined fraction in chimeric mutants. Similarly, a large number of studies have been carried out using CRISPR/Cas systems for various applications, such as improving the biotic and abiotic stress tolerance, enhanced yield performance, production of various bio-active metabolites, enhancement of plant quality, and biofortification (Ricroch et al. 2017). Reports also suggest the successful applications of CRISPR-Cas9 genome editing tool for editing carotenoid biosynthetic pathway genes in tomato (Pan et al. 2016; Hayut et al. 2017). Similarly, CRISPR/Cas9 tool has been utilized for targeted mutagenesis of the *DFR-B* gene locus (dihydroflavonol-4-reductase-B), a key gene of anthocyanin biosynthetic pathway in *Ipomoea nil* (Watanabe et al. 2017). The authors observed drastic changes in the stem color of the plant during the initial stages of transgenic plant development under in vitro conditions. About 75% of the transgenic plants exhibited anthocyanin-less white flowers with the biallelic mutations at Cas9 cleavage site in *DFR-B* gene obtained by either a single-base insertion or by deletions of more than two bases. *Agrobacterium*-mediated homologous recombination was achieved in tomato by targeted insertion of strong cauliflower mosaic virus 35S promoter upstream of the endogenous anthocyanin mutant 1 (ANT1) coding sequence using CRISPR/Cas9 system (Cermak et al. 2015). ANT1 encodes a MYB transcription factor and its overexpression resulted in intense purple coloration in various tissues like flowers, fruit, and foliage in the gene-targeted plants as compared to the wild type due to anthocyanin accumulation (Fig. 12.5).

12.6 Natural Bio-active Compounds: Current Market

A number of plant-derived bio-active compounds have been considered as pharmaceuticals, or nutraceuticals, and their production represents a challenge for the research to increase their market concern (Rea et al. 2010; Swamy and Sinniah 2016). During the year, 2004 plant-derived drugs were recognized to have the Food

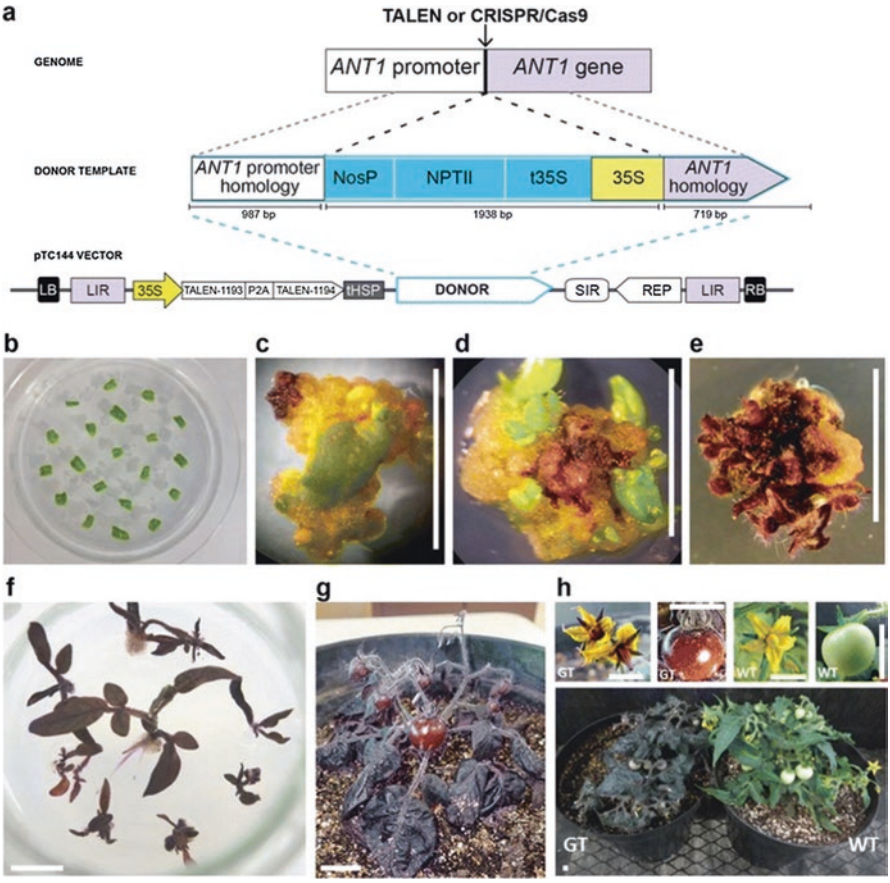

Fig. 12.5 Gene targeting upstream of the *ANT1* gene. (**a**) *Top*: illustration of the GT event. Upon cleavage by the nuclease and homologous recombination with the replicon, the donor cassette is inserted upstream of *ANT1*. *Bottom*: structure of the transfer DNA (T-DNA) vector, pTC144, which produces DNA replicons. *LB* left T-DNA border, *LIR* BeYDV large intergenic region, *35S* cauliflower mosaic virus 35S promoter, *tHSP Arabidopsis thaliana* heat shock protein 18.2 terminator, *SIR* BeYDV short intergenic region, *REP* coding sequence for Rep/RepA, *RB* right T-DNA border. Additional components of the donor include *NosP Agrobacterium tumefaciens* nopaline synthase promoter, *NPTII* neomycin phosphotransferase gene for kanamycin resistance, and *t35S* CaMV 35S terminator. For expression of CRISPR/Cas9 reagents, the TALEN coding sequence was replaced with a plant codon-optimized Cas9 gene, and the gRNAs were expressed from the AtU6 promoter (not shown). (**b**–**h**) Regeneration of tomato plants with targeted insertions. (**b**) Cotyledons of tomato cv. MicroTom after inoculation with *Agrobacterium*. (**c**) A recombinant explant 3 weeks after inoculation. Part of the developing callus accumulates anthocyanins due to the targeted promoter insertion and *ANT1* overexpression. (**d**) Explants 5 weeks after inoculation. Small shoots begin to develop on the purple callus. (**e**) Multiple shoots growing from the purple callus 10–12 weeks after inoculation. (**f**) Plantlets develop roots 12–14 weeks after inoculation. (**g**) Plantlet transplanted to soil. (**h**) Dark purple coloration in flowers, fruit, and foliage results from targeted promoter insertion. Flowers, fruit, and mature plants are compared between wild-type (WT) plants and those that have undergone GT. Scale bars = 1 cm. (Source: Cermak et al. 2015)

and Drug Administration (FDA) status. As a result, large pharmaceuticals companies started to develop plant-based drugs at a larger scale. The widespread application of plant-based drugs has attracted investment from both public and private players in the market. According to the British Chambers of Commerce (BCC), during 2015, the market value of the drugs derived from plants and botanicals was about $25.6 billion globally which will be increased up to $35.4 billion in 2020, and 6.6% will be the steady compound annual growth rate (CAGR) for the period 2015–2020. This remarkable growth includes an increasing faith among the people that plant-derived drugs are far better than those that are synthetic (Jamshidi-Kia et al. 2018). Other key factors include the exchange of materials between the Indian, Chinese, and other markets for their increased production. Increase in the market of plant-derived drugs helped the growth of pharmaceutical market which improves the overall economy (Singh et al. 2018). The entry of brands, such as Picato, Sativex, and Jevtana, will further continue to increase the herbal industry growth through 2020. This plant-derived drug market could be valued at approximately 25% of the prescription drug market across its entire timeline. Botanicals, not surprisingly, as a subgroup of all plant-based medicines sold as prescription drugs are expected to experience the growth at marginally higher levels comparative to the whole pharmaceutical segment at a CAGR of 6.4% from 2015 to 2020. In contrast to traditional drug development, botanical drug development is approximately $80 million over 10 years with potential average sales of $1 billion (http://blog.bccresearch.com/global-markets-for-botanical-and-plant-derived-drugs-to-reach-35.4-billion-by-2017).

During the period between 1994 and 2001, the worldwide demand for herb-based products has increased at an annual rate of 8%. According to the forecast of the World Health Organization (WHO), the worldwide market for herbal products would be worth $5 trillion by the year 2050. At present, the United States and Europe are the most important markets for herbal products in the world, with a market share of 20% and 41%, correspondingly. In India, the estimated annual market for Indian systems of medicine, which depend on herbs, is around INR. 5000 crores domestically and about INR. 500 crores globally. Overall, the Ayurveda medicines dominate with a share of over 85% of the total herbal market, which is followed by Homeopathy, Unani, and Siddha. Studies also suggest that the widely spread applications of herb-based medicines are not limited to only emerging countries but also used in the developed countries (Joshi and Nulkar 2018). Recent years have witnessed the growing interest among both medical practitioners and patients to make use of herbal approaches for health problems. Recently, FDA has relaxed the guidelines required to sell herbal supplements, which has encouraged the herbal industry to boom.

12.7 Conclusions and Future Prospects

With the advent of advanced metabolic, gene expression and computational tools, the characterization of key genes responsible for regulation and accumulation of a desired phytochemical has been attempted. The target gene for plant-derived bioactive constituents can be sequenced in a shorter time with the development of sequencing techniques. The progress in scientific developments certainly holds a great potential toward the application of plant metabolic engineering in future. Genomics methodologies help in identifying the regulatory genes. Likewise, transcriptomics allows to understand the expression or regulation of genes, while proteomic approaches delineate why the level of expression of few biosynthetic genes do not relate with the metabolites profile. The commercial demand for the phytocompounds is the core impetus for implementing massive research efforts toward understanding and manipulating plant biosynthetic processes using different physiological, chemical, and biotechnological ways. The exploitation of strategies for improving the productivity of plant bio-active compounds made within last few years has been well explained in this chapter.

In the coming years, unknown biosynthetic pathways, responsible for the production of bio-active compounds at the enzyme and gene level, will be unraveled. This all will lead to new possibilities for the production of fine chemicals with improved properties such as increased resistance, better taste and smell, other colors, increased levels of health-promoting characteristics, and decreased level of toxic or other unwanted compounds. Although the knowledge of the biosynthetic pathways, the genes involved, the promoters, and the precursors in plants has been well characterized, further work on functional characterization for optimizing the biotechnological production of the plant-derived compounds would definitely help in understanding posttranscriptional regulatory mechanisms behind the desired metabolite biosynthesis and accumulation. This information could be used for metabolic engineering of the entire pathway to increase the production of important bio-active compounds which can be further utilized for human benefits.

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13 Enhancement of Rosmarinic Acid Content by Biotechnological Approaches and Metabolic Engineering

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Abstract

Various secondary metabolites having medicinal values are isolated from different medicinal plants. Rosmarinic acid is a dimer of caffeic acid, and a vital antioxidant reported with numerous biological properties, such as anticancer, antimicrobial, analgesic, etc. Initially, rosmarinic acid was isolated from rosemary (*Rosmarinus officinalis*) belonging to the family, Lamiaceae. Subsequently, the existence of rosmarinic acid was identified in different medicinal plants, such as basil, salvia, lavender, etc., belonging to the families, Lamiaceae and Boraginaceae. The low content of rosmarinic acid in field-grown plant parts and seasonal variation are the major limitations of its continuous supply for medicinal purposes. Alternatively, plant tissue culture is a superior and attractive strategy to enhance the rosmarinic acid content. The cell culture technique facilitates sustainable production of rosmarinic acid in a controlled environment, and it is well established. To enhance the rosmarinic acid content in herbs different tissue culture approaches, such as callus induction, hairy root culture, *Agrobacterium* infection induced callus, precursors (phenylalanie and tyrosine) addition in callus suspension culture, and elicitation by supplementing yeast extract and methyl jasmonate in callus suspension have been followed by several researchers. Further metabolic engineering is the promising approach where expression of rate limiting enzymes of rosmarinic acid synthesis pathway is over expressed by molecular cloning. Phenylalanine ammonia-lyase (PAL) and tyrosine aminotransferase (TAT) are well-known enzymes involved in rosmarinic acid biosynthesis. PAL and TAT are well characterized at molecular level, and their overexpression facilitated increases in biosynthesis of rosmarinic acid (RA) in different plants. Thus, the purpose of this chapter is to explore the biological activity of rosmarinic acid and its production enhancement through various biotechnological approaches including plant cell culture, elicitation, hairy root culture, and metabolic engineering.

Keywords

Rosmarinic acid · Plant cell culture · Biotechnology · Metabolic engineering

13.1 Introduction

The flora and fauna of the world are providing several types of compounds with varied biochemical activity, which are readily used for various purposes by humans since thousands of years. Humans have been harnessing food and other requirements from jungles of the world almost from the start of human race on the planet earth. It has been observed that most of the cancers in humans have been caused by atmospheric constituents along with the food habits one is living with; it is assessed that almost third part of different types of cancers can be evaded simply by altering food habits! This knowledge has created a lot of influence on people; hence the interest in natural compounds is increasing for maintenance of good health and to

treat ailments. We have been getting all our requirements of food from plants. Additionally, the plants are providing various subsidiary metabolites products, being used in various applications like pharmaceuticals, agrochemicals, food additives (colours, fragrances, flavours and biopesticide). Currently 80% of the natural products belong to plant origin (Phillipson 1990), which amounts to 30,000 products. The number of compounds from plants is four times more than that from microbial origin. The 3500 new chemical compounds were discovered, from which 2600 were of plant origin in 1985. All over the world, 121 very effective medicines were of plant origin as per Payne et al. (1991). Currently about 75% of people worldwide show faith in natural medicines from plants. Plants have been providing food and useful medicines since ancient times and will keep providing novel drugs in the future, as the study of majority of plants has not been completed (Cox and Balick 1994). The development in the chemical analysis instrumentation for structure elucidation and identification has made it easier to correlate the activity with specific family of plants with experiments. Though there is sufficient advancement in chemistry, still we are dependent on biological sources for many secondary metabolites and pharmaceuticals (Pezzuto 1995).

Biotechnology, with regard to the above, opens up a great way to utilize cells, tissue and organ, as well as growing the organism in vitro to genetically modify them to get required chemicals generated. The basic metabolites generated as a result of photosynthesis later generate secondary metabolites, which are typical of plant origin and cannot be produced by microbes or animals. Although because of advanced genetic engineering it has become possible to generate specific chemicals which are not originally produced by plant. Utilization of genetic engineering methods like plant cell culture for synthesizing medicinal metabolites has been well known (Verpoorte et al. 2002). The in vitro methods have been developed essentially for plant biology, and scientists have utilized plant cell biosynthesis for obtaining medicines and the studying of plant metabolism (Verpoorte et al. 2002). Plant tissue culture make and emit several metabolites useful as medicines, which are of special interest; researchers are working on these aspects for getting the effective and special compounds for specific ailments. Several useful compounds have been retrieved by in vitro cultures like alkaloids, saponins, cardenolides, anthraquinones, polyphenols and terpenes and also reviewed many times (Exarchou et al. 2000; Vanisree and Tsay 2004).

Rosmarinic acid (Fig. 13.1) is a diphenolic chemical readily available in several types of plants, belonging to Boraginaceae and Lamiaceae families (Yang and Shetty 1998). Rosmarinic acid is synthesized through the phenylpropanoid pathway (Fig. 13.2). Its biological, chemical and therapeutic characteristics have been well studied by Petersen and Simmonds (1987). Rosmarinic acid is considered a very effective, therapeutic, and a well-known antioxidant (Exarchou et al. 2000).

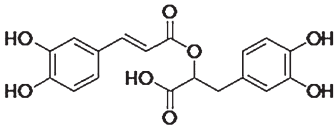

Fig. 13.1 Chemical structure of rosmarinic acid

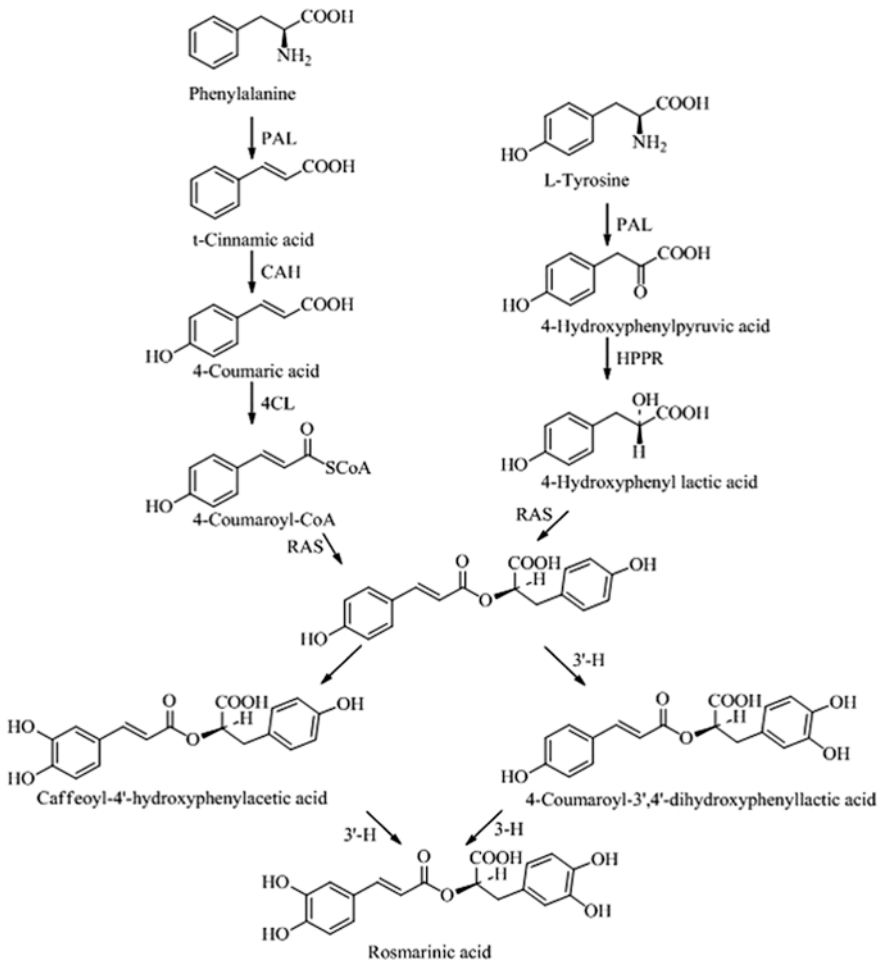

Fig. 13.2 Biosynthetic pathways for rosmarinic acid. *PAL* phenylalanine ammonia-lyase, *CAH* cinnamic acid 4 hydroxylase, *4CL* hydroxycinnamate:coenzyme A ligase, *TAT* tyrosine aminotransferase, *HPPR* hydroxyphenylpyruvate reductase, *HPPD* hydroxyphenylpyruvate dioxygenase, *RAS* hydroxycinnamoyl-CoA:hydroxyphenyllactate hydroxycinnamoyl transferase, *3-H, 3′-H* hydroxycinnamoyl- hydroxyphenyllactate 3- and 3′- hydroxylases

The herbs, which produce the rosmarinic acid, the dominant phenolic constituent, have been in use since the ancient times in classical medicines in Europe, India and Japan for treating ailments like diabetes mellitus, stomach ache, headache, insect bites and acne. Many rosmarinic acid-carrying extracts obtained from the foliage of spices and herbs are known to be antitumourigenic, antioxidant, antimutagenic, anti-HIV, anti-proliferative and anti-cyclooxygenase (Makino et al. 2000; Kelm et al. 2000). Amount of rosmarinic acid found in plants developed by tissue

cultures is much more compared to originally found from natural plant extract. The cultures produced for rosmarinic acid are made from *Anchusa officinalis* (De-Eknamkul and Ellis 1984, 1985), *Eritrichium sericeum* (Fedoreyev et al. 2005), *Lithospermum erythrorhizon* (Yamamoto et al. 2002) (Boraginaceae), *Coleus blumei* (Petersen et al. 1993), *Lavandula vera* (Georgiev et al. 2006), *Ocimum basilicum* (Kintzios et al. 2004), *Salvia officinalis* (Hippolyte et al. 1992) and *Zataria multiflora* (Mohagheghzadeh et al. 2004). Alternate estimation about the yield of rosmarinic acid is more than 5% of cell mass (dry) in *Anthoceros agrestis* (Vogelsang et al. 2006). The secondary method utilized in extraction of rosmarinic acid is transformation of roots of *Salvia miltiorrhiza* (Chen et al. 1999) and *S. officinalis* (Grzegorczyk et al. 2006, 2007). Product from RA concentrating lavender cells shows major foraging characteristics (Kovacheva et al. 2006). Much greater quantity of RA is concentrated in *Agrobacterium* modifying biomass of *C. blumei* (Bauer et al. 2004). The aim of this research is to know all biological activity of rosmarinic acid and its roles in various biological procedures like plant cell culture, elicitation, hairy root culture and metabolic technique by molecular cloning to enhance the rosmarinic acid content within the plant system.

13.2 Biological Activity of RA

Biochemical activity of RA has been comprehensively explored, since several years; the present document includes more than 250 references. Previous research revealed antibacterial, anti-inflammatory, antioxidant and antiviral properties of rosmarinic acid (Petersen and Simmonds 1987). The radical scavenging property of rosmarinic acid is because of membrane stabilization and obstruction to radical spread, which results in safeguarding the membrane from oxidant attack (Perez-Fons et al. 2010). Present research confirms the ability of rosmarinic acid to strengthen the structural and anti-oxidative durability of the liposomes (Panya et al. 2010). Rosmarinic acid can scavenge the Reactive Oxygen Species (ROS) and eliminated Interleukin-6 (IL-6) secretion, which later block UVB caused damage to human keratinocytes (Vostalova et al. 2010). Rosmarinic acid enhanced DNA repair mechanism and prevents chemically activated chromosome damage. Rosmarinic acid doesn't induce DNA damage and considerably reduces the nuclear fragmentation (Furtado et al. 2010). A significant performance of rosmarinic acid is in improving the cognitive performance (Park et al. 2010). Moreover, rosmarinic acid in reduced micromolar level considerably protects neurons (Fallarini et al. 2009). Amyotrophic lateral sclerosis (ALS) is a disease of degeneration of nerves, degenerating motor neurons; intraperitoneal injection of rosmarinic acid considerably prevented the disease progress, confirmed with paw-grip strength tests, reduced the damage of motor function nerves (neurons) and increased the duration of model mice life span (Shimojo et al. 2010). Kim et al. (2009) confirmed that rosmarinic acid has an anti-angiogenic property for retinal neovascularization in animals under test for retinopathy. Rosmarinic acid considerably prevented the spread of eye cells, which is concentration-based, but prevented the in vitro angiogenesis of artery generation.

The rosmarinic acid behaves like anti fibrosis drug. In a carbon tetrachloride (CCl_4)-induced rat liver fibrosis, rosmarinic acid decreased the fibrosis level and improved biomarker indicators and tissue morphology (Li et al. 2010). Rosmarinic acid causes apoptosis in active T cells in people with rheumatoid (Hur et al. 2007). The prolonged encounter to RA (Rosmarinic Acid) in food habit is enough for prevention of cancer in animals (Paluszczak et al. 2010). Rosmarinic acid prevents the movements of breast cancer cells and prevents spreading, chiefly by modulating signalling pathways (Xu et al. 2010).

13.3 Plant Cell Culture as a Tool to Enhance the Rosmarinic Acid Content

Rosmarinic acid is present in several tissue culture systems, like *Anchusa officinalis*, *E. sericeum*, *L. erythrorhizon* (Boraginaceae), *Coleus blumei*, *Ocimum basilicum*, *S. officinalis*, *S. miltiorrhiza* and *Anthoceros agrestis* (Anthocerotaceae) (Petersen and Simmonds 1987; Matkowski 2008). Rosmarinic acid may be produced using plant cell cultures in large quantity easily without specific efforts, because RA is formed as subsidiary metabolites and is continuously biochemically synthesized in the herbs species (Bais et al. 2002). The E-4 calli line with cuprum glycerate produces rosmarinic acid 2.04% and (−)- rabdosiin less than 1% of dry weight. However, the root culture of *E. sericeum* is better in yields by accumulating rosmarinic acid and rabdosiin at 4.50% and less than 2% of dry weight respectively. Eritrichin is considered a predecessor of rabdosiin biochemical synthesis, a missed out connection between rosmarinic acid and rabdosiin. The process of biochemical manufacture of rosmarinic acid is narrated by Petersen and Simmonds (1987), Park et al. (2008) and by Matkowski (2008). Specifically, 0.1 Kg of rosmarinic acid was produced from *C. blumei* suspended cells cultivation in 32 liter biochemical reactors in 2 weeks. In the biochemical reactor culture of *A. officinalis*, rosmarinic acid manufacture is getting reduced due to dissolved oxygen level, agitation and aeration conditions. At peak cell concentration of 35 g/l, the RA yield is around 4 g/l after controlling oxygen level in water to be more than 30% level (Su et al. 1995). Consistent control of permeability of *C. blumei* cells with (DMSO) dimethyl sulfoxide and cell stagnation showed promising results with increased release of rosmarinic acid in fed-batch biochemical reactors (Park and Martinez 1992, 1994).

13.3.1 Elicitation of Rosmarinic Acid

Plant cell cultures have been developed from several species, but in many cases, it does not produce adequate quantities of required metabolites (Vanishree et al. 2004). In several instances, the yield of required metabolites is increased by treating the basic cells with elicitors like heavy metals, methyl jasmonate, salicylic acid and chitosan (Poulev et al. 2003). An ‘elicitor’ is a molecule or compound inducing biochemical synthesis of secondary metabolites in plant cell culture system.

Elicitation alters or improves biochemical synthesis of required chemicals, because of introduction of elicitor at trace level (Radman et al. 2003). Elicitors are derived from different sources such as microbes, fungus, and plant cell wall fragments which has capacity to enhance secondary metabolite content. Scientists speculated about bonding of elicitor to membrane receptor for elicitation process (Hanania and Avni 1997). The Ca^{2+} ion induction to cytoplasm from outside the cell to the intra cell Ca^{2+} reserve was reported by Gelli et al. (1997). Apostol et al. (1989) studied the manufacture of free radicals like oxide anion and H_2O_2, which give a direct antibacterial activity and contribute in production of biologically active fatty acid subsidiaries. In line with this ROS function for joining cell membrane and attached proline-rich proteins, H_2O_2 functions as subsidiary message carrier and is functional for the first step of excitation of defence genes (Low and Merida 1996).

The influence elicitors such as yeast extract and methyl jasmonate on the enhance of rosmarinic acid content reported in plants namely *Orthosiphon aristatus* (Sumaryono et al. 1991) and *Lithospermum erythrorhizon* (Mizukami et al. 1992). In cell cultures of *L. erythrorhizon*, rosmarinic acid yield is enhanced two to three times by the essence of yeast, to the maximum levels in 24 h after addition, but if 100 μM methyl jasmonate is added, the production is increased by tenfold after 48–72 h (Szabo et al. 1999). Hippolyte et al. (1992) have studied the influence of phenylalanine on *Salvia officinalis* cell growth and have established the enhancing effect on cells and production of rosmarinic acid. Sucrose is considered the better carbon supply in proper development of cell suspensions; also sucrose level in the medium will highly affect the manufacture of secondary metabolites of the phenylpropanoid stages (Ibrahim 1987). Abundance of sucrose level in cell culture increases the rate of rosmarinic acid yield in *S. officinalis* than the control (Hippolyte et al. 1992). Liquid medium cell cultures of *C. blumei* added with product from the culture medium of phytopathogenic *Pythium aphanidermatum* as an elicitor improves product rosmarinic acid (Szabo et al. 1999). Rosmarinic acid accumulation is influenced with yeast extract to *L. erythrorhizon* (Mizukami et al. 1992) and *O. aristatus* (Sumaryono et al. 1991) suspension cultures. A number of experimentations indicate the existence of several paths for improving the production of secondary metabolites with the help of elicitors; as an example, methyl jasmonate has a great role in the pathways of rosmarinic acid (Zhao and Sakai 2003).

13.3.2 Hairy Root Culture System to Enhance Rosmarinic Acid Content

Agrobacterium rhizogenes is a gram-negative microbe found in soil, belonging to Rhizobiaceae family, responsible for causing hairy roots disease by attacking the wounded higher parts of the plants. These pathogens affect a DNA segment (T-DNA region bounded by 25 bp direct oligonucleotide repeats) because of its waste root-inducing (Ri) plasmid into the DNA of the diseased plant. The fresh hormonal homeostasis causes the generation of new roots, called hairy roots, which emerge at the wounded site (Gaudin et al. 1994). Because of high yield and stability, hairy root

cultures are studied since the last few years, as it shows capability to produce costly subsidiary metabolites from wild-type roots (Giri and Narasu 2000). Sterile hairy root cultures have been prepared from about 200 varieties of higher plants, because of their capability in synthesizing large range of subsidiary metabolites and also to study metabolite activity with inorganic and organic elicitors (Guillon et al. 2006). It is an established fact that rosmarinic acid production is greater in roots than in leaves or shoots (Bais et al. 2002). It is observed that sweet basil cell culture produces almost 10 mg/g (dry weight) of rosmarinic acid, 11 times higher than callus culture or in leaves of the plant (Kintzios et al. 2003). In vitro production of basil may be a solution for recovering essential oil, from the air inside the chamber of the plants being grown (Zeldin et al. 1988). Hairy root cultures are established in order to make high yielding product range for phenolic compounds like rosmarinic acid and lithospermic A and B acids (LAB) (Tada et al. 1996). Hairy root (HR) is observed to be fast initially till 21 days of culture; however the yield of rosmarinic acid (RA) and lithospermic acid (LA) is obtained after 48–56 days from the start and reached almost 3.5 times higher compared to amount achieved in controlled plants. The lithospermic acid B yield has no effect by hairy root culture (Tada et al. 1996). Additionally, high significance has been imparted for *A. rhizogenes* strains and the culture medium. It has been observed that the increase of root biomass without any lag time reached a maximum after about 2 weeks (since culture) in woody plant medium (WPM) (Tada et al. 1996). In most of the media, hairy root yielded sufficient quantity of RA; specifically, high levels (over 14% of dry wt) of yield was noted in MS (clone J-l, 14.1%, at eigth week) and B5 (clone A-2, 14.0%, at sixth week) media, being almost 3.5 times more than that (3.98% of dry wt, in leaf portion) of the intact plant and was similar to the what was obtained in suspension of *C. blumei* (Petersen et al. 1994) and *S. officinalis* (Hippolyte 2000), cultured under optimized conditions for rosmarinic acid production. In all experimented cultures, the maximum production of rosmarinic acid was 73.5 mg/flask produced by J-1 in MS medium at fifth week. Normal roots of *O. basilicum* produced peak quantity of rosmarinic acid (0.98% g fresh weight basis) compared to leaves and shoots. Hairy root cultures of *O. basilicum* attacked with *A. rhizogenes* indicated a threefold jump in growth and rosmarinic acid yield, if compared with normal roots. However, Srivastava et al. (2014, 2016) performed an in vitro experiment for finding the super (high rosmarinic acid producing) cultivation of *O. basilicum* and subsequently *Agrobacterium rhizogenes* attacked changes for the selection of three high rosmarinic acid producing transformed root lines.

13.4 Metabolic Engineering for Rosmarinic Acid Production

Biochemical synthesis of rosmarinic acid is successfully elucidated including the eight enzymes present in producing it has been well understood (Barberini et al. 2013). Aromatic amino acids phenyl alanine and tyrosine are precursors of rosmarinic acid synthesis (Fig. 13.2). These two amino acids are derivatized simultaneously by respective enzymes. Phenylalanine ammonia-lyase initiates the conversion

of phenylalanine into trans-cinnamic acid by deamination. Then trans-cinnamic acid is converted into 4-coumaric acid by cinnamic acid 4 hydroxylase. Further 4-coumaric acid is converted into 4-coumaroyl-CoA by hydroxycinnamate:coenzyme A ligase. On the other hand, L-tyrosine is converted in 4-hydroxyphenylpyruvic acid by tyrosine amino transferase. Then 4-hydroxyphenylpyruvic acid is converted into 4 hydroxyphenyllactate by hydroxyphenylpyruvate reductase. Rosmarinic acid synthase (RAS) catalyses the formation of 4-coumaroyl-4′-hydroxyphenyllactate and the producing ester, when hydroxylated by two cytochrome P450 monooxygenases to form rosmarinic acid. RA accumulation is always followed by PAL activity (Petersen and Alfermann 1988; Hausler et al. 1991; Kim et al. 2004).

Only four of the enzymatic reactions available in the rosmarinic acid biochemical synthetic pathway look very special for this synthesis (Hucherig and Petersen 2013). Enzymes involved in phenylpropanoid pathway such as phenylalanine ammonia-lyase, cinnamate 4-hydroxylase and 4-Coumaroyl-CoA ligase available normally in land plants and synthesizes the predecessor for the production of lignin and some aromatic compounds.

TAT (tyrosine aminotransferase) is an important enzyme which produces pHPP, required in the biochemical synthesis process for tocopherols and plastoquinones (Douce and Joyard 1996). Enhanced PAL activity and the activity of the rosmarinic acid biochemical synthetic enzyme RAS are observed in attacked cells by elicitor of *C. blumei* (Szabo et al. 1999). Supplementation of MeJA enhanced expression of PAL and TAT enzymes in *S. milthorriza* which significantly increase rosmarinic acid content (Xing et al. 2013), indicating the idea of using elicitors to up regualte the expression of rate limiting enzymes of rosmarinic acid biosynthetic pathway. Hairy root cultures of *S. miltiorrhiza* supplemented with yeast extract enhanced TAT expression and subsequently increased rosmarinic acid content in this culture system (Yan et al. 2006). The transformation of hydroxylpyruvate into glycerate by a NADH-dependent peroxisomal hydroxylpyruvate reductase (pHPR) or a cytosolic NADPH-dependent HPR2 during photo respiration (Timm et al. 2008) is equivalent to the stereo-specific decrease of pHPP by HPPR. In normal case HPR2 from *Arabidopsis thaliana* heterologously expressed in *Escherichia coli* accepted hydroxyphenyl pyruvate as a substrate, even though *A. thaliana* does not biochemically synthesize rosmarinic acid (Petersen 2013). Rosmarinic acid yield has increased in *S. milthorriza* when HPRR expression level is increased. However, it is not confirmed that HPPR is a rate-limiting enzyme of rosmarinic acid biosynthetic pathway because high rosmarinic acid yield was found to be strongly correlated with the high expression of enzymes involved in the tyrosine-derived pathway (Xiao et al. 2009; Zhang et al. 2014). The enzymes of the three precursor reactions for rosmarinic acid biochemical synthesis, rosmarinic acid synthase and the 3-30-hydroxylases were studied in cell culture systems of *C. blumei* (Petersen 1997). However, the hydroxycinnamoyl transferases and the meta-hydroxylases showed a quite higher degree of similitudes in sequences, hence considered in close relation to each other; expression studied in heterologous systems indicated that the enzymes from *C. blumei* are specific to substrates utilized in rosmarinic acid biochemical synthesis (Berger et al. 2006; Eberle et al. 2009). The rosmarinic acid

yield of 992 mg/l was obtained with hPPR and TAT genes co-overexpression (Xiao et al. 2011). These outcomes are partly established by rosmarinic acid biochemical synthesis in cell suspension cultures of *Salvia officinalis* compared with the expression of the hPPR gene (Barberini et al. 2013).

13.5 Conclusions and Future Prospects

Use of herbal-derived products is widely accepted because of their less toxic nature. Rosmarinic acid is a vital compound present in medicinal plants, and extraction of sufficient amount of rosmarinic acid from field-grown plants which meets the demand remains unanswered. Rosmarinic acid is present in field-grown plants in low amount, and isolation of rosmarinic acid from the plant tissues is expensive. Over the years plant tissue culture and metabolic engineering approaches are overcoming the hurdles. Complete characterization of rosmarinic acid biosynthetic pathway leads to identification of rate-limiting enzymes. Overexpression of rate-limiting enzymes along with precursor feeding (phenylalanine and tyrosine) in plant cell culture systems results in a significant amount of rosmarinic acid accumulation. Since, rosmarinic acid is a potent antioxidant and anticancer and radiation protection agent, its production needs to be further enhanced. In future bottleneck for rosmarinic acid production is further identification and characterization of enzymes of rosmarinic acid biosynthetic pathway. Consequently alter their expression in cell culture system through metabolic engineering approaches would yield significant details about involvement of key enzymes involved in rosmarinic acid biosynthetic pathway.

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