Sonia Malik *Editor*

Exploring Plant Cells for the Production of Compounds of Interest

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वक्रतुण्ड महाकाय सूर्यकोटि समप्रभ ।
निर्विघ्नं कुरु मे देव सर्वकार्येषु सर्वदा ॥

Vakra-Tunndda Maha-Kaaya Suurya-Kotti Samaprabha |
Nirvighnam Kuru Me Deva Sarva-Kaaryessu Sarvadaa ||

With a curved trunk, large body and the brilliance of a million Suns, O Lord, please make my work free of obstacles. Always remain with me in all actions.

Preface

Plants are able to produce a bewildering array of compounds, which are used as medicines, food additives, flavours, fragrances and agrochemicals. These compounds are produced during specific developmental stages of plants or under specific conditions. The demand for plant-derived natural compounds has been continuously increasing because of their significant value and new applications. There are various mechanisms involved in the production of these bioactive compounds from plant cells. It is important to exploit the potential of plant cells for the optimum production of compounds of interest by understanding the biosynthetic pathways and other factors. By employing different biotechnological methods, it is possible to improve the production of compounds of interest from plants. All these issues have been addressed in this book. Twelve chapters are written by globally renowned researchers working in plant biotechnology and natural products.

The Chapter "Secondary Metabolite Production in Plant Cell Culture: A New Epigenetic Frontier" by Brzycki et al. highlights the current strategies for plant metabolic engineering and reviews the current understanding of epigenetic regulation in plants, with emphasis on applications to secondary metabolic engineering. The current developments in bioprocess optimization, metabolic engineering strategies and bioreactor design for *in vitro* production of camptothecin via plant cell biofactories are discussed in the Chapter "Plant Cell Biofactories as In Vitro Production Platforms of the Anti-cancer Drug Camptothecin" by Sarayu et al. The changes in morphophysiological state of intact plants and plant cell cultures may affect secondary product metabolism, which can influence the overall production of bioactive compounds. This issue is addressed in the Chapter "Not One for All: The Interwoven Relationship Between Morphophysiology and Secondary Metabolite Production in Plant Cell Cultures" by Ping and Yee. The Chapter "Anthocyanins and Proanthocyanidins as Anti-cancer Agents" by Bhushan et al. gives information about potential resources of anthocyanins and their role and mechanism in cancer prevention, while successes and challenges of using plants of the *Lemnaceae* family for the development of plant-based expression systems are summarized in the Chapter "Duckweeds for the Production of Therapeutic Proteins" by Khvatkov. The Chapter "Essential Oils from Plants: Industrial Applications and Biotechnological

Production" by Andrade et al. aims to provide an overview of the composition in terms of active compounds, biological activities, and techniques to extract and evaluate the composition of different essential oils and their biotechnological production. The various biotechnological approaches, such as optimization of tissue culture conditions, elicitation, *Agrobacterium*-mediated transformation and metabolic engineering for the production of antistress compounds, are summarized in the Chapter "Biotechnological Production of Antistress Compounds: Current Status and Future Prospects" by Nayak et al. The Chapter "Elicitors as a Biotechnological Tool for In Vitro Production of Bioactive Phenolic Compounds" by Castro et al. provides insights into the effects of elicitation on secondary metabolites production. Nadaroglu in the Chapter "Immobilization and Application of Industrial Enzymes on Plant Based New Generation Polymers" highlights the immobilization and application of enzymes used in different industrial areas to new generation polymers based on plants. Lignans biosynthesis in plants, their health benefits and production by biotechnological means have been detailed in the Chapter "Recent Advances Towards Development Plant Cell Culture Process for Sustainable Production of Lignans and Its Health Benefits" by Gabr et al. The Chapter "Physiology of Camptothecin Synthesis in Plants and Root Organ Cultures of Ophiorrhiza Mungos L. and Its Production in Root Fermenters" by Wetterauer et al. discussed the production of anti-cancer drug camptothecin in *Ophiorrhiza mungos* with regard to concentration and localization of camptothecin and its derivatives within all plant parts. Nutritional and hormonal requirements of seedlings and shoots of *Haloxylon recurvum* and *Haloxylon salicornicum* are analysed under *in vitro* conditions in the Chapter "In Vitro Culture of Haloxylon Recurvum and Haloxylon Salicornicum: Valuable Source of Food Additives, Pharmaceutical and Nutritional Components from Extreme Arid Zone" by Goswami and Dagla.

Through this multi-authored book, efforts have been made to provide information about different strategies and methods to produce compounds of interest by employing various biotechnological means. This book will be a valuable reference for biotechnologists, plant tissue culturists, food technologists and researchers working in the area of natural plant products as well as medical and healthcare industries around the globe.

São Luís, MA, Brazil Sonia Malik

Contents

Secondary Metabolite Production in Plant Cell Culture: A New Epigenetic Frontier

Cassandra M. Brzycki, Eric M. Young, and Susan C. Roberts

Abstract Plant-derived secondary metabolites have many applications as fragrances, food additives, dyes, and bioactive medicinal compounds. Production of these compounds in plant cell culture has many advantages, and metabolic engineering strategies have been developed to increase yields. Yet, long-term subculturing can reduce productivity due to epigenetic downregulation of secondary metabolism. Innovative techniques that take advantage of new insights into epigenetic regulation have been developed for a variety of eukaryotes. Applying this epigenetic control to plant cell culture could restore and maintain high productivity, even in cultures that have been in suspension for many years. Here, we highlight current strategies for plant metabolic engineering and review the current understanding of epigenetic regulation in plants, with particular emphasis on applications to secondary metabolic engineering. We then discuss work in the emerging field of epigenetic engineering and postulate how epigenetic engineering techniques developed in mammalian systems could be used to overcome current limitations for industrial production of natural products in plant systems.

Keywords Metabolic engineering · Epigenetics · Synthetic biology · Epigenetic engineering · Gene expression · Secondary metabolism · DNA methylation · Histone modification · RNA interference · CRISPR

Abbreviations

Acetyl-CoA	Acetyl coenzyme A
Ago	Argonaute
BAPT	Baccatin III 13-O-(3-amino-3-phenylpropanoyl) transferase
Cas9	CRISPR-associated protein 9
Cas13	CRISPR-associated protein 13
CMT3	Chromomethylase 3
CRISPR	Clustered regularly interspaced short palindromic repeat

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CRISPRi	CRISPR interference
dCas9	Catalytically dead Cas9
DMAPP	Dimethylallyl pyrophosphate
DME	Transcriptional activator demeter
DML2	Demeter-like protein 2
DML3	Demeter-like protein 3
DNA	Deoxyribonucleic acid
DNMT	DNA methyltransferase
DNMT3A	DNA methyltransferase 3A
DRM	Domains rearranged methyltransferase
Erythrose 4-P	Erythrose 4-phosphate
Glucose 6-P	Glucose 6-phosphate
Glyceraldehyde 6-P	Glyceraldehyde 6-phosphate
GPP	Geranyl pyrophosphate
HAT	Histone acetyltransferase
HDAC	Histone deacetylase
HDAC3	Histone deacetylase 3
HDM	Histone demethylase
HMT	Histone methyltransferase
IPP	Isopentenyl pyrophosphate
JA	Jasmonic acid
LSD1	Lysine-specific demethylase 1
MAPK	Mitogen-activated protein kinase
MeJA	Methyl jasmonate
MET1	Methyltransferase 1
MEP	Methylerythritol 4-phosphate
miRNA	Micro RNA
MVA	Mevalonate
NADPH	Nicotinamide Adenine Dinucleotide Phosphate
PEP	Phosphoenolpyruvate
PRDM9	PR domain zinc finger protein 9
RdDM	RNA-directed DNA methylation
Ribose 5-P	Ribose 5-phosphate
RISC	RNA-induced silencing complex
RITA	RNA-induced transcriptional activation
RNA	Ribonucleic acid
RNAa	RNA activation
RNAi	RNA interference
ROS1	Repressor of silencing 1
SA	Salicylic acid
saRNA	Small activating RNA
siRNA	Small interfering RNA
SMYD3	SET and MYND domain-containing protein 3
TALEN	Transcription activator-like effector nuclease
TET1	Ten-eleven translocation methylcytosine dioxygenase 1
ZFN	Zinc finger nuclease

1 Introduction

Plants produce many different compounds to protect themselves from environmental stressors, predators, and pathogens (Sanchez-Muñoz et al. 2019). These natural products, also referred to as specialized or secondary metabolites, are an essential source of fragrances, food additives, dyes, and bioactive medicinal compounds (Eibl et al. 2018). Of over 1000 new drugs that were approved by the FDA from 1981 to 2010, half were derived from plant natural products (David et al. 2015), including paclitaxel, a chemotherapy medication derived from *Taxus* spp. (Onrubia et al. 2013). Despite the wealth of plant-derived natural products, production of these compounds at a commercial scale is difficult (Wilson and Roberts 2012). Several approaches have been established for industrial production of plant secondary metabolites, including natural harvest, total chemical synthesis, and plant cell culture.

Natural harvest, or extraction of natural products from the whole plant, is often impractical due to the large amount of plant matter that must be harvested to meet the demand. For example, approximately 38,000 trees must be harvested to meet the annual demand of only 25 kg of paclitaxel (Suffness 1993). Many medicinal plants, such as *Taxus* species, grow very slowly, and thus, overharvesting has a negative environmental impact and can result in endangerment of these plant species (Lu et al. 2016). Because of this lack of sustainability, natural harvest is not often a suitable method for industrial-scale production.

Total chemical synthesis, or chemical production from widely available precursors, has also been explored as a route for production of plant natural products. This approach has been successful for many simpler natural products, such as vanillin, which is most often produced from lignin, guaiacol, or eugenol (Schwab et al. 2008). However, for more complicated natural products with multiple chiral centers, chemical synthesis is not always possible, and if routes are established, they are rarely economically feasible at large scales. For example, while paclitaxel has been successfully chemically synthesized using two different total synthesis schemes (Holton et al. 1994; Nicolaou et al. 1994), both options have over 40 reactions, utilize many harsh chemicals, and have low yields. These factors make chemical synthesis of paclitaxel neither environmentally friendly nor economically feasible.

Heterologous production, or production in a non-native host, is a promising alternative to natural harvest and total chemical synthesis due to its increased sustainability and established bioprocessing methods for microbial and yeast systems. However, heterologous production is not always possible because the biosynthetic pathways for production of these natural products are often not fully characterized. Plant genomes are extremely large (often 10–20 Gb) and complex, making them difficult and expensive to sequence and limiting genomics-driven research (Jiao and Schneeberger 2017). However, even in cases where heterologous production of plant natural products is possible, production in a non-native host requires substantial genetic modification beyond simply importing the biosynthetic pathway (Komatsua et al. 2010). For example, heterologous production of lycopene in *S. cerevisiae* required both strain optimization and combinatorial optimization of

many different lycopene biosynthesis genes to reach reasonable titers (Li et al. 2019). The high degree of engineering and optimization required for heterologous production of secondary metabolites makes this approach not always suitable for highly complex products.

Plant cell culture, or the production of natural products typically using dedifferentiated plant cells, is the current state of the art for commercial production of several important plant-derived secondary metabolites, including paclitaxel (Sanchez-Muñoz et al. 2019). To establish plant cell lines, explants are taken from whole plants, which are then propagated in sterile media and treated with hormones to form dedifferentiated plant tissue. Similar to heterologous production, plant cell culture is a sustainable, environmentally friendly alternative to natural harvest or total chemical synthesis. Additionally, growth conditions are more easily controlled compared to whole plant systems, enabling more consistent product yields, and secondary metabolites can be produced from rare or endangered medicinal plant species without fear of overharvesting (Kolewe et al. 2008). Plant cell culture also has advantages over heterologous production because high-producing cell lines can be established without genomics and genetic engineering in cases where this is necessary. Further, because native hosts are already adapted to produce the secondary metabolite of interest, fewer genetic modifications are needed to achieve high yields (Wilson and Roberts 2014).

While production in plant cell culture is highly suitable for synthesis of complex natural products, there are still unique challenges that must be overcome to improve yield in industrial-scale production processes. While yields of secondary metabolites are typically highest shortly after plant cell cultures are initiated, production of secondary metabolites often fluctuates and gradually decreases over time (Sanchez-Muñoz et al. 2019). This is problematic because there are currently few methods for effective cryopreservation of plant cell lines, and initiating new cell lines would result in unpredictable fluctuations in secondary metabolite production (Reeves et al. 2018; Fenning et al. 2017; Volkova et al. 2015). Recent studies have shown that this decrease in secondary metabolite production is correlated with an increase in DNA methylation, suggesting that epigenetic modifications play an important role in regulation of secondary metabolite biosynthesis during initiation and maintenance of plant cell cultures (Fig. 1) (Sanchez-Munoz et al. 2018; Kiselev et al. 2013).

To date, most metabolic engineering strategies for plant cell culture systems have emphasized pathway engineering, which has shown some success in systems such as *Catharanthus roseus* (Sharma et al. 2018), *Vitis amurensis* (Veremeichik et al. 2017), and *Taxus* species (Li et al. 2012). However, to enable consistently high production of these secondary metabolites in plant cell culture, it is necessary to also understand and rationally control the epigenetic silencing mechanisms that regulate secondary metabolite biosynthesis. This chapter will begin with a review of current strategies for plant metabolic engineering, with particular emphasis on the impact these techniques have on the maintenance of productive cell lines during long-term subculturing. Next, we will review the current understanding of epigenetic regulation in plants and the role that epigenetic factors play in regulation of plant

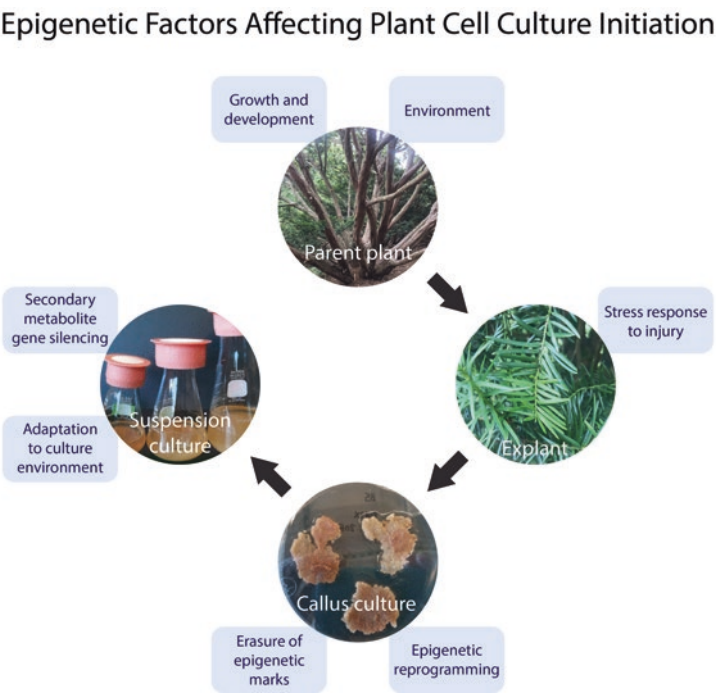

Fig. 1 Epigenetic factors influencing gene expression during initiation of plant suspension cultures
Epigenetic regulatory mechanisms (indicated by blue boxes) play an important role in plant stress response during generation of suspension cultures. To generate callus cultures, sterile explants are excised from a parent plant and cultured on solid media containing hormones that induce dedifferentiation, which triggers substantial epigenetic reprogramming. These callus cultures can then be transferred into liquid media to initiate suspension cultures. As suspension cultures are maintained, a gradual loss of secondary metabolite production is observed over time due to epigenetic regulatory mechanisms, such as increased global DNA methylation.

growth and development, including secondary metabolism. Finally, we will discuss work in the emerging field of epigenetic engineering and how epigenetic engineering techniques developed in mammalian systems could be used to overcome current limitations for industrial production of plant natural products.

2 Metabolic Engineering of Secondary Metabolism

Metabolic engineering is an important discipline that has developed tools to increase production of secondary metabolites in a variety of systems, including plant cell culture. These strategies are generally focused on the optimization of genetic and regulatory processes and have historically been used with great success in a variety of hosts, accelerating the development of industrial-scale plant cell culture processes.

In this section, we will review plant secondary metabolism, discuss current metabolic engineering methods used to increase production of secondary metabolites in plants, and consider how epigenetic factors may impact metabolic engineering efforts.

2.1 Plant Secondary Metabolism

Cell metabolism is broadly divided into primary (central) and secondary (specialized) metabolism (Pichersky and Gang 2000) (Fig. 2). Primary metabolism refers to the set of metabolic pathways that are absolutely required for survival, such as glycolysis and the citric acid cycle. Conversely, secondary metabolism refers to the set of pathways synthesizing metabolic products that may be involved in viability and defense but are not absolutely required for the survival of the organism. While secondary metabolites are produced in a number of bacteria, fungi, and animals, plants are well-known for their large variety of secondary metabolites that have societally relevant applications (Wilson and Roberts 2014). Plant secondary metabolites can serve a wide range of functions within the cell, including cell signaling (plant hormones, such as auxins and gibberellins), defense (paclitaxel, caffeine), and antioxidants (β-carotene, lycopene), and are often produced in response to environmental stressors (Pichersky and Gang 2000). Due to their complexity and structural diversity, these secondary metabolites often have significant commercial value as pigments, flavorings, dyes, medicines, and supplements (Wilson and Roberts 2014).

There is not a universal system for categorization of plant secondary metabolites, but many of the most important secondary metabolites are classified into one of the three categories: terpenoids, phenols, and alkaloids (Guerriero et al. 2018). Terpenoids, and terpenes, are a class of natural products derived from 5-carbon isoprene units and have the general formula $(C_5H_8)_n$, where n refers to the number of isoprene units (Cheng et al. 2007). Both terpenes and terpenoids are categorized by the number of isoprene units they contain, which is usually between 1 and 8. There are two discrete pathways that produce terpenoids within the cell – the mevalonate (MVA) pathway, which occurs in the cytosol, and the methylerythritol 4-phosphate (MEP) pathway, which occurs in the plastids (Martin et al. 2003). While in most organisms, only one of these pathways is active, plants are unique in that both the MVA and MEP pathways are active simultaneously (Hemmerlin et al. 2012), with transport of intermediates and products between the pathways. Once the terpene backbone is generated, terpenes can undergo further modifications (such as cyclization and oxygenation) to form terpenoids, a class of oxygen-containing molecules derived from terpenes (Davis and Croteau 2000). Examples of terpenoids produced by plant secondary metabolism include many plant essential oils responsible for fragrances, antioxidants (retinol, β-carotene), and drugs (artemisinin, cannabinoids, paclitaxel) (Guerriero et al. 2018).

Phenols (also referred to as phenolics) are a group of compounds derived from the phenol group and are characterized by the presence of one or more aromatic

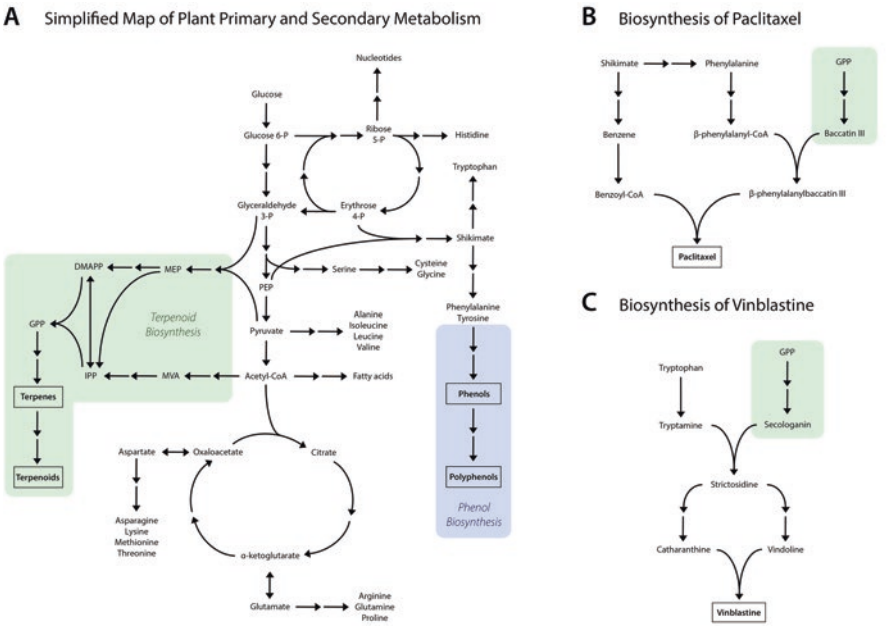

Fig. 2 Simplified maps of major primary and secondary metabolic pathways in plants
(**a**) General map of both primary and secondary metabolisms in plants, illustrating pathways for production of two major classes of natural products: terpenoids (green) and phenols (blue). Single arrows represent a single reaction, while two arrows represent two or more reactions. Black boxes indicate major products of secondary metabolism.
(**b**) Alkaloids and other more complex natural products can be synthesized in unique ways using precursors sourced from many different metabolic pathways. As an illustrative example, this panel depicts a simplified map of how paclitaxel is synthesized from both terpenoid and phenylalanine precursors.
(**c**) Indole alkaloids are a large class of secondary metabolites that includes many pharmaceuticals, such as catharanthine, vincristine, and vinblastine. As an illustrative example, this panel depicts a simplified map of how vinblastine is synthesized from both terpenoid and tryptophan precursors.
Abbreviations: *acetyl-CoA* acetyl coenzyme A, *DMAPP* dimethylallyl pyrophosphate, *erythrose 4-P* erythrose 4-phosphate, *glucose 6-P* glucose 6-phosphate, *glyceraldehyde 6-P* glyceraldehyde 6-phosphate, *GPP* geranyl pyrophosphate, *IPP* isopentenyl pyrophosphate, *MEP* methylerythritol 4-phosphate, *MVA* mevalonate, *PEP* phosphoenolpyruvate, *ribose 5-P* ribose 5-phosphate

rings containing hydroxyl groups (Guerriero et al. 2018). They are produced in plants from the aromatic amino acids phenylalanine and tyrosine, which are synthesized via the shikimate pathway (Vogt 2010). This group of compounds includes phenylpropanoids, coumarins, stilbenes, and flavonoids, which are often used as pigments or dietary supplements (such as resveratrol and anthocyanidins). Polyphenols, which consist of multiple polymerized phenolic groups, include compounds such as tannins that have antioxidant properties and lignins that have applications in bioenergy and advanced materials (Spiridon 2020).

Alkaloids are a broad category of natural products characterized by the presence of basic nitrogen atoms and are one of the largest sources of plant-derived

medicinally active compounds (Guerriero et al. 2018). Because of their diversity, there is not one universal metabolic pathway responsible for the creation of alkaloids, and they are often derived from offshoots of the terpenoid or phenol biosynthesis pathways (Rao and Ravishankar 2002). Many alkaloids are chimeric and are formed by combining terpenes or phenols with other primary or secondary metabolites. For example, indole alkaloids (one of the most important classes of alkaloids) are synthesized by combining tryptophan and terpenoid precursors (Fig. 2). Many common bioactive compounds are alkaloids, including recreational drugs (caffeine, nicotine, cocaine, methamphetamines, psilocybin), and commonly used medications (morphine, vincristine, vincamine, quinine) (Qiu et al. 2014).

In general, most of the compounds targeted for industrial production in plant cell culture are chemically complex alkaloids, but some terpenoids and phenols have also been considered. For plant cell culture to be the most feasible production process, generally the biosynthetic pathway must be complex and not fully characterized, and the product must be of substantial value per unit weight (Oksman-Caldentey and Inze 2004). Conversely, low-value compounds like pigments, dyes, and flavorings are difficult to produce at reasonable prices using plant cell culture (Eibl et al. 2018). Compounds that have successfully been commercialized using plant cell culture include the drugs paclitaxel, podophyllotoxin, and scopolamine (Wilson and Roberts 2012).

2.2 Tools for Metabolic Engineering in Plant Cell Culture

Because plant cell cultures naturally produce very low concentrations of secondary metabolites, metabolic engineering is a useful strategy to increase production of these metabolites to commercially feasible titers and promote the use of plant cell culture technology more broadly. Three techniques are currently commonly used in plant metabolic engineering – elicitation, pathway overexpression and knockout, and transcription factor engineering.

Elicitation

Elicitation is one of the most effective and widely used tools for increasing production of secondary metabolites in plant cell culture at both laboratory and industrial scales (Halder et al. 2019). In whole plants, secondary metabolites are most often produced either at specific developmental stages of the plant or in response to specific environmental stimuli (De Geyter et al. 2012). Elicitors are physical or chemical agents that take advantage of this mechanism by stimulating a defense response, causing plants to produce secondary metabolites that increase resistance to environmental stressors or pests (Narayani and Srivastava 2017) (Fig. 3). Substances originating from outside the cell are called exogenous elicitors, while substances

originating from inside the cell are called endogenous elicitors (Narayani and Srivastava 2017).

Elicitors can be classified into two general categories: biotic and abiotic elicitors. Biotic elicitors are products derived from a living organism, often one that is pathogenic to the particular plant species (such as yeast extract, chitosan, or purified non-native proteins) (Halder et al. 2019). In contrast, abiotic elicitors are stressors that are not biologically produced including heavy metal salts, osmotic stressors (such as sorbitol or sodium chloride), and intracellular signaling molecules (including jasmonates, salicylic acid, and systemin) (Halder et al. 2019). In particular, jasmonates (such as jasmonic acid and methyl jasmonate) are some of the most effective and widely used elicitors, due to their central and universal role as signaling molecules responsible for regulating the defense response in a variety of plant species (Nielsen et al. 2019).

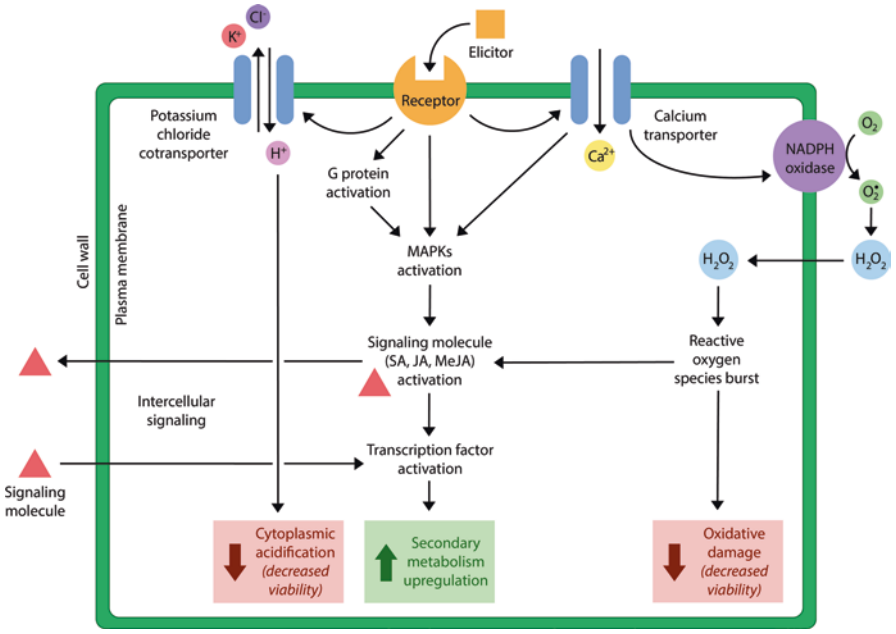

Fig. 3 Diagram of elicitation signal transduction mechanism in plants
In plant cell culture systems, elicitation triggers a complex signal transduction cascade that results in a variety of both positive and negative outcomes (indicated by green and red boxes). When elicitors bind to receptors, they trigger a signal transduction cascade that activates signaling molecules, such as salicylic acid (SA), jasmonic acid (JA), and methyl jasmonate (MeJA). These signaling molecules result in upregulation of secondary metabolism and can also leave the cell to trigger upregulation of secondary metabolism in neighboring cells. However, binding of elicitors to receptors also triggers increased hydrogen ion and calcium ion import, which results in cytoplasmic acidification and oxidative damage, respectively. These outcomes are associated with decreased cell viability, which is not desirable for plant cell culture biomanufacturing platforms.

Generally, elicitors are quite effective at increasing production of secondary metabolites in many different plant cell culture systems (Table 1). At laboratory scales, treatment with elicitors often increases production of secondary metabolites by approximately 2- to 8+-fold depending on a variety of factors, including species, elicitor concentration, and culture age (Halder et al. 2019). Elicitation is often less effective in older cell lines, suggesting that there may be some interaction between DNA methylation and transcriptional reprogramming triggered by elicitation (Sanchez-Munoz et al. 2018). However, there has not yet been substantial research on whether increased global DNA methylation in long-term suspension cultures is indeed responsible for this decreased efficacy.

One advantage of using elicitation as an approach for increasing production of secondary metabolites is that it quickly induces complex transcriptional reprogramming, resulting in concerted activation of the entire metabolic pathways only at the time that increased production is desired (De Geyter et al. 2012). This intricate upregulation and downregulation of all of the many relevant genes associated with elicitation is not yet feasible with current plant genetic engineering techniques (Chen et al. 2019). Despite these advantages, a significant disadvantage of elicitation is that it is not yet possible to decouple this increase in secondary metabolite production with other less desirable effects of elicitation, such as decreased cell growth and viability (Halder et al. 2019).

Overexpression and Knockout of Metabolic Pathways

Overexpression of rate-influencing biosynthetic pathway genes is a widely used, effective metabolic engineering approach to improve synthesis of a desired compound (Pickens et al. 2011). In plants, overexpression of biosynthetic pathway genes is generally achieved by transformation with a vector containing the gene of interest under control of a strong, constitutive promoter, such as the cauliflower mosaic virus promoter (CaMV35S) (Zhang et al. 2005). This method has been used successfully in a variety of species of medicinal plants, including *Taxus* spp. and *Catharanthus roseus.* For example, overexpression of DBAT (a gene controlling one of the steps in paclitaxel biosynthesis in *Taxus* spp.) resulted in a 1.7-fold increase in paclitaxel production (Zhang et al. 2011).

Another approach commonly used in metabolic engineering is deleting or knocking down expression of other undesirable metabolic pathways that compete for the same precursors (Pickens et al. 2011). By eliminating pathways that compete for the same precursors, more metabolic flux can be directed toward production of the secondary metabolite of interest. For example, by knocking out a pathway that competed for monoterpenoid precursors of menthol, menthol production in mint plants was significantly increased (Mahmoud and Croteau 2001). Similarly, GABA content was successfully increased in tomato plants by 6.8–9.2 times compared to wild-type plants by knocking down expression of a competing pathway (Koike et al. 2013).

Genetic engineering-based approaches have the potential to be more advantageous than elicitation for engineering plant cell cultures due to their specificity.

Table 1 Common elicitors used to enhance production of secondary metabolites in plant cell culture

Elicitor	Species	Secondary metabolite	Citation
Jasmonic acid	*Vitis vinifera* *Calendula officinalis* *Mentha x piperita*	Resveratrol Oleanolic acid Rosmarinic acid	Santamaria et al. (2012), Wiktorowska et al. (2010), and Krzyzanowska et al. (2012)
Methyl jasmonate	*Taxus chinensis* *Panax ginseng* *Mentha x piperita* *Gymnema sylvestre* *Vitis vinifera* *Rhazya stricta* *Plumbago indica* *Withania somnifera*	Paclitaxel Ginsenoside Rosmarinic acid Gymnemic acid Anthocyanin Terpene indole alkaloids Plumbagin Withanolides	Zhang et al. (2000), Thanh et al. (2005), Krzyzanowska et al. (2012), Chodisetti et al. (2015), Qu et al. (2011), Akhgari et al. (2019), Gangopadhyay et al. (2011), and Sivanandhan et al. (2013)
Salicylic acid	*Taxus chinensis* *Withania somnifera* *Linum album* *Vitis amurensis* *Brugmansia candida* *Azadirachta indica* *Salvia miltiorrhiza*	Paclitaxel Withanolides Podophyllotoxin Resveratrol Tropane alkaloids Azadirachtin Phenolic acid	Wang et al. (2004), Sivanandhan et al. (2013), Yousefzadi et al. (2010), Kiselev et al. (2015), Pitta-Alvarez et al. (2000), Satdive et al. (2007), and Dong et al. (2010)
Yeast extract	*Salvia miltiorrhiza* *Pueraria candollei*	Tanshinone Isoflavonoids	Zhao et al. (2010) and Udomsuk et al. (2011)
Chitosan	*Taxus chinensis* *Artemisia annua* *Plumbago indica* *Calendula officinalis* *Azadirachta indica* *Cistanche deserticola*	Paclitaxel Artemisinin Plumbagin Oleanolic acid Azadirachtin Phenylethanoid glycosides	Zhang et al. (2000), Putalun et al. (2007), Gangopadhyay et al. (2011), Wiktorowska et al. (2010), Prakash and Srivastava (2008), and Cheng et al. (2006)

Because the expression of specific genes can be regulated, production of secondary metabolites can be increased with fewer of the less desirable effects of elicitation (Pickens et al. 2011). However, genetic engineering approaches are inherently limited by the speed of plant transformation, and thus to date, most work on genetic engineering of plant cell cultures has only reported overexpression of a single gene (Nielsen et al. 2019). This is not usually as effective as elicitation, which upregulates many different genes involved in secondary metabolism (Kiselev et al. 2015; Goklany et al. 2013; Lenka et al. 2015). Luckily, recent advances in plant transformation and genome editing tools (such as CRISPR-enabled multiplex genome editing) have greatly improved the rate at which multiple edits can be made to plant genomes, making more complex genetic modifications now feasible (Minkenberg et al. 2017).

Transcription Factor Engineering

Transcription factors are proteins present in eukaryotes that bind to particular DNA sequences to control the rate of transcription (Latchman 1997). Groups of transcription factors work together to upregulate and downregulate expression of gene clusters in response to a variety of different stimuli, including environmental triggers (Zhao et al. 2005). They can either activate or repress gene expression, depending on the transcription factor, and often trigger signal transduction cascades that change the expression of other transcription factors or genes (Lee and Young 2000; De Geyter et al. 2012).

In several different plant species, there is evidence that many of the genes involved in secondary metabolite biosynthesis are controlled by transcription factors. For example, in *Catharanthus roseus* plant cell culture, elicitation with methyl jasmonate drastically upregulated expression of transcriptional activators, while expression of transcriptional repressors remained low (Goklany et al. 2013). Thus, underexpression of transcription factors that repress secondary metabolism-related gene expression (or conversely, overexpression of transcription factors that promote that gene expression) can result in an increase in secondary metabolite production (Butelli et al. 2008). For example, transient heterologous expression of a transcription factor isolated from *Tripterygium wilfordii* in *Catharanthus roseus* resulted in increased expression of vinca alkaloid biosynthetic genes and a corresponding severalfold increase in vinca alkaloid production (Han et al. 2020).

Upregulation of these transcription factors could result in more targeted upregulation of secondary metabolite biosynthesis without the negative effects on growth associated with elicitation. By upregulating one transcription factor, many related genes can be upregulated, making this approach much faster and more feasible than changing the expression of each individual gene (Tsang et al. 2014). However, the regulatory mechanisms governing secondary metabolite production are complex and not yet fully understood, making it difficult to determine appropriate transcription factor targets. Some studies have shown that silencing certain transcriptional repressors did not increase secondary metabolite production as predicted, likely due

to compensation by other epigenetic regulatory mechanisms (Rizvi et al. 2016). Additionally, binding of transcription factors to target DNA is often disrupted by DNA methylation, so even if the transcription factor were upregulated, it would not necessarily be able to upregulate target genes depending on its epigenetic status (Bell and Felsenfeld 2000; Lowder et al. 2015).

3 Major Mechanisms of Epigenetic Regulation

While metabolic engineering techniques have been moderately successful at increasing production of secondary metabolites in plant cell culture, it is clear that to control levels of production over time, epigenetics needs to be considered. Epigenetics play an important role in regulating gene transcription and a variety of cellular processes, including production of secondary metabolites. Recent research in epigenetics has improved our understanding of epigenetic regulatory processes, and in fact, we now know that many of the earliest and most effective metabolic engineering efforts for plant cell culture (such as elicitation and media optimization) worked by inducing complex epigenetic responses that were not yet fully understood. In order to increase rates of production of secondary metabolites, purely genetic manipulations are insufficient, since epigenetic mechanisms will still modulate gene expression over time in ways that are not yet fully appreciated (Sanchez-Muñoz et al. 2019). Understanding the role of epigenetics in secondary metabolite production will enable the development of engineering strategies to rationally control expression of important genes, making it possible to achieve significantly higher product yields.

The term "epigenetics" was first coined nearly 80 years ago by C. H. Waddington, who initially used it to refer to the complex interactions between the genome and the environment that control differentiation of cells during embryonic development (Waddington 2012). Today, epigenetics is defined as the study of heritable changes in phenotype that do not involve direct modifications to the DNA sequence (Miryeganeh and Saze 2020). These epigenetic modifications, such as DNA methylation, histone modification, and interfering RNA transcripts, play an important role in regulating gene expression in many eukaryotes, including plants (Bird 2007). In fact, as Waddington's original definition alludes to, it is now believed that many of the changes that complex organisms experience as they grow and develop throughout their lifespan are controlled by epigenetic factors (Kumar et al. 2017). Importantly, epigenetic changes can stem from both internal cellular signals as a normal part of growth and development or from external or environmental factors, such as exposure to stress (Sanchez-Muñoz et al. 2019).

Because epigenetics is so broadly defined, there are a wide variety of mechanisms that are considered to be examples of epigenetics. DNA methylation is one of the most widely studied epigenetic modifications, but histone modification and RNA interference also play well-established roles in regulation of gene expression (Kumar et al. 2017). Here, we will discuss the mechanisms of these three primary

epigenetic modifications, with a particular focus on their role in regulating plant growth and secondary metabolite production (Fig. 4).

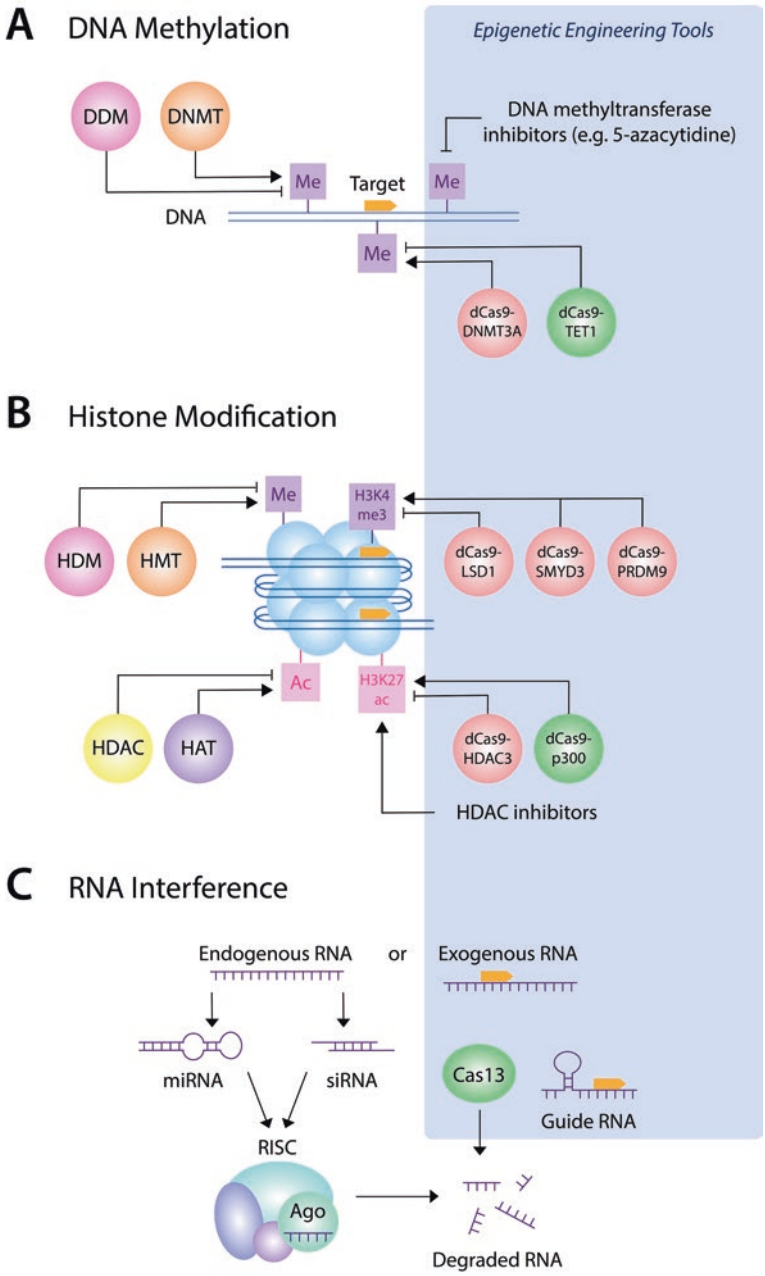

Fig. 4 Major epigenetic regulatory mechanisms and methods for epigenetic engineering in plant systems

3.1 DNA Methylation

DNA methylation is defined as the covalent addition of a methyl group to a nucleotide at the C5 position (Kumar et al. 2017). It is one of the most common and universal epigenetic modifications and is widespread among both prokaryotes and eukaryotes (He et al. 2011). The majority of organisms, including plants, can only methylate cytosine residues, but methylation of other nucleotides can occur in select circumstances (Heithoff et al. 1999). If this methylation occurs within a gene promoter, it reduces the rate of transcription by two related mechanisms. First, the bulk provided by the additional methyl groups blocks recognition and binding by certain transcription factors that increase the rate of gene transcription (Cedar and Bergman 2009). Second, DNA methylation recruits binding of other factors that specifically bind to the methylated DNA and block transcription factor access (Vanyushin and Ashapkin 2011).

In plants, there are three different sequence contexts in which cytosine methylation can occur: CG (sometimes referred to as CpG), CHG, and CHH, where H represents any nucleotide other than guanine (Law and Jacobsen 2010). This flexibility is in contrast to mammals, where methylation only occurs in the CG context (Bartels et al. 2018). Methylation *in planta* can further be categorized into two types: de novo methylation, which is the initiation of new methylation patterns, and maintenance methylation, which copies existing methylation patterns onto daughter DNA strands during DNA replication (Sanchez-Muñoz et al. 2019). Notably, *de novo* methylation by DRM methyltransferases is known to be one of the primary transcriptional silencing pathways in plants (Pribylova et al. 2019). Because of the robustness of the maintenance methylation pathway, changes in DNA methylation

Fig. 4 (continued) Three of the most prominent epigenetic regulatory mechanisms in plants are DNA methylation (**a**), histone modification (**b**), and RNA interference (**c**). Techniques have been developed for engineering each of these three epigenetic mechanisms (indicated by blue boxes). Enzymes in green have been used successfully in plant systems, while enzymes in red have not.
(**a**) DNA methylation is regulated by two families of enzymes: DNA methyltransferases (DNMTs) and DNA demethylases (DDMs), which methylate or demethylate DNA, respectively. DNMT inhibitors (such as 5-azacytidine) can be used to prevent the action of DNMTs, resulting in global DNA demethylation. Target DNA can also be selectively methylated or demethylated by the use of dCas9 proteins fused to the appropriate effector domains.
(**b**) Histone acetylation is regulated by histone acetyltransferases (HATs), which acetylate histones, and histone deacetylases (HDACs), which deacetylate them. HDAC inhibitors prevent the action of HDACs, resulting in global histone hyperacetylation, and methylation and acetylation can be selectively engineered using dCas9 fusion proteins.
(**c**) In RNA interference, RNA is cleaved into miRNA and siRNA, which can each be incorporated into an RNA-induced silencing complex (RISC). RNA complementary to the template incorporated into the RISC is then recognized and degraded. Exogenous RNA containing the engineering target can be added to activate the RNA interference pathway, or CRISPR/Cas13 systems can be designed to bind to and degrade the target

are heritable throughout multiple generations of organisms and remain generally stable over time (Martienssen and Colot 2001).

In plants, DNA methylation is performed by a family of enzymes called DNA methyltransferases (DNMTs) (Cedar and Bergman 2009). *De novo* and maintenance methylation mechanisms are controlled by a total of three different DNMTs: methyltransferase 1 (MET1), domains rearranged methyltransferase (DRM), and chromomethylase 3 (CMT3) (He et al. 2011). DRM is responsible for *de novo* methylation for all three cytosine contexts, while for maintenance methylation, MET1 maintains methylation of CG contexts and CMT3 maintains methylation of CHG contexts (Parrilla-Doblas et al. 2019). There is no known enzyme that specifically maintains methylation of CHH contexts, since methylation in the CHH context is accomplished solely by the de novo mechanism (Sanchez-Muñoz et al. 2019).

DNA can be demethylated both passively, through the failure of methylating enzymes to properly copy methylation patterns during replication, and actively, through the action of DNA demethylases (Parrilla-Doblas et al. 2019). Active DNA demethylation is often used by plants as a mechanism of quickly activating gene expression in response to a developmental trigger or environmental stressor (Law and Jacobsen 2010). Four enzymes are involved in active DNA demethylation in plants: repressor of silencing 1 (ROS1), transcriptional activator demeter (DME), demeter-like protein 2 (DML2), and demeter-like protein 3 (DML3) (Pribylova et al. 2019). Each of these four enzymes is involved in demethylating all three cytosine contexts.

Both DNA methylation and demethylation are closely involved in regulation of growth and development, cell differentiation, and adaptation to the environment (Gehring et al. 2009). Due to the role active demethylation plays in adaptation to environmental stressors, it is not surprising that increased DNA methylation is observed as a result of long-term suspension culture (Sanchez-Muñoz et al. 2019). Similar to how whole plants can adapt their physiology in response to environmental changes, plant cells in suspension culture can adapt their physiology to survive in suspension for long periods of time. In particular, studies have indicated that genes in secondary metabolite biosynthetic pathways are often more methylated in cultures that have been maintained in suspension culture for many years (Sanchez-Munoz et al. 2018). This is a significant barrier preventing continued production of plant secondary metabolites at scale.

3.2 Histone Modification

Histones are a family of positively charged proteins that associate with DNA to form structural units called nucleosomes (Bannister and Kouzarides 2011). The tightness with which DNA is wound around histones can vary depending on their posttranslational modifications, which makes these modifications play an important

role in regulation of gene expression (Bannister and Kouzarides 2011; Rea et al. 2000). There are five major families of histones: H1 and H5 (which are isoforms), H2A, H2B, H3, and H4 (Wang et al. 2008). H2A, H2B, H3, and H4 are similar in structure and are known as the "core" histones, which associate to form the nucleosome "bead," while H1/H5 histones are known as "linker" histones (Bhasin et al. 2006). During the formation of the nucleosome "bead," two dimers of H2A and H2B associate with two dimers of H3 and H4 to form a histone octamer (Luger et al. 1997). The DNA wraps around this octamer while the "linker" histones (H1 or H5) bind the DNA at their entry and exit from the nucleosome bead, locking the DNA into place (Ramakrishnan et al. 1993). The structure of the core histones is quite conserved across all eukaryotic species, while the structure of the linker histones can be more variable (Over and Michaels 2014).

There are a variety of posttranslational modifications of histone proteins that alter how they interact with DNA strands (Wang et al. 2008). Most modifications occur on the lysine and arginine-enriched N-terminal tail of the histone proteins, which is unstructured and protrudes from the nucleosome complex (Shilatifard 2006). These modifications affect the ability of the DNA to wrap around the histones, changing the accessibility of the DNA for transcription and thus, changing gene expression. The most well-characterized histone modifications are methylation and acetylation, but there are a wide variety of other known posttranslational modifications, including phosphorylation, citrullination, SUMOylation, ADP-ribosylation, and ubiquitination (Shilatifard 2006; Roth et al. 2001; Greer and Shi 2012). Because of the numerous possibilities for different combinations of histone modifications, a complete understanding of how each possible combination affects gene expression remains elusive. However, the more common combinations of histone modifications have been more extensively studied and have more predictable effects on gene expression (Strahl and Allis 2000; Wang et al. 2008). This knowledge of how combinations of different histone modifications affect gene expression is referred to as the "histone code" (Jenuwein and Allis 2001).

Histone methylation is one of the best understood histone modifications and involves the transfer of methyl groups to lysine or arginine residues on any of the four histone families (Greer and Shi 2012). The most commonly methylated residues are lysines on histones H3 and H4, including H3K4, H3K9, H3K27, H3K48, H3K79, and H4K20 (where the first number indicates the number of the histone and the second number indicates the number of the lysine being methylated) (Barski et al. 2007). Each lysine residue can be either mono-, di-, or trimethylated, with each level of methylation having different effects on expression of the DNA complexed to that region of the histone (Greer and Shi 2012). Whether or not expression of the relevant DNA is upregulated or downregulated is largely dependent on the location of the residue being methylated and the degree of methylation. Di- and trimethylation of H3K9 and H3K27 and dimethylation of H3K4 are consistently associated with gene repression, while generally most other histone methylation is associated with gene activation (Barski et al. 2007; Liu et al. 2019).

Methylation of histones is performed by a diverse family of enzymes called histone methyltransferases (Zhang and Reinberg 2001). Unlike DNA methylation,

which is controlled by only three enzymes, histone methylation is controlled by a much larger number of histone methyltransferases (HMTs) that all differ in both their substrate specificity (the location of the amino acid being methylated) and their product specificity (the number of methyl groups transferred); details are described elsewhere (Dillon et al. 2005). Histone methylation is more stable than many other histone posttranslational modifications, and thus for a number of years, it was thought to be irreversible, but in 2004, the first histone demethylase, KDM1A, was discovered (Shi et al. 2004). There are now known to be six families of histone demethylases that, like histone methyltransferases, have a wide range of both substrate and product specificities (Shi et al. 2004).

Another of the most well-characterized histone modifications is acetylation, which is the addition of an acetyl group to lysine residues (Bannister and Kouzarides 2011). Acetylation is considered to be more reversible and dynamic than methylation, and unlike methylation, which has a more complex relationship with gene expression, it is almost always associated with gene activation (Bannister and Kouzarides 2011). Histone acetylation is associated with regulation of a wide range of cellular processes, including DNA damage and repair, cell cycle progression, stress response, cell differentiation, and nuclear import (Roth et al. 2001). The most common sites for histone acetylation include H3K9, H3K14, H3K18, H3K23, H3K56, H4K5, H4K8, H4K12, and H4K16 (Bannister and Kouzarides 2011).

The enzymes that catalyze the addition of acetyl groups are called histone acetyltransferases (HATs), while the enzymes that catalyze their removal are called histone deacetylases (HDACs). Similar to histone methylation, there are a large number of different HATs and HDACs which have varying substrate specificities. Histone acetyltransferases fall under one of several different classes: GNAT, MYST, p300/CBP, SRC (nuclear receptor coactivators), and others (Roth et al. 2001), while histone deacetylases are categorized under one of the four classes: Class I, Class II, Class III, and Class IV (De Ruijter et al. 2003). Notably, because of the role that histone acetylation plays in cell cycle regulation, many anticancer drugs are histone deacetylase inhibitors (Bannister and Kouzarides 2011).

3.3 RNA Interference

In eukaryotic organisms, intergenic or antisense translation gives rise to a variety of RNA molecules that are not translated into proteins, which are referred to as noncoding RNA (Holoch and Moazed 2015). There are a wide variety of classes of noncoding RNA that play diverse roles in cellular function and signaling, such as transfer RNA, ribosomal RNA, and many different types of small RNA, including many whose functions are still unknown (He and Hannon 2004; Liu et al. 2017). We will discuss two specific types of small, noncoding RNA known as micro RNA (miRNA) and small interfering RNA (siRNA), each of which plays a central role in

gene repression through a process called RNA interference (RNAi) (Jones-Rhoades et al. 2006).

Micro RNA (miRNA) are small, noncoding RNA molecules that play diverse roles in cellular signaling and control, including RNA silencing pathways (Liu et al. 2017). They are approximately 22 base pairs long and fold back on themselves to form imperfect stem-loop secondary structures (Jones-Rhoades et al. 2006). To produce miRNA, genes are first transcribed by RNA polymerase II (Pol II) to form miRNA precursors called pri-miRNA. These pri-miRNAs are then further processed by Dicer endoribonucleases to form mature miRNA, which consist of a guide strand duplexed with a near-complementary "passenger" strand (referred to as miRNA*) (Liu et al. 2017). The guide strands then disassemble from the passenger strands to associate with argonaute (AGO) proteins in the RNA-induced silencing complex (RISC), which is a large complex consisting of many different proteins responsible for binding and cleaving RNA (Liu et al. 2017). Once incorporated into the RISC, the guide strand can then bind to complementary mRNA, following which it is cleaved by the RISC, resulting in reduced gene expression (Jones-Rhoades et al. 2006).

Similar to miRNA, small interfering RNA (siRNA) are short (about 20–25bp), double-stranded noncoding RNA molecules that play an important role in gene regulation (He and Hannon 2004). Unlike miRNA, they consist of a double-stranded, perfectly complementary RNA segment with no hairpins or mismatched bases (Carthew and Sontheimer 2009). They are produced by cleavage of long double-stranded RNA into short fragments by Dicer endoribonucleases, which then triggers the recruitment of various proteins to form the RISC (Matzke and Mosher 2014). Once the RISC is formed, the strand with the less stable 5′ end remains part of the complex (forming the "guide" strand), while the other strand is degraded, leaving a single-stranded RNA template (Holoch and Moazed 2015). The RISC can then bind and cleave complementary mRNA by the same mechanism as with miRNA.

Both miRNA and siRNA play important roles in regulation of plant growth and development. In addition to their direct roles in RNA silencing by cleavage of RNA transcripts, siRNA is involved with regulation of both DNA methylation and histone methylation, forming a complex regulatory loop (Holoch and Moazed 2015). RNA-directed DNA methylation (RdDM) requires specialized transcriptional machinery, including two plant-specific RNA polymerases: Pol IV and Pol V (Matzke and Mosher 2014). Transcripts from Pol IV are copied to form long double-stranded RNA, which are then processed by Dicer endoribonucleases into siRNA. When one of the siRNA strands complexes with AGO, it then guides the complex to complementary transcripts generated by Pol V, which ultimately recruits DNA methyltransferases to the aforementioned transcripts. This event triggers DNA methylation in all cytosine contexts (CG, CHG, and CHH) at the genomic loci transcribed by Pol IV, resulting in gene silencing (Wassenegger et al. 1994).

Overall, while RdDM is important to gene regulation in plants, it plays a less directly important role in gene silencing than endogenous de novo methylation pathways. For example, in one study in *Arabidopsis thaliana*, approximately 2000 genes were reactivated in *ddm1* and *met1* knockout mutants, while only around 40

genes were reactivated in an RdDM-defective mutant (Matzke and Mosher 2014). However, the true power of this mechanism lies in its ability to perpetuate positive feedback loops. In particular, the majority of genes targeted by RdDM have H3K9me modifications, which reinforce the gene silencing caused by DNA methylation (Matzke and Mosher 2014). In *Arabidopsis thaliana,* the enzymes that catalyze H3K9 methylation (SUVH histone methyltransferases) have a 5-methylcytosine binding domain, and similarly, one of the enzymes that catalyzes DNA methylation (CMT3) contains an H3K9me binding domain (Enke et al. 2011). This indicates that both H3K9me and DNA methylation reinforce the effects of each other, creating a strong, self-perpetuating feedback mechanism that maintains both epigenetic modifications.

In addition to RNA silencing pathways, there is evidence that small noncoding RNA are involved in similar pathways that regulate gene activation. This phenomenon, called RNA activation (RNAa), was first reported in 2006. When human cell lines were transfected with synthetic RNA complementary to promoter regions of certain genes, expression of those genes increased (Li et al. 2006). While these pathways have mostly been studied in mammalian cells, RNA activation is likely conserved across most eukaryotic species, including plants (Shibuya et al. 2009). Processing of RNA for RNA activation follows a mechanism analogous to the mechanism for RNA interference. Double-stranded RNA is first cleaved by Dicer into small activating RNA (saRNA), which is then bound by an AGO protein, similar to how siRNA is processed. However, the saRNA is then loaded into a different protein complex known as the RNA-induced transcriptional activation (RITA) complex, which recruits transcriptional activators and triggers epigenetic changes associated with DNA activation (Vaschetto 2018). While the exact role of RNAa in regulation of gene expression in plants is not yet well-established, it is likely that this mechanism plays a key role in concerted activation of multiple genes (Vaschetto 2018).

4 Tools for Epigenetic Engineering

Epigenetic engineering is an emerging field that seeks to modify the epigenome, analogous to how the field of genetic engineering modifies the genome (Pulecio et al. 2017). While genetic engineering involves directly editing the sequence of DNA bases itself, epigenetic engineering techniques do not change the DNA sequence, but instead, its expression level. Epigenetic engineering techniques fall into two general categories: global epigenetic engineering, which affects the entire epigenome nonspecifically, and targeted epigenetic engineering, which affects the expression of only specific genes (Vojta et al. 2016). Here, we will discuss tools that have been developed for both global and targeted epigenetic engineering approaches in a variety of host organisms.

4.1 Global Epigenetic Engineering

Global epigenetic engineering approaches manipulate the epigenome nonspecifically, often by the addition of chemicals that inhibit certain epigenetic modifications, such as DNA methylation or histone acetylation. In this section, we will review approaches used for both global DNA methylation engineering and global histone modification engineering and discuss their potential for engineering secondary metabolite production in plant cell culture.

Global DNA Methylation Engineering

Due to the role of DNA methylation in modulating gene expression in response to stress, modification of methylation patterns has been explored as a tool to engineer novel changes in plants, such as increased production of secondary metabolites (Li et al. 2013a). There are a variety of chemical agents that can be used to inhibit DNA methylation, each with different mechanisms of action. One of the first compounds used to inhibit DNA methylation is 5-azacytidine, which is a cytidine analog originally used as a chemotherapeutic for acute myelogenous leukemia and other cancers (Cihak and Vesely 1978). Because 5-azacytidine can be incorporated into both DNA and RNA but is unable to be methylated due to its chemical structure, it soon became used as a tool to study DNA methylation patterns in plants.

Early research on the effects of 5-azacytidine in plants determined that treatment with 5-azacytidine was correlated with decreased global DNA methylation in a variety of plants, including liverwort (Takio et al. 1999), petunia (Prakash and Kumar 1997), and potato (Prakash and Kumar 1997), and did not adversely affect plant growth at the concentrations used. It was also shown that this decreased methylation has enabled reactivation of previously silenced genes, which led to future work on treatment with 5-azacytidine as a way to increase production of secondary metabolites in plant cell cultures (Li et al. 2013a).

5-Azacytidine has been used successfully to promote higher levels of secondary metabolite production in plant cell culture for several different species. In particular, treatment of grape cell (*Vitis amurensis*) suspension cultures with 50–200 μM of 5-azacytidine (with fresh 5-azacytidine-spiked media added every 3 days) resulted in a 1.9–2.0-fold increase in resveratrol levels and significantly increased expression of several genes known to play important roles in resveratrol production (Tyunin, Kiselev and Zhuravlev 2012). However, these concentrations of 5-azacytidine also resulted in a significant decrease in accumulated biomass, with cells treated with 5-azacytidine accumulating 1.3–2 times less biomass over the course of 35 days (Kiselev et al. 2011). These results are similar to those found with triterpenoid production in birch (Zeng et al. 2019), cephalotaxine production in *Cephalotaxus mannii* (Wei et al. 2019), and paclitaxel production in *Taxus* species (Li et al. 2013a). In all cases, while treatment of plant cell cultures with 5-azacytidine did increase production of the secondary metabolites of interest, it also resulted in a corresponding decrease in cell growth.

An emerging alternative to 5-azacytidine is zebularine, a DNA methyltransferase inhibitor with a similar structure and mode of function (Cheng et al. 2003). Unlike 5-azacytidine, which prevents DNA methylation by incorporating into the genome, zebularine is a competitive inhibitor of DNA methyltransferases, reducing their ability to methylate DNA (Marquez et al. 2005). Additionally, zebularine is significantly less toxic and has a longer half-life in aqueous solutions at physiological temperatures (Baubec et al. 2009), making it potentially better suited for plant cell culture applications.

Initial studies of zebularine in animal models showed that similar to 5-azacytidine, it was able to both inhibit DNA methylation and reactivate previously transcriptionally silenced genes (Cheng et al. 2003). Despite its proven efficacy as a methyltransferase inhibitor (Cheng et al. 2003; Marquez et al. 2005; Yoo et al. 2004; Yoo and Jones 2006), there has so far been comparatively little research on the effects of zebularine treatment in plants. One of the first studies on zebularine treatment in plants found that similar to 5-azacytidine, *Arabidopsis thaliana* seedlings treated with 5-azacytidine exhibited a dose-dependent response (Baubec et al. 2009). At low concentrations of zebularine, there was a small decrease in both global methylation (as measured by UPLC) and cell growth, and at higher concentrations, there was a larger decrease in global methylation, but a corresponding larger decrease in cell growth. Compared to seedlings treated with 5-azacytidine, the seedlings treated with the same concentration of zebularine had slightly lower global methylation levels (4.0% vs. 4.8%), indicating that zebularine is at least as effective as 5-azacytidine as a demethylating agent.

Despite this seemingly promising preliminary research, treatment with global demethylating agents as a method of inducing secondary metabolite production in plant cell culture has some limitations. While both 5-azacytidine and zebularine do increase secondary metabolite production when normalized by cell dry weight, this increase in cell productivity is insignificant when the decrease in biomass accumulation is considered. Additionally, since methylation of genes plays an important role in a variety of cellular processes other than secondary metabolite production, global demethylation results in substantial off-target effects, such as the decrease in cell viability observed at certain concentrations of these demethylating agents. The inherent lack of specificity of this approach limits long-term applications of global DNA methyltransferase inhibitors.

Global Histone Modification Engineering

Similar to how 5-azacytidine and zebularine can demethylate DNA on a global level, there are compounds that can modify histones on a global level. Many of these compounds have been investigated as potential cancer therapeutics due to the role that histone modifications play in regulating cancer disease progression (Lee and Marks 2010). However, there is some evidence that these compounds could also be useful in regulating gene expression in plant cell culture. Histone acetylation is of particular interest because deacetylation is known to be a marker of cell death in elicited plant cells (Bourque et al. 2011). If high levels of histone acetylation could

be maintained under elicitation, higher levels of secondary metabolite production could potentially be achieved without a decrease in cell growth or viability.

Histone deacetylase (HDAC) inhibitors are drugs that inhibit histone deacetylation, resulting in hyperacetylated histones and transcriptional activation of genes. There is some evidence that histone acetylation may be implicated in the plant stress response. For example, in a study that involved submerging rice plants in water, when the rice was treated with trichostatin A (an HDAC inhibitor), genes associated with stress response were more acetylated (as measured by kinetic chromatin immunoprecipitation) and upregulated than the untreated group (Tsuji et al. 2006). This effect was shown to be dynamic and reversible, taking place within the span of several hours after the stress was induced. While there have not been studies on the effects of HDAC inhibitors on elicited plant cells, this suggests that treatment with HDAC inhibitors could potentially improve their ability to respond to stressors, such as elicitation.

To date, there is little information on the effects that HDAC inhibitors may have on secondary metabolite production in plant cell culture. Several studies have shown that treatment with HDAC inhibitors increases recombinant protein production in *Nicotiana tabacum* cell culture (Rebelo et al. 2020; Santos et al. 2017), which suggests that there may be a similar effect with native secondary metabolites. However, similar to global demethylating agents, many common HDAC inhibitors are also known to negatively affect plant growth and development. For example, several studies have shown that treatment of *Arabidopsis thaliana* with a variety of HDAC inhibitors generally results in abnormal phenotypes, arrested growth, and upregulation of embryogenesis-related genes (Lochmanova et al. 2019; Tanaka et al. 2008).Therefore, at this point, it is difficult to determine whether or not engineering global histone acetylation would have a significant impact on production of secondary metabolites in plant cell culture.

4.2 Targeted Epigenetic Engineering

In contrast to global epigenetic engineering approaches, which affect the entire genome, targeted epigenetic engineering approaches affect only specific genes, resulting in more specific effects on gene expression without some of the drawbacks of global approaches (such as abnormal or reduced growth) (Xu and Oi 2019). Genes can be rationally targeted using a variety of different DNA-binding proteins, including zinc finger nucleases (ZFNs), transcription activator-like effector nucleases (TALENs), and CRISPR-associated proteins, such as Cas9 (Miglani et al. 2020). Before the development of CRISPR/Cas9 technology, these localized epigenetic modifications were made using ZFNs or TALENs, and the slow and laborious process of protein engineering limited large-scale applications of these technologies (Pulecio et al. 2017). The advent of CRISPR/Cas9 technology was a major breakthrough for the field due to the ease of programming guide RNA to target specific genes (Jinek et al. 2012). Most current targeted epigenetic engineering approaches rely on the use of a catalytically dead Cas9 enzyme (dCas9), which

retains its ability to bind specific sequences of DNA but does not cleave them. The dCas9 enzymes can be fused to a variety of different effector domains, such as DNA demethylases or histone deacetylases, to make site-specific epigenetic modifications (Gilbert et al. 2013). This section will review current CRISPR/Cas9-based tools that have been developed for targeted epigenetic engineering (Table 2).

Targeted DNA Methylation Engineering

As previously described, methylation of DNA in eukaryotes is endogenously regulated by groups of DNA methyltransferases and demethylases. The methylation status of genes can be altered by using a dCas9-based system, where the dCas9 enzyme is fused with an endogenous DNA methyltransferase or demethylase. Tools for targeted DNA methylation engineering have been developed successfully for mammalian systems using the human homologs for previously discussed plant DNA methyltransferases and demethylases: DNMT3A for methylation and TET1 for demethylation (Liu et al. 2016; McDonald et al. 2016; Vojta et al. 2016). These dCas9-DNMT3A and dCas9-TET1 fusions have proven activity in mammalian cell lines. For example, in neuron cells, a dCas9-TET1 fusion was successfully used to demethylate the BNDF promoter, activating BNDF gene expression, while a dCas9-DNMT3A fusion was able to block binding of CTCF and interfere with gene looping (Liu et al. 2016).

While these tools utilize DNA methyltransferases and demethylases derived from human cells, they should be functional in plant cells as well. In one study, a zinc finger fused to the human demethylase TET1 was used to demethylate the promoter regulating the *FWA* gene in *Arabidopsis thaliana,* resulting in upregulation of *FWA* and a heritable late-flowering phenotype (Gallego-Bartolome et al. 2018). This result confirms the functionality of these human-derived enzymes in plants and

Table 2 Notable tools developed for targeted CRISPR/dCas9-based epigenetic engineering of DNA methylation and histone modification

Effector domain	Cell line	Epigenetic mechanism	Effect on gene expression	Citation
DNMT3A	Mouse fibroblasts	DNA methylation	Downregulation	Liu et al. (2016)
TET1	Mouse cortical neurons	DNA demethylation	Upregulation	Liu et al. (2016)
p300	Human HEK293T cells	Acetylation of H3K27	Upregulation	Hilton et al. (2015)
HDAC3	Mouse neuroblastoma cells	Deacetylation of H3K27ac	Downregulation	Kwon et al. (2017)
SMYD3	Many human cell lines	Trimethylation of H3K4	Upregulation	Kim et al. (2015)
PRDM9	Human HEK293T and A549 cells	Trimethylation of H3K4	Upregulation	Cano-Rodriguez et al. (2016)
LSD1	Mouse embryonic stem cells	Demethylation of H3K4me2	Downregulation	Kearns et al. (2015)

provides a proof of concept for the potential for these dCas9-based systems to regulate gene expression in plant cell culture.

Targeted Histone Modification Engineering

While the role that DNA methylation plays in decreasing secondary metabolite production in plant cell cultures over time is well-established, the role that histone modification plays in this phenomenon is less certain. However, this approach can still be used to increase or decrease expression of a variety of genes implicated in secondary metabolite production, analogous to typical metabolic engineering approaches. As with targeted methylation engineering approaches, targeted histone modification can be achieved by fusing the dCas9 protein with an enzyme that performs the histone modification of choice, most commonly methylation or acetylation (Tadic et al. 2019). In principle, any type of histone modification could be achieved, as long as the appropriate effector domain is fused to the dCas9 protein.

Similar to targeted methylation engineering, most tools developed for targeted histone modification engineering are centered around applications in mammalian cells, with a focus on histone methylation and acetylation (Xu and Oi 2019). The first paper describing CRISPR-based engineering of histone acetylation used a dCas9 protein fused to the catalytic core of p300 (a human histone acetyltransferase) to catalyze acetylation of histone 3 at lysine 27, leading to transcriptional activation of both genes and enhancers targeted by the guide RNA (Hilton et al. 2015). Gene activation by this p300 core fusion protein is advantageous because it can activate genes resistant to activation by other means, such as dCas9-VP64 activation (Hilton et al. 2015). This p300 core fusion system is also known to be functional in *Arabidopsis thaliana* (Paixao et al. 2019). Methods have also been developed for engineering histone methylation in mammalian cells, such as gene activation by fusion of dCas9 with the histone methyltransferase LSD1 (Kearns et al. 2015) or gene repression by fusion with the histone demethylases SMYD3 or PRDM9 (Kim et al. 2015; Cano-Rodriguez et al. 2016).

Targeted RNA Engineering

Targeted RNA engineering is one of the oldest forms of epigenetic engineering and has been widely used for many years. In a groundbreaking paper published in *Nature* in 1998, Andrew Fire, Craig Mello, and colleagues demonstrated that injecting exogenous double-stranded RNA into *C. elegans* resulted in silencing of genes with a complementary sequence (Fire et al. 1998). They found that by introducing foreign RNA into the cell, it is identified as a target for gene silencing, triggering the RISC to bind to and degrade the corresponding mRNA transcripts. This discovery ultimately led to them receiving the 2006 Nobel Prize in physiology or medicine for their work on RNA interference (RNAi).

Since then, RNAi has been proven to be a robust and powerful method of silencing undesirable genes in a variety of organisms, including model eukaryotic

systems such as *C. elegans* and *D. melanogaster*, mammalian cell culture, whole plants, and even plant cell culture. Using RNAi, a variety of different crops have been engineered to have more desirable traits by silencing key genes in various biosynthetic pathways (Mansoor et al. 2006). For example, by silencing the CaMxMt gene, which is part of the caffeine biosynthetic pathway in coffee bean plants, they can be engineered to produce decaffeinated beans (Ogita et al. 2004). Other examples of uses of RNAi in crops include improving shelf life in potatoes by reducing enzymatic browning (Wesley et al. 2001), increasing carotenoid and flavonoid content in tomatoes for increased health benefits (Davuluri et al. 2005), and improving the nutritional value of cottonseed oil by increasing stearic acid and oleic acid content (Liu et al. 2002).

While there is comparatively little work on applications of RNAi for plant cell cultures, there is still evidence that RNAi could become a powerful tool for engineering natural product metabolism, particularly for suppressing production of undesirable side products. For example, knocking down production of PLR, an enzyme that catalyzes production of lariciresinol (one of two lignin precursors), in *Forsythia koreana* plant cell culture led to an almost complete loss of lariciresinol and an overabundance of the other lignin precursor, pinoresinol (Kim et al. 2009). In another case, RNAi was used in *Catharanthus roseus* cell cultures to knock down tryptamine biosynthesis, leading to synthesis of novel alkaloid compounds (Runguphan et al. 2009). These results suggest that use of RNAi to engineer secondary metabolism in plant cell cultures is worth further investigation, with a focus on epigenetic engineering.

In addition to classical RNAi-based gene knockdown, CRISPR/Cas13-based methods have been developed for targeting and engineering RNA transcripts (Cox et al. 2017; Abudayyeh et al. 2017). Unlike many other Cas systems, which bind and cut DNA, Cas13 specifically binds and cuts RNA, making it suitable for posttranslational manipulation of gene expression (Wolter and Puchta 2018). These methods have been proven functional in several different eukaryotes, including plants (Abudayyeh et al. 2019). Cas13 can be used to knock down expression of certain genes similar to classical RNAi-based methods, but with superior specificity and the potential for multiplexing (Pickar-Oliver and Gersbach 2019). As with other CRISPR/Cas technologies, effector domains can also be fused to dCas13 proteins to enable applications such as RNA single-base editing and visualization (Schindele et al. 2018). While Cas13 technology has not yet been used for metabolic engineering applications in plant systems, as the technology becomes more well-developed, it is likely to enable many more complex and controlled changes in plant metabolism than the previous methods (Pickar-Oliver and Gersbach 2019).

5 The Future of Plant Epigenetic Engineering

Many of the tools developed for epigenetic engineering have been successful in other contexts, but underexplored in the context of engineering secondary metabolism in plant cell culture. With an improved understanding of how epigenetic

factors contribute to regulation of secondary metabolism, we can now start to rationally manipulate them on a mechanistic level to achieve high yields of secondary metabolites. These epigenetic approaches are not meant to replace traditional metabolic engineering approaches, but rather, to complement them. While classical metabolic engineering techniques can be used to increase production of secondary metabolites in the short term, complementary epigenetic engineering techniques can be used to ensure that those changes are stable over time and not shut down by compensatory regulatory mechanisms. This section will provide a vision for how targeted epigenetic engineering could be used in the near future to increase production of secondary metabolites in plant cell culture.

There are many different potential targets for DNA methylation engineering in plant cell culture systems that parallel those in classical metabolic engineering. Similar to how genes can be upregulated or downregulated using traditional overexpression or underexpression approaches, they can also be upregulated or downregulated using either a dCas9-TET1 fusion or a dCas9-DNMT3A fusion, respectively. In general, genes in the biosynthetic pathways that produce secondary metabolites become more highly methylated over time, so selectively demethylating these genes with a dCas9-TET1 fusion construct would likely increase production of secondary metabolites, at least up to the levels observed in recently initiated cultures. For example, in *Taxus* plant cell culture, the BAPT gene in the paclitaxel synthesis pathway is highly upregulated when newly initiated cultures are elicited, but only slightly upregulated when 10-year-old cultures are elicited (Sanchez-Munoz et al. 2018). In the 10-year-old cell lines, the promoter regulating expression of BAPT was shown to be significantly more methylated (specifically in the CHH context) than the newly initiated cultures, suggesting that this may be a cause for this discrepancy in gene expression (Sanchez-Munoz et al. 2018) and a potential epigenetic regulatory mechanism for the paclitaxel biosynthetic pathway. Therefore, if the BAPT promoter could be selectively demethylated using a CRISPR-based approach, paclitaxel production in older cell cultures could potentially be restored. Using a similar approach, by methylating competitive pathways using a dCas9-DNMT3a fusion, production of secondary metabolites could also be increased.

While the potential of this approach is promising, an often-cited problem is that the changes made to DNA methylation will not be stable over time. This is a reasonable concern, because while methylation in the CG and CHG contexts is relatively stable and inheritable (being controlled by maintenance methylation pathways), CHH methylation is solely controlled by the de novo methylation mechanism, which is dynamic and reversible in response to stress (Sanchez-Muñoz et al. 2019). One potential solution to this problem is to manipulate the DNA methyltransferase production pathways themselves. In studies comparing older and newly initiated plant cell lines, the levels of CG and CHG methylation were largely unchanged between the lines, while the levels of CHH methylation increased significantly in the older cell lines (Sanchez-Munoz et al. 2018). It is therefore likely that increased CHH methylation is what causes this phenomenon of decreased secondary metabolite production in older cultures. Underexpression of methyltransferases responsible for CHH methylation (such as DRM) could result in greater resistance to gene

silencing in older cultures while maintaining the methylation patterns responsible for normal growth and development. This could be accomplished by traditional methods (such as RNAi) or by selectively methylating the gene controlling production of DRM methyltransferases using a dCas9-DNMT3A fusion.

Using targeted epigenetic engineering approaches in combination with elicitation or transcription factor engineering could result in concerted activation of many biosynthetic pathway genes with fewer negative side effects, such as decreased growth. For example, in *Taxus* species, there are a variety of transcription factors, such as the WRKY and bHLH families, that are known to be upregulated under elicitation and are likely involved in regulating paclitaxel biosynthesis (Li et al. 2013b; Lenka et al. 2015). Upregulation of these transcription factors using any activating epigenetic approach (such as DNA demethylation, histone trimethylation, histone acetylation, or RNAa) would increase their expression and allow them to correspondingly upregulate expression of biosynthetic pathway genes. This approach would be advantageous because by upregulating a single transcription factor, many genes with roles in paclitaxel biosynthesis would be simultaneously activated or repressed, including genes whose roles in paclitaxel biosynthesis may not yet be known. A further extension of this approach would be to more specifically tune gene expression by methylating or demethylating particular genes under control of the transcription factor of interest. Targeted methylation could be used to inhibit expression of genes that have undesirable effects (such as growth inhibition), while targeted demethylation could be used to ensure that genes known to play key roles in secondary metabolite synthesis (such as BAPT for *Taxus* species) are highly expressed.

While selective activation of desirable pathways and deactivation of unfavorable pathways may be able to restore secondary metabolite production in older cultures to baseline levels observed in newly initiated cultures, this approach would not be able to significantly increase production of secondary metabolites on its own. To increase production beyond baseline levels, this epigenetic engineering approach needs to be used in combination with traditional metabolic engineering and cellular engineering approaches. For example, to increase the expression of the BAPT gene beyond baseline levels, elicitation, transcription factor overexpression, or overexpression of the BAPT gene itself will need to be performed. In this way, the combined efforts of metabolic engineering and epigenetic engineering could have profound effects on cellular metabolism, beyond the effects of either technique in isolation.

6 Conclusions

Production of secondary metabolites in plant cell culture is regulated by a variety of complex epigenetic mechanisms, including DNA methylation, histone modification, and interfering RNA transcripts, which results in decreased production of secondary metabolites in long-term plant cell culture. While metabolic engineering approaches

(such as elicitation, overexpression of biosynthetic pathway genes, competing pathway knockouts, or transcription factor engineering) are powerful tools for increasing secondary metabolite production, their effects are not stable as cultures age. This is a significant barrier in plant cell culture biotechnology, preventing reliable commercial production of more compounds using plant cell culture. While global epigenetic engineering approaches (such as treatment with DNA methyltransferase inhibitors or HDAC inhibitors) have been investigated as a possible solution to this problem, treatment with these agents results in a significant decrease in cell growth, making them not suitable for industrial applications. However, epigenetic engineering approaches that target specific genes are emerging as a promising potential solution to this problem. By engineering the epigenome via targeted CRISPR-based approaches, high levels of secondary metabolite production can be maintained, even in cultures that have been in culture for many years. When used in combination with traditional metabolic engineering approaches, epigenetic engineering could also be a powerful tool to stably increase secondary metabolite production beyond baseline levels. With the advent of higher throughput techniques for plant transformation, such as multiplex CRISPR/Cas9 approaches, creating cell lines with more complex genetic and epigenetic changes is now becoming a possibility. This new strategy will enable us to not only make current plant cell culture products more affordable but help bring new products to market that cannot be produced by other means. By harnessing the power of epigenetic engineering, production of societally valuable pharmaceuticals and other plant natural products in plant cell culture can become more accessible to industry.

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Plant Cell Biofactories as In Vitro Production Platforms of the Anticancer Drug Camptothecin

Sarayu Murali, Hemalatha Rajendran, and Smita Srivastava

Abstract Plants have been a treasure trove for products traditionally used in healthcare from time immemorial. Their contribution to the pharmaceutical, nutraceutical, and chemotherapeutic armamentarium has been unequivocally significant. Plant products, specifically secondary metabolites, have been under the limelight for their extensive use in the pharmaceutical industry with a plethora of applications ranging from treating deadly diseases like cancer to minor ones such as common cold. Camptothecin, an anticancer drug, was first identified and isolated from *Camptotheca acuminata*, a medicinal tree of China. The chief function of the metabolite in planta is to offer protection against herbivores. It acts on the replicating fork of DNA-topoisomerase I complex and inhibits their dissociation further arresting the cell division and cancer growth. Apart from anticancerous activity, camptothecin possesses antiviral, antifungal, and anti-parasitic activity. So far, camptothecin has been commercially extracted only from trees, *C. acuminata* and *Nothapodytes nimmoniana* (native to Indo-China region). However, the quantity of camptothecin present in these trees is subjected to seasonal and geographic variations. Furthermore, due to high demand of camptothecin, extraction from natural sources has led to significant decrease in the number of trees in their natural habitat and adulteration of the raw material during collection. Chemical synthesis of camptothecin has its own limitations such as high cost, harsh chemical and physical reaction conditions, low product yield, and tedious downstream processing. As a promising alternative, in vitro plant cell/tissue culture technology may be utilized to achieve a large-scale production of camptothecin for sustainable and continuous production to meet the ever-increasing market demand. Thus, apart from an overview of camptothecin discovery, structure, and mechanism of action, this chapter aims to highlight and discuss the current developments in bioprocess optimization and metabolic engineering strategies culminated with a discussion on bioreactor design for the in vitro production of camptothecin via plant cell biofactories.

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Abbreviations

HCPT	10-Hydroxy-CPT
10-HGO	10-Hydroxygeraniol oxidoreductase
DXS	1-Deoxy-D-xylulose-5-phosphate synthase
DXP	1-Deoxy-D-xylulose-5-phosphate
2,4-D	2, 4-Dichlorophenoxyacetic acid
MECS	2-C-Methylerythritol-2,4-cyclodiphosphate synthase
HMGR	3-Hydroxy-3-methylglutaryl-CoA reductase
HMGS	3-Hydroxy-3-methylglutaryl-CoA synthase
CMS	4-(cytidine 5-diphospho)-2-C-methylerythritol synthase
CMK	4-(cytidine 5-diphospho)-2-C-methylerythritolkinase
MCPT	9-Methoxy CPT
ABA	Abscisic acid
AACT	Acetyl-CoA: acetyl-CoA C-acetyltransferase
ASA	Anthranilate synthase
BAP	Benzylaminopurine
CPT	Camptothecin
DMAPP	Dimethylallyl diphosphate
DW	Dry weight
DXR	DXP reductoisomerase
G10H	Geraniol 10-hydroxylase
GES	Geraniol synthase
GPP	Geranyl diphosphate
GAP	Glyceraldehyde-3-phosphate
GPPS	GPP synthase
Gy	Gray (unit of absorbed dose of ionizing radiation)
HDR	Hydroxy methyl butenyl 4-diphosphate reductase
HDS	Hydroxy methyl butenyl 4-diphosphate synthase
HMBPP	Hydroxy methyl butenyl diphosphate
IPP	Isopentenyl diphosphate
JA	Jasmonic acid
MeJA	Methyl jasmonate
MEP	Methylerythritol phosphate
MDC	Mevalonate 5-diphosphate decarboxylase
MK	Mevalonate kinase
MVA	Mevalonic acid
MS	Murashige-Skoog medium
2ip	N^6-(2-isopentenyl) adenine
CPR	NADPH-cytochrome P450 reductase

NAA	Naphthalene acetic acid
NN	Nitsch & Nitsch medium
PMK	Phosphomevalonate kinase
PGR	Plant growth regulators
PEG	Polyethylene glycol
ROS	Reactive oxygen species
SA	Salicylic acid
SLS	Secologanin synthase
SGD	Strictosidine beta-glucosidase
STR	Strictosidine synthase
TOP1	Topoisomerase 1 enzyme
TDC	Tryptophan decarboxylase
TSA	α-Subunit of tryptophan synthase
TSB	β-Subunit of tryptophan synthase

1 Introduction

Cancer has been one of the world's largest health issues over the past few decades. It has been one of the leading causes of death in the world. In 2017 alone, nearly 24.5 million cancer cases and 9.6 million deaths have been reported worldwide (Fitzmaurice et al. 2018). Although there have been several technological and pharmacological developments, there has been a gripping need for the production of anticancer drugs to meet the ever-increasing demand. Most of the drugs used in chemotherapeutics have been derived from plant sources (Korkina and Kostyuk 2012). These phytochemicals supporting the survival of plants, termed as secondary metabolites, are sought-after by humankind for cancer treatments.

Camptothecin (CPT) (a modified monoterpene indole alkaloid) is the third most promising and sought-after secondary metabolite used by the pharmaceutical industry around the globe after taxol and vinca alkaloids. It is the lead molecule for the commercial drugs (irinotecan and topotecan) used in the treatment of colon, lung, breast, uterine, and cervical cancers. CPT is commercially extracted from two plants, *Camptotheca acuminata* and *Nothapodytes nimmoniana* (both native to Southeast Asia). The annual production of CPT throughout the world in 2015 was reported to be only 600 kg, while approximately 3000 kg of CPT was needed in international markets (Cui et al. 2015).

Over the years, CPT has been mainly extracted from natural plant resources. However, owing to unavailability of the plant material, seasonal and geographic variations in the CPT yield in plants, difficulties in extraction, and purification from the whole plant, utilization of natural plants as sources for CPT has been an arduous task. There have been some reports on the establishment of total synthesis of CPT by chemical methods at a commercial level. However, they suffer from limitations like low product yield, being labor intensive, high cost of production, and harsh

physical and chemical conditions (like sub-zero temperature) (Venugopalan and Srivastava 2015). Moreover, the downstream processing (purification) involves multiple steps, sometimes resulting in toxic intermediates and by-products (Buyel 2018).

Bioreactor cultivation of different plant cell/tissue cultures has been used in the past decades to achieve increased production of commercially important metabolites such as food additives, nutraceuticals, and therapeutic proteins. Well-known examples include the anticancer drug, paclitaxel production from *Taxus* species; dietary and nutritional supplement, ginseng production from *Panax ginseng*; and therapeutics, recombinant protein α-galactosidase A production from carrot suspension cultures (Kolewe et al. 2008).

In this regard, large-scale cultivation of high-yielding plant cell/tissue cultures in bioreactors (an aseptic and controlled environment) can be an excellent alternative for sustainable and continuous production of CPT at industrial scale. Moreover, plant cell/tissue cultures are amenable to process optimization tools, scale-up, and metabolic engineering strategies for enhanced productivity of CPT (Habibi et al. 2018). Thus, the proposed chapter aims to highlight and discuss the current developments and challenges in the in vitro production of CPT via plant cell biofactories. The information can be of interest and use to academic and industrial researchers and scientists working toward the development of a sustainable and economical plant cell-based bioprocesses for CPT production at large scale.

2 Camptothecin

2.1 Discovery, Structure, and Role of CPT in the Natural System

CPT is a naturally occurring cytotoxic monoterpene indole alkaloid, first isolated from the Chinese happy tree *Camptotheca acuminata* during a project involved in screening for antitumor agents by the National Cancer Institute in the United States (Wall et al. 1966). Although CPT had shown phenomenal anticancer activity in numerous cancer cell lines, its poor solubility and toxic side effects, which majorly included hemorrhagic cystitis and myelosuppression, resulted in abandoning of its preclinical trials (Li et al. 2017).With biotechnological advancements in the past decades, CPT derivatives have gained immense popularity making them among the most prescribed anticancer drugs in the society.

With a molecular formula of $C_{20}H_{16}N_2O_4$ and a molecular weight of 348.352 g mol^{-1}, CPT (IUPAC - (*S*)-4-ethyl-4-hydroxy-1H-pyrano [3′,4′:6,7] indolizino [1,2-b] quinoline-3,14-(4H,12H)-dione) is a slightly yellow-colored powder characterized by poor aqueous solubility and a high melting point ranging between 275 and 277 °C. It is structurally a planar hetero-pentacyclic ring. In the five-ringed structure, while A, B, D, and E rings are hexacyclic, the ring C is pentacyclic

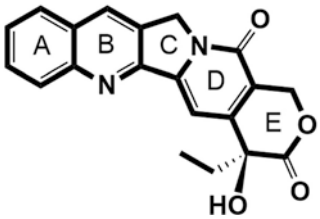

Fig. 1 Structure of camptothecin. (Adapted from Li et al. 2017)

(Fig. 1). A, B, and C rings form a pyrrolo [3,4-beta]-quinoline moiety, while the D ring forms a conjugated pyridine moiety. The E ring contains the alpha-hydroxy lactone ring with an *S* configuration and a chiral center at the 20th position.

2.2 Natural Sources of CPT and Biosynthesis Pathway in Plants

Although CPT is being chiefly extracted from *C. acuminata* and *N. nimmoniana*, it has been reported to be produced in many other tropical plants. Nearly 34 members from the Rubiaceae, Icacinaceae, Nyssaceae, Cornaceae, Apocynaceae, and Loganiaceae families have been recorded with CPT content (Lorence et al. 2004). A brief overview of the content of CPT and some of its derivatives reported in different plant parts has been listed in Table 1.

Biosynthesis Pathway in Plants

Although CPT is one of the most promising anticancer drugs of plant origin (Yamazaki et al. 2003), its biosynthetic pathway is a complex mechanism involving many distinct enzymatic steps. Moreover, the regulatory mechanism of its synthesis has not been deciphered completely (Lorence et al. 2004). All monoterpenoid indole alkaloids (MIA), including CPT, are synthesized from strictosidine, a universal biosynthetic precursor that is formed by the condensation of the indole amino acid derivative tryptamine with a monoterpene glucoside, secologanin, catalyzed by the enzyme strictosidine synthase (STR) (Lu et al. 2009). These two precursors for strictosidine biosynthesis, tryptamine and secologanin, are synthesized via different pathways – tryptamine from the shikimate pathway and secologanin from isoprenoid biosynthesis pathway (Lorence et al. 2004). Two different isoprenoid biosynthesis pathways exist in higher plants, namely, mevalonic acid pathway (MVA) which occurs in the cytosol and methylerythritol phosphate pathway (MEP) which occurs in the plastids (Fig. 3). They are important for the synthesis of isopentenyl pyrophosphate (IPP), a five-carbon intermediate in the production of several isoprenoids including CPT, tanshinone, taxol, etc. (Kai et al. 2011).

Table 1 Yield of camptothecin and its derivatives from natural plant parts/in vitro cultures

S No.	Family	Species	Nature of tissue	Content of CPT and/or derivatives (μg g^{-1} DW)	References
1.	Apocynaceae	*Ervatamia heyneana*	Wood and stem bark	MCPT – 400, CPT – 1300	Gunasekera et al. (1979)
		Chonemorpha fragrans	Stem bark	CPT – 140	Kedari and Malpathak (2014)
			Hairy root	CPT – 240–300	
			Adventitious root	CPT – 330	
		Chonemorpha grandiflora	Callus	CPT – 3	Malpathak et al. (2010)
			Stem with bark	CPT – 13	
2.	Betulaceae	*Alnus nepalensis*	Leaves Stem	CPT – 920 CPT – 190	Pu et al. (2019)
3.	Gelsemiaceae	*Mostuea brunonis*	Total plant	CPT-20-O-β-glycoside – 100	Pu et al. (2019)
4.	Icacinaceae	*Apodytes dimidiata*	Stem bark	CPT – 50	Ramesha et al. (2013)
		Gomphandra comosa	Fruit	CPT – 30	Ramesha et al. (2013)
		Gomphandra coriacea	Seed coat	CPT – 5	Ramesha et al. (2013)
			Endosperm	CPT – 8	
			Twig	CPT – 20	
			LeafRoot	CPT – 30CPT – 286	
		Gomphandra polymorpha	Fruits	CPT – 110	Ramesha et al. (2013)
		Gomphandra tetrandra	Leaf Stem bark	CPT – 4.5 CPT – 60	Ramesha et al. 2013
		Merrilliodendron megacarpum	Leaf and stem	CPT – 530, MCPT – 170	Arisawa et al. (1981)
		Miquelia dentata	Twig	CPT – 30	Ramesha et al. (2013)
			Immature fruit	CPT – 80	
			Leaf	CPT – 240	
			Seed coat	CPT – 540	
			Bud	CPT – 580	

			Root	CPT – 1530	
			Fruit coat	CPT – 1990	
			Mature fruit	CPT – 6300	
			Fruit	CPT – 12200	
			Cotyledon	CPT – 14180	
		Miquelia kleinii	Fruit	CPT – 1530	Ramesha et al. (2013)
		Nothapodytes nimmoniana	Callus from root organs	CPT – 1.7	Padmanabha et al. (2006), Kavitha et al. (2010), Karwasara and Dixit (2013) and Manjunatha et al. (2016)
			Callus	CPT – 9.5	
			Untransformed root	CPT – 100	
			Embryo	CPT – 110	
			Immature seeds	CPT – 400	
			Mature seeds	CPT – 540	
			Leaves	CPT – 580	
			Seedlings	CPT – 610	
			Shoot	CPT – 750	
			Bark	CPT – 600–1000	
			Hairy roots	CPT – 500–1500	
			Stem wood	CPT – 1400–2400	
			Stem	CPT – 1780	
			Root	CPT – 15,590	
		Nothapodytes pittosporoides	Root	CPT – 5300	Ramesha et al. (2013)
		Pyrenacantha volubilis	Cotyledon Fruit Root	CPT – 13,500 CPT – 6000 CPT – 4880	Ramesha et al. (2013)

(continued)

Table 1 (continued)

S No.	Family	Species	Nature of tissue	Content of CPT and/or derivatives (μg g^{-1} DW)	References
5.	Meliaceae	*Dysoxylum binectariferum*	Bark	CPT – 1045	Jain et al. (2014)
6.	Nyssaceae	*Camptotheca acuminata*	Bark	CPT – 1800–2000	Sakato et al. (1974), van Hengel et al. (1992), López-Meyer et al. (1994) and Wiedenfeld et al. (1997)
			Stem	CPT – 490	
			Leaves	CPT – 4000–5000	
			Root	CPT –520	
			Seeds	CPT – 3000	
			Fruits	CPT – 2362	
			Callus	CPT – 2040–2360, HCPT – 80–100	
			Cell cultures	CPT – 2.5–4	
			Hairy roots	CPT – 1000	
		Camptotheca lowreyana	Young leaves Old leaves	CPT – 3913–5537 CPT – 909–1184	Li (1997)
		Camptotheca yunnanensis Dode	Young leaves Old leaves	CPT – 2500–4500 CPT – 590	Li (1997)
7.	Rubiaceae	*Ophiorrhiza alata*	Leaf Root In vitro leaf In vitro adventitious root Hairy root	CPT – 83 CPT – 388 CPT – 94 CPT – 556 CPT – 785	Ya-ut et al. (2011)
		Ophiorrhiza eriantha	Plant	CPT – 0.3	Rajan et al. (2013)
		Ophiorrhiza grandiflora	Plant	CPT – 1.07–1.34	Rajan et al. (2013)
		Ophiorrhiza japonica	Plant	CPT – 50–60	Krishnan et al. (2014)
		Ophiorrhiza kuroiwae	In vitro shoot In vitro root Hairy root	CPT – 370 CPT – 250 CPT – 219	Asano et al. (2004)

		Ophiorrhiza liukiuensis	In vitro shoot In vitro root Hairy root Plant	CPT – 40 CPT – 80 CPT – 83 CPT – 126, MCPT – 126, 10-MCPT – 29.6	Asano et al. (2004)
		Ophiorrhiza mungos L.	Callus culture Cell suspension Root Shoot Plant	CPT – 40–100 CPT – 1120 CPT – 860 CPT –96 CPT – 170, MCPT – 10.4	Deepthi and Satheeshkumar (2016), (2017a, b) and Tafur et al. (1976)
		Ophiorrhiza prostrata D. Don	Root	CPT – 280–1900	Martin et al. (2008)
		Ophiorrhiza pumila Champ.	Seedlings	CPT – 333, MCPT – 232	Kitajima et al. (1997), Saito et al. (2001) and Cui et al. (2015)
			Leaves	CPT – 300–400	
			Young roots	CPT –1000	
			Hairy roots	CPT – 1000	
			Transgenic hairy roots	CPT – 680–1640	
			In vitro root	CPT – 225	
			In vitro shoot	CPT – 760	
		Ophiorrhiza rugosa	Root Shoot	CPT – 20 CPT – 10	Roja (2006)
		Ophiorrhiza shendurunii	Plant	CPT – 0.05	Rajan et al. (2013)
		Ophiorrhiza trichocarpos	Plant	CPT – 19–28	Rajan et al. (2013)

MCPT 9-methoxy CPT, *HCPT* 10-hydroxy CPT, *DW* dry weight

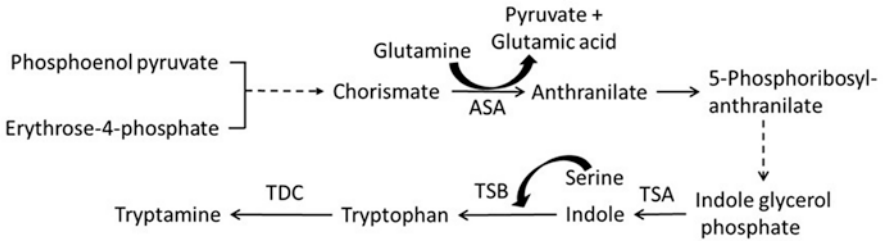

Fig. 2 Formation of tryptamine in the camptothecin biosynthesis pathway in plants. Dotted lines indicate multiple steps involved. (Adapted from Kai et al. 2015)

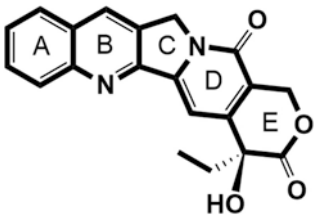

Fig. 3 Formation of IPP in the camptothecin biosynthesis pathway in plants. (Adapted from Rather et al. 2019, Kai et al. 2015)

Extensive CPT biosynthesis studies have revealed that a possible cross talk between MVA and MEP provides common terpene precursors for CPT; however MEP remains the main source (Kai et al. 2011). For the ease of understanding, CPT biosynthesis can be divided into two major parts.

(a) Pre-strictosidine Pathway.

(i). Formation of Tryptamine (Shikimate Pathway).

In 1979, Hutchinson et al. first established the involvement of secologanin and tryptamine in CPT biosynthesis using radioactive precursor feeding experiments in *C. acuminata.* Three biosynthetic enzymes involved in the shikimate pathway have been characterized in CPT producing plants (Fig. 2):

– Anthranilate synthase (ASA).
– α- and β-Subunits of tryptophan synthase (TSA and TSB, respectively),
– Tryptophan decarboxylase (TDC).

In the first step of the indole pathway, chorismate is converted to anthranilate catalyzed by ASA. (Herrmann and Weaver 1999). Anthranilate gets converted to the intermediates 5-phosphoribosylanthranilate and indole-3-glycerol phosphate. In the presence of TSA, indole-3-glycerol phosphate gets converted to indole. Indole is later condensed to form tryptophan in the presence of serine and TSB. Formation of tryptamine has been depicted in Fig. 2.

(ii). Formation of Isopentenyl Diphosphate (MVA and MEP Pathway).

The precursor of terpenoid biosynthesis, isopentenyl diphosphate (IPP), can be formed via MVA pathway and the MEP pathway. In higher plants, both the pathways can operate simultaneously leading to the formation of IPP (Fig. 3).

In the MEP pathway, condensation of glyceraldehyde-3-phosphate (GAP) and pyruvate in the presence of 1-deoxy-D-xylulose-5-phosphate synthase (DXS) leads to the formation of 1-deoxy-D-xylulose-5-phosphate (DXP). In the presence of DXP reductoisomerase (DXR), DXP is converted to 2-C-methyl-D-erythritol 4-phosphate (MEP). After a series of enzymatic stages, hydroxy methyl butenyl diphosphate (HMBPP) is formed. HMBPP leads to the production of IPP and its isomer dimethylallyl diphosphate (DMAPP) catalyzed by HMBPP reductase (HDR).

In the MVA pathway, acetyl CoA is converted to acetoacetyl-CoA which is subsequently converted to 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA) with the help of 3-hydroxy-3-methylglutaryl-CoA synthase (HMGS) (Kai et al. 2006). Full-length cDNA of HMGS has been successfully isolated from young leaves of *C. acuminata* based on homology cloning strategy by rapid amplification of cDNA ends (RACE) (Kai et al. 2011). 3-Hydroxy-3-methylglutaryl-CoA reductase (HMGR) catalyzes conversion of HMG-CoA to MVA. This step has been considered the first key step in the MVA pathway of plants by Liao et al. (2014). After a series of reactions, MVA gets converted to IPP in the presence of mevalonate 5-diphosphate decarboxylase (MDC).

(iii). Formation of Secologanin (Iridoid Pathway).

Condensation of IPP and DMAPP formed in the MEP/MVA pathway led to the formation of geranyl diphosphate (GPP). In the subsequent steps, GPP gets converted to geraniol with the help of geraniol synthase (GS). Some cytochrome P450 monooxygenases are involved in the formation of secologanin from geraniol. This includes geraniol 10-hydroxylase (G10H) and secologanin synthase (SLS) (Fig. 4).

(b) Post-Strictosidine Pathway.

Strictosidine, the key intermediate for most MIA synthesis, is formed by the condensation of secologanin and tryptamine in the presence of STR (Stöckigt and Ruppert 1999). Hutchinson et al. (1979) reported that intramolecular cyclization of strictosidine could result in the formation of strictosamide. However, the other intermediates between strictosamide and CPT have not been completely defined. Some reports on the probable sequential reactions from strictosamide to CPT have been postulated. According to Kitajima et al. (2005), pumiloside and deoxypumiloside isolated from *Ophiorrhiza* species could be possible intermediates in the formation of CPT. The comprehensive pathway has been shown in Fig. 5, and the enzymes reported in CPT biosynthesis of plants have been shown in Table 2.

2.3 Mechanism of Action against Tumors

Topoisomerases are enzymes that unwind the DNA double helix to release tensed supercoils present at the upstream of DNA, during replication and transcription, by cleaving and religating the DNA strands. CPT belongs to the class of topoisomerase poisons which binds to the DNA-TOP1 complex (not to individual DNA or TOP1) forming a stable ternary complex and blocks the ability of DNA Topoisomerase-I

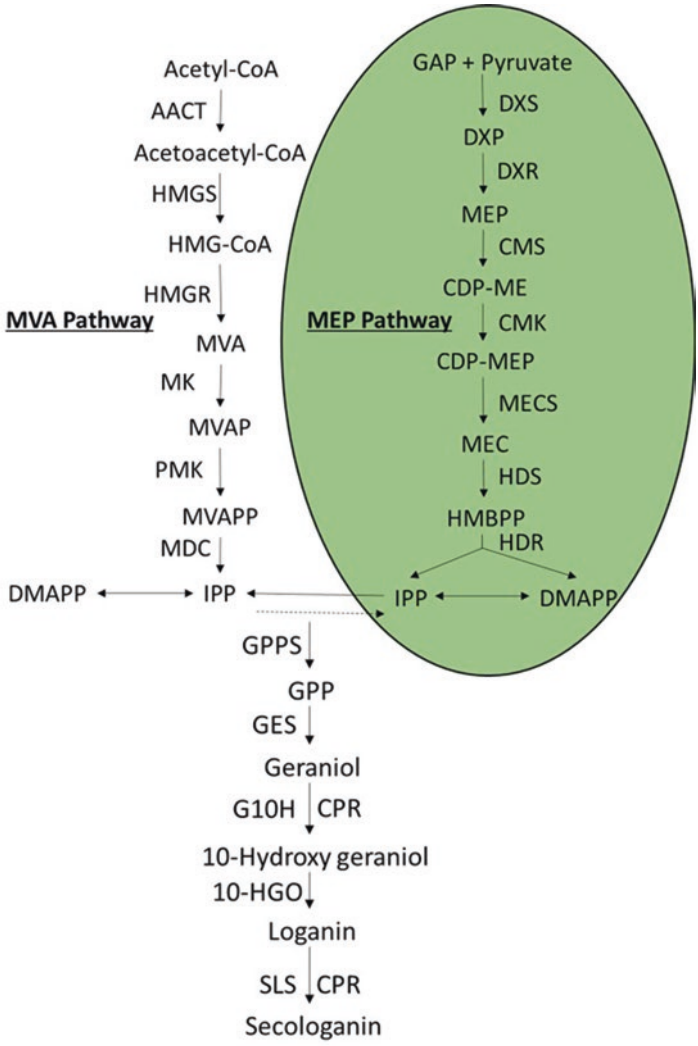

Fig. 4 Formation of secologanin in the camptothecin biosynthesis pathway in plants. (Adapted from Rather et al. 2019, Kai et al. 2015)

(TOP 1) to religate and repair the single-strand breakage (Hsiang et al. 1985; Redinbo et al. 1998). The five-ring planar structure of CPT with one chiral carbon at 20th position in α-hydroxyl lactone ring and the pyridine moiety are crucial for the inhibition of TOP1 (Fig. 1). The *S* isomer of CPT is abundant in natural sources and more active compared to *R* isomer of CPT (Lu et al. 2007). D and E rings of CPT form hydrogen (H)-bonding with the amino acid residues of TOP1 and nucleosides of DNA (Fig. 6). CPT-DNA-TOP1 complex blocks further progression of replication fork, thereby arresting the cell cycle and division.

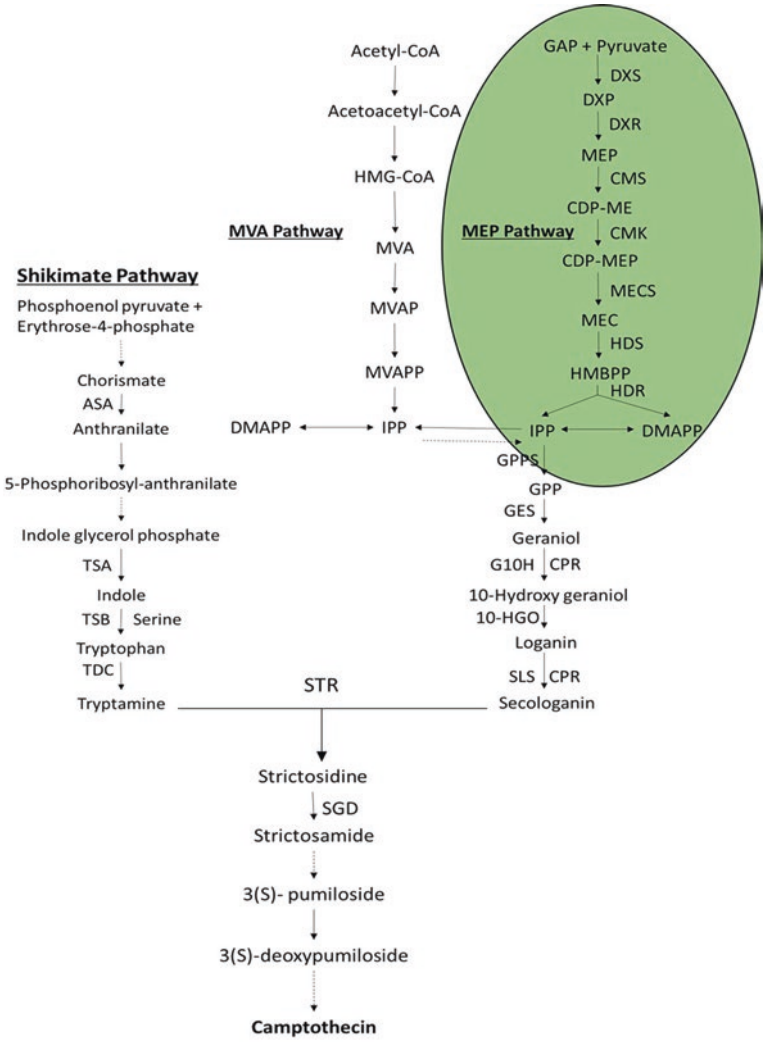

Fig. 5 Camptothecin biosynthesis pathway in plants. (Adapted from Rather et al. 2019, Kai et al. 2015)

Table 2 Enzymes involved in the camptothecin biosynthesis pathway of plants

S No.	Enzyme	Species	Expression profiling	Reference
1.	Anthranilate synthase alpha (CaASA1)	*C. acuminata*	Associated with CPT biosynthesis, regulated by seedling development, constitutively expressed	Lu et al. (2005)
2.	Anthranilate synthase alpha (CaASA2)	*C. acuminata*	Not regulated by seedling development, constitutively expressed with low levels	Lu et al. (2005)
3.	Tryptophan synthase beta subunit (CaTSB)	*C. acuminata*	Isolated and characterized with higher levels in apex, bark, young stem, and young leaves	Lu and McKnight (1999)
4.	Tryptophan decarboxylase 1 (CaTDC1)	*C. acuminata*	Associated with CPT biosynthesis and regulated by developmental stages, did not match accumulation sites of CPT indicative of a likely intracellular CPT transport mechanism in *C. acuminata*	López-Meyer and Nessler (1997) and Valletta et al. (2010)
5.	Tryptophan decarboxylase 2 (CaTDC2)	*C. acuminata*	Involved in the plant defense system for being responsive to methyl jasmonic acid (MeJA) or fungal elicitors	López-Meyer and Nessler (1997)
6.	Tryptophan decarboxylase (OpTDC)	*O. pumila*	Expressed in roots, stem, and hairy roots	Yamazaki et al. (2003)
7.	1-Deoxy-D-xylulose 5-phosphate reductoisomerase (CaDXR)	*C. acuminata*	Strongly expressed in stem, weakly expressed in root and leaf	Yao et al. (2008)
8.	3-Hydroxy-3-methylglutaryl-CoA synthase (CaHMGS)	*C. acuminata*	Strongly expressed in hypocotyls and cotyledons, responsive to MeJA	Kai et al. (2013)
9.	3-Hydroxy-3-methyl glutaryl-CoA reductase (CaHMGR1)	*C. acuminata*	Responsive to wounding and MeJA	Burnett et al. (1993)
10.	3-Hydroxy-3-methyl glutaryl-CoA reductase (CaHMGR2) & (CaHMGR3)	*C. acuminata*	Unresponsive to MeJA and wounding	Maldonado-Mendoza et al. (1997)
11.	Hydroxy methyl butenyl diphosphate reductase (CaHDR)	*C. acuminata*	Maximum expression in flowers, responsive to MeJA, unresponsive to salicylic acid (SA)	Wang et al. (2008)
12.	Isopentenyl diphosphate isomerase (CaIPP)	*C. acuminata*	Expressed in stems, young leaves, and roots, unexpressed in mature leaves and fruits	Pan et al. (2008)

(continued)

Table 2 (continued)

S No.	Enzyme	Species	Expression profiling	Reference
13.	Geraniol 10-hydroxylase (CaG10H)	*C. acuminata*	Expressed constitutively and responsive to MeJA	Sun et al. (2011)
14.	NADPH cytochrome P-450 reductase (OpCPR)	*O. pumila*	Constitutively expressed in roots, stems, leaves, and hairy roots, unresponsive to SA	Yamazaki et al. (2003)
15.	Secologanin synthase (CaSLS)	*C. acuminata*	High expression in young leaves and petioles, responsive to MeJA	Sun et al. (2011)
16.	Strictosidine synthase (OpSTR)	*O. pumila*	Expressed in stems, roots, and hairy roots	Yamazaki et al. (2003)
17.	Strictosidine synthase (OjSTR)	*O. japonica*	Expressed constitutively and responsive to elicitors MeJA and SA	Lu et al. (2009)
18.	Strictosidine synthase (CaSTR)	*C. acuminata*	Responsive to MeJA and constitutively expressed	Sun et al. (2011)

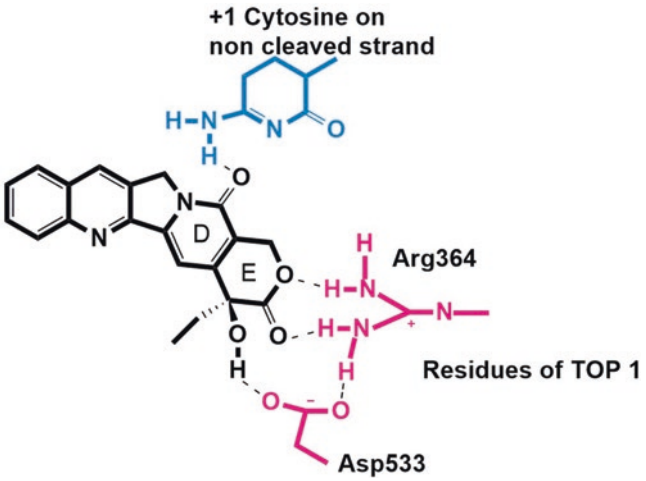

Fig. 6 Action of camptothecin on DNA-TOP1 complex: formation of stable ternary complex by camptothecin with DNA-TOP1. (Adapted from Redinbo et al. 1998)

2.4 CPT Derivatives and Their Commercial Applications

The unmodified CPT molecule has poor solubility in aqueous solutions making it difficult for intravenous administration to patients. Later, modifications were done on A and B rings of CPT which led to enhanced aqueous solubility and reduced the cytotoxicity of CPT. A and B ring-modified CPTs like topotecan, irinotecan (FDA approved in 1996), and belotecan (marketed in South Korea) are prescribed for cancer chemotherapy, and more CPT derivatives are being designed and are in various phases of clinical trials to increase the pharmacological properties in different cancer cells (Giannini 2014) (Fig. 7).

In order to establish the DNA-TOP1 blocking, the E ring of the CPT molecule has to be intact. But, in physiological pH, the E ring opens and gets hydrolyzed to form a carboxylate (Thomas et al. 2004). The carboxylate form readily binds with serum albumin, an abundant protein in human blood. Since ring and carboxylate forms of CPT are in chemical equilibrium, concentration of available CPT molecule

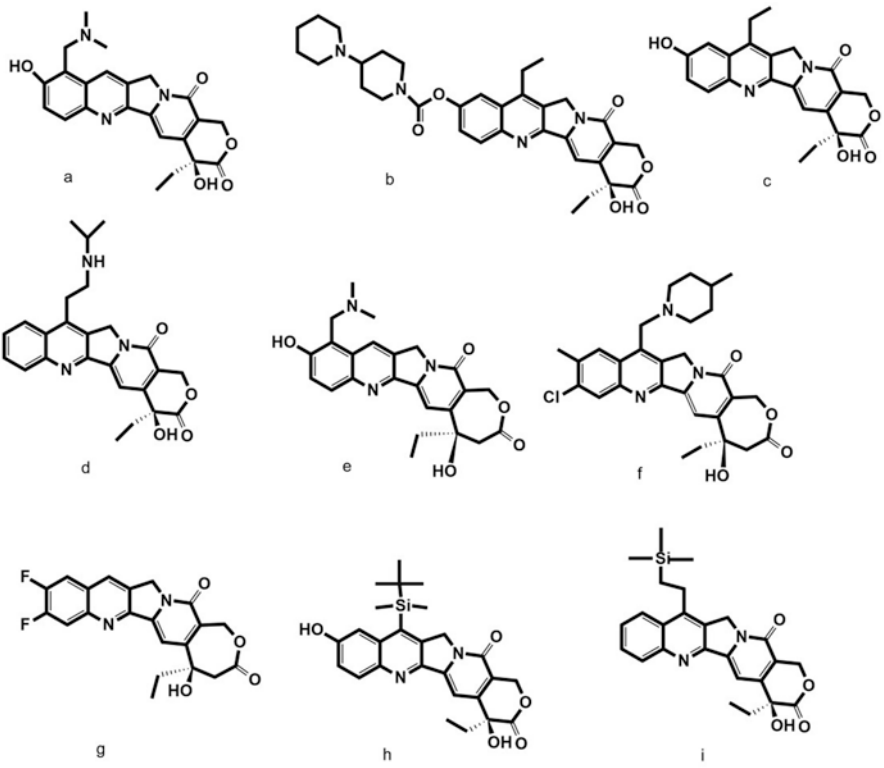

Fig. 7 Pharmacologically important camptothecin derivatives which are used commercially or in clinical trials. (Adapted from Giannini 2014; Lavergne et al. 1997; Xu et al. 2016). (**a**) Topotecan; (**b**) Irinotecan; (**c**) SN-38, an active metabolite of Irinotecan; (**d**) Belotecan; (**e**) Homocamptothecin; (**f**) Elomotecan; (**g**) Diflomotecan; (**h**) Silatecan; (**i**) Karenitecin.

decreases due to constant formation of CPT-albumin complex (Lavergne et al. 1997). Modifications in the E ring were studied to increase the stability of CPT in physiological conditions. Homocamptothecins are such modifications, in which the number of carbon atoms in the E ring is increased without affecting the H-bond interactions with DNA-TOP1 complex. Homocamptothecins are more stable than CPTs with unmodified E ring at physiological pH (Lavergne et al. 1997). Diflomotecan and elomotecan are such homocamptothecins, which are under clinical trials for treating colon, breast, and prostate cancers (Xu et al. 2016).

3 In Vitro Plant Cell Cultures for CPT Production

Plant cell cultures have become increasingly attractive cost-effective alternatives to classical approaches for the mass production of plant-derived metabolites. Plant cell culture is the only economically feasible way of producing high-value metabolites from rare and/or threatened plants uninfluenced by contamination and climatic variations and is capable of generating sustainable yields. The use of metabolic engineering strategies for further yield enhancement and scale-up to bioreactors can be done for consistent in vitro production of CPT in large scale.

3.1 Callus and Cell Suspension Cultures for CPT Production

Callus is a mass of undifferentiated cells, mostly parenchymatous, in response to wounding in plants. In laboratory conditions, callus can be induced by the manipulation of specific plant growth regulators (PGRs) on an excised plant part placed on a solidified basal medium. This callus can be transferred to a liquid basal medium with same combination of plant growth regulators to generate cell suspension cultures. Plant cell suspension cultures mimic microbial cultivation in liquid and can be grown in shake flasks under controlled conditions of a rotary incubator. Callus and cell suspensions of CPT-producing plants were established around 45 years ago in 1970s (Table 3), where *C. acuminata* cell suspension culture was reported by Sakato et al. (1974). However, the amount of CPT produced by the in vitro cells was 2.5 μg g^{-1} DW which is 1000 times less than the amount present in leaves of *C. acuminata*. Later attempts on cell suspension culture of *C. acuminata* achieved higher concentration up to 100 μg g^{-1} DW (Pan et al. 2004) but failed to achieve CPT content as high as that in the natural tissues. Moreover, attenuation in production of CPT was observed over repeated subculturing of *C. acuminata* cell suspension in same media composition (Yang et al. 2017).

Similarly, cell cultures of the second major CPT-producing plant, *N. nimmoniana*, were established for production of CPT from plant cell cultures. Along with CPT, these *N. nimmoniana* callus cultures were also reported to synthesize 9-methoxycamptothecin in low concentrations (Roja and Heble 1994). In another

Table 3 Callus and plant cell suspension cultures with camptothecin production

Culture type	PGR combination in growth medium (concentration given in brackets)		Media composition	CPT yield/ production	Reference
	Auxin (mg L^{-1})	Cytokinin (mg L^{-1})			
1.	*Nothapodytes nimmoniana*				
Callus	2,4-D (2)	BAP (1)	MS medium with 3% (w v^{-1}) sucrose	Traces	Roja and Heble (1994)
	Picloram (2)	–	MS medium with 3% (w v^{-1}) sucrose	9.5 µg g^{-1}DW	Ciddi and Shuler (2000)
	2,4-D(3)	BAP (1)	MS medium with 2% (w v^{-1}) sucrose	13.06 x 10^3µg g^{-1}DW	Thengane et al. (2003)
	Picloram (2)	BAP (1)	MS medium with 3% (w v^{-1}) sucrose and gibberellin (1 mg L^{-1})	29 µg g^{-1}DW	Sundravelan et al. (2004)
	NAA (2)	BAP (0.5)	MS medium with 3% (w v^{-1}) sucrose	49 µg g^{-1}DW	Fulzele et al. (2015)
	2,4-D (0.5)	BAP (3)	MS medium	5.9 µg g^{-1}DW	Patil et al. (2016)
	NAA (2)	BAP (1)	MS medium	50 µg g^{-1}DW	Isah (2017)
Cell suspension	NAA (2)	BAP (0.5)	MS medium with 3% (w v^{-1}) sucrose	35 µg mL^{-1} in spent medium and traces in biomass	Fulzele et al. (2001)
	NAA (2)	Kinetin (0.2)	MS medium with 3% (w v^{-1}) sucrose	8.8 µg mL^{-1} in spent medium and 28.5 µg g^{-1}DW in biomass	Karwasara and Dixit (2013)
2.	*Camptotheca acuminata*				
Callus	NAA (4)	BAP (2)	MS medium	2.32x 10^3 µgg^{-1}DW	Wiedenfeld et al. (1997)
Callus	NAA (5) and 2,4-D (0.3)	BAP (0.5)	MS medium with 3% (w v^{-1}) sucrose	7 µg g^{-1}DW	Pi et al. (2010)
	–	2ip (1)	Modified Gamborg's B5 medium with 3% (w v^{-1}) sucrose	43 µg g^{-1}DW	Park et al. (2003)
	NAA (2)	BAP (0.25)	MS medium with 3% (w v^{-1}) sucrose	4.5 µg g^{-1}DW	Yang et al. (2017)
Cell suspension cultures	NAA (0.5)	BAP (2)		2 µg mL^{-1}in spent medium	

(continued)

Table 3 (continued)

Culture type	PGR combination in growth medium (concentration given in brackets)		Media composition	CPT yield/ production	Reference
	Auxin (mg L^{-1})	Cytokinin (mg L^{-1})			
	2,4-D (0.1)	Kinetin (3)	MS medium with tryptophan (23.5 g L^{-1})	2.54 µg g^{-1}DW	Sakato et al. (1974)
	NAA (0.4)	–	Modified MS medium with 3% (w v^{-1}) sucrose	36 µg g^{-1}DW	van Hengel et al. (1992)
	2,4-D (0.1) and NAA (0.5)	BAP (0.5)	MS medium with 4% (w v^{-1}) sucrose	107 µg g^{-1}DW	Pan et al. (2004)
	2,4-D (0.1) and NAA (0.1)	Kinetin (0.1)	MS medium with 3% (w v^{-1}) sucrose	1.65 µg g^{-1}DW	Zhang et al. (2017)
3.	*Ophiorrhiza mungos*				
Callus	NAA (2)	BAP (1)	Half strength MS medium with 3% (w v^{-1}) sucrose	230 µg g^{-1}DW	Krishnan et al. (2019)
Cell		–		310 µg g^{-1}DW	
suspension	NAA (3) and 2,4-D (1)	Kinetin (0.5)	Half strength MS medium with 3% (w v^{-1}) sucrose	60 µg g^{-1}DW	Deepthi and Satheeshkumar (2016)
4.	*Chonemorpha grandiflora*				
Callus	2.4-D (1)	–	MS medium	3 µg g^{-1}DW	Kulkarni et al. (2010)

study, callus induced from immature cotyledons of *N. nimmoniana,* using plant growth regulators 2,4-D (3 mg L^{-1}) and BAP (1 mg L^{-1}), was reported to have 1.3% DW (13 mg g^{-1} DW) of CPT (Thengane et al. 2003). Table 3 gives different compositions of PGRs for the cultivation of callus and cell suspension cultures of plant species which synthesize CPT under lab conditions.

Though these therapeutic molecules are intracellular in nature, the cells in suspension are known to release the bioactive components into the liquid medium, making the extraction easier. Likewise, *C. acuminata* cell suspension cultures were also reported to secrete CPT up to 2 mg L^{-1} in the spent medium after the cells reached their death phase (10 days after culture initiation) (Yang et al. 2017). The concentration of CPT in spent medium was severalfold higher than in cell pellet. However, in a separate study with *N. nimmoniana* cell suspension culture, extracellular CPT concentration increased from the beginning of exponential phase and reached maximum (8.8 µg mL^{-1}) at stationary phase (20 days after initiation) (Karwasara and Dixit 2013).

3.2 Organ Cultures (Hairy Root, Somatic Embryos, Shoot Cultures)

A plethora of valuable secondary metabolites/phytochemicals have been produced in unorganized callus and suspension cultures in the past century. However, in some cases the production of these valuable secondary metabolites requires more differentiated organ cultures than callus (Dörnenburg and Knorr 1997). Among organ cultures, hairy roots, somatic embryos, and shoot cultures have been extensively used in the production of phytochemicals.

Hairy Roots

When plant parts are infected with *Agrobacterium rhizogenes*, a gram-negative soil bacterium, genetically transformed root cultures called hairy roots are obtained. In addition to fast and hormone-free growth, these are suitable for process optimization, exhibiting high levels of stability and producing high yields of phytochemicals. They have been known to be excellent platforms for production of plant secondary metabolites in laboratory and bioreactor level (Srivastava and Srivastava 2007).

With regard to CPT production, hairy root cultures of different CPT-producing plants have been reported (Saito et al. 2001; Sudo et al. 2002). *O. pumila* hairy roots were established by transformation with *A. rhizogenes* ATCC 15834 mediated transformation as reported by Saito et al. (2001). The hairy root cultures produced up to 0.1% (w w^{-1}) CPT. The authors also reported that the culture medium accumulated considerable amounts of CPT, which was further increased with help of Diaion HP-20, a polystyrene resin that absorbed the extracellular CPT released in the medium, which could later be recovered from the resin. Sudo et al. (2002) successfully scaled up *O. pumila* hairy roots in a modified 3 L bioreactor, and CPT concentration of 0.0085% (w w^{-1}) FW was reported after the 8 weeks of cultivation. Development of hairy root cultures of *Ophiorrhiza* species – *O. liukiuensis* and *O. kuroiwai* – was reported by Asano et al. (2004), where both CPT and 10-methoxycamptothecin were found to accumulate in *O. liukiuensis* and *O. kuroiwai* hairy roots up to 83 μg g^{-1} DW and 219.3 μg g^{-1} DW, respectively. Hairy root cultures have also been induced using *A. rhizogenes* TISTR 1450 in *Ophiorrhiza alata* Craib (Ya-ut et al. 2011) where 785 μg g^{-1} DW of CPT has been reported in hairy roots, which was nearly twice as that found in the natural soil grown plants (388 μg g^{-1} DW).

Hairy roots of *C. acuminata* have also been established from plant tissues transformed with *A. rhizogenes* ATCC15834 and R1000 (Lorence et al. 2004) and were found to produce 1 mg g^{-1} DW CPT and 10-hydroxy CPT that was secreted into the medium with 1 mg g^{-1} and 0.15 mg g^{-1} DW concentration, respectively.

Shoot Cultures and Somatic Embryogenesis.

Somatic embryogenesis is an in vitro plant regeneration technique used to reduce multiplication time which offers a potentially efficient system for large-scale clonal propagation (Evans et al. 1981). Since some medicinal plants have become endangered as a result of excessive deforestation for extraction of phytochemicals and their multiplication through cutting or grafting either has not been reported or is extremely laborious, plant regeneration through somatic embryogenesis is a crucial approach which can be implemented for species propagation leading to enhanced production of phytochemicals (Fulzele and Satdive 2003).

Nothapodytes Species

In 1994, Roja and Heble reported the presence of CPT and 9-methoxy CPT in different developmental stages of somatic embryos and different parts of regenerated plant parts of *N. nimmoniana.* In the in vitro shoots, 0.075% DW of CPT and 0.013% of 9-methoxy CPT had been obtained. Fulzele and Satdive had reported that MS media devoid of any plant growth regulators favored the development of globular somatic embryos in *N. foetida* which could be used for further differentiation into plantlets and nearly 0.011% DW of CPT and 0.0028% DW of 9-methoxy CPT from somatic embryos was obtained (Fulzele and Satdive 2003). Further, the plantlet regeneration efficiency could be effectively increased by supplementing MS medium with BAP. It was observed that over 90% of the in vitro plantlets survived when transferred to soil. Interestingly, the study also reported that 2-year-old field-grown plants obtained from somatic embryos exhibited high levels of CPT (0.20% DW) and 9-methoxy CPT (0.097% DW) accumulated in roots, stem, and leaves. Owing to the critical endangering of *N. foetida*, recently an efficient protocol for regeneration via direct somatic embryogenesis was developed (Khadke and Kuvalekar 2013). Somatic embryos were induced from stem and leaf explants cultured on MS media supplemented with thidiazuron and 20% coconut water.

Camptotheca Species

Numerous methods have been developed to establish shoots and somatic embryos in *Camptotheca* species (Wang et al. 2006). Owing to the high demand of CPT and its low concentration in free grown *Camptotheca* plants, the clonal propagation of this important medicinal plant could open the door for large-scale production of CPT.

In 1996, Jain and Nessler reported strategies to develop multiple shoots from shoot buds of *C. acuminata.* From 25–30-day-old seedlings, shoot buds were excised and presoaked in different liquid basal media containing BAP, kinetin, or TDZ in different combinations. Subsequently they were cultured in semisolid media of the same composition. While several shoots developed, the maximum number of shoots was initiated from buds. To enable rooting, individual shoots were separated

from clusters and grown on Gamborg's B5 basal medium supplemented with indole-3-butyric acid (IBA). Nearly 82% of the plantlets exhibited rooting in Gamborg's B5 basal medium and IBA (4.9 μM) with the shortest root initiation period of 18 days (Jain and Nessler 1996).

Organogenesis and plantlet regeneration in *C. acuminata* Decne were reported by Wiedenfeld et al. in 1997. Nitsch & Nitsch (NN) medium supplemented with 0.05 mg L^{-1} IBA, 0.05 mg L^{-1} BAP, and 10 mg L^{-1} adenine sulfate was reported to be the best combination for shoot tip development. Interestingly, their buds were used to form somatic embryos after encapsulation in calcium alginate. In the *C. acuminata* Decaisne variety, micropropagation from axillary buds and seed embryos has been established and reported by Li and Liu (2001).

There have also been some reports on whole plant regeneration from calli. According to Wang et al. (2006), leaf explants were used to generate callus of *Camptotheca acuminata* in Woody Plant Medium (WPM) supplemented with 6-BAP, NAA, or 2,4-D. These calli induced shoot regeneration with a high regeneration frequency of 70.3% in WPM with 19.8 μM BAP and 5.8 μM NAA. The calli produced nearly 11.2 shoots per explant. The authors also reported induction of roots (~98% rooting percentage and 5.9 root per shoot) on WPM supplemented with 9.6 μM IBA.

Ophiorrhiza Species

Ophiorrhiza is another camptothecin-producing plant that has come into the forefront with several reports discussing strategies to enhance CPT in the plant.

High-frequency rapid regeneration of *O. prostrata*, via shoot organogenesis from leaf and internode explants, has been reported by Shahanaz Beegum et al. (2007). MS medium supplemented with 8.87 μM BAP and 2.46 μM IBA resulted in the highest number of shoots within 40 days.

Micropropagation in *O. mungos* from seedling cultures was reported by Jose and Satheeshkumar in 2004, where MS medium supplemented with 2.2 μM BAP led to the maximum production of adventitious shoots (~10.4 shoots) after 3 weeks.

4 Strategies for Enhanced CPT Production in Plant Cell Biofactories

4.1 Overexpression of Rate-Controlling Genes in the Biosynthetic Pathway

Although plant cell/tissue cultivation is an attractive platform for large-scale, sustainable production of commercially important phytochemicals, the ever-increasing demand for plant-based products, together with inflating industrial production costs,

has led to the search for integrative approaches to conventional bioprocess optimization to enhance product titers. In this regard, plant metabolic engineering has been a paradigm shift for systematically generating transgenic plant cell lines with increased biosynthetic capabilities.

To enhance the production of alkaloids like CPT, genetic engineering through gene overexpression could be used. Overexpression of genes involved in the indole alkaloid biosynthetic pathway in different plant species has been reported for the enhancement of secondary metabolite production (Cui et al. 2015).

Canel et al. reported the effects of tryptophan decarboxylase and STR individually and synergistically on alkaloid production in cell cultures of *C. roseus* in 1998. The authors established plant cell cultures through *Agrobacterium*-mediated transformation which showed wide phenotypic diversity. Transgenic cultures with overexpressed STR alone showed tenfold higher STR activity than wild-type cultures. Two cell lines accumulated over 200 mg L^{-1} strictosidine and/or strictosidine-derived TIAs – ajmalicine, catharanthine, serpentine, and tabersonine – while tryptophan decarboxylase activity levels were maintained equivalent to wild-type levels. They also reported that transgenic calli with overexpressed TDC not only showed decreased productivity but was also detrimental to normal culture growth. Similarly, overexpression of geraniol 10-hydroxylase (G10H) and octadecanoid-derivative responsive *Catharanthus* AP2-domain (ORCA3) has been reported in hairy roots of *C. roseus* for enhancement of catharanthine production (Wang et al. 2010). Furthermore, ORCA3 and G10H overexpression regulate alkaloid biosynthesis and metabolism with supporting evidence from NMR metabolomics (Pan et al. 2012).

In 2003, Yamazaki et al. discussed the cloning, characterization, and differential expression of camptothecin biosynthetic genes in hairy roots of *O. pumila.* The authors could characterize two key enzymes – STR and TDC – from the hairy roots. The recombinant STR and TDC proteins could exhibit STR and TDC activities when expressed in *Escherichia coli*. The high expression levels of STR and TDC cDNA observed in hairy roots, stems, and roots could be correlated with STR protein accumulation using immunoblot analysis.

The co-overexpression of G10H and STR enhanced CPT content in the hairy roots of *O. pumila*, as reported by Cui et al. (2015). STR and G10H from *C. roseus* were individually and simultaneously introduced into *O. pumila.* Overexpression of only G10H significantly improved CPT production as compared to the non-transgenic hairy root culture implying that G10H could be playing an imperative role in CPT accumulation in *O. pumila.* These studies have also shown significant increase in CPT production where, co-overexpression of G10H and STR has shown an increase in the yield of CPT by 56% as compared to single overexpression lines and non-transgenic lines (Cui et al. 2015). This seems to be the first report on CPT enhancement using a metabolic engineering strategy, opening new avenues for creation of transgenic plant cell/tissue producing higher amounts of CPT without violating natural flora.

4.2 *Effect of Medium Components on CPT Production*

Nutrients in the culture medium greatly control the growth of plant cells and production of secondary metabolites/phytochemicals. Plant nutrient medium contains macroelements such as carbon, nitrogen, phosphorus, potassium, calcium, and sulfates, along with microelements such as iron, manganese, copper, boron, and molybdenum which are essential for metabolism of plant cells. Carbon and nitrogen sources are building blocks of cells, and certain metal ions are important for enzyme activities as cofactors, maintaining osmosis, and are involved in synthesis of proteins, nucleic acids, and membranes. Apart from these, some vitamins like nicotinic acid, thiamin, and pyridoxine also present in plant tissue culture medium. Optimum composition of these chemicals significantly influences the growth and production of secondary metabolites/phytochemicals in in vitro cultures.

Most plant cell/tissue cultures induced from CPT-producing plants were maintained in MS medium (Table 3). *C. acuminata* callus cultures were found to grow and give high yields of CPT when MS basal salt medium supplemented with Gamborg's B5 vitamins was used, compared to callus grown on MS medium and Gamborg's B5 medium alone (van Hengel et al. 1992). Sucrose favored the growth and alkaloid production by *C. acuminata* and *N. nimmoniana* cells in suspension in comparison to other carbon sources such as glucose, fructose, and maltose (Sakato and Misawa 1974; Karwasara and Dixit 2013). In few studies, high concentration of sucrose (40–60 g L^{-1}) was used to increase CPT production in *C. acuminata*, *N. nimmoniana*, and *O. mungos* callus and cell suspensions (Wiedenfeld et al. 1997; Pan et al. 2004; Karwasara and Dixit 2013; Kim et al. 1999; Deepthi and Satheeshkumar 2016). However, high sucrose concentration (50 g L^{-1}) was found to have inhibitory effect on biomass accumulation in *C. acuminata* cell suspension (Sakato and Misawa 1974). In *N. nimmoniana* cell suspensions and *O. mungos* adventitious roots, 50 g L^{-1} sucrose increased the biomass production along with CPT content (Deepthi and Satheeshkumar 2016).

The nitrogen source in plant culture medium which will be in the form of nitrate and ammonium mixture greatly influences the growth and secondary metabolite production. In MS medium, nitrate to ammonium ratio is 4:2, and total nitrogen concentration is 60 mM. When nitrate was used as sole N source in culture medium, biomass of *C. acuminata* cell suspension cultures was increased by threefold with very low CPT when compared to other nitrate-ammonium combinations used, and a high ratio of ammonia-nitrate (5:1) led to increased CPT accumulation (Pan et al. 2004). Similar trend was also observed in *N. nimmoniana* and *O. mungos* (ammonium-nitrate ratio 4:2) cell suspension cultures, suggesting that higher ammonium content is required by the cells for enhanced CPT content/production (Karwasara and Dixit 2013; Deepthi and Satheeshkumar 2016).

Phosphates are important component of nucleic acids, and they are involved in photosynthesis and energy transports in plant cells. Phosphate composition in MS medium is 1.25 mM. In *O. mungos* cell and adventitious root suspension culture, high biomass was obtained when phosphate concentration was 1.25 mM, and high

CPT content was observed at phosphate concentrations between 0.312 and 1.25 mM (Deepthi and Satheeshkumar 2017b). When phosphate concentration was increased to 2.5 mM, it negatively affected the root biomass and CPT content. On the contrary, Karwasara and Dixit (2013) reported that at 2.5 mM phosphate concentration, *N. nimmoniana* achieved higher biomass compared to other concentrations and maximum CPT content was observed at 0.5 mM phosphate concentration in the medium. Based on these observations, it can be hypothesized that phosphate requirement differs for different types of in vitro cultures for CPT production.

All these studies state that it is necessary to narrow down the mineral requirements for successful growth and production by in vitro cultures of CPT-producing plants. For this purpose, statistical methods of nutrient optimization have been performed in plant tissue culture of different species to obtain maximum production in terms of biomass and secondary metabolites (Prakash and Srivastava 2005; Chattopadhyay et al. 2002b; Srivastava and Srivastava 2012b; Schmitz et al. 2016).

Besides C and N sources and other macroelements, microelements also influence the secondary metabolite production. Increasing the concentration of iodide, cobalt, molybdate, and cupric salts in MS medium increased the CPT production in *C. acuminata* cell suspension cultures by 2.7-fold along with increase in biomass (Pan et al. 2004). These salts can also function as elicitors to enhance the CPT-producing capacity of the cells.

4.3 Effect of Environmental Parameters on In Vitro Cultures of CPT-Producing Plants

Apart from the chemical composition of the medium, parameters such as pH, temperature, agitation speed, light intensity, wavelength and photoperiod play a significant role in growth and production of secondary metabolites by in vitro plant cultures. pH plays a major role in the transport of solutes across cell membranes. Hence, it is vital to monitor the pH change during the course of the culture, which is not feasible in shake flask cultures. Therefore, the initial pH of the medium should be maintained at an optimal range so that the growth of cells/tissue is not hampered. Generally, initial pH of the plant nutrient medium is maintained between 5.6-5.8 before autoclaving. In a study performed in *C. acuminata* cell suspension with initial medium pH varying from 3 to 9 (after autoclave), pH 4.3 was reported to provide the highest biomass concentration (Sakato and Misawa 1974). In the same study, cell suspension was exposed to different white light intensities (0, 200 and 2000 lux) and maximum biomass concentration was acheived at 200 lux exposure.

In addition to light intensity, the wavelength of light also plays a key role in biomass and metabolite production. In the visible spectrum, red light influenced the biomass production positively followed by yellow light and blue light (Sakato and Misawa 1974). This was further confirmed in *C. acuminata* seedlings, where red light increased the leaf biomass and under blue light, CPT content and the

expression of CPT biosynthetic pathway enzymes increased by two fold (Liu et al. 2015) compared to white light. Hence, it is crucial to optimize the physical parameters in order to attain maximum biomass and secondary metabolite production in plant tissue cultures.

4.4 Elicitation and Precursor Feeding

Elicitors are stress inducers/signaling molecules which activate plant defense mechanism against stress or unfavorable conditions. Stress in plant or in vitro plant systems is known to significantly increase the secondary metabolite production (Narayani and Srivastava 2017). Elicitors, such as hormones, can be produced by the plant itself (endogenous) or received from outer environment (exogenous), like pathogen attack or physiochemical changes. The chief stress signaling molecules are jasmonic acid (JA), salicylic acid (SA), and abscisic acid (ABA), which trigger

Table 4 Elicitors used in in vitro plant cell/tissue cultures for camptothecin enhancement

Plant species and in vitro culture type	Elicitor	Fold enhancement in CPT yield/titer in response to elicitor	Reference
N. nimmoniana, callus culture	Gamma irradiation 20 Gy[a]	20	Fulzele et al. (2015)
N. nimmoniana, cell suspension culture	Yeast extract[b]	4.2	Isah (2017)
	Vanadyl sulfate[a]	3.03	
C. acuminata, cell suspension culture	Jasmonate[a]	11	Song and Byun (1998)
C. acuminata suspension, culture	Fungal elicitor PB90[b]	3	Lu et al. (2011)
C. acuminata, cell suspension culture	Sorbitol[a]	500	Yang et al. (2017)
C. acuminata, seedlings	Abscisic acid[a]	1.44	Kai et al. (2014)
C. acuminata, seedlings	Salicylic acid[a]	High expression of iridoid pathway genes in leaves	Liu et al. (2018)
C. acuminata, callus culture	Mechanical stress 2 Hz hr. $^{-1}$ [a]	4	Kang et al. (2011)
O. mungos, shoot culture	Methyl jasmonate[a]	7	Krishnan et al. (2019)
O. mungos, cell suspension culture	Yeast extract[b]	13.3	Deepthi and Satheeshkumar (2016)
	Silver nitrate[a]	8.7	
	Jasmonic acid[a]	18.66	Deepthi and Satheeshkumar (2017a)

[a]Abiotic elicitors
[b]Biotic elicitors

systemic response in the plant system. Concentration of elicitor, time of addition of elicitor to in vitro cultures, and exposure time of the cultures toward the elicitors are important factors influencing not only the secondary metabolite accumulation but also the viability of cells (Narayani and Srivastava 2017). Hence, these factors have to be optimized to balance the secondary metabolite production and survival of plant cells long enough for them to synthesize the metabolites in elicited conditions.

Elicitation can be used to trigger the CPT production in in vitro cultures of CPT-producing plant species. The plant signaling molecules such as JA, SA, and methyl jasmonate (MeJA) were used in callus, cell suspension, and hairy root cultures of CPT producers which enhanced the CPT production by multiple folds (Table 4). JA at a final concentration of 50 μM, when added to *C. acuminata* cell suspension culture, increased the CPT concentration by 11 times compared to unelicited cultures. SA, when added to in vitro shoots of *C. acuminata*, increases the expression of genes in iridoid biosynthetic pathway (Liu et al. 2018).

Some non-plant-based biotic (biological in origin) elicitors are found using the ability of plant cells to sense pathogen-associated molecular patterns and to trigger the defensive responses. Several studies have been done to increase the secondary metabolite production by mimicking the pathogen attack on plant in vitro cultures (Narayani and Srivastava 2017). Yeast extract has been used for elicitation of CPT production in *C. acuminata* (Song and Byun 1998), *N. nimmoniana* (Isah 2017), and *O. mungos* (Deepthi and Satheeshkumar 2016) cell suspension culture. Similarly, a protein secreted by plant pathogenic fungi named *Phytophthora boehmeriae*, PB90, was found to increase the CPT content by threefold in *C. acuminata* cell suspension culture (Lu et al. 2011).

High-energy radiations such as gamma and ultraviolet radiation induce the production of reactive oxygen species (ROS) which in turn results in the oxidative stress inside the cell. The cells which undergo oxidative stress start secreting enzymes which counteract the ROS generation (Vardhan and Shukla 2017). Plant cells which were exposed to low doses of gamma radiation (less than 40 Gy units) tend to produce secondary metabolites to thwart the oxidative stress caused by radiation. After exposure to 20 Gy gamma radiation, the CPT yield in *N. nimmoniana* callus increased up to 20-fold and cell growth by two-fold (Fulzele et al. 2015). UV radiation is known to increase the expression of tryptophan decarboxylase in *C. roseus* (Ouwerkerk et al. 1999). When *C. acuminata* cell suspension cultures were exposed to UV-B radiation at 5 μmol m^{-2} s^{-1} for 1 hr., the CPT production was increased by 3.3-fold (Ruan et al. 2014).

Herbivory/folivory insects like caterpillars, when feeding on plant parts, create vibration on the plant. This vibration can be sensed by the plant system and activates their systemic response against leaf feeders. Therefore, by mimicking this vibration in in vitro system, defense molecules such as CPT production might be enhanced. Callus cultures of *C. acuminata* when subjected to mechanical vibration on a vibrating platform at the order of 2 Hz for 1 hour every day increased the CPT content by fourfold (Kang et al. 2011). Heavy metals such as vanadium, silver, and copper also induce oxidative stress in plant cells and initiate defense response. Vanadyl sulfate enhanced CPT content in *N. nimmoniana* cell suspension culture by threefold (Isah

2017). Similarly, 8.7-fold increment in CPT yield was observed when silver nitrate was used as an elicitor in *O. mungos* cell suspension cultures (Deepthi and Satheeshkumar 2016). Apart from these elicitors, osmotic stress inducers such as sorbitol, mannitol, and polyethylene glycol (PEG) have also been used to attain higher CPT production in plant in vitro cultures. Osmotic stress mimics the drought condition in the system which in turns activates the secondary metabolism. In *C. acuminata* cell suspension culture, addition of sorbitol to the medium increased the CPT content by 500 times compared to unelicited suspension culture (Yang et al. 2017).

Precursors are intermediates in a metabolic pathway upstream of the end product. Exogenous addition of precursors increases the local concentration of intermediates, driving the biosynthetic pathway toward end product (Whitmer et al. 2002). This aspect of secondary metabolite productivity enhancement was explored for CPT production in vitro cultures. When secologanin and tryptamine were added separately as precursors to *O. pumila* hairy roots, the CPT production was not observed. It is possible that precursors were not absorbed by hairy roots (Saito et al. 2001). But addition of secologanin to *C. acuminata* cell suspension cultures increased the CPT content by three times (Yang et al. 2017). In the same study, addition of tryptamine alone did not influence the CPT production; instead, the precursor was used in kynuramine synthesis pathway suggesting that secologanin might be limiting precursor for CPT biosynthesis or the alternate pathway might be much more active than the MIA pathway. In *C. roseus* cell suspension culture, combined addition of shikimate and iridoid precursor increased the production of ajmalicine by six times. Studies on combining precursor feeding (loganin and tryptamine) and elicitation (MeJA) for secondary metabolite production were also done in *C. roseus* suspension cultures. This strategy increased the final metabolite concentration by severalfold (Lee-Parsons and Royce 2006).

5 Bioreactor Cultivation for Mass Production of CPT

Recently, large-scale production of plant-based secondary metabolites in bioreactors has been drawing huge attention. Paclitaxel, shikonin, and ginseng are some notable examplesof plant metabolites produced in Industrial-scale bioreactors. Even though plant cell cultures are lucrative options for large-scale production of secondary metabolites, several challenges during scale-up have to be overcome. While biological challenges include slow growth rates, physiological heterogeneity, and low metabolite content, operational challenges include prevention of wall adhesion to bioreactors, adequate light requirement, shear sensitivity, and appropriate mass transfer. Although recent years have seen a lot of progress in design of different kinds of bioreactors and optimization of media components, environmental factors such as shear stress and oxygen transfer call for more detailed assessment with each type of plant cell/tissue culture. Choosing a proper cultivation strategy like batch, fed-batch, and two-stage reactors also plays a vital role in increasing the

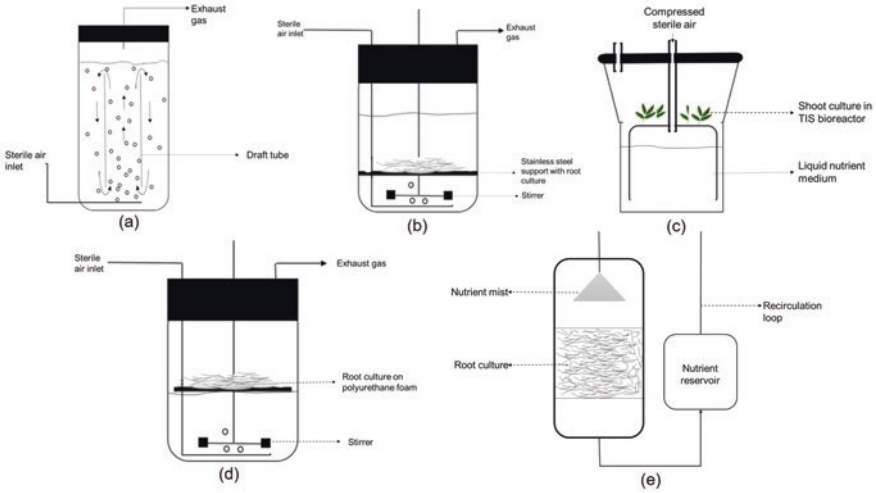

Fig. 8 Bioreactor configurations used for the cultivation of in vitro cultures of camptothecin-producing plant species. (**a**) Airlift bioreactor. (Adapted from Yu et al. 2005); (**b**) stirred tank reactor with stainless steel support. (Adapted from Sudo et al. 2002); (**c**) temporary immersion system bioreactor. (Adapted from Sankar-Thomas and Lieberei 2011); (**d**) stirred tank reactor with foam support. (Adapted from Thakore et al. 2017); (**e**) nutrient mist bioreactor. (Adapted from Wang and Qi 2010; Srivastava and Srivastava 2012a)

productivity. A methodical analysis of these parameters would pave way for successful establishment of plant cell biofactories.

When cell suspension/organ cultures are grown in reactors, stress on cells due to mechanical agitation is undesirable; however, agitation is an important factor to achieve homogeneity in the growth medium. To overcome this challenge, most plant cell suspensions in reactors are accompanied by air-driven agitations as in airlift and bubble column bioreactors (Srikantan and Srivastava 2018). 20 L airlift bioreactors using *C. roseus* cell cultures have been reported for the production of ajmalicine and catharanthine (Zhao et al. 2001). With regard to CPT production, 5 L airlift bioreactors with *C. acuminata* cell suspension cultures have been reported (Fig. 8a) to produce isocamptothecines (camptothecins without A ring) up to 4.7% DW (Yu et al. 2005).

For growing organ cultures like hairy roots, reactors with supports have been used for the production of secondary metabolites. Hairy roots of *O. pumila* have been grown in a 2.5 L working volume stirred tank reactor with a stainless steel support (Fig. 8b). Around 22 mg of CPT was obtained from biomass and spent medium after 8 weeks of cultivation (Sudo et al. 2002). When organ cultures are submerged, cellular damage due to agitation, poor uptake of oxygen, and hyperhydricity impedes metabolite production. Certain reactor configurations provide controlled contact of organ cultures with nutrient medium. For example, as described by Thakore et al. (2017), adventitious/hairy roots of *C. roseus* were allowed to grow on a floating support on the liquid medium (Fig. 8d). The nutrient-rich medium gets absorbed by

the floating support which, in turn, is taken by the roots. Volumetric productivity of ajmalicine doubled in this configuration when compared with bubble column. In nutrient mist bioreactors (Fig. 8e), adventitious/hairy roots placed on solid support were sprayed with nano-sized droplets of nutrient medium at regular intervals (Wang and Qi 2010; Srivastava and Srivastava 2012a). In these ways, aeration to the roots was not restricted, and tissue damage or cell death due to shear stress will be avoided (Srikantan and Srivastava 2018).

Similarly, for shoot and embryo cultures of *C. acuminata*, temporary immersion system (TIS) bioreactors were studied to provide nutrition along with necessary aeration (Fig. 8c). *C. acuminata* shoots produced 2.5 mg/g DW of CPT when grown in TIS reactor with eight immersion cycles per day (Sankar-Thomas and Lieberei 2011).

The physicochemical environment of nutrient medium can be continuously monitored and controlled in a bioreactor which is an advantage over shake flask cultures. The knowledge obtained from shake flask cultures of CPT-producing plants can be utilized in bioreactor cultivations. From data acquired from batch cultivation in reactor studies, one can construct a mathematical model which can be further used in fed-batch and continuous cultivation to enable non-inhibitory and non-limiting concentrations of limiting nutrients throughout the cultivation for achieving maximum productivities (Chattopadhyay et al. 2002a; Ferri et al. 2011).

Though bioreactor cultivation of CPT producers has been a less explored part in the history of camptothecin, various yield and productivity enhancement techniques can be studied with cues from similar secondary metabolite production systems using plant cell cultures in bioreactor conditions for better production of the metabolite in the large scale.

6 Conclusion

Camptothecin is one of the most in demand secondary metabolites for the treatment of cancer, and its commercial production is heavily dependent on the natural resources, i.e., plants. With the aim to fulfill the demand and reduce this dependency, it is necessary to increase the research on developing in vitro cultures of these natural resources. In the recent years, the scientific community has documented a remarkable growth in using the in vitro plant systems for the production for valuable secondary metabolites. In order to achieve profitable production of these metabolites under controlled conditions from in vitro cultures, in-depth understanding of their biosynthetic pathway is required. With current information regarding the upstream biosynthetic enzymes, it has been possible to overexpress and to metabolically engineer CPT-producing cell/organ cultures for high production. However, enzymes in later stages have to be elucidated for further development in this direction. Also, apart from strain improvement techniques, consolidated process enhancement approaches such as modifying media components, culture condition optimizations, co-cultivation, elicitation treatment, and exogenous precursor

addition can be used. With modern technological advancements, genome-scale and mathematical modeling approaches can be used to rationally identify bottlenecks and knockout targets for further CPT enhancement, thereby reducing the production costs. Scale-up of plant cell/tissue cultures in bioreactors is a crucial step to meet the high demand for anticancer drugs in the medicinal field. Bioreactor cultivation of different plant cell/tissue cultures has been used in the past decades to achieve increased production of commercially important metabolites such as food additives, nutraceuticals, and therapeutic proteins. However, production of CPT using plant cell cultivation in bioreactors is still at research level. Considering the ever-increasing requirement of anticancer drugs, future studies on large-scale production of CPT through high-yielding plant cultures incorporating productivity enhancement strategies are extremely important.

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Not One for All: The Interwoven Relationship Between Morphophysiology and Secondary Metabolite Production in Plant Cell Cultures

Winnie Yap Soo Ping and Melissa Kam Yit Yee

Abstract Although plant cell culture has the inherent capacity to enhance the production of secondary metabolites and amenable for industrial scale-up via bioreactors, not all plant secondary products can be synthesized and accumulated in its respective plant cell cultures. Obviously, there is not one perfect protocol that is suited for all plant species to establish cell cultures for production of compounds of interest. As cell cultures represent a lower level of differentiation from their tissues of origin, the mechanism cells employ to accomplish spatiotemporal regulation of secondary metabolism can be rather different from its mother plant. The focus of this chapter is, therefore, to draw attention on the differential production of secondary metabolites between intact plants and plant cell cultures, how changes in their morphophysiological state may affect secondary product metabolism in plant cell cultures, and how this may affect the overall production efficiency of a culture. We believe that this knowledge is especially relevant to understand and to plan for future biotechnological research relating to manipulation of the biosynthetic potential of plant cells in the production of plant secondary products.

Keywords Artemisinin · Morphophysiology · Secondary metabolite · Cytodifferentiation · Feedback inhibition · Cellular degradation

1 Introduction

As sessile as they are, the plant defense systems are elegant examples of how nature devices highly efficient solutions to overcome vexations. Across evolutionary timescale, the co-evolution between host plants and invading phytopathogens or phytophagous organisms to constantly enhance their defense and counter-defense

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mechanisms, leading to persistent co-evolutionary turnover in defense and adaptive radiation, has in turn ultimately led to the diversification in plant secondary metabolites (Speed et al. 2015). With the ability to perceive an external stimulus and to anticipate and respond to adverse environmental conditions via sophisticated cross-talk strategies between the different defense/stress response mechanisms, plants can adapt to their ecological niches and maximize survival. In this massive chemometabolic diversity, secondary metabolites are commonly categorized into phenolics, terpenes, and nitrogen- or sulfur-containing compounds (Guerriero et al. 2018; Zaynab et al. 2018). Although an estimation of 200,000 up to 1,000,000 secondary metabolites (also known as specialized metabolites or natural products) are produced in higher plants, having relieved from major roles in plant growth and development, these compounds typically occur in low concentrations in planta (Fiehn 2002; Saito and Matsuda 2010; Afendi et al. 2012). While some phytocompounds are synthesized almost universally by plants, others may confer an expression of individuality with its production specific to certain organisms or species.

The fact that multicellular organisms share a common ancestry that endowed them with a broad range of conserved cellular processes and common biochemical architecture and molecular functioning, phytochemicals involved in plant defense will also interact with human bodily functions (Caporale 1995; Kennedy and Wightman 2011; Atanasov et al. 2015). This biological similarity across taxa is exemplified by the jasmonates of plant signaling molecules and mammalian paracrine molecules. Both chemical groups are enzymatically derived from the same genetically preserved pathways in response to biotic and abiotic stresses (Wasternack and Hause 2002; Iriti and Faoro 2007). In the vast majority of cases, the functions and benefits of these plant secondary metabolites to their producers are of obvious reasons (such as deterrent to predators and volatile cues for communication) (Altindal and Altindal 2017; Zaynab et al. 2018), while some with yet unknown functions, it is this area of secondary metabolism which provides most of the bioactive natural products that find application in pharmaceuticals, crop protection, dyes, flavors, and fragrances (Facchini et al. 2012). A classification of phytochemicals and their versatile uses is detailed in Table 1.

2 Is Plant Cell Culture the Epitome of Phytochemical Production?

Given the importance of plant-derived natural compounds that became indispensable for modern pharmacotherapy, an associated challenge is often the struggle to meet global demand. Due to the naturally low availability in medicinal plants, the exhaustive and unsustainable harvesting of plant materials will lead to ecological-related concerns threatening the wild populations. As if that were not enough, geographical and seasonal variations often result in highly variable chemical signatures and contents among populations and over short spatial distances within the populations which, in turn, affect

Table 1 Examples of plant-derived secondary metabolites and their related applications

Class	Secondary metabolite	Plant species	Uses/biological actions	References
Phenolics	Capsaicin	*Capsicum* spp.	Analgesic; antiobesity; gastroprotective; antidiabetic; anti-inflammatory	Fattori et al. (2016) and Zhang et al. (2017)
	Catechin	Originally derived from *Senegalia catechu*, found abundantly also in tea (e.g., *Camellia sinensis* L.), cocoa, and berries	Antioxidant; antihypertensive; antiproliferative; antithrombogenic; antihyperlipidemic	Zanwar et al. (2014)
	Hypericin	*Hypericum perforatum*	Antibacterial; antiviral; anticancer (photosensitizer); antioxidant; anti-inflammatory; wound healing; antinociceptive; antidepressant	Wang et al. (2010) and Klemow et al. (2011)
	Rosmarinic acid	*Rosmarinus officinalis*; *Salvia officinalis*; *Salvia lavandulifolia*; *Ocimum tenuiflorum*; *Origanum vulgare*; *Origanum majorana*; *Melissa officinalis*	Antioxidant; anti-inflammatory; antibacterial, antiangiogenic; antimutagenic; antidepressant; antiallergenic; neuroprotective	Alagawany et al. (2017)
	Shikonin	*Lithospermum* erythrorhizon Sieb. et Zucc.; *Arnebia euchroma* (Royle) Johnst.; *Arnebia guttata* Bunge	Colorant (in textile, food and cosmetic industries); antibacterial; anti-inflammatory; anticancer; antioxidant; anti-HIV	Chen et al. (2003) and Wang et al. (2019)
	Silymarin	*Silybum marianum*	Hepatoprotective	Hellerbrand et al. (2016)

(continued)

Table 1 (continued)

Class	Secondary metabolite	Plant species	Uses/biological actions	References
Terpenes	Artemisinin	*Artemisia annua* L.	Antimalarial; anticancer; antiviral; antiparasitic	Krishna et al. (2008)
	Azadirachtin	*Azadirachta indica*	Insecticidal	Dawkar et al. (2019)
	Cannabidiol[a]	*Cannabis sativa* L.	Antianxiety; antinausea; antiarthritic; antipsychotic; anti-inflammatory; immunomodulatory; antiseizure (Epidiolex® oral solution; FDA approved); insecticidal; antiaddiction	Pisanti et al. (2017) and Park et al. (2019)
	Forskolin	*Coleus forskohlii*	Anticancer; antiglaucoma; antiobesity	Kamohara (2016) and Srivastava et al. (2017)
	Ginsenoside	*Panax* spp.	Antioxidant; anti-inflammatory; vasorelaxant; antiallergic; antidiabetic; anticancer; antiaggregant	Kim (2018)
	Paclitaxel	*Taxus brevifolia* Nutt.	Anticancer (chemotherapy drug, Taxol® and Onxal™); anti-inflammatory; axon regeneration; antiproliferative	Zhang et al. (2014)

(continued)

Table 1 (continued)

Class	Secondary metabolite	Plant species	Uses/biological actions	References
Nitrogen-containing heterocycles	Berberine	*Berberis vulgaris*, found also in several other plant families and genera	Antibacterial; anti-inflammatory; antioxidant; antihyperlipidemic; hepatoprotective; nephroprotective; immunomodulatory	Neag et al. (2018)
	Caffeine	*Coffea* spp.	Antimicrobial; insecticidal; analgesic; stimulant	Pech-Kú et al. (2018)
	Camptothecin	*Camptotheca acuminata*	Anticancer (chemotherapy drugs Hycamtin® and Camptosar®, derived from camptothecin analogs topotecan and irinotecan, respectively)	Huryn and Wipf (2008)
	Codeine/ morphine	*Papaver somniferum*	Analgesic; antitussive	Heard and Fletcher (2011) and Resnik (2018)
	Theobromine	*Theobroma cacao L.*	Antitumor; anti-inflammatory; vasodilator; diuretic; myocardial stimulant	Martinez-Pinilla et al. (2015)
	Vincristine	*Catharanthus roseus*	Anticancer (chemotherapy drug Oncovin® and Vincasar Pfs®); immunosuppressive	Rubio et al. (2004) and Aniszewski (2015)
Sulfur-containing compounds	Allicin	*Allium sativum* L.	Antibacterial; antifungal; as dietary supplement	Marchese et al. (2016)
	Glucosinolate[b]	Brassicaceae family	Anticarcinogenic; antifungal; antibacterial; antiproliferative[c]	Barba et al. (2016)

[a]Cannabidiol is a C21 terpenophenolic compound (WHO 2017), synthesized from geranyl diphosphate (terpene) and olivetolic acid (phenol)

[b]Glucosinolates are sulfur- and nitrogen-containing glycosides (Bruneton 2013), though often classified as sulfur-containing compounds

[c]Listed biological properties are a contribution of both glucosinolates and their breakdown products

production quality. Codeine in opium poppy plants (*P. somniferum*), for instance, varies throughout development whereby rainfall and low temperatures may reduce accumulation during capsule maturation (Dittbrenner et al. 2009). Furthermore, attempts at commercializing natural products via total chemical synthesis or semi-synthesis to tackle these complications were hampered. This is mainly ascribed to the high degree of structural complexity of secondary metabolites having numerous oxygen-containing substituents and chiral centers (Atanasov et al. 2015). In a scenario when these approaches are successful, the multiple chemical conversion procedure of the manufacturing system demands high financial and environmental costs. An example is the semi-synthetic production of artemisinin by Sanofi. Having a production capacity of manufacturing approximately 60 tons of antimalarial drugs annually, a financial trade-off that came with it was that Sanofi's semi-synthetic artemisinin (US$ 350–400 per kg) was not competitive in price when excess agricultural artemisinin bloomed (< US$ 250 per kg) (Peplow 2016). In this context, the growing demand for pharmacologically active plant compounds has made these metabolites enthralling targets for their in vitro production.

The in vitro cultivation of isolated plant cells, known as plant cell culture, has been regarded as a convenient biotechnological technique to produce plant metabolites. The substantial cell fate plasticity and totipotency of plant cells in response to metabolite biosynthesis under controlled conditions; production of structurally complex metabolites; independence of geographical and seasonal variations; continuous and uniform production in terms of quality and yield; exclusion of agrochemical application; shortened growth cycles; and a production process aligned with current good manufacturing practices are several advantages associated with the use of plant cells as biofactories (Rao and Ravishankar 2002; Wilson and Roberts 2012; Xu and Zhang 2014; Ochoa-Villarreal et al. 2016). The commercial successes of plant cell-derived products that garnered most attention were by Phyton Biotech (paclitaxel for Taxol® formulation), Samyang Biopharmaceuticals (paclitaxel for Genexol® production), and Mitsui Chemicals (shikonin as colorant), to name a few (Ochoa-Villarreal et al. 2016). Yet this well-established technology platform is not without its problems, particularly one associated with unsatisfactory low yield of production. It is undeniable that dedifferentiated calli or suspension cells can synthesize a wide range of phytocompounds, in the strictest sense, that involve less complicated natural biosynthesis. However, the discussion of experimental results reporting the lack of stimulation in cell culture-based metabolite production has not been given much attention. The implications of these negative results, in our opinion, can be extensive and deserve further investigation to fully understand the underlying causes restricting efficient secondary metabolite production. On the basis of our recent studies, we highlight several physiological attributes that possibly explain the roadblock responsible for failure relating to plant cell-based production of natural products. These include (i) the lack of cytodifferentiation in cell cultures; (ii) negative feedback regulation of metabolite biosynthesis; and (iii) sequestration of phytocompound by cellular detoxification (Fig. 1). It should be acknowledged that these attributes are closely related and interdependent. It is, therefore, profitable to discuss these factors collectively instead of individual elements. The following pages will then present a specific case study that details these morphophysiological events and their effects on product synthesis.

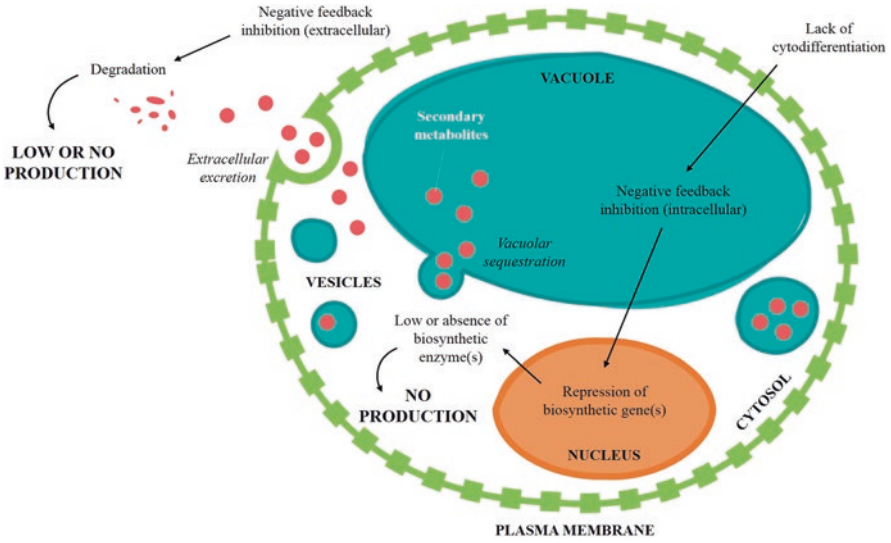

Fig. 1 Schematic representation of secondary metabolism in cultured plant cell. Improper accumulation of metabolites in cytosol or sequestration in vacuole, due to the lack of specialized cells or tissues, may trigger the self-regulatory feedback inhibition mechanism that leads to metabolic detoxification and synthesis inhibition via suppression of biosynthetic genes. Absence of relevant enzymes along the biosynthetic pathway may truncate product synthesis

3 What Makes Plant Cell Culture?

Although some secondary metabolites are detected in cells of the whole plant, secondary metabolism as we know in its natural settings is associated with an array of cell types, and metabolite synthesis and accumulation do not always occur in the same location. In most cases, the formation of precursors to intermediates along the biosynthetic pathway up till the final product is placed in different subcellular membranous organelles, in highly specialized cells or in different plant tissues or organs (Roze et al. 2011). These "plant divisions" are rather diverse and multileveled, reminiscent of a matryoshka doll. Take the monoterpenoid indole alkaloids of *C. roseus* as an example. While these alkaloids accumulate in specialized cells called idioblasts and laticifers, the pathway enzymes are found in the roots, in the photosynthetic organs, in the epidermis of young leaves and stems, and in the flower buds (St-Pierre et al. 1999). In these structures, the biosynthetic enzymes are then localized to different subcellular compartments including cytosol, vacuole, endoplasmic reticulum, vesicles, and thylakoid membrane (Roytrakul and Verpoorte 2007; Ziegler and Facchini 2008). In view of this intricate spatial distribution of key elements in secondary metabolism and translocation, such metabolites would be difficult to detect, if not found in in vitro cell cultures, though they often maintain the inducibility of secondary biosynthesis upon contact with biotic or abiotic elicitors. The question is, why is that the case and how does it affect cell culture performance?

As presently understood, plant cell culture technology stems from the derivation of de-differentiated cells from a selection of starting plant materials (termed explants), and it involves the reversion of developmental reprogramming through in vitro cell fate switch that results in the loss of phenotypic specialization and reversion of cells to a less differentiated state (Miguel and Marum 2011). Simply put, cell cultures often lack differentiation and are devoid of organs, while the production and accumulation of certain metabolites require a degree of differentiated micro plant or organ cultures. For example, the production of nicotine in *Nicotiana tabacum* occurs at the distal ends of roots and shoots, whereby the biosynthesis of lysine to anabasine happens in the former and conversion of anabasine to nicotine in the leaves. As a result, only trace amounts of nicotine were detectable in *N. tabacum* callus and shoot cultures due to the lack of organ-specific anabasine, and nicotine accumulation is regulated by the interaction of both root and shoot tissues (Saunders 1998). The undifferentiated *Papaver* spp. cell cultures failed to produce morphine, as it was noted that laticifers are absent from these cultures but present in seedling cultures (Kutchan et al. 1983; Rush et al. 1985; Weid et al. 2004). Another related example of tissue-specific metabolite is the antidepressant hypericin and hyperforin derived from *H. perforatum*. The undifferentiated cells are unable to produce these phytochemicals without a certain extent of morphogenesis (Kirakosyan et al. 2004).

Looking at the associations of spatial distribution of metabolite biosynthesis in whole plants, the correlation of metabolite synthesis and accumulation with cytodifferentiation in cell cultures seemed mundane for proper production, but there are exceptions whereby biosynthetic production occurs indiscriminative of tissue type. In *Rauvolfia* spp., reserpine was detected in whole plant cultures, calli, and even cell cultures, disregarding the fact that reserpine is derived naturally from the plant roots (Yamamoto and Yamada 1986; Anitha and Kumari 2006; Panwar and Guru 2015). In this regard, it would seem unreasonable to determine for certain that plant cells lacking differentiation are the primary factor for incapability in biosynthesizing secondary metabolites. In instances relative to that of *Rauvolfia* spp., it appears that some cell cultures can synthesize phytocompounds in liquid suspension, but it is the accumulation and subsequent total content that are far too low. In fact, we believed that the general lack of cellular differentiation could be one of the factors that, in a cascading effect, affected how cells may accumulate and store their products. Without specialized partitioning, plant cells would rely on vesicular and vacuolar sequestration, since vacuoles occupy 90% of the cell's volume, for the accumulation of toxic secondary metabolites to avoid autointoxication (Martinoia et al. 2000; Yazaki et al. 2008). Such occurrence was demonstrated by Alcantara et al. (2005) in which cultured opium poppy cells undergo major ultrastructural modification that resulted in the formation of large dilated endoplasmic reticulum-derived vesicles to sequester the elicitor-induced sanguinarine. Interestingly, it was noted that the localization and accumulation of sanguinarine in both cultured cells and intact plants were highly similar. The benzylisoquinoline alkaloid pathway is localized to the parietal sieve element reticulum in the plant, and the large, alkaloid-containing vesicles found in opium poppy laticifers emerged in the same manner as cultured

cells, from dilations of the endoplasmic reticulum. Another example is berberine sequestration in cultured cells of *Coptis japonica*, where its accumulation in the vacuolar matrix is mediated by an ABC transporter and H+/berberine antiporter (Otani et al. 2005). The ability of plant cells to accumulate metabolites intracellularly usually reflects the effective secondary compound production in high amounts, but not all cells in a culture will display physiological homogeneity in terms of accumulation. In the same study by Alcantara et al. (2005), only a subset of elicitor-treated opium poppy cells (approximately 20%) was identified to contain detectable amounts of sanguinarine. Similarly, subpopulations of *Taxus media* cells exhibited different DNA contents due to a change in ploidy after a year of subculture (Baebler et al. 2005). This heterogeneity within cell populations is potentially linked to the genetic and epigenetic instability of plant cells grown in culture. Tanurdzic et al. (2008) and Gao et al. (2019) pointed out that the differential expression of genes (those associated with secondary metabolite biosynthesis) and epigenetic variation during dedifferentiation and redifferentiation processes might affect cell fate and distort cellular functions.

Phytotoxins not accumulated in the vacuoles of cultured plant cells are instead directed to the cellular surface and via extracellular exudation are released into the surrounding medium to prevent the build-up of metabolites in the cytosol (Procházková et al. 2011). In such cases, it is conceivable that the decreased productivity of secondary metabolites is associated with feedback inhibition of product synthesis. As plant cells are constantly bathed in the liquid medium, the release secondary metabolites into the medium is likely to trigger the negative feedback mechanism to inhibit excess production of phytotoxic metabolites (Misawa 1994). In this self-regulating system, the output response diminishes the initial stimulus to maintain fluctuations within limits acceptable to the function of organisms. For instance, the immediate removal of products excreted into the culture medium has been emphasized as a measure to reduce feedback inhibition and product degradation (Wang et al. 2001). On a side note, the repression of secondary metabolite production may also occur intracellularly. Additionally, enzymatic or non-enzymatic degradation in the medium may negatively influence metabolite productivity in cell suspension cultures (Rao and Ravishankar 2002). As the culture fluid functions as an extracellular lytic compartment due to the release of catabolic enzymes (peroxidases, glucosidases, lipases, etc.) and acids from cells (Dörnenburg and Knorr 1995), the exudates will be rapidly metabolized. Such detoxification of excreted phytotoxic compounds has been described by Weiss et al. (2006) in cultured cells of *Eschscholzia californica.* With the addition of sanguinarine to the suspension culture, a rapid decline and eventual disappearance of the benzophenanthridine alkaloid from the medium coincided with an increase of dihydrosanguinarine, a form of a less cytotoxic precursor of sanguinarine. On such basis, it was concluded that cells are capable of reabsorbing and through reduction the substance is rendered less harmful, which is then recycled for further biosynthetic reactions.

Faced with the dilemma of wondering if your work has been sloppy and your metabolite has gone undetected or is your cell cultures really not synthesizing products? If you were certain of the reliability of the former, perhaps a look at the bio-

synthetic pathway from a molecular aspect would be beneficial. Relating this final point of discussion to the genetic instability of cell cultures, dedifferentiated plant cells may possess a complete set of secondary metabolite biosynthetic genes, but it is also highly possible that some cell cultures will retain only a small fraction of genes. In *Lupinus* spp., for instance, it was ascertained that the biosynthetic enzymes of quinolizidine alkaloids and its precursor lysine are localized in chloroplasts and gene activation would require light (Wink and Hartmann 1982). A comparison between light- and dark-incubated *C. roseus* cell cultures has shown that the former had a higher accumulation of serpentine due to higher vacuolar peroxidase activity (20-fold) that plays a role in the conversion of ajmalicine to serpentine (Sierra 1991). This can be inferred that while biosynthetic genes or enzymes are present in cell cultures, a stimulus would be required to activate the necessary genes for production. Another example would be that of morphine accumulation in opium poppy cell cultures whereby morphine was not detected in neither induced nor non-induced cultures (Facchini et al. 1996; Alcantara et al. 2005). Although the enzyme codeinone reductase (COR) responsible for the production of morphine was present in non-induced cells, but it remained low and did not increase in response to elicitor treatment, transcriptionally or post-transcriptionally. The silencing of *COR* genes in transgenic opium poppy plants that resulted in the accumulation of (S)-reticuline rather than codeinone (Allen et al. 2004) supports the fact that the absence of a single enzyme or enzymes will impede metabolite production. In this case, the inactive or repressed COR may have prevented intermediates from general benzylisoquinoline alkaloid biosynthesis from entering the morphine-specific branch pathway. Furthermore, the improper compartmentalization of COR to the cytosol in cultured cells also indicates the inability of cell cultures to form a complex of morphine branch pathway enzymes (Allen et al. 2004). Hence, for the efficient operation of the secondary metabolite pathway, plant cell cultures should possess the essential regulatory, cellular, and metabolic components to support the biosynthesis and accumulation of secondary products. It must also be expected that proteins related to compartmentation, transport, and detoxification are co-expressed with biosynthetic enzymes in order to keep secondary metabolite production compatible with the fitness of the producing cell. In short, the spatiotemporal regulation of secondary metabolism is an important recurring factor in both plant cell cultures and intact plants.

4 Contributing to the Biochemistry of Artemisinin Metabolism in Cell Culture: Case Analysis of *Artemisia annua*

In this section, we will take a closer look at the interaction between morphophysiological changes in *A. annua* plant cell culture and artemisinin production and their differences to that of intact plants as an exemplary case study.

Exclusively derived from the aerial parts of plants, artemisinin (an endoperoxide sesquiterpene lactone) is synthesized and sequestered specifically in the glandular

secretory trichomes (GSTs) on leaf, stem, and floret surfaces (Brown 2010; Olsson et al. 2009; O'Neill et al. 2010; Rizzello et al. 2014). Localized in the cytosol, artemisinin biosynthesis occurs in the sub-apical and apical cells of GST (Xie et al. 2016). The first committed step of artemisinin biosynthesis begins with farnesyl diphosphate being converted to amorpha-4,11-diene by the enzyme amorpha-4,11-diene synthase (ADS). Through sequential enzymatic oxidation catalyzed by cytochrome P450 monooxygenase (CYP71AV1), amorpha-4,11-diene is successively converted into artemisinic alcohol, artemisinic aldehyde, and artemisinic acid, with artemisinic aldehyde as the last common intermediate of a branching pathway leading into two different routes producing either artemisinin or arteannuin B. For artemisinin production, the enzymes artemisinic aldehyde Δ11(13) reductase (DBR2) and aldehyde dehydrogenase 1 (ALDH1) are involved to yield its direct precursor dihydroartemisinic acid (DHAA), while ALDH1 and CYP71AV1 partake in the formation of artemisinic acid the precursor of arteannuin B (Zhang et al. 2008; Teoh et al. 2009; Yang et al. 2015). In the final conversion step of non-enzymatic photooxidation reaction (Brown 2010), artemisinin and arteannuin B are synthesized from their respective precursors and sequestered in the subcuticular space of the GST (Fig. 2a). Considering the main ecological role of artemisinin in plant/plant allelopathy by repressing germination and growth of plant competitors, it is unsurprising that the autotoxicity of artemisinin has constricted evolution of its storage to non-living plant parts rendering it harmless to the producing plant (Jessing et al. 2014).

Due to its enormous impact as malaria treatment, the most common research focus associated with *A. annua* plants and in vitro cultures is the development of strategies to boost artemisinin production that are amenable to industrial scale-up. Unlike other cell culture systems such as *Lupinus* spp., *Cytisus* spp., and other Leguminosae whereby the underlying causes responsible for failure in producing substantial amounts of quinolizidine alkaloids are studied physiologically and biochemically, little is known about the metabolism of artemisinin in *A. annua* cell cultures. Thus, improving the performances of *A. annua* cell cultures would, in this sense, require more effort. The full potential of cell suspension culture of *A. annua* as an alternative source of artemisinin is, in our opinion, still far reaching based on the contradicting results that are reported. While Baldi and Dixit (2008), Chan et al. (2010), Caretto et al. (2011), and Salehi et al. (2019) successfully stimulated artemisinin production by several folds via the incorporation of elicitors, Woerdenbag et al. (1992) and Ferreira and Janick (1996) reported either trace or no artemisinin in cell cultures. On the basis of such evidence and our recent observation, the lack of cellular or tissue differentiation dominates as one of the reasons for reduced artemisinin production. As mentioned earlier, the multicellular GSTs are the main site of biosynthesis and localization of enzymes specific to artemisinin biosynthesis in the apical cells of GSTs is evident. Comparing this to the cultured cells of *A. annua* consisting of only single and aggregated cells, the differentiation and development of specialized cell types crucial for their defensive function are yet to occur unless otherwise induced to undergo in vitro differentiation to develop GSTs (Fig. 2b) (Duke et al. 1994; Olsson et al. 2009; Chezem and Clay 2016).

Bearing in mind of the positive results on plant cell-derived artemisinin, it can be argued that cultured cells of *A. annua* can synthesize artemisinin in its less differenti-

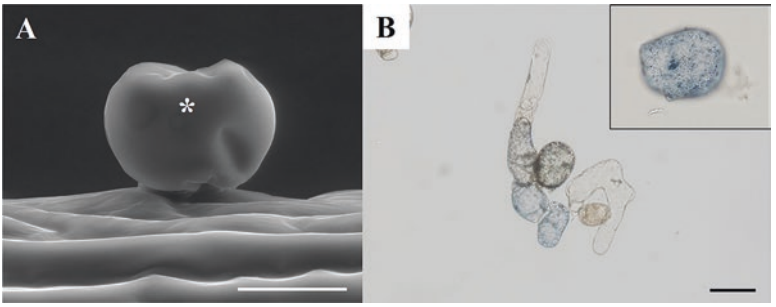

Fig. 2 Morphological differences of glandular secretory trichome and cultured cell of *Artemisia annua*. (**a**) The overall view of the 10-celled glandular secretory trichome obtained from scanning electron microscopy (SEM), showing the subcuticular space of the bilobed sac (asterisk) where artemisinin sequestration occurs. (**b**) Light microscopy of stained cultured cells showing aggregates and single cell (inset). Scale bars, 30 μm; 0.1 mm

ated state and it would suggest that the cells are also able to prevent autointoxication in this case. Assuming that artemisinin biosynthetic genes are localized in the cytosol of the cultured cells, similar to its localization in apical and subapical cells in GSTs, artemisinin should be stored either intracellularly via vacuolar sequestration or extracellularly by releasing artemisinin into the outer medium to maintain homeostasis in the cells. In both cases, the high levels of artemisinin will undoubtedly trigger the feedback inhibition mechanism to inhibit its own synthesis. Such phenomenon is clearly demonstrated by the application of exogenous artemisinin on mature *A. annua* plants, acting through the repression of transcription of cytochrome P450, *CYP71AV1*, and/or *ADS* genes (Arsenault et al. 2010). It is also important to note that poor artemisinin accumulation in cell suspension cultures can be attributed to the enzymatic or non-enzymatic degradation in the medium. When excreted out from the cells, artemisinin is highly vulnerable to catabolism by hydrolytic and oxidizing enzymes (e.g., released by ruptured cells) present in the culture medium. Evidence for the rapid decomposition of artemisinin has been demonstrated by Woerdenbag et al. (1992) when incubated with a cell homogenate of *A. annua* or spent culture medium and in a solution of commercial horseradish peroxidase as control. To reaffirm these postulations, the presence of artemisinin in the liquid medium was verified by Kam and Yap (2019), and it showed no trace of artemisinin. This has not only confirmed that artemisinin is not released into the medium, but it also suggests a possibility of vacuolar sequestration of artemisinin or it would also mean that artemisinin was not synthesized by the cells.

It is no surprise that repression of metabolic pathway genes can arise in plant cell cultures as a consequence of negative feedback triggered by increased levels of phytotoxin intracellularly or extracellularly. But it also appears that there could be an association between the extent of cytodifferentiation and the spatial-temporal regulation of artemisinin-specific biosynthetic pathway genes. This was perfectly illustrated by previous descriptions of cell suspension cultures of *Cytisus scoparius*, *Laburnum alpinum*, and *Lupinus polyphyllus*. Owing to the low expression of related genes, quinolizidine alkaloids were produced in much lower amounts (0.01 – 5 μg g^{-1} fresh weight) compared to those of differentiated plants (500 – 5000 μg g^{-1} fresh weight)

(Wink 1989). In the case of *A. annua* cell culture established from a low artemisinin-producing chemotype, the genes *ADS*, *DBR2*, and *ALDH1* were found to be absent (Kam and Yap 2019). Similar results of the inability to detect *ADS* expression in *A. annua* cell cultures have also been reported by Jing et al. (2009) and Caretto et al. (2011). Unfortunately, comparison of results was not possible since not all genes of the pathway were studied in the latter. Lacking genes encoding both DBR2 and ALDH1 enzymes would prevent the intermediate artemisinic aldehyde from entering the artemisinin-specific branch pathway. In addition, as *ADS* is an essential gene for the first committed step of artemisinin formation (Mercke et al. 2000), its absence in cultured *A. annua* cells would likely lead to the truncation of artemisinin biosynthesis pathway, which in either case will lead to no production of artemisinin. Clearly, this is only the beginning of the study of artemisinin metabolism in cultured cells. More detailed analysis on the adaptive mechanisms to achieve temporal and spatial regulation of artemisinin synthesis at the level of the single cell should be warranted, and details of the intriguing possibilities of enzymatic hydrolysis of artemisinin and export mechanism in cell culture systems await further studies. Addressing these conjectures may provide opportunities for further improvement in reducing artemisinin loss in such in vitro production system, as interest in plant cell-derived artemisinin has recently been renewed with Phyton Biotech onboard (Peplow 2018).

5 Conclusion

In summary, the production of secondary metabolites in plant cell cultures is not a straightforward process but one that is temperamental and involves various interactions with the chemical, physical, and environmental factors of growth. The above discussion and case study illustrate the point that the response of plant cell cultures to secondary metabolite production may be quite different from the responses observed in an intact plant. A change in morphophysiological state of a plant cell, as simple as being less differentiated, will inevitably affect the related downstream biochemical processes (Fig. 1). To fully utilize the potential of plant cells for production of compound of interest, our understanding of the intricate and self-regulated biosynthetic pathway, albeit close monitoring and precise manipulation, is utmost important. In addition, factors that affect the spatiotemporal dynamics and regulation of secondary metabolite biosynthesis and accumulation must not be ignored when evaluating the efficiency of plant cell cultures as an alternative production system to generate plant secondary products.

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Anthocyanins and Proanthocyanidins as Anticancer Agents

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Abstract Plants over the course of evolution have customized their genomes and produce an enormous variety of specialized phytochemicals termed as secondary metabolites. Secondary metabolites are required by the plants for their interaction with environment and their survival in adverse conditions or stresses. They are derived by unique biosynthetic pathways using primary metabolite and their intermediates. These are mostly synthesized in the cytosol (anthocyanins), chloroplasts (terpenoids), or mitochondria (some amines) but targeted for storage in vacuole for utilization under need. Anthocyanins provide a great economic value for mankind as drugs, food supplement, and dyes. The health benefits of anthocyanins are numerous which have been extrapolated by the scientific community on the basis of their antioxidant capacity. Here potential resources and their role in cancer with biochemical mechanisms have been summarized. The research efforts and accomplishments for the novel anthocyanins and functional food are expected to lead to a sustainable agriculture, health, and environment.

Keywords Anthocyanins · Cancer · Apoptosis · Angiogenesis · Antioxidative

1 Introduction

The most dreadful disease on this earth is cancer. Notably, only 10% at the most of all cancers are due to genetic factors, while 90% are directly or indirectly correlated with an individual's lifestyle and dietary habits, stress, and environmental conditions. It covers more than 100 types of disorders in animal system. According to WHO, 9.6 million persons were estimated to have died from one or the other type of cancer worldwide in 2018. The Asia accounts for the 48% of total cancer inci-

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dence. In males, the lung cancer is the most common followed by prostate cancer, while in females the breast cancer is of most common occurrence followed by lung cancer (Bray et al. 2018).

Plants have evolved to produce a diverse array of chemical cornucopia to support their life cycles. Most are products of secondary metabolism, that is, biochemical pathways that are not involved in the synthesis of essential cellular components and not nutritious, in general, but more complex molecules that provide additional functions. Examples of these metabolites are flavonoid, isoprenoids, terpenoids, anthocyanins, etc. Function of these is to attract pollinators or act as oviposition stimulants. These may repel pests and provide resistance against pathogens, e.g., antimicrobials and phytoalexins. They are also helpful in dissipating extra heat received from sunlight and protect the photoreceptors (Nabavi et al. 2020).

The cell produces a number of reactive species during its normal functioning. Production of these free radicals cannot be avoided, and the cell has internal antioxidative mechanism to balance them. There is delicate balance of production and scavenging of free radicals in any biological system. Excess of these radicals creates stress on the cell functioning. The oxidative stress, environmental conditions, and oncogene activation predispose the cell to grow in abnormal fashion. These dysregulated cells are known to be cancer cells.

The selection of healthy diet having anthocyanins helps the cell to combat free radicals generated inside and prevent undesirable consequences. This linkage of anthocyanins and free radical sequestration system motivates the research community to discover anticancer compounds from the plants. Many reports have shown that a healthy lifestyle, including a colorful diet, such as purple corn, fruits, and vegetables, can help reduce the risk of cancer or decelerate the rate of cancer progression (Xu et al. 2016; Sun et al. 2017; Reale et al. 2018). Hence, humans are more inclined toward healthy food having medicinal or pharmaceutical significance.

Earlier aromatic and medicinal plants have long been exploited as a source of pharmaceutical compounds, and a number of species are cultivated specifically for the purpose of extracting drugs and other valuable molecules. However, unique germplasm collection of staple crops like wheat, maize, and sorghum can provide metabolites or compounds which are of high value. The use of plant color extract in folk medicine since ancient time has encouraged scientists to discover their compounds and role in various ailments. The extraction, identification, and functional evaluation of anthocyanins from different plants or plant products have been a subject of research (Dimitrios, 2006; Dai and Mumper, 2010).

The anthocyanin biosynthesis pathway is conserved across plant kingdom so most plants produce the same basic molecular skeletons of flavans, but these are decorated with functional groups in a highly specific way, so that unique molecules may be found in only one or a few plant species. Furthermore, these compounds are often produced in extremely low amounts, so extraction and purification can be expensive. Alternative strategies like cell culture can be applied for higher yield at pilot scale. The following sections will highlight the diversity of these compounds, e.g., anthocyanins from different sources and their role and mechanism in cancer prevention.

2 Structural Features of Anthocyanins

The anthocyanins (Greek *anthos*, flower, and *kyaneos*, blue) are basically glycosylated polyhydroxy and polymethoxy derivatives of 2-phenylbenzopyrylium salts. These molecules commonly share the same generic structure, the flavan nucleus, consisting of two aromatic rings (A and B) linked by an oxygen-containing pyran ring (C) (Fig. 1). Anthocyanin belongs to the class of flavonols having flavylium ion that possess no ketone oxygen at the C-4 position. Till date, 23 anthocyanidins and a total of 700 different anthocyanins have been identified of which only 6 are the most common in vascular plants, cyanidin (Cy), pelargonidin (Pg), peonidin (Pn), malvidin (Mv), petunidin (Pt), and delphinidin (Dp), as shown in Table 1 (He and Giusti, 2010; Smeriglio et al. 2016). In nature, cyanidin is a reddish-purple (magenta) pigment. Delphinidin, common among all, appears as a blue-reddish or purple pigment in the plant. Brick red pelargonidin differs from most of the anthocyanidins (Gebhardt et al. 2020). Each anthocyanin has its own color shade, but a slight modification in the substitution pattern may allow it to change to another. For example, increasing hydroxylation (pelargonidin → cyanidin → delphinidin) and acylation of the B ring shifts anthocyanins to bluer shades (bathochromic effect), while the presence of many methoxyl groups (cyanidin → peonidin → malvidin) shifts the color toward red (hypsochromic) (Grotewold, 2006). The glycosylation of A ring provide deeper blue hue, while glycosylation at other sites increases color intensity. The number and type of sugar further add to more discolor diversity. The whole color chemistry of anthocyanin revolves around the positive charge on C ring and the double bond present therein. At an acidic pH (pH < 3), anthocyanins appear to be red and violet at pH 7–8; at a very high pH (pH > 11), it is blue in color.

The biosynthesis of anthocyanins takes place through the phenylpropanoid pathway in which rate-limiting enzymes are chalcone synthase and chalcone isomerase (Fig. 2). The branching of the pathways starts from naringenin, whose transformation under flavanone hydroxylase leads to formation of dihydroflavonols. The different dihydroflavonols under the action of dihydroflavonol reductase (DFR), anthocyanidin synthase (ANS), and UDP-glucose flavonoid transferase (UGFT) enzymes finally convert into anthocyanins. Although the major enzymes operating

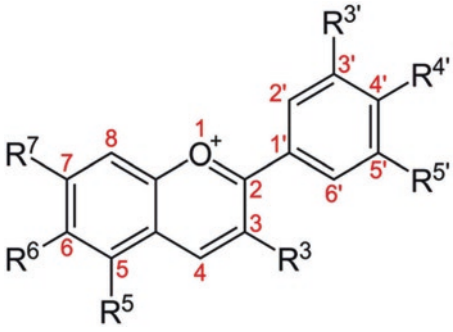

Fig. 1 Flavylium cation: basic skeleton of anthocyanidin

Table 1 Categorization of major anthocyanins based on color and substituents

	3’	5’	λmax	Spectrum
Pel	H	H	494	Orange
Cy	OH	H	506	Orange-Red
Dp	OH	OH	508	Blue-Red
Pn	OMe	H	506	Orange-Red
Pet	OMe	OH	508	Blue-Red
Mv	OMe	OMe	510	Blue-Red

Pel pelargonidin, *Cy* cyanidin, *Dp* delphinidin, *Pn* peonidin, *Pet* petunidin, *Mv* malvidin. The 3’ and 5’ positions on B ring (shown in Fig. 1) determine the color type and intensity

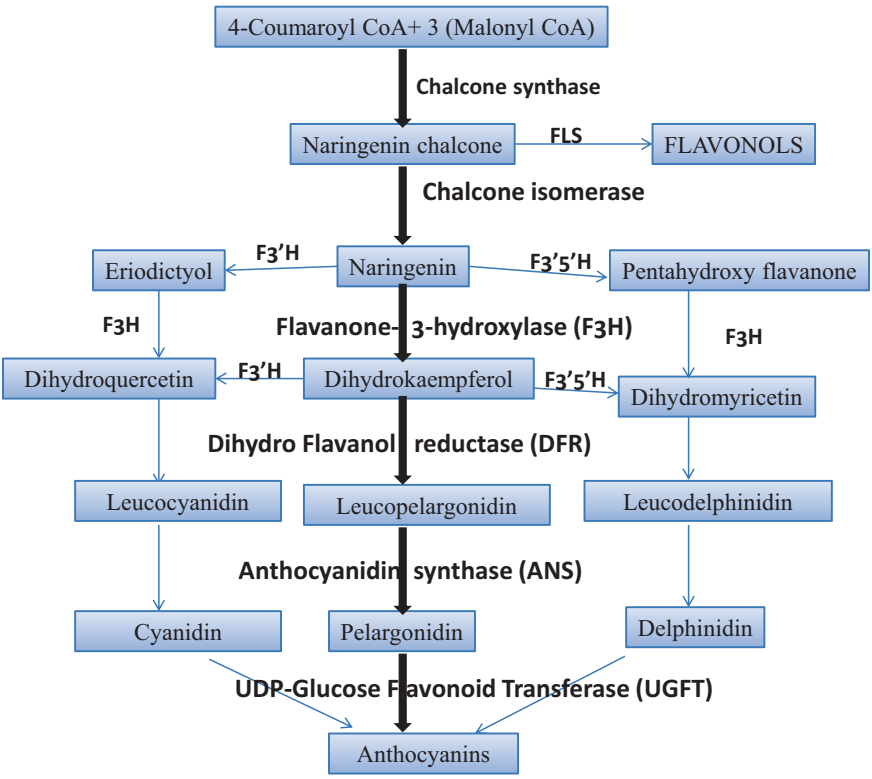

Fig. 2 Biosynthetic pathway of anthocyanins through phenylpropanoid pathway. *F3’H* Flavonoid 3’ hydroxylase, *F3’5’H* Flavonoid 3’5’ hydroxylase

in the flavonoid pathway are well-known and their encoding genes have been identified through model plant system like maize, many aspects underlying the synthesis of these pigments have yet to be fully elucidated (Andersen et al. 2008).

The anthocyanidins and their glucosides are further modified with in vivo reactions such as acylations. The acylation of anthocyanin glucoside, either aromatic or aliphatic generates further diversity. The common organic acids used in aliphatic

acylations are malonic and succinic acid, while aromatic acids are coumaric and caffeic acid. Glucosylation at C-3 as well as the acylation of carbohydrate residues with phenolic (p-coumaric, caffeic, ferulic) or aliphatic (acetic, malonic, succinic) acids increases the stability and solubility of anthocyanins, whereas the glycosylation at C5 favors degradation (de Pascual-Teresa et al. 2013).

3 Source Diversity in Type and Content of Anthocyanins

Anthocyanins are generally found in three colors, i.e., red, blue, and purple shade of flowers. Traditionally, these are being used in color preparation and folk medicines. In addition to flowers, they are present in berries (elderberry, raspberry, cranberry, etc.), leafy vegetables (cabbage), tubers (potato), root (carrot), cereals (maize, rice, etc.), and some tropical drupes (plum, peach). The content and type of anthocyanin present in these sources have been mentioned in Table 2.

The maize crop is well-known for its diverse and fascinating colors in different parts of its product. A huge pool of color maize germplasm is available in nature. An excellent example of rich germplasm prediction is transfer of blue color from maize to petunia flower in the late 1980s when DFR gene from maize was introduced into petunia. Cyanidin is the abundant one, and peonidin is the least abundant but is of specific interest because it gives the darkest shade of bluish red, as shown by its λmax (Ananga et al. 2013). However, along with pelargonidin, some amount of delphinidin also has been found in some red maize, whereas blue maize has cyanidin as major share. The magenta red maize has as minor share of peonidin and cyanidin (Salinas-Moreno et al. 2005; Abdel-Aal et al. 2008; Zhao et al. 2009; Zilic et al. 2012).

The content of acylated anthocyanin in blue maize is more than red or purple corn (Collison et al. 2015; Žilić et al. 2019). In flower, it occupies more than 40% share, whereas in kernels it has been found >90 % share of blue maize (Fossen et al. 2001; Harakotr et al. 2014). In maize single and dimalonylated cyanidin glucoside are uniquely and predominantly present (Salinas-Moreno et al. 2012). Other miscellaneous anthocyanins are mentioned in Table 2. A novel maize anthocyanin cyanidin 3-disuccinyl glucoside has been identified which is stable in the presence of enzymes and solubility in water (Nankar et al. 2016). Some color maize has been known to contain flavan-3-ol condensed anthocyanins with catechin and afzelechin (Cuevas Montilla et al. 2011; Dia et al. 2015; Chatham et al. 2018).

The pigmented rice has been admired for its history and heritage all over the world. Anthocyanins, which are responsible for purple to red pigmentation, represent the bulk of the flavonoids present in pigmented rice (Yang et al. 2009a). The compounds cyanidin-3-O-glucoside, peonidin-3-O-glucoside, cyanidin-3-O-rutinoside, and cyanidin-3-O-galactoside are the most prominent. Their aliphatic and aromatic versions are also present in minor forms (Limtrakul et al. 2019). The phenotypically red and brown were relatively supposed to be lacking anthocyanins (Gunaratne et al. 2013; Xiongsiyee et al. 2018), but a low level in both red and

Table 2 The diversity of anthocyanin in various plant sources

Source	Anthocyanin	Content (mg/100 g FW)	Reference
Corn (purple, blue, red)	Cy-3-glu, Pg-3-glu, Pn-3-glu, Cy-3-(6' mal)G, Pg-3-(6' mal)G, Pn-3-(6' mal)G; cyanidin 3-(6″-suc)G, cyanidin 3-(3', 6'-mal- suc)G, and peonidin 3-(6'-suc)G	300-850 (~4000 in dried cob)	Harakotr et al. (2014) and Galvez Ranilla et al. (2017)
Rice bran (black, red)	Cy-3-glu, Pn-3-glu, cyanidin-3-O-rut, cyanidin-3-O-gal	10-493 (~1250 in dried bran)	Pereira-Caro et al. 2013, Gunaratne et al. (2013) and Limtrakul et al. (2019);
Sorghum (black)	7-Methoxy-apigeninidin,7-methoxy-luteolinidin, and 5-methoxy-luteolinidin, apigeninidin 5-glucoside, luteolinidin 5-glucoside, 7-methoxy-apigeninidin 5-glucoside, 7-methoxy-luteolinidin 5-glucoside, and 5-methoxy-luteolinidin 7-glucoside	20-450 (180-612 in black)	Dykes et al. (2013) and Petti et al. (2014)
Wheat (blue, purple, red)	Cy-3-glu, Del-3-rut, Mal-3-glu, Pn-3-ara, ProPel-Dimer, ProDp/Cy-oligomer (ProCyB3)	0.5-16	Dinelli et al. 2011, Gamel et al. (2019) and Khlestkina et al. (2019)
Barley bran (black, blue, purple)	ProDel-Dimer, trimer, tetramer, pentamer	6-35	Verardo et al. (2015)
Soyabean (black)	Cy3G, Pel3g, Dp3G, Pet3G	150-350	Kim et al. (2012), Koh et al. (2014), Park et al. (2015), Hashimoto et al. (2015) and Jhan et al. (2016)
Black raspberry	Cy-3-rut, Cy-3-xyl-rut, Cy-3-glu, Cy-3-sam	76-428	Lee et al. (2016) and Jiang et al. (2020)
Blueberry	Mv-3-gal, Dp-3-gal, Dp-3-ara, Pt-3-gal, Pt-3-ara,Mv-3-ara	60-480	Lee et al. (2016) and Jiang et al. (2020)
Bilberry	Dp-3-gal, Dp-3-ara, Dp-3-glu, Pt-3-glu, Mv-3-glu	300-770	Müller et al. (2012) and Jiang et al. (2020)
Elderberry, chokeberry, cranberry	Cy-3-gal, Cy-3-ara	500-1000	Lee et al. (2016)
Grapes (red)	Cy-3-glu, Dp-3-glu, Pn-3-glu, Pt-3-glu, Mv-3-glu, Mv-3-glu-acetate	33-751	García-Beneytez et al. (2002) and Jiang et al. (2020)

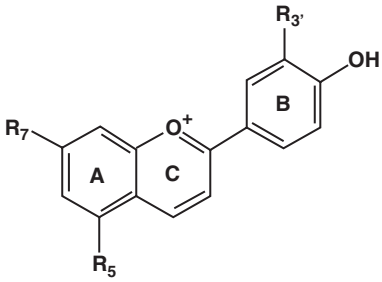

Fig. 3 Structure of 3-deoxycyanidin

brown rice accessions have been reported (Boue et al. 2016; Ghasemzadeh et al. 2018). Unstable anthocyanidins can be converted into the colorless flavan-3-ols catechin, epicatechin, and epigallocatechin through the action of anthocyanin reductase, and when glycosylated, a wide array of distinct molecules like proanthocyanidins are generated (Ko et al. 2006; Sasaki et al. 2014). Catechin was the most abundant in the red rice bran with a concentration of 98 mg/g.

The majority of natural anthocyanins in plants are C-3-hydroxylated anthocyanins; however, the anthocyanins found in sorghum are almost exclusively the C-3-deoxylated analogs, that is, 3-deoxyanthocyanidins (Fig. 3), which are a rare subclass of anthocyanins found in bran layer (Awika 2011; Kawahigashi et al. 2016; Xiong et al. 2019). The best part of these pigments is that they are stable to pH, heat, and bleaching agents. The black sorghum (red genotype) bran has the highest levels of 3-deoxyanthocyanidins (1790 to 6120 μg/g), followed by brown (red genotype) and red (both genotypically and phenotypically red) bran (Dykes et al. 2009; Awika 2011; Dykes et al. 2013). In addition, 3-deoxyanthocyanidins are also distributed in other plant tissues of sorghum such as the sheath and leaves, with a concentration of up to 90 mg/g under normal conditions or more under pathogen infection (Liu et al. 2010; Geera et al. 2012; Petti et al. 2014).

The content of anthocyanins is not only dependent on source but also on harvesting stage, soil nutrition, sunlight, and temperature. For example, some cereals in the milky stage generally have high anthocyanins, whereas some have maximum in the mature stage. The temperature and oxygen also have some effect on its content. For example, the aging of rice culminates in more proanthocyanidins because anthocyanins are degraded. The low temperature storage of purple potatoes converts starch into sugar which may upregulate the anthocyanin biosynthesis genes.

4 Role of Anthocyanin in Cancer

At present, surgery, radiotherapy, and chemotherapy management methods are limited for affluent persons because cytotoxic drugs are not easily affordable or available in retail shops. In addition, above treatment methods are associated with pain and adverse effects (Chidambaram et al. 2011). It is better to identify the benign

form of cancer, e.g., polyps, at an early stage and go for chemoprevention drugs or supplements which can stop the metastasis, progression into new sites, and conversion into malignant cancer. A number of reports are available in the literature where a population taking anthocyanins have substantially prevented the metastasis.

Healthy diet has been long proposed as an alternative strategy to reduce the risk of cancer; 10%–70% of all cancers were correlated with diet (Bishayee et al. 2015). The logic behind this is simple antioxidative mechanism of anthocyanins which helps in sequestration of free radicals (Zhu 2018). Different anthocyanidin molecules have different reducing power. Among several anthocyanidins, Dp followed by Cy and Pg has been found to be the most effective in scavenging superoxide anion, whereas Pg was the most powerful against the hydroxyl radical. Regardless of the sugar attached, potency of anthocyanins toward the superoxide radical and peroxynitrite radical scavenging was in the following order: Dp > Pt > Mv Cy > Pn > Pg (Liobikas et al. 2016).

Anthocyanins in purified form have been extensively studied for their anticancer properties (Lin et al. 2017; de Sousa Moraes et al. 2019). The types of substituents, sugars, and acylated acids and their position and degree of modifications are the main factors which influence the anticancer property. Earlier it was documented that acylated and non-acylated anthocyanins have same anti-proliferation capability (Zhao et al. 2004). But recently, it has been suggested that non-acylated monoglycosylated anthocyanins had greater anticancer property, while anthocyanidins have been demonstrated to be better inhibitors of cell proliferation than anthocyanins, with Dp having the best growth inhibition property owing to the presence of hydroxyl groups on the B ring of the anthocyanidin core. Nevertheless, the structure–activity relationship of diversified anthocyanins across the plant kingdom for multiple modes of cancer prevention remains to be further elucidated. The non-anthocyanin flavonoids are involved in co-pigmentation pattern and are diverse in nature. They also possess anticancer activities, but the detail and significance of each molecule is away from the scope of this chapter, so we have focused on anthocyanin and proanthocyanidin molecules here.

5 Mechanism of Action

Biologically active phytochemicals such as anthocyanins or anthocyanidins have exhibited many possible multiple and sometimes additive mechanisms to prevent a different type of cancer, as shown in Table 3. These mechanisms include induction of detoxification phase II enzymes, inhibition of oxidative damage, cell cycle arrest (G1/G0 and G2/M), apoptosis, and anti-angiogenesis and anti-proliferation (Shih et al. 2007; Singletary et al. 2007; Srivastava et al. 2007; Henderson et al. 2012; Forster et al. 2013; Chatthongpisut et al. 2015; Roleira et al. 2015).

At a first instance, the role of anthocyanin has been proposed as prevention of procarcinogen to carcinogen. This inhibition may be mediated through modulation of cytochrome p450 system and other detoxifying enzymes. The detoxification

Table 3 Biochemical mechanisms of action adopted by anthocyanidins and proanthocyanidins

Sr. no.	Strategy highlighted by the researchers	Reference
1.	Regulating the expression of anti-oncogenes and tumor suppression genes	Malik et al. (2003), Xie et al. (2019) and Upanan et al. (2019)
2.	Detoxification of free radicals, inactivation of procarcinogen, induced cytochrome P4501A1 expression	Zhang et al. (2013) and Zhang et al. (2014)
3.	Anti-invasive potential (MMP inhibition) and and anti-angiogenesis (VEGF blocked): anti-proliferation	Lala et al. (2006), Wang et al. (2009), Shin et al. (2009a), Faria et al. (2010) and Lim et al. (2013)
4.	Induction of apoptosis (COX-2 expression) : Anti-inflammatory	Tsoyi et al. (2008) and Hui et al. (2010)
5.	Insensitive signaling pathways (MAPK, ERK, JNK/p38, β-catenin Akt-mTOR) blocked: cell cycle arrest	Shin et al. (2009b), Kim et al. (2012), Choe et al. (2012), Chen et al. (2015), Bontempo et al. (2015), Park et al. (2015), Hashimoto et al. (2015) and Limtrakul et al. (2016)

enzymes facilitate their conjugation with glucuronic acid and their easy secretion from the body. The formation of new compounds (xenobiotic) may also boost up the immune system to tackle with inflammatory cytokines (Scalbert et al. 2005). Inhibition of cecal β-glucuronidase and activation of detoxification enzymes in the liver and colon is majorly responsible for anticancer activity of anthocyanidin in colon cells (Reynoso-Camacho et al. 2015).

Angiogenesis is another important step in the transition of tumors from a benign state to a malignant one. In angiogenesis, vascular endothelial growth factor (VEGF) and matrix metalloproteinase (MMP) are key factors which help in formation of new blood vessels for supply of nutrients and degradation of collagen, extracellular matrix. Anthocyanins are quite successful in attenuating the induction of VEGF and hypoxia-inducible factor (HIF-1a) under hyperglycemic and hypoxic conditions ex vivo (Wang and Stoner, 2008; Kang et al. 2013). In a mechanistic study, it was indicated that C3G could inhibit migration and invasion of A549 tumor cells (Ding et al. 2006). Similarly, it was demonstrated that C3G inhibited the migration and invasion of Lewis lung carcinoma cells both in vitro and in vivo in C57BL/6 male mice (Chen et al. 2005). Cyanidin is involved in inhibition of uncontrolled cell growth and division through cyclooxygenase (COX-2) and inducible nitric oxide synthase (iNOS) gene expression in colon cancer cells (Kim et al. 2010). It stops the synthesis of COX-2 enzyme in colon cancer and stops MMP-9 expression in bladder, stomach, and lung cancer (Singh et al. 2011; Filipiak et al. 2014; Luthra and Lal 2016).

In a cell predisposed to cancer, i.e., premalignant cell, there is activation of some transcription factors which stops the cross talk of immune cells and premalignant cells to generate inflammatory response. Nuclear factor kappa light chain-enhancer of activated B cells (NF-κB), activator protein-1 (AP-1), signal transducer and acti-

vator of transcription-1 (STAT-1), and octamer-binding protein (OCT-1) are some of the transcription factors that are activated in cancer cells as well as other cells of the body under distress and disease conditions. Evidence suggests that metabolites of anthocyanins can reduce the activation of these transcription factors. The inflammatory microenvironment represses the immune system and stimulates the production of inflammatory cytokines and some factors such as tumor necrosis factor (TNF), interferon (IFN-γ), and p65. This promotes the survival and proliferation of abnormal cells (Fan et al. 2013).

The insensitivity toward anti-growth signals also makes the cancer cells to grow irrespective of the growth conditions and local environment. The progression through various phases of cell cycle using cyclins and cyclin-dependent kinases (CDKs) without controls leads to cancer. On the other hand, anthocyanins have been shown to stimulate the expression of tumor suppressor genes via demethylation of promoters of CDK inhibitor 2A (CDKN2A) and Wnt inhibitor factor-1 (WIF1) (Wang et al. 2013). Inhibitors of CDK activity, specifically p21 and p27, also appear to be sensitive to anthocyanin and anthocyanidin molecules promoting cell cycle arrest at either G1/G0 or G2/M and apoptosis in carcinoma cells (Malik et al. 2003). Similarly, the anthocyanin-rich extract from potato also led to G1/G0 cell cycle arrest in prostate cancer LNCaP and PC-3 cells with the higher p27 protein levels.

Apoptosis is the usual way of programmed cell death under tumor surveillance mechanism, but the cancer cells escape this route and undergo uncontrolled cell divisions to increase their number. The imbalance of pro-apoptotic and anti-apoptotic proteins is another marker of malignant cells. The anthocyanin pigments are capable of inducing the expression of anti-apoptotic genes, e.g., Bcl-2 and p53 (Jeong et al. 2016; Xie et al. 2019). The use of chemotherapeutic agents induces apoptosis of cancerous cells. This fact led scientists to search novel drugs. The anthocyanin-rich extract caused caspase-dependent apoptosis in LNCaP cells with the induction of poly ADPribose polymerase (PARP) cleavage and the activation of caspase 3 (cleavage), as well as caspase-independent cell death in PC-3 cells with mitochondrial release and nuclear uptake of the pro-apoptotic endonuclease G (Endo G) and apoptosis-inducing factor (AIF) proteins (Bendokas et al. 2019). In addition, anthocyanin-induced autophagy pathway has also been reported to be a novel therapeutic target for cancer treatment. Longo et al. (2008) found that this autophagy was switched to apoptosis as characterized by the upregulation of Bax and caspase-3 and downregulation of mammalian target of rapamycin (mTOR) in human liver cancer cells. Inhibition of autophagy by either 3-methyladenine or autophagy-related gene 5 (Atg5) small interfering RNA enhanced anthocyanin-triggered apoptosis in liver cancer PLC/PRF/5 cells.

The disturbed signaling pathways which are linked with cell cycle, hormonal response, and transporters lead to cancer. The cascade of responses is presumed to be initiated by ligand–receptor interactions. Hence, these interactions, signaling pathways, and their factors can be considered as target for cancer prevention and chemotherapeutics. Majority of the investigations have indicated that anthocyanins can inhibit several signaling pathways involved in tumor growth. In such studies,

mitogen-activated protein kinase (MAPK) pathway, c-Jun N-terminal kinase (JNK), retrovirus-associated DNA sequences (RAS), and rapidly accelerated fibrosarcoma (RAF) were used for prevention of breast cancer cell proliferation through black rice extract (Xia et al. 2006; Chen et al. 2015). Thus, the specific sequences may be useful to treat patients at an advanced cancer stage.

The Wnt pathway is crucial for cell proliferation, differentiation, and survival. β-Catenin is a key component of the Wnt signaling pathway, and the overactivation of β-catenin in cytosol is related to cancer metastasis. The translocation of β-catenin to nucleus and transcription activation of c-myc and cyclin-D1 is key factor for metastasis (Pramanik et al. 2015). Under normal conditions, β-catenin is controlled by dephosphorylated glycogen synthase kinase 3β (GSK3b). When GSK3β is phosphorylated, GSK3β loses its activity and no longer controls β-catenin. An anthocyanin extract from Korea wild berry Meoru effectively inhibited liver cancer Hep3B cell migration and invasion by decreasing the expression of phospho-GSK3β and β-catenin. Moreover, AMP-activated protein kinase (AMPK) activation induced by anthocyanins might be an upstream regulator of the GSK3β/β-catenin pathway (Park et al. 2014).

Notably, apoptotic pathways depended on upstream activation of mitogen-activated protein kinase (MAPK) and c-Jun N-terminal kinase (JNK) pathways, indicating that MAPK signaling pathway might participate in the molecular mechanisms of anticancer activity (Reddivari et al. 2007; Chen et al. 2015). The properties of antioxidant and blocking activation of the MAPK pathway lie in the hydroxylation pattern of anthocyanin molecule. In addition, the activation of NF-*k*B pathways also might be shown to be involved in tumor growth and development (Ho et al. 2017). Afaq et al. (2005) reported that the animals pretreated with anthocyanin-rich extract from pomegranate fruit resulted in substantially reduced skin tumor incidence and lower tumor body burden in 7,12-dimethylbenz(α)anthracene or 12-O-tetradecanoylphorbol-13-acetate-initiated CD-1 mouse. This is mediated through the inhibition of phosphorylation of extracellular signal-regulated kinase (ERK)1/2, p38, and Jun N-terminal kinases (JNK1/2), as well as the activation of NF-kB and IkKα and phosphorylation and degradation of IkBα in stomach cancer (Sehitoglu et al. 2014; Luthra and Lal 2016). Besides inhibiting the phosphorylation and degradation of IκBα, delphinidin was able to suppress activation of I kappa B kinase-α (IKKα), important to trigger IκBα activation in a dose-dependent way. As a result, phosphorylation of NF-κB/p65 was also inhibited by delphinidin, hence reducing the nuclear translocation of NF-κB/p65. The role of pomegranate juice in preventing the reoccurrence and metastasis of prostate cancer after surgery has been observed (Folmer et al. 2014).

The devastating effects of ultraviolet radiations are due to its oxidative DNA damage by cyclobutane pyrimidine dimer generated reactive species (De Gruijl 2002). UV-B radiation causes inflammation to the skin surface which in turn mediates the phosphorylation of protein kinases (Sharma et al. 2007) and promotes carcinogenesis (Vaid et al. 2010). These radiations also activate COX-2 expression and prostaglandins (PGE2) secretion. External application of cyanidin-3-O-glucoside inhibits the UV-caused epidermal thickening and decreases the mRNA and protein

expression of proliferating cell nuclear antigen (PCNA) and cyclin by regulating UV-induced inflammation-mediated signaling pathways such as phosphorylation of ERK, MEK, MKK4, and nuclear factors like NF-κB and AP-1 (Kim et al. 2010; Pratheeshkumara et al. 2014). The action of delphinidin and cyaniding as topoisomerase inhibitor has been proposed as a tool in anticancer therapy (Esselen et al. 2011). The inhibition generates double-strand (ds-) and single-strand (ss-) breaks in the genome and activates the apoptotic pathway.

6 Maize Anthocyanins and Cancer

The anticancer effects of color corn achieved in vitro which might also accrue in vivo are uncertain, but almost every study of anthocyanin extract in rodents recommends that anthocyanins that reach the colon may protect cells from carcinogens, although the human epidemiological evidence for protection against cancer is mixed. Purple corn anthocyanin was shown to suppress the incidence, number, and activity of aberrant crypts, of colorectal adenomas and carcinomas induced in rats by various mutagens (Hagiwara et al. 2001; Lim et al. 2013; Reynoso-Camacho et al. 2015).

The oxidative and nitrosative stress for longer duration creates inflammation, and this is strictly tied to occurrence of cancer (Roleira et al. 2015). It was interesting to read the study in which corn diet reduced the mRNA expression of various cytokines (TNF-α, IL-6), reduced Ras protein levels, and inflammatory enzymes like inducible NOS while augmenting mRNA expression of free radical detoxification system. At the molecular level, purple corn activated caspase-3, a key protease associated with DNA fragmentation and apoptosis, and reduced Ras protein levels in tumor cells (Fukamachi et al. 2008; Kaspar et al. 2011; Singh et al. 2011; Nunes et al. 2013; Guillen-Sanchez et al. 2014; Guo et al. 2017; Wu et al. 2019).

Anthocyanin and their condensed forms may help in decrease VEGF expression and preventing the invasive potential of angiogenesis in tumorous cells (Wang and Stoner 2008; Finkel et al. 2013; Mazewski et al. 2017). The Mexican, Chinese, and Peruvian purple corn extracts which are rich in cyanidin-3-glucoside and its acylated forms are highly (77% as compare to control) effective in suppressing hepatic (HepG2) and colon (Caco2 and HT29) carcinogenesis (Jing et al. 2008; Zhao et al. 2009; Urias-Lugo et al. 2015). The comparison of purple corn with different berries has revealed its ultimate anticancer potency (Jing et al. 2008). Purple corn color demonstrated significant inhibition activity on 7,12-dimethylbenz[α]anthracene-induced mammary carcinogenesis development in human c-Ha-ras proto-oncogene transgenic (Hras128) rats in a dose-dependent manner, supported by the incidence of middle-sized (0.5 to 2.0 g) mammary tumors being significantly decreased in purple corn color-fed rats. In vitro, purple corn extract was found to inhibit cancer cell viability and induce apoptosis in mammary tumor cells. The number and incidence of small-sized (<0.5 g) mammary tumors was not suppressed by purple corn color, indicating it was not able to inhibit the emergence of mammary tumors in these rats (Fukamachi et al. 2008). In a study with male F344/DuCrj rats, purple corn color (5% dietary level) showed significant reduction of colorectal carcinogen-

esis induced by 1,2-dimethylhydrazine (DMH) and 2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine (PhIP) (Hagiwara et al. 2001). Long et al. (2013) showed the proliferation androgen-dependent prostate cancer in the cell line LNCaP was inhibited by purple corn color through limiting the expression of cyclin D1 and inhibiting the G1 stage of the cell cycle. In this research, male TRAP transgenic rats for adenocarcinoma of the prostate gland that consumed purple corn pigments showed lower percentages of adenocarcinoma and higher percentages of low-grade prostatic intraepithelial neoplasia.

Antimutagenic activity of processed corn against aminoanthracene (Mendoza-Diaz et al. 2012) and of blue corn against aflatoxin B1 has been established (Loarca-Pina et al. 2019). An interesting feature of anthocyanins is their capability to absorb UV radiation. Anthocyanin-rich products like lipsticks help in absorption of UV and minimizing melanin formation from tyrosine (Murapa et al. 2012; Westfall et al. 2019). The monomeric anthocyanins present in color corn tortilla have more selective anti-proliferative activity than unprocessed corn (Reynoso Camacho et al 2015; Herrera-Sotero et al. 2017, 2019). Purple corn tortilla was better than yellow and red tortillas in anti-carcinogenic effect. The corn peptides generated through endogenous proteases may also have pro-apoptotic role in conjunction with some anthocyanins (Li et al. 2013). The evaluation of extracted color from various parts of corn cob, i.e., silk, tassel, or cob itself, for the selective or broad-spectrum role in cancer prevention needs more epidemiological studies.

7 Rice Anthocyanins and Cancer

The anthocyanins of red, purple, and black rice bran have been shown to act as anti-angiogenesis, anti-mutagenesis, and anti-invasive and thus are potential suppressors of cancer (Xia et al. 2006; Verma and Srivastav, 2020). The targeted actions of rice bran extract like cell cycle arrest making cancer cell unviable, apoptosis, and its autophagy are dependent upon its inherent composition of anthocyanins, spectrum of phenolic compounds in rice seed, and target cell. All the three breast cancer cell lines MCF-7, MDA-MB-231, and MDA-MB-453 were inhibited for migration, invasion, and proliferation, and only MDA-MB-453 undergoes apoptosis (Hui et al. 2010; Chen et al. 2015).

The mechanistic way of anthocyanins in cancer prevention lies with cytochrome C release-triggered apoptosis (Hui et al. 2010), suppressed expression or antagonization of angiogenesis factor matrix metallopeptidase (MMP)-9, known zinc-dependent endopeptidase, VEGF (Chen et al. 2006a; Murapa et al. 2012; Tanaka et al. 2012; Filipiak et al. 2014), and blockage of carcinogenetic cytochromes P450 (CYP1A1 or CYP1B1) and/or by effectively scavenging free radicals (Insuan et al. 2017; Ghasemzadeh et al. 2018). The limitation of metastatic cell invasion (Chen et al. 2006a) and growth inhibitory effect on human hepatocellular carcinoma cells through blocking insensitive signaling (Banjerdpongchai et al. 2013; Chen et al. 2015) and inhibiting key metabolic activating enzymes at first stage of induced hepatocarcinogenesis by various colored rice varieties has been reported (Suwannakul et al. 2015).

The proanthocyanidin-rich red rice has profound anticancer effect on stomach and cervical cancer and leukemia as compared to purple ones (Chen et al. 2012; Forster et al. 2013). Proanthocyanidin-rich polar fraction (PRFR) from red rice's germ and bran at 20 and 40 μg/mL concentrations was found capable in reducing cell viability of HepG2 cells without posing any alterations in p53 (Upanan et al. 2019). The multifarious activities like its impact on DNA-binding activity of NF-*k*B and AP-1, degradation of the extracellular matrix-associated protein, and collagenase activity and inhibition of TNF-α, IL-6, and intracellular adhesion molecule (ICAM-1) have advocated the role of proanthocyanidins as chemopreventive drugs (Pintha et al. 2015; Limtrakul et al. 2016). The migration and invasion of MDA-MB-231 human breast and human fibrosarcoma HT1080 cell lines is interrupted by proanthocyanidins (Pintha et al. 2014, 2015). The proanthocyanidin-rich fraction could inhibit cell proliferation and induce cell apoptosis by increasing the apoptotic proteins, i.e., Bax, such as cleaved PARP-1 and cleaved caspases, and decreasing the anti-apoptotic protein *survivin* without p53 protein changes. The cleavage of PARP is essential for prevention of depletion of NAD and induced necrosis. The anthocyanin and its metabolite, e.g., protocatechuic acid activity, by regulating NF-κB translocation and MAPK activation shows anti-inflammatory activity both in vitro and in vivo (Min et al. 2010; Limtrakul et al. 2016).

8 Sorghum Anthocyanidins and Cancer

The characteristic feature of 3-deoxyanthocyanidins is their effective cytotoxicity as compared to anthocyanidins. For instance, apigeninidin and luteolinidin were reported to be more cytotoxic than their anthocyanin analog pelargonidin and cyanidin, respectively, to human intestinal epithelial (HEP-G2) and leukemia (HL-60) cancer cells (Shih et al. 2007). These compounds also act directly against cancer by inducing cell apoptosis and inhibiting the proliferation and metastasis of cancer cells (Shih et al. 2005; Shih et al. 2007; Yang et al. 2009b; Awika 2011; Devi et al. 2011; Woo et al. 2012; Suganyadevi et al. 2013; Massey et al. 2014). The proanthocyanidins of sorghum are highly polymerized and diverse. The inhibition of aromatase by the proanthocyanidin-rich fraction from sorghum seed extract has paved the way for its anti-invasive property against including colon, hepatoma, and esophageal, intestinal epithelial, leukemia, breast, and stomach cancer cells (Hargrove et al. 2011; Pintha et al. 2015).

9 Fruit Anthocyanins and Cancer

The anticancer activities of anthocyanins and proanthocyanidins from colored fruits like black currant and vegetables like purple potato have been demonstrated to inhibit the initiation, promotion, and progression of several cancers, such as breast cancer (Singletary et al. 2007; Hui et al. 2010; Devi et al. 2011; Kapinova et al.

2018), prostate cancer (Reddivari et al. 2007), liver cancer (Yokohira et al. 2008; Bishayee et al. 2010, 2011), colorectal and intestinal cancers (Hagiwara et al. 2001; Lala et al. 2006; Srivastava et al. 2007; Dinicola et al. 2012; Lim et al. 2013; Grimes et al. 2018), cervical cancer (Rugina et al. 2012; Pan et al. 2019), lung cancer (Ho et al. 2010; Singh et al. 2011; Aqil et al. 2012), pancreatic cancer (Guttenplan et al. 2017), fibrosarcoma (Filipiak et al. 2014), and metastatic melanoma (Bunea et al. 2013). Berries had preventive effects against various cancer through modulated multiple signaling pathways such as NF-κB, Wnt/β-catenin, PI3K/AKT/PKB/mTOR, and ERK/MAPK (Afrin et al. 2016). The action of different anthocyanidins and non-anthocyanidins is either synergistic or additive.

Most berries, e.g., black raspberries, red raspberries, strawberries, blueberries, and blackberry, are rich in bioactive substances (Szajdek and Borowska, 2008), the antioxidant activity of berry bioactive substances involved in scavenging or preventing further generation of ROS (Tulipani et al. 2014), regulating cell cycle and apoptosis, inhibiting carcinogen-induced DNA adduct formation, and enhancing DNA repair (Stoner et al. 2008; Dai et al. 2009; Cho et al. 2015; Riaz et al. 2016). For cancer prevention, berries' ability to decrease DNA damage (the first step of benign cancer initiation) is seen as a good way (Paredeslopez et al. 2010). These genes include XPA, DNL3, ERCC5, CYP450, CYP3A4, MAPK14, and MAPKK (Aiyer et al. 2008). In healthy female volunteers, supplementation with 1 L/d of a mixture of blueberry and apple juices for 4 weeks, the levels of H_2O_2-induced DNA damage was significantly reduced (Wilms et al. 2007).

Black raspberries (BRB) have the potential to prevent cancer through its anthocyanins, which have chemopreventive potential (Cerda et al. 2005; Wang et al. 2009). Dietary freeze-dried powder of black raspberries has prevented carcinogen-induced esophagus (Stoner et al. 2010; Teng et al. 2017), colon (Kresty et al. 2001) and estrogen-induced mammary gland (Aiyer et al. 2008; Aqil et al. 2016) tumors in the rat. The micro RNAs which are abnormally overexpressed or under-expressed are protected by the anthocyanin-rich diet (Aqil et al. 2016). The overexpression of COX-2 in benign polyp or adenocarcinoma is well-known. In preneoplastic tissues of NMBA-treated esophagus, the 5% black raspberry diet downregulated the mRNA and protein expression levels of COX-2 and c-Jun (a component of AP-1). The proliferation index was positively correlated with both of these components. The extracts of black raspberry and black currants induced the cytotoxic effects in colon cancer HT-29, HCT-116, and HepG2 cells and exerted important pro-apoptotic functions of the COX-2 expressing HT-29 cells. The ratio of pro-apoptotic and anti-apoptotic was high for hepatoprotection (Seeram et al. 2006; Bishayee et al. 2010, 2011). The expression of c-Jun and VEGF, a potent angiogenesis factor, was also inhibited by BRB in mutagenized animals (Chen et al. 2006b, 2016). The raspberry extract had repressed the invasion DNA damage and G1, cell preparation phase of colon cancer cells were significantly reduced HT-29 (Coates et al. 2007). Lyophilized black raspberries (LBRs) inhibited azoxymethane (AOM)-induced colon carcinogenesis in rats, when fed rats at 10% LBRs of the diet, the incidence of tumor did not reduced, but the total tumor (adenoma and adenocarcinoma) multiplicity was reduced by 71% relative to AOM controls, respectively (Harris et al. 2001).

Blueberry had a very significant inhibitory effect for breast cancer in the rat model (Aiyer et al. 2008; Ravoori et al. 2011). Induction of apoptosis in mouse melanoma B16-F10, human colon cancer HT-29, and human fibrosarcoma HT-1080 cells through anthocyanin extract has been testified (Srivastava et al. 2007; Bunea et al. 2013; Filipiak et al. 2014). Blueberry malvidin and anthocyanin-pyruvic acid adduct extracts (250 μg/ml) demonstrated anti-invasive potential in both of the breast cancer cell lines, MDA-MB-231, and MCF7 and mouse melanoma cells. The former cell line behaves independently of estrogen receptors (Faria et al. 2010; Bunea et al. 2013). The chemo-inhibiting extracts inhibited proliferation of cancer cell through apoptosis. The enzymes lactate dehydrogenase and quinone reductase were activated and inhibited, respectively (Srivastava et al. 2007; Bunea et al. 2013). The gallic acid and MMP interactions studied through NMR show inactivated enzyme core with gallic acid (Filipiak et al. 2014).

The growth inhibition of human HT-29 colon cancer cells with 50 μg/mL chokeberry anthocyanin/ml have revealed enhanced expression of tumor suppression genes ($p21^{WAF1}$ and $p27^{KIP1}$:cdk inhibitors) and a decreased expression of cyclooxygenase-2 gene (Rugina et al. 2012). After 48 h of treatment, the production of ROS has been increased. This resulted in cell cycle arrest at G1/G0 and G2/M stage and restrains the uncontrolled proliferation. As expected, the extracts have no obvious growth inhibition on normal colonic cell NCM460 (Malik et al. 2003; Hsu et al. 2012). The in vivo H1299 xenografts were interrupted for further growth through bilberry extract (Kausar et al. 2012; Ha et al. 2015). The levels of the apoptotic markers like Bcl-2 were also reduced in the study, which was the prediction of the inhibitory effect of chemopreventive agents on rat esophageal carcinogenesis (Kristo et al. 2016; Teng et al. 2017). Previously, bilberry extracts for 1 week were effective in decreasing Ki-67 and increasing cleaved caspase-3 expression, ultimately reduced proliferation index in colorectal cancer patients (Thomasset et al. 2009). Comparison of bilberry with blueberry extract has shown the more active malvidin than cyanidin in suppressing colon carcinoma cells (Zu et al. 2010).

The chokeberry extracts, containing high levels of monoglycosylated cyanidin derivatives, showed a stronger chemoprotective activity than grape and bilberry extracts (Zhao et al. 2004; Jing et al. 2008). Moreover, degrees of HT29 growth inhibitory activity were as follows: chokeberry and bilberry > purple carrot and grape > red radish and elderberry. In another study, extracts of bilberry, chokeberry, and grape (containing 3.85 g anthocyanins per kg diet) for 14 weeks significantly reduced azoxymethane-induced aberrant crypt foci by 26–29% in 3–4-week-old male-specific pathogen-free F344 rats (Lala et al. 2006). This reduction is associated with reduced cell proliferation and decreased expression of the COX-2 gene. Moreover, the less common anthocyanin source from vine was reported having anti-invasive property in human hepatoma Hep3B cells in a cancer study (Kamei et al. 1998; Shin et al. 2009a, b). The grape delphinidin have the capability to prevent carcinogen-DNA adduct formation and modulates its metabolism (Singletary et al. 2007). A mixture of diverse anthocyanidins in equimolar and suboptimal concentration had therapeutic effect for non-small-cell lung cancer and prevented its future recurrence and metastasis (Kausar et al. 2012). Similar study on oral, prostate, and

colon cancer cell line was conducted to evaluated combined effect of cranberry anthocyanin with its proanthocyanidins (Seeram et al. 2006).

The outstanding actions of the combinatorial therapy probably resulted from its effects on the oncogenic Notch and Wnt pathways and their downstream targets (β-catenin, c-myc, cyclin D1, cyclin B1, pERK, MMP-9, and VEGF proteins), increased cleavage of the apoptotic mediators Bcl-2 and PARP, and increased suppression of TNF-α-induced NF-kB activation (Hsieh et al. 2013). The combined effects of anthocyanins and other phenolics from a number of anthocyanin-rich fruits and vegetables are mainly additive rather than synergistic or antagonistic (Jing et al. 2008). However, there are limited studies available about synergistic or antagonistic effects of various anthocyanins on inhibiting the initiation, promotion, and progression of carcinogenesis, which should be highlighted in the future. Cyanidin-3-O-rutinoside, cyanidin-3-O-glucoside of fruit berry, selectively inhibits cell growth and induced apoptosis in a highly tumorigenic rat esophagus RE-149 DHD cell line (Kim et al. 2010). The intake of purple-fleshed sweet potato (clone P40) significantly suppresses formation of aberrant crypt foci in the colons of female CF-1 mice coincided with a greater expression of apoptotic caspase-3 in the colon mucosal epithelial cells (Lim et al. 2013). Another study also proves the role of purple potato anthocyanin extracts (2 and 5 mg/ml) in inducing maturation of acute myeloid leukemia cells via TNF-related apoptosis-inducing ligand (Bontempo et al. 2015).

10 Metabolic Engineering

For the large-scale production of these molecules, we can transfer the genes into microbes like *Escherichia coli* or yeast. But it requires introduction and expression of 11 transgenes for production of simplest anthocyanin molecule like pelargonidin from phenylalanine. As the preference for specific molecule is desired, more genes would be required to introgressed into one circular genome. Thus it would be a tedious process to engineer microbial cells with multiple genes and their control system altogether. Secondly, it requires sufficient cofactor pool, protein folding, and target trafficking machinery. Thirdly, production of these molecules needs different specialized cells which are not accomplished by unicellular microbes (Oksman-Caldentey and Inzé 2004).

The key hurdles are absence of F3'H and F3'5'H monoxygenases in *E. coli* which results in pelargonidin derivatives only. The production of cyanidin 3-glucoside (C3G) from (+)-catechin and naringenin with an *E. coli* strain bearing the leucoanthocyanidin dioxygenase (LdOx/ANS), dihydroflavonol reductase, and glycosyl transferase (3-GT) genes was reported (Yan et al. 2008). Progress has been made in engineering anthocyanin production in *E. coli* by feeding of eriodictyol or catechin and their conversion to CG (Chouhan et al. 2017). More recently, peonidin 3-glucoside (P3G) was recovered in *E. coli* cultivated in shake flasks with a titer of 56 mg/L (Cress et al. 2017). The construct included the following genes: ANS, anthocyaniDIN-O-methyl transferase (AOMT), and 3-GT. A CRISPRi system was

used as well, to silence the transcriptional repressor MetJ to improve the intracellular pool of S-adenosyl methionine (SAM), thus insuring the efficient conversion of C3G to P3G (Cress et al. 2017). High production titers obtained after improving substrate uptake, increasing the intracellular availability of UDP-glucose, inhibition of the UDP-glucose degradation pathway, and construction of artificial enzyme clusters to avoid fast degradation of anthocyanidin aglycones (Leonard et al. 2008; Yan et al. 2008).

In contrast to microbes, plant cells contain all the genetic information required to produce and store anthocyanins and are not limited to the synthesis of anthocyanin monoglucosides. There is no requirement of feeding precursors or metabolic engineering of genes to improve flux. The main strength of plant cell cultures is their ability to produce rare anthocyanins with highly decorated side chains that are currently unavailable, commercially. The plant cells grow more slowly than *E. coli* and, consequently, must have higher yields to match the production rates of microbial systems. Plant cell cultures are typically heterogeneous in their cell composition, as a result of selection during callus formation and de-differentiation, which can cause variation in growth rates and product yields (Ochoa-Villarreal et al. 2016). The major limitation in conventional cell cultures is that anthocyanins, similar to most natural compounds, accumulate in specialized differentiated cells, but fast and continuous growth of suspension cultures requires undifferentiated cells. The conflict between growth rate and anthocyanin production requires the maintenance of a differentiation state that is an effective balance between the two extremes.

The plant cell cultures can be used in the same manner as microbial cultures for the production of important phytochemicals at large scale (Appelhagen et al. 2018). The exploitation of plant cells for the high value compounds through adoption of good manufacturing practice was allowed by the food and drug regulatory authority (FDA). However, industrial application of plant cell cultures for secondary metabolite production, including anthocyanins, is still rare, mostly as a result of the limited stability of cultures long-term, variable product yields, and high costs. However, cell suspension cultures often do not produce the downstream products made by the parent plant system. However, advances in cell line selection strategies, efficient non-thermal extraction protocols, and scale-up using high-capacity bioreactors are driving forces which can increase the product yields.

In the cell suspension culture, the production of anthocyanin is dependent upon precursors, light, temperature, and sugar. A number of plant systems have been used such as *Vitis vinifera*, *Daucus carota*, strawberry, and *Perilla frutescens* for biosynthesis of anthocyanins. Several strategies have been used to increase anthocyanin yields in plants, mostly through abiotic elicitation of methyl jasmonic acid, biotic elicitation through *Aspergillus*, or hyperactivation of the biosynthetic pathway by ectopic expression of regulatory genes. A cell suspension developed from line *Panax sikkimensis* has been reported to produce 199 mg/L at shake-flask level (Cai et al. 2012). Recently, stable tobacco lines have been used for the production of decorated anthocyanins like cyanidin 3-O-(coumaroyl) rutinoside through cell suspension culture (Appelhagen et al. 2018).

Fortunately, advances in plant transformation have made it possible to carry out metabolic engineering in plants themselves. Metabolic engineering need complete dissection of the pathway and understanding the flux and transport of molecules from one cell to another and one organelle to another within the cell. The metabolite profiling offers unprecedented possibilities to explore the extraordinary complexity of the plant biochemical capacity. In plants, anthocyanin pathway is well-known, and as more plant genomes are sequenced, we are likely to learn much more about their pathways too.

11 Perspective

Anthocyanins have been receiving enormous recognitions in the field of cancer research. The chemotherapeutic properties of anthocyanins studied in vitro (cell line studies) and in vivo (animal model) have been discussed. We briefly introduced the structure and associated activity, sources, and bioactivity. Though chemopreventive role of these color molecules has always been advocated in view of its antioxidative nature, some of the unresolved issues like structure–function relationship, role of co-pigments in cancer prevention, and drug–anthocyanin interactions are being studied worldwide for better understanding. A thousand numbers of anthocyanin and proanthocyanidins are known till date, but there are a huge number of unexplored anthocyanins. These unexplored molecules may provide additional benefits under nutritional enrichment. The substitutions and modifications of known basic molecules cannot be the sole determinant of the color type, pigment stability, and function. Hence, we have to find some more clues to develop structure–function relationship. There are a number of target organs whose dysfunctionality activates different genes and proteins on which anthocyanins may have direct or indirect effects. These indirect effects can have more physiological relevance on complete elucidation of signal transduction pathways. The dose of anthocyanins under cancer treatment is another field of study in which interaction of anthocyanins with anticancer drugs shall be studied in an exhaustive manner. The utilization of anthocyanin in functional food preparation is dependent upon the source availability and the yield. The production can be enhanced through metabolic engineering of source cells. All in all, anthocyanins are the defense molecules for our body, and in our stressful life, it requires their application in fancy food preparation and their consumption for disease prevention.

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Duckweeds for the Production of Therapeutic Proteins

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Abstract The production of various biopharmaceuticals in transgenic plants (biofarming) is becoming an increasingly popular trend in modern biotechnology. To date, the expression of recombinant proteins in transgenic plants is becoming a powerful alternative to classical expression methods. This is due to the fact that the use of plant expression systems can provide a significant reduction in the cost of recombinant protein production. The main efforts in the field of genetic engineering of *Lemnaceae* were aimed at obtaining plants-producers for the production of recombinant proteins for various functional purposes, primarily medical. Plants expressing monoclonal antibodies, viral and bacterial antigens, and various therapeutic and industrial proteins have already been obtained. Their biological activity has been confirmed, and researches in the field of the extraction and purification of target proteins have begun. Moreover, some efforts have already been

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directed at the development of the methods for humanizing the glycosylation of recombinant antibodies in transgenic plants-producers. Given the current progress and pace of development of research in various areas of *Lemnaceae* biology, we have no doubt that in the near future, we can expect the emergence of efficient and competitive duckweed-based expression systems for the production of recombinant proteins for various purposes.

Keywords Biopharming · Recombinant proteins · Transgenic duckweed · Therapeutic and veterinary substance · Transgene expression system

1 Introduction

Currently, the main efforts in the field of pharmaceutical research are focused on the development of drugs based on recombinant proteins – antibodies, cytokines, enzymes, etc. Of the 71 new biopharmaceutical substances permitted for use in 2014–2018, 62 are recombinant proteins, primarily antibodies and their derivatives (Walsh 2018).

For the production of recombinant proteins, expression systems based on mammalian cell culture and various microbiological (bacterial, yeast) systems are currently mainly used.

Plant expression systems are very promising, but they have limited application (less than 1% of the total number of registered drugs in 2017 https://www.americanpharmaceuticalreview.com/). However, the production of various biopharmaceuticals in transgenic plants (biofarming) is becoming an increasingly popular trend in modern biotechnology. This is due to the fact that the use of plant expression systems can provide a significant reduction in the cost of recombinant protein production. The degree of this reduction is estimated differently, depending on the type of target protein, and averages around 30% (Kaufman and Kalaitzandonakes 2011; Tusé et al. 2014). However, despite the obvious progress in this area, relatively few examples of successful commercialization of recombinant proteins obtained in plant expression systems are known (Park and Wi 2016; Schillberg et al. 2019).

One of the main drawbacks of plant expression systems is the possibility of uncontrolled release of recombinant DNA into the environment in the composition of pollen, seed, and various plant residues (Franconi et al. 2010; Kirk et al. 2005). Such fears are especially strong when various human proteins for medical purposes are expressed in plants. Therefore, in many countries, the cultivation of genetically modified plants for commercial purposes is prohibited by law.

The problem of environmental risks can be successfully solved by developing expression platforms that use closed cultivation systems (greenhouses, bioreactors), thus reliably preventing the leakage of recombinant DNA into the environment (Franconi et al. 2010).

Very significant progress has been made with the use of such platforms as plant cells suspension culture (Santos et al. 2016).

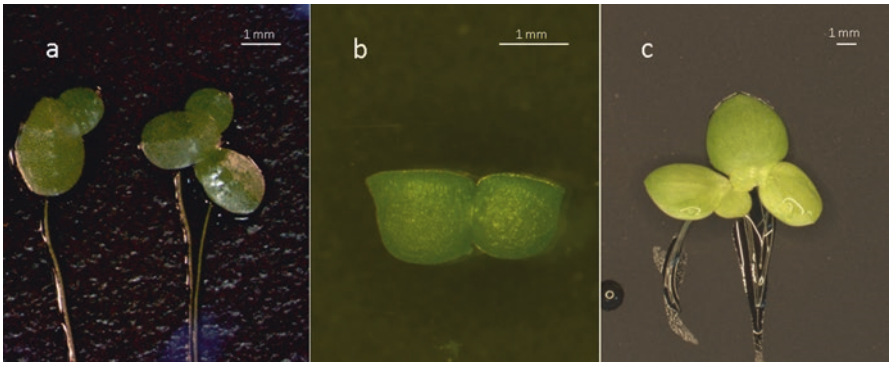

Fig. 1 Showed 3 of 5 *Lemnaceae* genus collected in Russia. *Lemna minor* (**a**), *Wolffia arrhiza* (**b**) and *Spirodela polyrhiza* (**c**) are members of family *Lemnaceae* and commonly known as duckweeds

The first recombinant protein of medical purpose (Elelyso/taliglucerase alfa) was obtained in carrots cell suspension culture (Walsh 2014).

Along with this, the possibility of using expression systems based on microalgae and bearded root culture has been studied; encouraging results have been obtained in this direction (Georgiev et al. 2012; Gurusamy et al. 2017). However, the application of cell culture technology requires the use of complex bioreactors (Murthy et al. 2014; Xu et al. 2016). On the other hand, rhizosecretion systems do not provide a sufficiently high yield of the target product.

The plants belonging to the family of *Lemnaceae* are a promising platform for the production of recombinant proteins in closed expression systems. They are small aquatic monocotyledonous plants and are members of family *Lemnaceae* and commonly known as duckweeds (Fig. 1), characterized by a high growth rate and vegetative reproduction under artificial cultivation conditions. The plants of *Lemnaceae* family can be grown in cultivators of various designs, particularly in completely isolated conditions, to exclude the penetration of recombinant DNA into the environment. They are photo-autotrophic organisms; their cultivation does not require expensive components. An important feature of the duckweeds is their ability to secrete recombinant proteins into the culture medium, which allows the significant simplification of their subsequent isolation and purification. The set of these features makes the plants of Lemnaceae family to be considered as unique objects for biofarming.

The successes and challenges of using the plants of *Lemnaceae* family for the development of plant-based expression systems are discussed below.

2 Botanical Description of Duckweeds (Plants of *Lemnaceae* Family)

Lemnaceae includes 5 genera [*Spirodela* Schleiden (Fig. 1c), *Landoltia* Les and Crawford, *Lemna* L. (Fig. 1a), *Wolffiella* Hegelmaier, *Wolffia* Horkel and Schleiden (Fig. 1b)] and about 37 species which are found on all continents of our planet

(Appenroth et al. 2013; Les and Crawford. 1999; Pena et al. 2017; Sree et al. 2016; Stockey et al. 1997). The taxonomic status of *Lemnaceae* has not been fully defined yet. For example, many taxonomists consider Lemnaceae as a separate taxon within the kindred family of Araceae (Davis 1995; French et al. 1995; Stockey et al. 1997; Rothwell et al. 2004). According to other classifications, *Lemnaceae* is assigned the status of an independent family (Appenroth et al. 2013; Cronquist 1988; Hutchinson 1973; Les et al. 2002; Sree et al. 2016; Thorne 1992).

Duckweeds are most common in South Asia, North and South America, Europe, South and Central Africa, and southern Australia, mainly in cultivated areas (Leng 1999). About half of the species inhabit the tropics and subtropics; the rest live in the temperate zone.

Lemnaceae unites aquatic, free-floating, mostly perennial herbaceous plants. As a result of hydrophilic evolution, they have reached the extreme degree reduction of all their organs; therefore they rank first among flowering plants by the simplicity of their structure (Kuehdorf and Appenroth 2012; Landolt 1986). Duckweeds are the smallest flowering plants in the world (Landolt 1986; Wolff 1992). Plants of this family form one leaf-like organ (from 0.3 mm to 12 mm), called as fronds (Li et al. 2004; Wayne and Thorne 1984). It is a symmetrical or asymmetrical plate, mostly green and flattened from the adaxial side (Landolt 1986; Leng 1999; Leng et al. 1995; Les et al. 2002; Wolff 1992). Plant roots are either absent (*Wolffiella* and *Wolffia*) or poorly developed and do not reach the ground (*Spirodela*, *Landoltia*, and *Lemna*). Duckweeds have mainly vegetative reproduction. They are able to double the mass of the population in 1−6 days and the doubling of the fronds number occurs in 2–3 days (Armstrong and Thorne 1984; Li et al. 2004; Khvatkov et al. 2019; Ziegler et al. 2015).

3 The Practical Application of *Lemnaceae* (Duckweed Plants)

Plants of the duckweed family have high potential for practical application (Appenroth et al. 2015; Lam et al. 2014; Zhao et al. 2012). In some Asian countries, duckweeds are used for human nutrition as a high-protein diet (Appenroth et al. 1982; Cheng and Stomp 2009; Van der Spiegel et al. 2013). As a source of protein, duckweeds can substitute soy bean products, at least in part (Sree et al. 2016). Duckweeds are effective for feeding domestic animals (Cheng and Stomp 2009; Landolt and Kandeler 1987; Van et al. 1997), poultry (Akter et al. 2011; Anderson et al. 2011; Men et al. 1995; Muztar et al. 1979), and pond fish (Hassan and Edwards 1992; Pipalova 2003). The integration of duckweeds into agricultural production gives the chance to receive cheap forage (Sree et al. 2016).

Under appropriate conditions of cultivation (Sree and Appenroth, 2014; Sree et al. 2015a, b), duckweed biomass with high starch content can be used as a raw material for biogas plants and biofuel production (Cui and Cheng 2015; Jain et al. 1992; Su et al. 2014). Thus, the cultivation of duckweed can significantly reduce the use of arable land in the production of biofuels.

A wide range of researchers showed the possibility of using duckweed for water decontamination from various kinds of pollution (Appenroth and Lam 2012; Bergmann et al. 2000; Chaudhary and Sharma 2014a, b; Xu et al. 2012; Zhao et al. 2015). In particular, duckweeds were successfully applied in purification of waters from metal salts (Chaudhary and Sharma 2014a, b; Sree et al. 2015b), organic compounds of nitrogen and phosphorus (Ansal et al. 2010; Körner and Vermaat 1998; Mohedano et al. 2012), various waste products of animals (Karpiscak et al. 1996; Van et al. 1997), and even phenolic compounds (Radulovic et al. 2019).

4 *In Vitro* Culture of *Lemnaceae* (Duckweed Plants)

First protocol of agrobacterial transformation of duckweed was developed in 2001, and after a while it was successfully adapted to the physiological characteristics of different species and geographical isolates of this plant family. Most studies on agrobacterial transformation of duckweeds used a scheme, including induction and cultivation of organogenic callus, its transformation by agrobacteria and subsequent regeneration of transformants in the presence of selective antibiotics.

2,4-D in concentrations from 1.0 mg l^{-1} (Li et al. 2004) to 20.0 mg l^{-1} is most often used for callus induction in duckweeds, less commonly picloram (4-amino-3,5,6-trichloropyridine-2-carboxylic acid) (Edelman et al. 1998; Firsov et al. 2015; Friedrich 2005; Khvatkov et al. 2015b; Li et al. 2004; Thu et al. 2010; Stomp and Rajbhandari 2000) or dicamba (3,6-dichloro-2-methoxybenzoic acid) (Li et al. 2004) in concentrations from 15.0 mg l^{-1} to 50.0 mg l^{-1}. Normally 2,4-D and dicamba are used together with BA in concentrations from 0.5 mg l^{-1} (Heenatigala et al. 2018; Khvatkov et al. 2015b; Thu et al. 2010; Stomp and Rajbhandari 2000) to 2.0 mg l^{-1} (Edelman et al. 1998; Heenatigala et al. 2018; Khvatkov et al. 2015b; Thu et al. 2010; Stomp and Rajbhandari 2000); in some cases it is possible to use TDZ (thidiazuron or 1-phenyl-3-(1,2,3-thiadiazol-5-yl) urea), 2iP (2-isopentenyladenine) (Li et al. 2004), or kinetin (Friedrich 2005).

The carbohydrate nutrition of explants significantly affects the process of callus genesis. Sucrose (Edelman et al. 1998; Firsov et al. 2015; Li et al. 2004; Stomp and Rajbhandari 2000; Vunsh et al. 2007) and glucose (Heenatigala et al. 2018; Khvatkov et al. 2015b) are most commonly used in concentrations from 1% to 3% (v/w) for the induction of callusing in duckweeds.

The addition of polyols (mannitol and sorbitol) at the concentration of 2% (v/w) to the medium had a positive effect on the induction of callusogenesis in plants of the *Wolffia* genus, *S. oligorhiza*, and *Landoltia punctata* (Li et al. 2004; Vunsh et al. 2007). The possibility of duckweeds callus structures subculturing on the medium with the reduced auxin content for the induction of callusogenesis is also shown (Khvatkov et al. 2015b; Stomp and Rajbhandari 2000).

Mainly plant regeneration arises on media lack of growth regulators and with a sucrose content of 1–2% (v/w) (Friedrich 2005; Heenatigala et al. 2018; Khvatkov et al. 2015b; Li et al. 2004; Thu et al. 2010; Stomp and Rajbhandari 2000). However,

a number of researchers have noted the positive effect of TDZ (for *Spirodella oligorhiza*, *Lemna gibba*, and *Lemna minor*), 2iP (for *Landoltia punctata*) in concentration of 1.0 mg l^{-1} (Li et al. 2004), and BA (for *Lemna gibba* G3) in concentration of 0.5 mg l^{-1} (Stomp and Rajbhandari 2000).

Various media can be used for in vitro culture and multiplication of duckweed family plants, for instance, Hoagland and Arnon medium (Chhabra et al. 2011, Kittiwongwattana and Vuttipongchaikij 2013, Radulovic et al. 2019), Steinberg medium (Khvatkov et al. 2013, 2019), Hutner's medium (Levard et al. 2013; Li et al. 2004), Schenk and Hildebrandt medium (Heenatigala et al. 2018; Khvatkov et al. 2015a, b; Wang 2016; Yamamoto et al. 2001; Zhang et al. 2010), Kuhl's modified medium (Appenroth et al. 1996; Ziegler et al. 2015), or modeled cultivation medium (for *W. arrhiza* - W3M and for *L. minor* – L4M; Khvatkov et al. 2019).

5 Genetic Transformation of *Lemnaceae* (Duckweed Plants)

Until 2000, researches on the *Lemnaceae* biotechnology mainly were carried out with such a species as *Lemna gibba* (Chang and Chiu 1976, 1978; Moon and Stomp 1997). In 1988, it was first reported that *Spirodela punctata* transgenic plants had been received (Edelman et al. 1998). Another member of the *Spirodela* genus, *S. oligorrhiza*, was transformed in 2007 (Vunsh et al. 2007), and a year later, an expression system based on this species was developed to produce recombinant aprotinin (Rival et al. 2008). The protocol for *Agrobacterium*-mediated transformation of *Spirodela polyrhiza* was developed by Wang (2016) and further improved by Yang et al. (2018).

The first report on the development of biotechnology methods for *Lemna minor* L. refers to 1991 (Frick 1991). An effective and reproducible method of genetic transformation of this species was developed in 2001 (Stomp and Rajbhandari 2000; Yamamoto et al. 2001). As a result of these researches, transgenic lines transformed by the *gus* gene and various selective markers were obtained. Further the *Lemna minor* agrobacterial transformation protocol was adapted to the genetic and physiological features of its various isolates (Cantó-Pastor et al. 2015; Chhabra et al. 2011; Li et al. 2004).

In the last 5–6 years, the special attention of researchers is drawn by representatives of another genus of a duckweed family, namely, *Wolffia*. Efficient and reproducible protocols for *Agrobacterium*-mediated transformation of *Wolffia* genus have been developed for *Wolffia arrhiza* (Khvatkov et al. 2015a) and *Wolffia globosa* (Heenatigala et al. 2018). Heenatigala et al. (2018) and Boehm et al. (2001) studied the features of transient expression in *Wolffia columbiana*; however this direction of researches didn't gain further development. The results of research on the genetic transformation of *Lemnaceae* are presented in Table 1.

In most studies on the genetic transformation of *Lemnaceae* expression, the vectors included strong constitutive promoters: 35S cauliflower mosaic virus promoter (Edelman et al. 1998; Firsov et al. 2015; Thu et al. 2010; Rival et al. 2008; Vunsh et al. 2007), nopaline synthase promoter (Firsov et al. 2015; Thu et al. 2010; Rival

et al. 2008; Stomp and Rajbhandari 2000; Vunsh et al. 2007), and octopine synthase promoter (Vunsh et al. 2007). These promoters have demonstrated the ability to provide a high level of heterologous genes expression in transgenic plants. The terminator sequences of octopine synthase genes (Vunsh et al. 2007), nopalin synthase genes (Firsov et al. 2015; Thu et al. 2010; Rival et al. 2008; Stomp and Rajbhandari 2000; Vunsh et al. 2007;), and 35S cauliflower mosaic virus terminator (Thu et al. 2010; Vunsh et al. 2007) have been successfully used as terminators in the vectors for transformation of duckweeds.

Table 1 Basic research on genetic transformation of *Lemnaceae*

Species of duckweed	Callusogenesis/ regeneration	Transformation	Reference
Spirodella oligorrhiza	+/+	–	Li et al. (2004)
S. oligorrhiza	+/+	Stable lines with *gfp* gene	Vunsh et al. (2007)
S. oligorrhiza	+/+	Stable lines with synthetic aprotinin gene	Rival et al. (2008)
Landoltia punctata (or *S. punctate)*	+/+	Stable lines with *gus* gene	Edelman et al. (1998)
Lemna gibba	+/-	–	Chang and Chiu (1976)
L. gibba	+/+	–	Chang and Chiu (1978)
L. minor	+/-	–	Frick (1991)
L. gibba	+/+	–	Moon and Stomp (1997)
L. gibba	+/+	Stable lines with *gus*, P-hemoglobin and P450 oxidase genes	Stomp and Rajbhandari (2000)
L. minor	+/+		
L. gibba	+/+	Stable lines with *gus* gene	Yamamoto et al. (2001)
L. minor	+/+		
L. minor	+/+	Stable lines with *gus* gene	Mourenets and Dolgov (2002)
L. minor	+/+	Stable lines with interferon, human growth hormone, Fab fragments, and monoclonal antibodies genes	Gasdaska et al. (2003)
Wolffia columbiana	-/-	Transient expression of *gus* gene	Kruse et al. (2001)
W. columbiana	-/-	Transient expression of *gus* gene	Boehm et al. (2001)
W. australiana	+/+	Transient expression of *gfp* gene	Friedrich (2005)
W. australiana	+/+	Transient expression of *gus* gene	Pham et al. (2010)
W. globosa	+/+		
W. arrhiza	+/+	Stable lines with *gus* gene	Khvatkov et al. (2015b)
W. globosa	+/+	Stable lines with *gus* gene	Heenatigala et al. (2018)

Selective *hpt* and *npt* II genes have been successfully applied to selection of transgenic plant tissues (Edelman et al. 1998; Firsov et al. 2015; Friedrich 2005; Heenatigala et al. 2018; Khvatkov et al. 2018; Thu et al. 2010; Rival et al. 2008; Stomp and Rajbhandari 2000; Vunsh et al. 2007). As a reporter gene, *gus* (*uidA*) was most often used (Boehm et al. 2001; Edelman et al. 1998; Firsov et al. 2015; Kruse et al. 2001; Thu et al. 2010; Stomp and Rajbhandari 2000; Yamamoto et al. 2001), less often *gfp* because of the autofluorescence of the explant tissues (Friedrich 2005; Vunsh et al. 2007).

6 Plants of Duckweed Family as Platforms for Recombinant Protein Expression

6.1 Features of Duckweeds as Objects of Biotechnology

Plants of the duckweed family have a number of valuable properties in terms of biotechnology. Duckweeds are high in protein – up to 40% of the dry weight of the plant. They can be cultivated at inexpensive mineral media of simple composition, carrying out photosynthesis throughout the plant, including in the roots. Meanwhile, the plants are able to secrete certain substances, including peptides, into the culture medium. This property is their protective mechanism to suppress the growth of algae and bacteria on the surface of fronds. Duckweed plants grow very quickly; the doubling time of their biomass is about 36 h under optimal conditions. Such growth rate combined with high protein content makes the duckweed culture comparable to yeast expression systems. The vegetative way of reproduction is also of a great advantage, since in conditions of artificial cultivation, duckweed doesn't produce pollen that allows to prevent environmental contamination reliably (Chang et al. 1977; Landolt 1957). Thus, plants belonging to the *Lemnaceae* family have a number of biotechnological indicators that bring them close to fungal systems. At the same time, they are the higher plants capable of carrying out a full range of proteins posttranslational modifications. Such a combination of properties makes the duckweeds promising objects for use in various fields of biotechnology, including biopharming (Everett et al. 2012; Gasdaska et al. 2003).

6.2 Expression of Recombinant Proteins in Duckweeds

Despite the obvious interest in duckweeds as expression platforms for the production of recombinant proteins, relatively few studies are still known in this area. Based on advances in genetic engineering of *Lemna minor* L. and *Lemna gibba* L., Biolex Therapeutics (USA) has developed the Lex SystemSM expression platform, where the plants of *Lemna minor* were used to produce recombinant proteins. A

group of proteins, including α-2b-interferon, insulin, human growth hormone, β-glucocerebrosidase, retinoblastoma protein, p53, angiostatin, leptin, serum albumin, hemoglobin, collagen, p450 oxidase, and monoclonal antibodies, was selected for the study. Growth hormone expressed in duckweed at the level of 800 mg kg^{-1} wet weight, monoclonal antibodies accumulated in the plant in an amount of up to 4.0% of the total soluble protein (8.62 g kg^{-1} dry weight), and the accumulation of Fab fragments was 2.6% of the total soluble protein (5.6 g kg^{-1} dry weight) (Gasdaska et al. 2003). In the same studies, secretion of recombinant proteins into the culture medium was shown. Thus, the growth hormone accumulated in the medium at the level of 609 mg l^{-1} and α-2b human interferon in the amount of 15 mg l^{-1}. It should be noted that these data are derived from information materials published on the Biolex Therapeutics website and from its patents (Dickey et al. 2009, 2011; Stomp 2005). Descriptions of these researches and data on the expression features of the studied proteins are not presented in the scientific literature. Currently, the rights for the LEX SystemSM expression platform are owned by Synthon company (Netherlands), which continues to improve it. In particular, the company was developing a technology to produce recombinant antibodies for the treatment of non-Hodgkin lymphoma (Yao et al. 2015).

In addition to commercial companies, works in the field of recombinant proteins expression in duckweeds were carried out in a number of scientific organizations. The main efforts were aimed at the expression of various antigens and industrial enzymes in duckweed. The results of these studies are presented in Table 2. Thus, the protective antigen of porcine epidemic diarrhea virus (Ko et al. 2011), protein Ag85B-ESAT6 of *Mycobacterium tuberculosis* (Peterson et al. 2016), and avian influenza virus H5N1 hemagglutinin H5N1 (Bertran et al. 2015) were expressed in *Lemna minor*. The ability of a recombinant hemagglutinin to induce an immune answer in hens, sufficient for their complete protection against lethal doses of a virus of flu, was confirmed in experiments of Bertran et al. (2015). The M2e peptide of the avian influenza virus H5N1 in fusion with the ricin subunit B (RTB) has been successfully expressed in transgenic *Lemna minor*; oral immunization of mice showed induction of specific antibodies against M2e (Firsov et al. 2018). Such industrial enzymes as endoglucanase E1 from *Acidothermus cellulolyticus* and phytase from *Aspergillus nidulans* have been successfully expressed in *Lemna minor* besides various antigens (Ghosh et al. 2018; Sun et al. 2007).

Experiments on the expression of heterologous proteins were also carried out with other species of duckweed – *Spirodela polyrhiza* L. and *Wolffia arrhiza* L. Recombinant aprotinin and H5N1 avian influenza virus hemagglutinin have been successfully obtained in transgenic *Spirodela* plants (Rival et al. 2008; Thu et al. 2015). Recombinant granulocyte colony-stimulating factor (G-CSF) was successfully expressed in transgenic plants of *Wolffia*; target protein accumulation reached 35.5 mg kg^{-1} wet weight of plants (Khvatkov et al. 2018).

As a rule in studies on the expression of recombinant proteins, the gene of the target protein was under the control of 35S cauliflower mosaic virus promoter; rarely ubiquitin promoters were used (Cox et al. 2006; Thu et al. 2015). In most

Table 2 Expression of recombinant proteins for various purposes in duckweed (*Lemnaceae* family)

Species	Target protein/vector features	Expression level	Remark	Reference
Lemna minor	Humanized monoclonal antibodies (mAb) against CD30 (H-chain is under the control of an octopine-mannopene synthase chimeric promoter; L-chain is under *L. minor* polyubiquitine promoter LmUbq; RNAi cassette is harboring *S. polyrhiza* polyubiquitine promoter SpUbq. H chain is fused with the 5'-leader of ribulose bisphosphate carboxylase small subunit	Up to 2.1% of TSP	Optimization of glycans composition	Cox et al. (2006)
Lemna minor 8627	Endoglucanase E1 from *Acidothermus cellulolyticus* (35S CaMV promoter)	Up to 0,24% of TSP	The activity of recombinant endoglucanase is shown	Sun et al. (2007)
Lemna minor	Protective antigen of swine epidemic diarrhea virus (35S CaMV promoter)	ND	–	Ko et al. (2011)
Lemna minor	Ag85B and ESAT6 *Mycobacterium tuberculosis* proteins (35S CaMV promoter)	0.4–0.5 μg g^{-1} of plants wet weight	–	Peterson et al. (2016)
Lemna minor	Hemagglutinin of the avian influenza virus H5N1 (expression system LEX System™, Biolex Therapeutics, Pittsboro, NC)	~0.280 mg g^{-1} of plants wet weight (12% TSP)	Full protection of chickens from lethal doses of influenza virus	Bertran et al. (2015)
Lemna minor	M2e peptide of avian influenza virus H5N1 in fusion with β-glucuronidase (35S CaMV promoter)	Up to 0.97 mg g^{-1} of plants wet weight (up to 2.0% TSP)	–	Firsov et al. (2015)
Lemna minor	Phytase *Aspergillus nidulans*	ND	It has been shown that phytase is suitable for use as a feed additive for chickens	Ghosh et al. (2018)

(continued)

Table 2 (continued)

Species	Target protein/vector features	Expression level	Remark	Reference
Lemna minor	M2e peptide of avian influenza virus H5N1 in fusion with ricin B subunit (35S CaMV promoter, tobacco PR1a signal peptide)	0.25–2.5 μg g^{-1} of plants wet weight (up to 0.01% TSP)	Adjuvanticity of the fused protein M2e - ricin B subunit of has been confirmed	Firsov et al. (2018)
Spirodela oligorrhiza SP	GFP (35S CaMV promoter)	>25% TSP	–	Vunsh et al. (2007)
Spirodela polyrhiza L	Aprotinin (35S CaMV promoter, tobacco PR1a signal peptide)	3.7% of the secreted into the medium protein (0.65 mg/l in the culture medium)	The correct splicing of recombinant aprotinin and its secretion into the culture medium have been confirmed	Rival et al. (2008)
Spirodela polyrhiza L	Hemagglutinin of the avian influenza virus H5N1 (ubiquitin promoter)	ND	–	Thu et al. (2015)
Wolffia arrhiza	Human granulocyte colony-stimulating factor (35S CaMV double promoter, rice α-amylase signal peptide)	Up to 35.5 μg mg^{-1} of plants wet weight (0.2% TSP)	–	Khvatkov et al. (2018)

studies, target proteins had cytoplasmic localization, less often apoplastic; in this case, the target protein was fused with the signal peptide of the small subunit of ribulose-1,5-bisphosphate carboxylase (RuBisCO) (Cox et al. 2006), tobacco PR1a protein (Firsov et al. 2018; Rival et al. 2008), or rice α-amylase (Khvatkov et al. 2018).

Data on the accumulation of target proteins varied significantly in different studies. Thus, in the study of Sun et al. (2007), endoglucanase E1 was expressed in *Lemna minor* tissues at the level of 0.24% of the total soluble protein (TSP). The M2E peptide of avian influenza virus H5N1, fused with the ricin subunit B, expressed at a low level – up to 2.5 μg g^{-1} of duckweed wet biomass, which is probably due to the instability of RTB in the plant cells secretory pathway (Firsov et al. 2018). At the same time, the accumulation of peptide M2e, fused with a more stable partner of β-glucuronidase, was much higher in duckweed plants – up to 0.97 mg g^{-1} wet weight (Firsov et al. 2015). The Ag85B and ESAT6 proteins of *Mycobacterium tuberculosis* expressed in *Lemna minor* at low levels (0.4–0.5 μg g^{-1} wet weight of duckweed) (Peterson et al. 2016).

In the study of Bertran et al. (2015), H5N1 avian flu virus hemagglutinin expressed at a fairly good level – 0.28 mg g^{-1} wet weight of duckweed (about 12% of TSP, according to the densitometric analysis). The hemagglutination test confirmed the high activity of the resulting recombinant protein, which was equal to

400.000 HAU ml^{-1}. As it was noted above, the recombinant hemagglutinin induced an immune response in chickens sufficient to protect them from lethal doses of the virus. The recombinant human G-CSF accumulated in *Wolffia arrhiza* tissues at a rather high level – up to 35.5 mg kg^{-1} wet weight (corresponding to 0.2% of TSP), which is consistent with the level of endoglucanase E1 accumulation according to Sun et al. study (2007).

More than a half of the studies have demonstrated the level of target protein accumulation above 2% of TSP (table. 2). High levels of target protein accumulation were characteristic of the experiments where degradation-resistant reporter proteins, such as GFP (Vunsh et al. 2007), β-glucuronidase (Firsov et al. 2015), as well as proteinase inhibitor aprotinin (Rival et al. 2008), were expressed.

High levels of expression were obtained using the LEX SystemTM optimized for the expression of such recombinant proteins as monoclonal antibodies and avian influenza virus hemagglutinin in *Lemna minor* (Bertran et al. 2015; Cox et al. 2006). Endoglucanase E1 from *Acidothermus cellulolyticus* and human G-CSF accumulated in the duckweeds at a lower level – about 0.2% TSP – that corresponds to a few dozen milligrams of the target protein in one kilogram of plants wet weight (Khvatkov et al. 2018; Sun et al. 2007). Based on the published data, it can be concluded that the potential level of recombinant proteins expression in plants of the duckweed family is quite high. However, the need for further improvement of expression platforms on their basis is quite obvious, in particular, selection of optimal target proteins for expression in duckweeds, definition of optimal cell compartments for their accumulation, and choosing of suitable conditions for cultivation of plants as the producers.

An important aspect of research is studying of a possibility of recombinant proteins secretion into the culture medium. The ability to secretion is an undeniable advantage and a distinctive feature of expression systems based on duckweeds, as it makes significant simplification of the target proteins extraction and purification (Everett et al. 2012; Gasdaska et al. 2003). However, the issue remains virtually unexplored to date. The secretion of recombinant aprotinin from transgenic *Spirodela polyrhiza* plants into the culture medium was shown in the Rival et al. study (2008). Aprotinin (molecular mass 6,5 kda, serine proteinase inhibitor) accumulated in the medium at the level of 3.7% of the total secreted protein, which corresponded to 0.65 mg of recombinant aprotinin per 1 l of the medium. In the rest of available studies, there is no data on the secretion of recombinant proteins into the culture medium. In particular, it remains unclear whether the proteins larger than aprotinin can be secreted into the medium.

In our research, we studied the secretion of recombinant G-CSF into the medium for cultivation of producing plants. Transgenic plants of *Wolffia arrhiza* (Khvatkov et al. 2018) and *Lemna minor* expressing human G-CSF were used in the experiments. G-CSF secretion (molecular mass 19,6 kda) was observed in experiments with *Wolffia arrhiza* only (up to 1.5 mg l^{-1}); G-CSF was not detected in the *Lemna minor* culture medium (unpublished data). The reason of such distinction isn't clear yet and demands further studying.

The peculiarities of target proteins purification from the producing plants are insufficiently studied yet. In a study by Rival et al. (2008), aprotinin derived from the culture medium was purified by affinity chromatography using mice anti-aprotinin antibodies. Further MALDI-TOF/MS and LC/MS/MS analyses and sequencing of the N-terminal region of aprotinin confirmed its complete natural identity.

Few studies have explored some aspects of purification of recombinant mAb derived from the biomass of duckweed (Barros et al. 2011; Woodard et al. 2009). Particular attention in these studies was paid to the development of methods for removing phenolic compounds from plant extracts. Optimization of the extraction buffers composition and extraction conditions (pH, duration of extraction, selection of anion exchange resins) made it possible to achieve the removal of contaminating phenolic compounds from the extracts without significantly increasing the cost of purification procedures.

6.3 Improvement of Duckweed Plants as Producers of Recombinant Proteins

Certain efforts were directed at improving *Lemna minor* as an expression platform. Cox et al. (2006) investigated the possibility of optimizing the composition of glycans in *Lemna minor*. Plants expressing mAb against human CD30 were transformed by construction for RNA interference comprising the antisense sequences of α-1,3-fucosyltransferase and β-1,2-xylosyltransferase genes. As a result, it was shown that recombinant anti-CD30 mAbs did not contain detectable amounts of plant-specific N-glycans. It was also shown that the resulting antibodies had better receptor-binding properties and cytotoxicity indexes than the corresponding antibodies obtained in the CHO cell culture.

To date, *Lemna minor* (Van Hoeck et al. 2017) and *Spirodela polyrhiza* (An et al. 2018; Hoang et al. 2018; Michael et al. 2017) genomes have been sequenced. The study of transcriptomes of *Spirodela*, *Landoltia*, and *Lemna* genera has been started.

The relationship between gene expression and the influence of environmental factors such as radiation, heavy metals, starvation, etc. was mainly studied in transcriptomics researches (An et al. 2018; Wang et al. 2016). In the near future, genomic studies will allow significant progress in understanding of various aspects of genetics, biochemistry, and physiology of Lemnaceae, including with regard to the development of expression systems based on these plants. The solution of these tasks will be promoted by the development of genome editing methods for duckweeds. In the research of Liu et al. (2019), an efficient genomic editing system was developed for *Lemna aequinoctialis*. It is based on plants' stable genetic transformation with the use of agrobacteria and CRISPR/Cas9 system; the efficiency of the developed protocol was about 14% (Liu et al. 2019).

7 Conclusion

To date, significant progress has been achieved in the field of biotechnology and genetic engineering of duckweeds. Methods for their in vitro cultivation have been developed; effective and reproducible genetic transformation protocols have been created; transgenic plants of six species have been obtained. The main efforts in the field of genetic engineering of *Lemnaceae* were aimed at obtaining plants-producers for the production of recombinant proteins for various functional purposes, primarily medical. Plants expressing monoclonal antibodies, viral and bacterial antigens, and various therapeutic and industrial proteins have already been obtained. Their biological activity has been confirmed, and researches in the field of the extraction and purification of target proteins have begun. Moreover, some efforts have already been directed at the development of the methods for humanizing the glycosylation of recombinant antibodies in transgenic plants-producers. The "omics" studies of *Lemnaceae* have begun; in particular, the full genomes of *Lemna* and *Spirodela* have already been sequenced. Thus, current advances in biotechnology, genetic engineering, and genomics of duckweeds open up new and diverse opportunities for the development of duckweed-based expression systems. Nevertheless, research efforts in developing of duckweed-based expression systems are in fact at the beginning. Further researches are needed to increase the accumulation of recombinant proteins in plants-producers. Still there are practically no data on secretion of target proteins into the culture medium and about the nature and dynamics of their accumulation in the medium. No data are available for the assessment of plants-producers cultivation conditions impact on the accumulation of target proteins. Reliable studies on the optimization of cultivation parameters in order to increase their accumulation are absent.

Advances in genetic engineering, genomics and proteomics knowledge of duckweeds are opening new prospects in the field of humanization of posttranslational modifications of target proteins. First of all it concerns the glycosylation of recombinant proteins for medical purposes, but also relates to other posttranslational modifications, such as phosphorylation and sulfation.

In addition, systematic researches are needed to develop methods for the extraction and purification of recombinant proteins, both from the biomass of the producers and from the culture medium. Progress in this area can be facilitated by advances in the genomics and proteomics of *Lemnaceae*, which open up the possibility of modifying plant metabolome to reduce the content of substances complicating purification of target proteins. The development of cultivation methods for *Lemnaceae* implies the need to prevent the leakage of recombinant DNA into the environment. In particular, this may be facilitated by obtaining auxotrophic or polyauxotrophic variants of duckweeds for biofarming purposes.

Given the current progress and pace of development of research in various areas of *Lemnaceae* biology, we have no doubt that in the near future, we can expect the emergence of efficient and competitive duckweed-based expression systems for the production of recombinant proteins for various purposes.

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Essential Oils from Plants: Industrial Applications and Biotechnological Production

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Abstract Essential oils have been used since the discovery of fire by many civilizations. Alchemists used to produce the *Quinta essentia* by distillation, which now is known as *essential oils*. This review aims to provide a systematic overview of the composition in terms of active compounds and main biological activities of different essential oils. In general terms, these include anti-inflammatory, antidepressant, antioxidant, antitumor, antimicrobial, anticancer, and antimutagenic activities.

In addition, the techniques to extract and evaluate their composition are discussed. Moreover, their main industrial applications, especially the application as flavors and fragrances, in pharmaceutical and medicinal industry, in alternative medicines, in cosmetic industry, and in food industry are also reviewed and discussed in order to identify the future trends. Finally, the biotechnological production of essential oils and their components was also assessed.

Keywords Biological properties · Biosynthesis · Biotechnology · Chemical analysis · Essential oils · Industrial applications

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1 Introduction

Essential oils (EOs) are natural organic molecules, from aromatic plant materials including buds, flowers, fruits, seeds, leaves, twigs, bark, wood, and roots containing volatile compounds (Dvaranauskaité et al. 2009; Aidi Wannes et al. 2010; Lv et al. 2012; Hill et al. 2013; Ribeiro-Santos et al. 2015, 2017a, 2018a). Alchemists used to produce the spirit or *Quinta essentia* by distillation, which now is known as *essential oils* (Chemat 2010). They are important secondary metabolites, biosynthesized in specialized cells of certain aromatic plants present in different parts of these plants, involving different enzymatic reactions. These cells have a central role in EOs biosynthesis, accumulation, and secretion into the environment, being the natural "factories" for the synthesis of EOs. Numerous environmental factors also affect biosynthesis of EOs and their secretion into the environment, such as ultraviolet radiation/light, developmental stage of the plant, salt stress, and fertilizers (Rehman et al. 2016a, b). Knowledge about biosynthesis of EOs constituents paves the way for improving the production of a given compound(s) through traditional breeding or through modern plant biotechnology (Woronuk et al. 2011).

The extraction of the EOs from plants can occur by different methods, being the steam distillation and hydrodistillation the most commonly used methods for the extraction at laboratory scale (Ribeiro-Santos et al. 2018a).

A wide variety of EOs, such as bergamot (*Citrus bergamia*), lemongrass (*Cymbopogon flexuosus*), clove (*Syzygium aromaticum*), mustard (*Brassica nigra*), lemon (*Citrus limon* L.), thyme (*Thymus vulgaris*), eucalyptus (*Eucalyptus globulus*), basil (*Ocimum basilicum* L.), oregano (*Origanum vulgare* L.), cinnamon (*Cinnamomum zeylanicum* Blume), mint (Mentha piperita), and rosemary (*Rosmarinus officinalis* L.), are known and applied in several areas of interest (Brud 2012; Ribeiro-Santos et al. 2015, 2017a, 2018b). In the cosmetic industry, EOs can be used due to their well-known fragrance properties. In addition, their properties allow the optimization of cosmetic products properties and their preservation (Carvalho et al. 2016). Regarding the food industry, EOs are used to extend food's shelf life, because of their antioxidant and antimicrobial activities, and as a natural flavor (Zinoviadou et al. 2009; Viteri Jumbo et al. 2014; Gaio et al. 2015, Ribeiro-Santos et al. 2017b, 2018b). The biological properties of EOs are also applied and studied in the pharmaceutical industry because they have therapeutic value and can also be used as flavors in medicines for skin and mucous applications. The pharmaceutical industry also uses some EOs to isolate their natural compounds (Cavaleiro 2007). Complementary therapies as aromatherapy use EOs as the main therapeutic agents to treat several diseases (Ali et al. 2015).

The therapeutic, preservative, medicinal, and flavoring properties of EOs are a result from the biological activity of their constituents, which includes terpenes (monoterpenes and sesquiterpenes) and phenylpropanoids. Their compositions are extremely complex due to the presence of highly functionalized chemical substances of different chemical classes (Temraz et al. 2009; Teixeira et al. 2013; Ribeiro-Santos et al. 2017c, 2018a).

In this line of thought, the technique used to identify and quantify the compounds of the numerous EOs is chromatography techniques, in particular, gas chromatography coupled with mass spectrometry detector (Ribeiro-Santos et al. 2018a).

In this review, research studies regarding EOs are discussed. Special focus is given to the analytical methods to identify the EOs origin and their main active components. Moreover, insights about their metabolic pathways in plants and biotechnological production are presented. Finally, their potential industrial applications are also addressed.

2 Biosynthesis

Existing more than 300 000 plants on planet earth and being the base of food chains, they play an important role in the capture of greenhouse gases, producing oxygen, constituting habitats, and serving as a shelter for a variety of species. Plants are linked to the humankind since the beginning of our existence. The primitive man used plants for several purposes, including clothing, food, and food packages (Heywood 2011; Mora et al. 2011; Pichersky and Lewinsohn 2011; Shikov et al. 2014).

The biological properties of plants are well known. Since ancient times, a selection of plants and their oils in traditional medicines and cosmetic and gastronomy applications is used. Although aromatic plants and spices are mostly used for culinary applications, they are also used for cosmetic and pharmaceutical applications. These plants present several biological properties, like antioxidant, antimicrobial, and antitumoral activities, being used in Chinese and Indian (Ayurvedic) traditional medicines (Ribeiro-Santos et al. 2017d; Sartoratto et al. 2004).

Algae play a very important role in nature, since they capture a major portion of the greenhouse gases and provide food and shelter for many marine organisms. In addition, several algae are used in the food industry for the manufacture of agar-agar and gelling applications. In the culinary world, algae are mostly used in Asian gastronomies, especially in Japanese cuisine. In Europe, its use is mostly reserved for food supplementation and industrial applications. Algae are also known to have several biological and powerful properties (Chan et al. 1997; Peinado et al. 2014).

Plants synthesize several metabolites (natural products) that protect them against UV radiation, pathogenic organisms, and natural predators. There is a great variety of these small molecular weight compounds with several biological properties like antitumoral, antiviral, antimicrobial, and antioxidant activities (Sun et al. 2016; Yang et al. 2016).

The chemical constitution of the plants depends, essentially, on the edaphoclimatic conditions to which the plant was exposed such as temperature, soil composition, and the time of harvest. All these factors will affect the production of secondary metabolites, specifically, the chemical constitution of essential oils (Ribeiro-Santos et al. 2017a; Yang et al. 2016).

According to the ISO 9235:2013, an essential oil is "product obtained from a natural raw material (2.19) of plant origin, by steam distillation, by mechanical processes from the epicarp of citrus fruits, or by dry distillation, after separation of the aqueous phase — if any — by physical processes" (International Organization for Standardization 2013). They have in their constitution several polar and nonpolar compounds that, usually, are divided into major and minor compounds. The major compounds, usually two or three, constituting approximately 85%, are responsible for the principal biological activities, and the minor compounds, although present in trace levels, can interact among each other or with the major compounds, producing synergic effects that can maximize some biological activities (Bakkali et al. 2008; Ribeiro-Santos et al. 2017a; Sangwan et al. 2001; Tongnuanchan and Benjakul 2014). The composition of essential oils is extremely complex, being a mixture of compounds with powerful biological activities, responsible for the plant's defenses. Usually, this mixture of low molecular weight compounds is divided into terpenes and aromatic compounds. Originated through the mevalonate pathway, terpenes are formed by isoprene units which, in turn, are formed by five carbon base units. There are innumerous combinations of isoprene units. Terpenes can be constituted by more than eight isoprene units, being the most common terpenes found in essential oils the monoterpenes (two isoprene units), which composed 90% of essential oils (Bakkali et al. 2008; Tongnuanchan and Benjakul 2014). With more than 80 000 known structures identified, terpenoids are present in most of living organisms and, since ancient times, have several applications in the human world, such as medicines, fragrances, and insect attractants or repellents (Bian et al. 2017; Gershenzon and Dudareva 2007; Pemberton et al. 2017; Pichersky and Raguso 2016; Sangwan et al. 2001). On the other hand, aromatic compounds occur less frequently than terpenes and are derived from phenylpropane which play a vital role in plant's life. The metabolic pathways of terpenes and aromatic compounds are different but can occur at similar times in the same plant, with one major pathway taking over (Bakkali et al. 2008; Herrmann and Weaver 1999; Sangwan et al. 2001). On the other hand, phenylpropanoids originate through the shikimate pathway. This pathway, used by a diverse group of organisms such as bacteria, fungi, algae, protozoan parasites, and plants, consists in metabolic route composed of seven steps to synthetize folates and aromatic amino acids (phenylalanine, tyrosine, and tryptophan).

According to data from the World Health Organization (2010), the major market for medicinal plants is the European. Europe also produces a great variety of medicinal plants and herbs, and the consumers often complement conventional medicine treatments with this alternative (WHO 2010).

2.1 Most Commonly Used Plants

More than 25% of all the medicines produced in the world are manufactured or derivatives from plants. For instance, paclitaxel (Taxol®) used for the treatment of several cancers is a derivate from the bark of *Taxus brevifolia*, and vinblastine, also

a drug used in chemotherapy of several cancers, is extracted from *Catharanthus roseus*, commonly known as Madagascar periwinkle (Lubbe and Verpoorte 2011). The use and application of essential oils by the human civilization dates back more than 5000 years, from the ancient civilization of Mesopotamia. Throughout history, these compounds passed for the Egyptians, Indians, Greeks, and Romans, being the most used the lavender, chamomile, peppermint, tea tree, eucalyptus, geranium, jasmine, rose, lemon, orange, rosemary, frankincense, and sandalwood essential oils (SADC Trade Development Project 2006). Currently, there are approximately 3000 known essential oils among which 300 are very important for the several industries that work with essential oils. Each year, approximately 50000 tons of essential oils are produced representing a market value of 700 million USD (Bakkali et al. 2008; Dima and Dima 2015).

Nowadays, the most used plants for the extraction of EOs are from the Lamiaceae, Apiaceae, Rutaceae, and Verbenaceae. The plants from these families are known to have powerful biological activities (Elshafie and Camele 2017).

Rosemary (*Rosmarinus officinalis* L.), thyme (*Thymus vulgaris* L.), oregano (*Origanum vulgare* L.), basil (*Ocimum basilicum* L.), and sage (*Salvia officinalis* L.) are some of the plants belonging to the Lamiaceae family known for their antioxidant and antimicrobial activities (Nieto 2017; Whiley et al. 2017).

From the Rutaceae family, the most known plants with interesting biological activities are from the genus *Citrus*. *Citrus aurantifolia*, commonly known as lime, has several biological properties, namely, anticancer activity once the effects of lime against carcinogenesis are proved (Narang and Jiraungkoorskul 2016). The action of the *Citrus limon* essential oil against the toxicity of acetylsalicylic acid on human liver was also proved by Bouzenna et al. (2016).

On the Apiaceae family, *Pimpinella anisum* L. (anise) is used since ancient times by the Iranian traditional medicine, and its essential oil has proved beneficial effects against nonalcoholic fatty liver disease (Asadollahpoor et al. 2017) (Table 1).

2.2 Extraction Methods

As mentioned before, EOs and extracts can be obtained by several extraction methods (Table 2). The selection of the extraction method is very important once its conditions can directly influence the composition and the quality of the essential oil and, consequently, their biological activities (Cerpa et al. 2008; Masango 2005; Nakatsu et al. 2000; Pisoschi et al. 2018). The chemical composition of EOs depends also on the part of the plant that is being extracted (such as flowers, bark, leaves, and seeds), age, and the vegetative state of the plant at the time of the harvest (Asbahani et al. 2015; Bakkali et al. 2008). Due to this, the extraction method is selected according to the oil purpose. For instance, to be used by the pharmaceutical or food industry, for their antibacterial properties, the most used/preferred methods are steam distillation or expression/cold pressing. On the other hand, in the cosmetic industry, the EOs used for fragrances are extracted with lipophilic solvents and with supercritical carbon dioxide (Bakkali et al. 2008).

Table 1 Some plants used for the extraction of essential oils and their biological activities

Plant species	Family	Common name	Biological activity	Reference
Arctotis arctotoides (L.f.) O. Hoffm	Asteraceae	–	Traditionally used for treatment of indigestion and catarrh of the stomach, epilepsy, topical wounds, and skin disorders. Possess antioxidant, antibacterial, antifungal, and anticancer activities	Saleh-e-In and Van Staden (2018)
Cinnamomum (genus)	Lauraceae	Cinnamon	Antioxidant, antimicrobial, insecticidal, antitumor, antidiabetic, and anti-inflammatory activities	Ribeiro-Santos et al. (2017b)
Citrus limon L.	Rutaceae	–	Treatment for obesity, diabetes, blood lipid lowering, cardiovascular diseases, brain disorders, and certain types of cancer	Bouzenna et al. (2016)
Echinophora (genus)	Apiaceae	–	Antifungal, antioxidant, anticancer, antimutation, and antimicrobial activities	Hosseini et al. (2017)
Lavandula (genus)	Lamiaceae	Lavender	Possess calming and sedative effects; anticancer and antimutagenic activities	Woronuk et al. (2011)
Ocimum basillicum L.	Lamiaceae	Basil	Treatment of headaches, coughs, diarrhea, constipation, warts, worms, and kidney malfunctions	Araújo Silva et al. (2016)
Rosmarinus officinalis L.	Lamiaceae	Rosemary	Anti-inflammatory, antidepressant, antioxidant, and antimicrobial activities; used in the treatment of headache, abdominal pain, and arthritis	Ribeiro-Santos et al. (2015)
Thymus vulgaris L.	Lamiaceae/ Labiatae	Thyme	Antiseptic, carminative, antimicrobial, and antioxidant	Golmakani and Rezaei (2008)
Zornia brasiliensis	–	"Urinária"	Diuretic and has effects against venereal diseases. Antitumoral activity	Costa et al. (2015)

Distillation is probably the most ancient and traditional method for extraction of EOs from plants. Regarding plant materials, such as aromatic plants, the high temperatures cause the release of EOs in vapor form from the plant cell membranes. These vapors are condensed and the essential oil is obtained in liquid form. It should be noted that in the distillation process of an essential oil, shattering "hard" parts of the plants (seeds, roots, stalks) is necessary in order to facilitate the liberation of the oil's vapors (Busato et al. 2014).

One of the most used methods for essential oil extraction is hydrodistillation. In this process, the plant parts are mixed with water and placed into an alembic. Then, the solution is submitted to high temperatures and boiled. The vapors from the water

Table 2 Some extraction methods of essential oils

Method	Breve description	Advantages	Disadvantages	Reference
Hydrodistillation	Plant materials are mixed with water and submitted to high temperatures and boiled. The water vapors with compounds from the plant material evaporate, pass through a condenser, and are recovered as a liquid	Simple method, easily installed and applied Does not require expensive material The required material can be distilled at temperatures below 100 °C	Water's boiling point is much lower than certain EOs compounds Long extraction time Low yield Chemical alterations of terpenoids Overheating can cause partial or full degradation of compounds (monoterpenes) Loss of polar molecules due to the use of water	Asbahani et al. (2015), Dima and Dima (2015), Gavahian et al. (2012), and Tongnuanchan and Benjakul (2014)
Vapor-hydrodistillation	Water does not come in contact with the plant material that is placed on a grid, and thus the water vapors drag the volatile compounds	Extraction time is reduced (compared to hydrodistillation) Artifacts are minimized Loss of polar molecules is reduced (compared to hydrodistillation)	Overheating Low yield Water's boiling point is much lower than certain EOs compounds	Asbahani et al. (2015)
Steam distillation (vapor-distillation)	Most used technique Water and plant material still do not come in contact, but the vapors occur inside the alembic, being possible for the steam to be superheated	Extraction time is reduced (compared to hydrodistillation) Artifacts are minimized Loss of polar molecules is reduced (compared to hydrodistillation) Less artifacts than the vapor-hydrodistillation	Overheating	Asbahani et al. (2015)

(continued)

Table 2 (continued)

Method	Breve description	Advantages	Disadvantages	Reference
Hydrodiffusion	Similar to steam distillation or hydrodistillation but the plant material is placed above the condenser, being the process executed from up to down	–	–	Asbahani et al. (2015)
Organic solvent extraction	The plant material is mixed with an organic solvent. Then the solvent is evaporated in lower pressure	Chemical alterations and chemical artifacts are minimal	Long extraction process The resulting essential oil is more expensive than other methods Solvent residue can be present in the final product, which can lead to toxicity	Pisoschi et al. (2018) and Tongnuanchan and Benjakul (2014)
Cold pressing	Traditional method of extraction of EOs from citrus fruit zest The extraction is mechanical, so the EO is obtained by cold pressing a mixture of water and plant material The oil is recovered by centrifugation			Asbahani et al. (2015)
Microwave-assisted hydrodistillation	The process is very similar to the hydrodistillation method, but the samples are mixed with water and heated in a microwave oven	The sample is heated almost simultaneously and at a higher rateHigher yield (compared to hydrodistillation)	Increased extraction of unwanted compounds due to severe thermal stress and localized high pressures	Golmakani and Rezaei (2008)

and oil evaporate, passing through a condenser, and are recovered as a liquid. Since oil and water are not miscible substances, the essential oil is easily obtained. This method is also very economic since it relies on non-expensive equipment and has a simple installation. Regarding the disadvantages, the boiling point of water is much lower than the boiling point of some EOs compounds, so the extraction may not

include some compounds with interesting biological properties. Also, in addition to the low yield, since it takes a lot of plant material to get a small amount of oil. Another disadvantage is the long extraction time that is achieved up to 24 hours (Asbahani et al. 2015; Dima and Dima 2015).

Unlike the previous descripted method in which the water is directly mixed with the plant material and heated, there are other methods that are made through the entrainment of the water steam. These sets of methods are quite similar to the hydrodistillation, but the water does not enter in contact with the plant material, reducing the chemical alterations and the extraction time (Asbahani et al. 2015; Masango 2005). These groups of extraction methods by water steam include vapor-hydrodistillation, steam distillation, and hydrodiffusion. These methods are described in Table 2.

Although distillation is the most used and traditional method of EOs extraction, new methods using more advanced technologies are being applied in order to decrease the time of the extraction, increase the extraction yield, and reduce the extraction cost and environmental impact. The microwave-assisted extraction and extraction using ultrasounds are two examples of more advanced methods that can reduce the extraction time and increase the essential oil yield (Golmakani and Rezaei 2008; Pisoschi et al. 2018).

Regarding the extraction of an essential oil using a simple solvent extraction process, the vegetal material is mixed with the solvent, and then, heat is applied in order to break the cell structure of the vegetable material to release the aromatic compounds. The solution is then filtrated and the solvent is evaporated to concentrate the essential oil. The remaining concentrate is denominated by resin (resinoid) or concrete, which is a combination of wax, fragrance, and essential oil. The concentrate is mixed with pure alcohol and then submitted to a distillation process at low temperatures in which the alcohol will absorb the fragrances and after the alcohol is evaporated, only the oil remains. The use of solvents for essential oil extraction, although a viable option, is quite limited. If the purpose of the essential oil is human consumption, the use of most organic solvents is discouraged, as it may result in toxic compounds to human health (Tongnuanchan and Benjakul 2014; Yara-Varón et al. 2017).

In order to improve the yields, the time of extraction, and the biological activities of EOs, advanced techniques are being developed. One of them is the supercritical extraction, which is normally made using carbon dioxide. This method is based on the fluid supercritical state of the used liquid (CO_2) which is reached at determined conditions of pressure and temperature. At this state, there is no distinction between the liquid and the gaseous state. At this state, fluids can present interesting characteristics such as low viscosity and high diffusivity. Regarding the extraction of EOs, the most common chosen fluid is the CO_2 because at its supercritical state (pressure 72.9 atm; temperature 31.2 °C), it is chemically inert, nontoxic, nonflammable, and unaggressive for thermolabile molecules of the plant's essence and can be obtained at a relatively low cost (Asbahani et al. 2015; Lepojević et al. 2017).

3 Chromatographic Analysis of EOs

Nowadays, the tampering or adulteration of EOs is very common; therefore techniques are necessary to determine the purity of the oils. These techniques can be separated in two categories: the classic analytical techniques and the modern analytic techniques. In the first category, the applied tests are focused, mainly, in the identification and the purity of the essential oil. On the other hand, the second category is more focused on the identification of the EOs components, so the two groups should be applied together to determine the authenticity of the EO (Baser and Buchbauer 2010; Elshafie and Camele 2017).

In the classic analytical techniques, there are some methods which can be highlighted. The specific gravity and the refractive index can be determined to evaluate the physical properties of an EO, and the determination of the presence of polar substances or the measurement of the points of the physical changes (melting and congealing points) can be used to evaluate the purity of the oils. In addition, the quality of an EO can be also evaluated through a solubility test in ethanol. Regarding the chemical properties of EOs, the toxicity can be evaluated through the determination of halogenated hydrocarbons, heavy metals, and esters derived from phthalic acid. The chemical properties can also be identified by the determination of chemical groups, such as the determination of aldehydes through the bisulfite method, in aldehyde-rich EOs, and the neutral sulfite test, in ketone-rich EOs (Elshafie and Camele 2017).

Regarding the more modern techniques, based on the compound identification methods, several chromatographic techniques can be emphasized. In general, chromatography is based on the compounds separation of a mixture due to its different affinity for two phases (stationary phase and mobile phase). The chromatographic techniques have three major objectives: the separation of compounds of a certain homogenized mixture, the identification of those compounds, and their quantification. For the determination of EOs compounds, the most used technique is gas chromatography (GC), coupled with mass spectrometry (MS) which is characterized for having the mobile phase in gas form. Liquid chromatography (LC) is also widely used (Elshafie and Camele 2017; Marriott et al. 2001). Some EOs and their major compounds are compiled in Table 3.

4 Potential Industrial Applications

EOs have been used for centuries as perfume fragrance, and many ancient civilizations have used this as a popular complementary and alternative therapy. There is a growing interest in the study of EOs not only due to their natural nature but also because they have demonstrated benefits in food and in human health (Ribeiro-Santos et al. 2015, 2017a, 2018a). Currently, EOs are the focus of extensive research due to their well-known biological properties derived of the great variety and diver-

Table 3 Major compounds of some essential oils and respective determination methods

Plant species	Common name	Extraction method	Major compounds	Method/ detector	Conditions	Analytical column	Ref.
Thymus vulgaris L.	Thyme	Microwave-assisted hydrodistillation	Thymol (40.20 %) *p*-Cymene (17.57 %) γ-Terpinene (8.54 %) Carvacrol (6.84 %)	GC-MS	Injector temp: 290 °C Injector mode: split (ratio: 1/10) Injected volume: 1 µl Carrier gas: helium (0.8 ml/min) Oven ramp: hold for 5 min at 50 °C, rise to 240 °C at 3 °C/min, rise to 300 °C at 5 °C/ min, hold for 3 min	HP-5MS (Agilent Technologies) capillary column (30 m x 0.25 mm i.d. 0.25 µm film thickness)	Golmakani and Rezaei (2008)
		Hydrodistillation	Thymol (37.20 %) *p*-Cymene (16.85 %) γ-Terpinene (9.06 %) Carvacrol (6.81 %)				
Mentha spicata L.	Spearmint	Microwave hydrodiffusion and gravity	Carvone (40.5 %) *cis*-Carveol (15.3 %) Limonene (10.3 %)	GC-FID GC-MS	**GC-FID** Vector gas: helium Injector and detector temp: 250 °C Injected volume: 0.4 µl Split ratio: 1:50 Oven ramp: 40 °C to 250 °C at 2 °C/min and 250 °C for 60 min **GC-MS** Carrier gas: helium Constant flow: 1 ml/min Injector temp: 250 °C Split ratio: 1:50 Oven ramp: 45 °C to 250 °C or 230 °C (2 °C/min), then hold isothermal (20 min) at 250 °C (apolar column) or 230 °C (polar column) Ion source temp: 230 °C Transfer line temp: 250 °C (apolar column) or 230 °C (polar column) Ionization energy: 70 eV	**GC-FID** HP1 column (J&W Scientific), polydimethylsiloxane (50 m × 0.20 mm i.d., 0.5 µm film thickness, constant flow 1ml/min) and Innowax (Interchim, Montlucon, France) poly(ethylene glycol) (50 m × 0.20 mm i.d., film thickness 0.4 µm) **GC-MS** Fused-silica capillary column HP-1 (Interchim) polydimethylsiloxane (50 m × 0.20 mm i.d., 0.50 µm film thickness) and Innowax (Interchim) poly(ethylene glycol) (50 m × 0.20 mm i.d., 0.4 µm film thickness)	Vian et al. (2008)
		Hydrodistillation	Carvone (45.5 %) *cis*-Carveol (16.9 %) Limonene (10.06 %)				
Mentha pulegium L.	Pennyroyal	Microwave hydrodiffusion and gravity	Pulegone (87.8 %) Menthone (3.1 %) Eucarvone (2.3 %)				
		Hydrodistillation	Pulegone (83.7 %) Linalool (3.3 %) Menthone (2.8 %) Eucarvone (1.4 %)				

(continued)

Table 3 (continued)

Plant species	Common name	Extraction method	Major compounds	Method/ detector	Conditions	Analytical column	Ref.
Zygophyllum album L.	–	Simultaneous distillation-extraction (SDE)	3-Nonen-2-one (3.4 %) α-Ionone (5.3 %) (E,β)-Damascenone (6.7 %) 2-Oxabicyclo [4,4,0] dec-9-ene-1,3,7,7-tétraméthyl (8.1 %) δ-Décalactone (11.8 %)	GC-MS GC-FID	**GC-MS** Carrier gas: helium Flow rate: 0.3 ml/min Spilt-less mode Injection volume: 1 µl Injection temp: 250 °C Oven ramp: 60 °C for 8 min, increased to 250 °C at 2 °C/min and held for 15 min Ionization mode: electronic impact at 70 eV **GC-FID** Column temp: 60 °C for 8 min, increased to 250 °C at 2 °C/min, held for 15 min Injection temp: 250 °C Split-less mode Injection volume: 1 µl Flow rate: 0.3 ml/min Carrier gas: nitrogen Flame ionization temp: 320 °C	**GC-MS** Nonpolar fused-silica capillary column HP5MS™ (30 m × 0.25 mm × 0.25 µm film thickness) and a polar fused-silica capillary column Stabilwax™ consisting of Carbowax™-PEG (60 m × 0.2 mm × 0.25 mm film thickness) **GC-FID** Fused-silica capillary column with an apolar stationary phase HP5MS™ (30 m × 0.25 mm × 0.25 µm film thickness)	Ferhat et al. (2007)
		Microwave-assisted simultaneous distillation-solvent extraction (MW-SDE)	α-Cadinol (4.1 %) Geraniol (4.3 %) A-Bisabolol (4.7 %) trans-Caryophyllene (4.7 %) trans-α-Bergamotene (5.4 %) Geranyl acetate (6.1 %) β-Bisabolene (17.8 %)				
Echinacea purpurea L.	Purple coneflower	Supercritical extraction processes	Torilenol (4.84 %) Widdrol (5.75 %) *cis*-Dihydro-mayurone (6.85 %) Hexahydrofarnesyl acetone (19.06 %)	GC-MS	Split-splitless injector Automatic liquid sampler Carrier gas: helium Flow rate: 1 ml/min Injector temp: 250 °C Detector temp: 260 °C Column temp: from 40 to 260 °C (at the rate of 4 °C/min), held isothermally at 260 °C for 25 min	HP-5MS column (30 m × 0.25 mm, 0.25 µm film thickness)	Lepojević et al. (2017)

Baccharis trimera (Less.) DC.	"Carqueja"	Steam distillation of vegetative organs (cladodes and roots)	Geranial (3.90 %) Cuminaldehyde (4.20 %) β-Eudesmol (4.80 %) Palustrol (7.80 %) Carquejyl acetate (23.50 %)	GC-MS	Ionization energy: 70 eV Injection volume: 1 µl Split ratio: 25:1 Carrier gas: helium Flow rate: 1 ml/min **Column 1** Injector, interface, and ion source temp: 280 °C Oven ramp: 40 °C (4 min), 40–180 °C at 4 °C/min, 180 °C (2 min), 180–280 °C at 10 °C/ min, 280 °C (10 min) **Column 2** Injector, interface, and ion source temp: 230 °C Oven ramp: 60 °C (4 min), 60–160 °C at 2 °C/min,160–230 °C at 20 °C/min, 230 °C (10 min) **Column 3** Injector, interface, and ion temp: 250 °C Oven ramp: 40 °C (4 min), 40–180 °C at 5 °C/min, 180–220 °C at 10 °C/min, 220 °C (10 min), 180–240 °C at 20 °C/min, 240 °C (10 min)	Three cross-linked capillary columns of equal dimensions (30 m × 0.25 mm i.d. × 0.25 µm film thickness): Column 1: Nonpolar HP-5MS (95 % dimethyl-5 % diphenylpolysiloxane) Column 2: Medium-polar OV-225 (50 % methyl-25 % cyanopropyl-25 % phenylsilicone) Column 3: Polar Stabilwax (100 % polyethylenglycol)	Minteguiaga et al. (2018)
		Steam distillation of blooming stage	Palustrol (4.40 %) Carquejyl acetate (71.40 %)				
Allium genus (Alliaceae family)	–	Hydrodistillation	Dimethyl trisulfide (4.5 %) Tricosane (5.2 %) Pentythiophene (5.4 %) Methylthiomethyl disulfide (28.2 %) 5-Chloroorcylaldehyde (45.60 %)	GC-MS	Injection volume: 0.2 µl Carrier gas: helium Flow rate: 1.1 ml/min Ionization energy: 70 eV	-	Alizadeh Behbahani and Imani Fooladi (2018)

(continued)

Table 3 (continued)

Plant species	Common name	Extraction method	Major compounds	Method/ detector	Conditions	Analytical column	Ref.
Asarum heterotropoides var. *mandshuricum*	–	Dynamic nitrogen-protected microwave-assisted hydrodistillation connected liquid-liquid extraction	Myristicin (8.60 %) 3,4,5-Trimethoxytoluene (11.00 %) 3,5-Dimethoxytoluene (12.73 %) Safrole (25.60 %) Methyl eugenol (26.80 %)	GC-MS	Ionization mode: 70 eV	HP-5MS (Agilent Technologies) capillary column (30 mm × 0.25 mm, 0.25 μm film thickness)	Chen et al. (2018)
Achillea wilhelmsii K. Koch	Iranian yarrow	–	*trans*-a-Necrodol acetate (3.70 %) Borneol (8.80 %) 1,8-Cineole (10.40 %) *trans*-Pinocarveol (17.50 %) Camphor (15.90 %)	GC-MS	Oven ramp: 60–250 °C at the rate of 5 °C/ min and finally held isothermally for 10 min. Helium was used as carrier gas	DB-5 (30 m × 0.25mm i.d., 0.25 mm film thickness), coupled with a TRACE mass ion trap detector	Saeidi et al. (2018)

GC-MS gas chromatography-mass spectrometry, *GC-FID* gas chromatography with a flame ionization detector

sity of their chemical compositions (Cavaleiro 2007; Ribeiro-Santos et al. 2018a, b). EOs have many active functions and compounds, which can be very attractive to use in cosmetic and pharmaceutical preparations, as well as in the food industry.

4.1 Application as Flavors and Fragrances

Despite their application for their biological properties (Table 3), the greatest use of EOs is as flavoring agents; more oils are used in flavors than in fragrances. Citrus EOs (e.g., lemon, lime, and orange) are the most widely used EOs worldwide and are used in beverages, candies, and gelatins besides being applied in various food products. The soft drinks industry is a major consumer of EOs of citrus origin (Bruds 2012; Mustafa 2015). Other brands of the food industry use substantial quantities of EOs in sweets, ice creams, confectionery and bakery products, and a variety of fast foods (where spice oils are used). Another relevant application of EOs, such as mint, eucalyptus, and some other herbal and fruity oils, is in oral care products, like chewing gums and all kinds of mouth refreshing confectioneries (Brud 2012). Perfumes, cosmetics, toiletries, detergents, cleaning and household products, and related products are perfumed with EOs due to their fragrances (Brud 2012). One of the best natural perfumes itself is geranium oil, generally used in soaps and detergents because its unique nature is never changed with alkalinity of soaps (Babar Ali et al. 2015).

The EOs that have highest production and market value worldwide are orange oil, cornmint, peppermint, eucalyptus, lemon, citronella, and clove (Brud 2012).

4.2 Application in Pharmaceutical and Medicinal Industry

The recent increase in the popularity of natural alternative medicines to conventional therapy has renewed interest in several EOs, which have long been considered potential natural remedies for various illnesses. Used directly or included in drug formulations, EOs are especially required for their pharmacologic effects on the digestive system and respiratory system, due to the analgesic and anti-inflammatory activity, inhibition of cancer cells proliferation, and glucose control in diabetes or due to effects on skin and exposed tissues. The different medicinal activities attributed to EOs result of the contribution of their different compounds. Some of them are compiled in Table 3. Pharmaceutical industry found in the EOs of some plants an answer to bacteria resistance to antibiotics. They are being studied to verify their application on patient treatments (Kaskatepe et al. 2016). Anti-inflammatory property of *Cymbopogon citrates* and *Eucalyptus citriodora* EOs was demonstrated in in vivo analysis, suggesting their potential role as therapeutic alternatives in dealing with inflammatory-related diseases (Gbenou et al. 2013).

4.3 Application in Alternative Medicines

Aroma and massage therapy are the practice of using EOs for psychological and physical well-being via inhalation and massage, respectively. They are a form of complementary and alternative medicine. Fatigue, agitation, and inflammatory diseases, such as allergy, rheumatism, and arthritis, are often eased using EOs massage therapy (Edris 2007). The inhalation practices of a number of EOs which are currently in use as aromatherapy agents to relieve anxiety, stress, and depression are showing positive effects in the treatment of epilepsy, menopausal disorders, and tuberculosis, exhibiting sedative properties and analgesic activity, acting against insomnia, memory loss, pain management, mental exhaustion, and burnout (Edris 2007; Babar Ali et al. 2015). EO as lavender EOs possesses potent calming and sedative effects, making them popular in aroma-therapeutic practices (Woronuk et al. 2011). Various other EOs are also used in aromatherapy, including peppermint, rosemary, orange, bergamot, lemon, cinnamon, jasmine, geranium, rose, citronella, clary sage, grapefruit, eucalyptus, tea tree, and chamomile (Edris 2007; Setzer 2009; Babar Ali et al. 2015).

4.4 Application in Cosmetic Industry

Despite the fact that the cosmetic industry uses EOs due to their fragrance properties, their preservative properties have drawn the attention of this industry. This capacity can be applied to skin products because of their ability to capture free radicals associated with skin aging process. EOs possess antimicrobial and insect repellent activities, and can be efficient in cleansing for over-oily skin, against acne and eczema, in the treatment of skin inflammation and prevention of other skin disorders. Furthermore, EOs possess anti-inflammatory activities, which can help prevent wrinkles and increase blood circulation in the skin and scalp (Batish 2008; Carvalho et al. 2016). EOs can be present in several formulations of various cosmetic categories. Examples of these are the moisturizers, sunscreens, antiaging and anti-acne creams, shampoos and other hair products, soaps and cleaning formulations, perfumes, deodorants, and antiperspirants, among others (Carvalho et al. 2016). Flower EOs, such as rose, tuberose, narcissus, gardenia, jasmine, and lavender, remain the most popular aroma ingredients in the cosmetic industry. Other commonly used EOs are mint, thyme, garlic, sage, clove, aloe vera, citronella, and rosemary (Muyima et al. 2002; Martins et al. 2014; Carvalho et al. 2016). *Lavandula officinalis*, *Rosmarinus officinalis*, *Artemisia afra*, and *Pteronia incana* EOs are used as fragrance and additionally as natural preservatives in an aqueous cream formulation in cosmetic industry (Muyima et al. 2002). EOs as preservative, anti-inflammatory, insecticidal, and antioxidant actions is presented in Table 3.

4.5 Application in Food Industry

Regarding the food industry, EOs can extend the storage stability of food, by inhibiting the growth of microorganisms and protecting food from oxidation due to their antimicrobial and antioxidant activities, respectively. They can be also applied as natural flavors to food products. Several authors have tested EOs as an alternative to the synthetic additives used in foods (Oussalah et al. 2004, 2007; Zinoviadou et al. 2009; Ribeiro-Santos et al. 2017e). Fruits, vegetables, minimally processed products, seafood, meat, poultry, sausages, dairy products, and cereals were tested with different EOs (Ribeiro-Santos et al. 2017e). New food packaging has been developed to incorporate EOs in order to protect different types of food (e.g., meat, fish, fruits, processed and raw food) with effective results (Ribeiro-Santos et al. 2017e, 2018a).

Essential oil of basil exhibited positive effect on shelf life of Italian-type sausage due to their antibacterial activity (Gaio et al. 2015). Combined EO of rosemary and cinnamon incorporated in food package showed protection of salami fat salami of oxidation (Ribeiro-Santos et al. 2017d). EOs as antimicrobial and antioxidant functions can be seen in Table 4.

4.6 Other Applications

Eucalyptus globulus and *Lavandula officinalis* EOs were tested in leather tanning industry in order as alternative preservatives for various tanned leather (Sirvaityte et al. 2011). In agricultural, *Eucalyptus* sp. EO showed to be a natural pesticide (Batish et al. 2008), and oregano (*Origanum vulgare*), savory (*Satureja montana*), and thyme (*Thymus vulgaris*) EOs have been reported to protect stored commodities from deterioration through postharvest control (Lopez-Reyes et al. 2010).

EOs are also used in the paint industry, which capitalizes on the exceptional cleaning and embalming properties of certain oils (Lis-Balchin 2012). Clove leaf and peppermint EOs were reported as natural flavor in the tobacco industry (Brud 2012).

The microencapsulation of EOs gives a more predictable and long-lasting effect of the products, multiplying their number of applications including, among others, biotechnology, pharmaceuticals, electronics, photography, chemical industry, and textile industry. The encapsulation is the process in which a liquid EO is enclosed in a carrier matrix to provide a dry, free-flowing powder. One needs to encapsulate the volatiles into a polymer matrix to lower their volatility in order to change the impact of fragrance and flavors, add fragrance to textiles, stabilize specific compounds, and tailor the fragrance to the intended use of a product, lowering the volatility and thereby prolonging the shelf life of a fragrance product (Karlsen 2012).

Table 4 Several biological/medicinal properties of different essential oils[a]

Essential oils	Biological activity/medicinal properties	Possible application[b]
Agarwood	Anti-pancreatic cancer	Pharmaceutical
Basil	Antitrypanosomal and antiplasmodial	Pharmaceutical
Black cumin	Breast cancer	Pharmaceutical
Cedarwood	Sedative effect	Aromatherapy
Checkerberry; chamomile, peppermint, roman chamomile, *bushy* lippie	Anti-inflammatory and analgesic	Pharmaceutical, cosmetic
Cumin, peppermint, fennel	Hepatoprotective	Pharmaceutical
Eucalyptus, cajaput	Mucolytic and bronchodilator effect	Pharmaceutical
Fennel, chamomile, peppermint, garlic, sage, roman chamomile, lemon balm, g*eranium*, rosemary	Carminative effects and intestinal antispasmodic	Pharmaceutical
Ginger, gentian, juniper	Gastric protection and treatment of dyspepsia	Pharmaceutical
Lavender	Analgesic	Pharmaceutical
Lavender	Treatment of menopausal disorders and relaxed effect	Aromatherapy Massage therapy
Lavender, monarda	Reduced the cholesterol content in the aorta	Aromatherapy
Lavender, peppermint, rosemary and clary sage, chamomile	Decrease symptoms associated with anxiety and/or stress	Aromatherapy
Lemon balm, rosemary	Antidiabetic	Pharmaceutical
Lemon balm, rosemary	Treatment from dementia	Massage therapy
Lemon balm, thyme, tea tree	Antiprotozoals	Pharmaceutical
Mexican tea, spearmint	Antihelminthic	Pharmaceutical
Nazar Panra	Antinociceptive and antipyretic	Pharmaceutical
*Onion, c*innamon, savory, thyme, clove, lavender, rosemary	Antimicrobial	Pharmaceutical, food, and cosmetic
Oregano, Himalayan cedar, rosemary, peppermint	Anti-inflammatory	Pharmaceutical Cosmetic
Pennyroyal, apple mint, fennel, eucalyptus	Insecticidal	Cosmetic Household
Pepper, estragon, fennel, grapefruit, rose, patchouli	Modulate sympathetic activity	Aromatherapy
Sacaca	Gastric protection Antiulcer	Pharmaceutical
Sandalwood, Greek sage	Antiviral	Pharmaceutical
Spanish sage	Treatment of neurodegenerative diseases	Pharmaceutical
Star anise	Colon cancer	Pharmaceutical
Tasmanian blue gum, rosemary, cinnamon	Antioxidant	Pharmaceutical, food, and cosmetic

(continued)

Table 4 (continued)

Essential oils	Biological activity/medicinal properties	Possible application[b]
Ylang ylang	Depression, anxiety, hypertension, frigidity, stress, and palpitations	Aromatherapy

[a]Adapted from Cavaleiro (2007); Edris (2007) and Ribeiro-Santos et al. (2018a)
[b]It depends on the essential oils

5 Biotechnological Production

EOs contain monoterpenoids (C10 terpenoids), sesquiterpenoids (C15 terpenoids), phenylpropanoids, and benzenoids. EOs are produced just in specific plant tissues which correspond to small percentages (in terms of weight) of the whole plant; therefore the obtainment of volatiles from cultivated plants is an expensive process. Usually plant seeds, flowers, stems, and roots only contain 0.1–10% v/w fresh weight of EO, although cases with < 0.1% or > 20% are also known.

Many times, it is cheaper to obtain plant volatiles through a chemical process; therefore the natural product just occupies a small part of the market.

The quality and quantity of EOs depends not only on the chemotype, ecological edaphoclimatic conditions, harvest time, vegetative development, and part of the plant but also on the extraction method and laboratory protocols; therefore many efforts are being made to develop new production methods which allow to obtain higher yields and low environmental impact (Calvo-Irabien 2018).

Moreover, nowadays, there is an increasing interest by natural products because consumers believe they are free of potential harmful traces of chemicals used in the manufacturing process. Natural products have generally higher biological activity because chemical synthesis often results in racemic mixtures. Consumers are also interested in low environmental impact processes; therefore nonchemical methods are preferred. On the other hand, industry is interested in ensuring a stable supply of EOs because some plants are rare and difficult to cultivate and present slow growing and to guarantee their quality and safety.

Biotechnology has been proposed as a valid alternative to produce EOs and their components at low process and employing environmentally friendly methods. These include de novo synthesis or biotransformation of cheap precursors into high-value products involving either fungi, yeast, bacteria, or enzymes (crude or purified).

Gouranis (2010) carried out an excellent review on the various methods using biotechnology to produce EOs and their components. Several reviews have already addressed biotechnology production of flavors (Mulder-Krieger et al. 1988, Krings and Berger 1998; Serra et al. 2005).

In vitro culture cells increase the biomass propagation rate and have it available and under controlled conditions. However, because they are influenced by a variety of physicochemical factors, it is not considered a promising alternative, even with inclusion of inducers. Mulder-Krieger et al. (1988) carried out an extensive review on methods to improve EOs yield in cell cultures.

The genetic transformation of plant tissues such as the insertion of T-DAN region of the RI plasmid (which has genes for auxin synthesis) of *Agrobacterium rhizogenes* results in the formation of "hairy roots." These hairlike structures are stable and have the T-DNA integrated, and they grow as fast or faster than normal roots and produce secondary metabolites similar to normal roots and metabolites produced in the aerial parts of the plants. Figueiredo et al. studied plant species (e.g., *Pimpinella anisum*, *Achillea millefolium*) whose hairy root cultures have the ability of producing EOs with similar or higher yields that those obtained with parent plants.

Besides the de novo synthesis of EOs or their volatile compounds involving plant cell cultures, it is also possible to use microorganisms for the production of all classes of volatiles specially aldehydes, alcohols, and organic acid esters. For instance, 2-phenylethanol, a rose-like aroma, can be produced by *Kluyveromyces* and other yeasts from phenylalanine.

Regarding the biotransformation of cheap, abundant precursors into high-value products, they can occur by plant and microorganisms cultures or by enzymes. Microorganisms cultured in supplemented media are more successful than plant cultures due to present higher yields of volatiles production. The theoretical limit of 1g/L is the required to consider a new procedure a good alternative from the commercial point of view.

One of the most important volatiles is vanillin followed by benzaldehyde, capsaicin, γ-decalactone, and 2-phenylethanol. Vanillin, a food additive, is mostly produced from guaiacol rather than from vanilla orchids (generally *Vanilla planifolia*) (less than 0.2%), but it can also be produced by ferulic acid from *Amycolatopsis* or *Streptomyces* species.

Capsaicin, another food additive, is produced by the same biosynthetic path of vanillin. Coumaric acid can be biotransformed into capsaicin using placenta cultures of *Capsicum frutescens*.

In a work carried out by Molina et al. (2013), fungal strains (e.g., *Penicillium* sp., *Aspergillus* sp.) were isolated from Brazilian fruits rich in terpenes and selected as biocatalysts for the bioconversion of citronellol into rose oxide, of limonene into carvone and α-terpineol, of geraniol into methyl-3-penten-1-ol and 6-methyl-5-hepten-2-one, and of linalool into linalool oxide, ocimenol, geraniol, and α-terpineol.

2-Phenylethanol can be obtained from phenylalanine using *Kluyveromyces marxianus* or *Saccharomyces cerevisiae*, or it can also be obtained from oleic acid using *S. cerevisiae*.

Benzaldehyde and γ-decalactone can also be obtained from biotransformation, the first from phenylalanine using *Ischnoderma* and the second from ricinoleic acid using *Candida sorbophila*, *Mucor circillenoides*, or *Yarrowia lipolytica*. Other γ-lactones besides γ-decalactone can also be obtained from fatty acid esters using *Mucor circillenoides* or from methyl ricinoleic acid using *Yarrowia lipolytica*.

Regarding the biotransformations involving enzymes, these are much less used than those involving whole cells systems. The yield of the process can be improved through encapsulation of the enzymes or immobilization of the enzyme on polymer, glass, or other supports.

The most interesting use of enzyme-catalyzed biotransformation in the case of phenolics and terpenoids is the liberation from their glycosylated forms, for example, vanillin can be liberated from glycovanollin by the action of commercial pectinase and cellulose.

Another approach for the obtainment of EOs or their volatile compounds is the genetic engineering of plant metabolic pathways in general and of terpenoids paths in particular. For instance, vanillin can be produced by *E. coli* carrying the 3-deoxyarabino-heptulosonic acid 7-phosphate synthase gene (Frost 2002).

6 Conclusion

It is consensual the numerous benefits of EOs due to their active biological properties, namely, antimicrobial and antioxidant activities. Therefore, it is of major importance the standardization of the production methods in order to guarantee their availability as well as their characteristics. The undesirable organoleptic effects of EOs can be minimized through a careful selection of the type of food. Moreover, synergetic effects between the components of formulations containing EOs shall be evaluated before becoming commercial applications. For instance, it has been reported synergism between carvacrol and its precursor *p*-cymene (Ultee et al. 2000) and between eugenol and cinnamaldehyde (Moleyar and Narasimham 1992). Special care shall be given to the use of EOs components applied to food for flavoring due to the need of accomplishing restricting regulations. The application of biotechnology to the production of EOs can greatly help to obtain them or their components at low prices and guarantee their constant availability. In the near future, it is expected the increase of the biotechnological production of EOs and their components.

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Biotechnological Production of Antistress Compounds: Current Status and Future Prospects

Sanghamitra Nayak, Asit Ray, Ambika Sahoo, Sudipta Jena, and Jeetendranath Patnaik

Abstract Stress and stress-related disorders are a major cause of diseases in modern times and contribute to around 75% of all illness. Recently antistress compounds (adaptogens) from plants have been gaining increasing attention due to their ability to attenuate stress effects. They regulate the immune response and the hormonal changes due to stress, thereby maintaining homeostasis. In the present review, some of the active constituents of adaptogenic plants, such as phenylpropanoid derivatives (rosin, rosavin, syringin, triandrin; salidroside, tyrosol), tetracyclic triterpenes (gingenosides, sitoindosides), and lignans (eleutherosides, schisandrin B), are discussed. Increasing demand for antistress compounds and their limited availability from natural resources due to overexploitation has necessitated their biotechnological production as an attractive alternative. This review summarizes various biotechnological approaches such as optimization of tissue culture conditions, elicitation, *Agrobacterium*-mediated transformation, and metabolic engineering using microbial systems for production of antistress compounds. Bioreactor cultures and various parameters that would enhance the biomass and accumulation of some antistress compounds are thoroughly discussed. Future research should focus on the identification of additional functional enzymes that are involved in their biosynthesis as well as product scale-up of antistress compounds for industrial applications.

Keywords Antistress compounds · Bioreactors · Biotransformation · Elicitor · Metabolic engineering · Plant cell culture

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1 Introduction

Depression is a serious, high-incidence, worldwide health condition. Approximately 300 million people are nearly affected worldwide due to mental disorders (WHO 2017). At its worst, depression can also lead to suicide. As per the World Health Organization (WHO) Global Burden of Disease Survey statistics, it is estimated that stress-related mental disorders would be the second major cause of disability by the year 2020 (Murray and Lopez 1996). A National Institute of Mental Health and Neurosciences survey estimates around 70 million Indians suffer diseases having physical symptoms originating from psychological or emotional causes, most common of which are stress (20%), anxiety (24.4%), and depression (18.5%) (Tripathi et al. 2019).

Current treatment regimens for treating depression are not ideally targeted in most cases. Although several synthetic antidepressants are presently available for the treatment of the mental disorder, they suffer severe drawbacks such as adverse reactions, poor tolerance, and a higher risk of relapse. Therefore, there is a clear need for therapeutic drugs which provide higher efficacy and better tolerance and have low toxicity. Numerous studies have demonstrated the use of herbal medicine for treating depression-related disorders in psychiatric patients, thereby signifying the need for alternative treatments other than pharmacotherapy and psychotherapy (Qureshi and AI-Bedah 2013).

Antistress compounds also known as adaptogens are natural herbal constituents known to improve the adaptability and survival of organisms under stress and tolerance of the body, not only against mental and behavioral disorders such as depression, anxiety, stress-induced chronic fatigue, bipolar disorders, etc. but also for aging-associated disorders including neurodegeneration, atherosclerosis, diabetes, heart diseases, high blood pressure, chronic inflammation, osteoarthritis, etc. They respond to stress stimuli by maintaining homeostasis and modulating the biosynthesis of eicosanoids. Numerous clinical trials suggest that these compounds exert an antistress effect that increases attention and endurance in stress and fatigue and reduces stress-related disorders of the immune system (Panossian and Wikman 2009). The mediators of the adaptive stress response and longevity signaling pathways (ASRSPs) have been reported to be positively influenced by certain adaptogenic plant extracts, while at the same time these adaptogens significantly affect the expression of genes encoding neurohormones and a whole range of other transmembrane receptors.

Research studies have shown that these antistress compounds are proven to be safe in acute and subacute toxicity studies by the US Food and Drug Administration (FDA). Another survey reported that nearly 53.6% of people suffering from self-reported depression in the United States used herbal medicines to treat depression (Kessler et al. 2001). More recent reports throw light on how the adaptogens impact stimulation and release of stress hormones, namely, NPY and Hsp72, into systemic circulation during innate defense response against mild stressors (ADAPT-232), which increase tolerance and adaptation to stress (Panossian et al. 2012). Particular emphasis has been placed on the effects of adaptogens on extracellular and intracellular communication mediators responsible for maintaining homeostasis of the neuroendocrine-immune complex (stress system) while activating the expression of stress-activated proteins and neuropeptides (Tripathi et al. 2019).

As a result, there is a global resurgence in the trade of antistress compounds, thereby necessitating in a stable raw material supply of the drug from natural sources. The extensive demand for antistress compounds greatly exceeds the supply through isolation from plant sources, and substitute ways need to be sought for the production of these antistress compounds from medicinal plants. Further due to the intensive collection, natural populations of the plant are dwindling from the wild, and it has become difficult to obtain the raw material in sufficient amounts to meet the increasing demands. Moreover, occurrence of secondary metabolites is quite species specific, and distribution of these plant species is limited to a particular geographical region for which obtaining a huge amount of natural resources of the particular plant is highly difficult. Despite the promises all the available biotechnology tools hold for metabolomics studies with respect to industrial production of plant bioactive compounds, very little is explored up to industrial scale. Additionally, the spatial-temporal regulation of metabolite production and storage in plants is gene or tissue specific. Moreover chemical synthesis of these complex natural compounds by synthetic approach is also not economically viable.

Considering all the above challenges, it is necessary to increase the production yield of these compounds in native plant species as well as to develop an alternative system for its production. Plant cell and organ cultures are being used as alternatives for the production of value-added bioactive compounds (Wu et al. 2007; Paek et al. 2009; Thanh et al. 2014). They offer the following benefits: easy extraction and purification techniques free from interfering matrices, non-dependence on environmental and edaphic factors, manipulation of biosynthetic routes for getting the desired bioactive compound, less production time, easy to fulfill the industry GLP and GMP regulatory requirements, and shunning hazardous restrictions against potential introduction of genetically modified organism into the natural ecosystem. Apart from use of many abiotic and biotic elicitors, bioprocessing pathway elucidation has been used as a useful strategy to trigger the production of bioactive compounds, in vitro (Gandhi et al. 2015; Saito 2018). Synthetic biology approaches such as the use of microbial or yeast heterologous hosts have emerged as a promising biological platform for improving the biotechnological production of these compounds in the future. In this chapter, we provide a comprehensive and systemic overview of existing and emerging biotechnological approaches such as tissue culture, elicitors, transgenic plants, and metabolically engineered microbial cells for improving the yield of antistress compounds. The various strategies adopted for scale-up production of antistress compounds such as the use of bioreactors are also elucidated in this chapter.

2 Chemical Classes of Antistress Compounds

Chemically, the antistress compounds are complex phenolics, tetracyclic triterpenoids, and oxylipins (Panossian et al. 2007; Ray et al. 2016) derived from plants. The phenolic compounds usually include phenylpropanoids glycosides, phenylethane derivatives such as rosin, rosavin, syringin, triandrin, salidroside, tyrosol, and lignans such as eleutheroside E and schisandrin B (Fig. 1). These compounds are

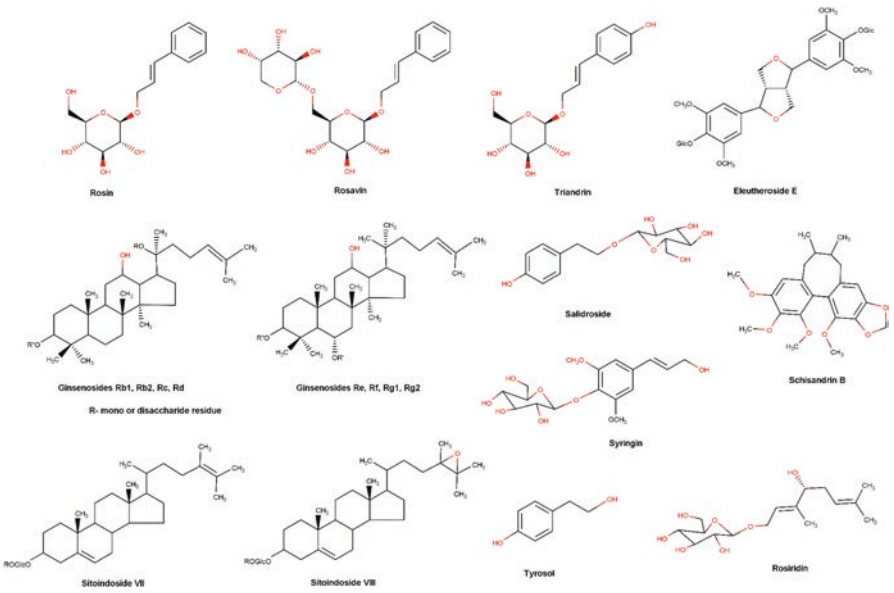

Fig. 1 Major antistress compounds isolated from plant

abundantly found in plants, namely, *Eleutherococcus senticosus*, *Rhodiola rosea*, and *Schisandra chinensis*. The antistress activities of phenylpropanoid derivatives of *R. rosea* were attributed to its effect on the levels and activity of monoamines and opioid peptides, such as β-endorphins (Kelly 2001). Salidroside, another phenylpropanoid glycoside, exhibits antidepressant mechanisms by inducing pro-inflammatory cytokine and regulating hypothalamic-pituitary-adrenal axis in rats (Yang et al. 2014). Tyrosol, a phenylethanoid derivative, shows a significant increase in phosphorylation of Akt, eNOS, and FOXO3a, key molecular targets involved against ischemia injury, while schisandrin B extracted from the fruit of *Schisandra chinensis* exerts neuroprotective activity by expressing heat shock proteins Hsp70. Another study showed that treatment with schisandrin B could improve the mitochondria antioxidant status of the brain and protect against cerebral damage in rats (Chen et al. 2008).

The tetracyclic triterpenes compounds such as cucurbitacin R diglucoside, ginsenosides, sitoindosides, and withanosides have structural similarity with corticosteroids and are associated with those found in plants like *Panax ginseng* and *Withania somnifera*. Ginsenosides are major constituents present in ginseng species such as *Panax ginseng* and *Panax quinquefolius* and are categorized broadly into two classes based on carbon aglycone moiety: dammarane family (four-ring structure) and the oleanane family. The dammarane-type ginsenosides contain three groups of compounds: protopanaxadiols, protopanaxatriols, and ocotillols. Ginsenosides Re, Rf, Rg1, and Rg2 belong to protopanaxatriol group, whereas ginsenosides Rb1, Rb2, Rb3, Rc, and Rd belong to protopanaxadiol group.

Acylsterylglucosides such as sitoindoside VII and sitoindoside VIII isolated from roots of *Withania somnifera* exhibit antistress activity (Bhattacharya et al. 1987), while glycolwithanolides like sitoindoside IX and sitoindosides X display immunomodulatory and antistress activity by increasing the learning ability and memory retention in albino rats (Ghosal et al. 1989).

3 Biotechnological Approaches for Production of Antistress Compounds

3.1 Optimization of In Vitro Culture Conditions

The production of secondary metabolites in tissue cultures can occur via two phases, i.e., biomass production and bioactive compounds synthesis, and both these phases need to be optimized independently for enhanced production of compounds (Murthy et al. 2014a; Isah et al. 2018). Optimization of the culture medium is an imperative step for enhancement of target bioactive compounds which leads to the maximum biomass accumulation and productivity (Isah et al. 2018). Several studies have been extensively carried out on the optimization of culture condition and media composition for the production of plant secondary metabolites (Wang et al. 2001; Wu et al. 2006; Satdive et al. 2007; Cui et al. 2010). In this chapter, various parameters on the accumulation of several antistress compounds have been discussed.

The physical factors such as light and temperature are known to increase the growth and metabolites productivity of the in vitro cultures (Yang et al. 2018). Park et al. (2012) reported that the roots of *P. ginseng* treated with 450 and 470 nm light emitted from light-emitting diodes (LED) resulted in the increase of 64.9% and 74.1% of ginsenoside, respectively, as compared to control. The red light can stimulate the accumulation of antistress compound like eleutheroside E in *Eleutherococcus senticosus* (Shohael et al. 2006), whereas blue light enhanced the production of schisandrin B in *Schisandra chinensis* (Szopa and Ekiert 2016). A study by Yu et al. (2005a, b) showed the effect of temperature on biomass accumulation and enhancement of ginsenoside in hairy roots of *P. ginseng* grown in bioreactors. Ginsenoside production was highest when the culture was incubated at 25°C as the optimum temperature for most of the strains are in the range of 24–28°C (Wu and Zhong 1999).

Biotechnological approaches such as cell culture and callus cultures have been successfully applied for the production of several antistress compounds like ginsenoside, salidroside, and eleutherosides (Park et al. 2005; Jeong et al. 2009; Bai et al. 2014). Similarly, production of antistress compounds has been successfully reported in plant cell suspension culture system in species like *Panax ginseng* (Wang et al. 2013), *Panax notoginseng* (Hu et al. 2007), *Eleutherococcus sessiliflorus* (Shohael et al. 2008), and *Eleutherococcus senticosus* (Shohael et al. 2014) (Table 1). Salidroside has been produced by plant cell cultures in different medici-

Table 1 Production of antistress compounds using plant cell suspension culture

Compound	Plant species	Culture condition			Reference
		Photoperiod	Medium	Culture type	
Eleutheroside E	*Eleutherococcus senticosus*	16/8 h (day/night)	MS medium + 1 mg/L 2,4-D + 3% sucrose	Bioreactor	Shohael et al. (2006)
Eleutherosides	*Eleutherococcus sessiliflorus*	Darkness	MS medium + 1 mg/L 2,4-D	Bioreactor	Shohael et al. (2005)
Eleutherosides E and B	*Eleutherococcus senticosus*	Darkness	MS medium + 1 mg/L 2,4-D +30% sucrose	Bioreactors	Shohael et al. (2014)
Eleutherosides E and B	*Eleutherococcus chiisanensis*	16/8 h (day/night)	MS medium + 4.5 μM 2,4-D + 30 g/L sucrose	Bioreactor	Jeong et al. (2005b)
Ginsenoside	*Panax ginseng*	Darkness	MS medium + 4.52 μM 2,4-D + 10 % coconut water	Bioreactor	Langhansova et al. (2005)
Ginsenoside	*Panax notoginseng*	Darkness	MS medium +2 mg/L 2,4-D + 0.7 mg/L kinetin + 60 mM KNO_3	Shake flask	Zhang et al. (1996)
Salidroside	*Rhodiola sachalinensis*	Darkness	MS medium + 3% sucrose + 0.3 mg/L NAA + 3 mg/L BAP	Bioreactor	Jianfeng et al. (1998)
Salidroside	*Rhodiola sachalinensis*	Darkness	$2B_5$ liquid medium +0.5 mg/L NAA+ 1 mg/L BA	Shake flask	Kim et al. (2004)
Salidroside	*Rhodiola sachalinensis*	16/8 h (day/night)	MS medium + 5 mg/L BA + 2.5 mg/L IBA	Gyratory shaker	Wu et al. (2003)
Salidroside	*Rhodiola sachalinensis*	Darkness	MS medium + 40 g/L sucrose+ 1.5 μM NAA + 15 μM BA	Shake flask	Xu et al. (1998)

nal plants such as *R. quadrifida* (Sheng et al. 2005), *R. sachalinensis* (Wu et al. 2003), and *R. kirilowii* (Krajewska-Patan et al. 2009). Similarly, the successful production and enhancement of ginsenoside content have also been established in different *Panax* species such as *Panax japonicus* (Kochan and Chmiel 2011; Kochkin et al. 2013) and *Panax quinquefolius* (Szymańska et al. 2013; Biswas et al. 2019).

The different combinations and concentrations of plant hormones in the culture medium are responsible for the cell growth, differentiation, and production of bio-

active compounds under in vitro condition (Shi et al. 2013; Park et al. 2019). Zhou et al. (2017b) studied that the MS medium fortified with BA (1.0 mg/L) and 2,4-D (0.3 mg/L) was found to be suitable for the induction of callus, whereas MS liquid medium containing BA (1.0 mg/L), 2,4-D (0.2 mg/L), and KN (0.5 mg/L) was optimal culture condition for schisandrin B production through suspension cells. However, the MS medium supplemented with 0.5 mg/L TDZ (thidiazuron) and 0.5 mg/L NAA was found to be the most effective combination of growth regulator for the production of salidroside and its aglycone tyrosol through callus subculture and subsequent cell suspension culture in *Rhodiola crenulata* (Shi et al. 2013).

3.2 Elicitation

Elicitors can be used to activate the pathway of secondary metabolism and promote the biosynthesis of target antistress compounds. It can be categorized into biotic elicitor components such as chitin, chitosan, and abiotic elicitors such as metal ions, UV radiation, and inorganic compounds (Karuppusamy 2009; Coste et al. 2011; Chen et al. 2001). Elicitation is a possible way of enhancing the production of commercially important secondary metabolites from plant by improving their survival in adverse condition (Narayani and Srivastava 2017). In an attempt to enhance the productivity of secondary metabolites, different biotic and abiotic elicitors have been extensively used in the hairy root and plant cell cultures of several medicinal plants (Thakur et al. 2018; Safaeizadeh and Boller 2019; Pinneh et al. 2019). Signaling molecules such as methyl jasmonate and salicylic acid have been extensively used for the enhancement of secondary metabolites in cell and organ cultures (Shabani et al. 2009; Zare-Hassani et al. 2019; Gai et al. 2019). Methyl jasmonate promoted the accumulation of eleutherosides in the embryonic suspension culture of *Eleutherococcus senticosus* (Shohael et al. 2007).

Methyl jasmonate is known to be strong and effective elicitor stimulating biosynthesis of several antistress compounds such as ginsenosides in *Panax ginseng* (Wang et al. 2005; Bae et al. 2006; Kim et al. 2009; Paek et al. 2009; Murthy et al. 2014a), pinoresinol in *Forsythia x intermedia* (Schmitt and Petersen 2002), and eleutherosides in *Eleutherococcus senticosus* (Shohael et al. 2007). The combination of salicylic acid and methyl jasmonate significantly improved the production of ginsenoside in adventitious root of *P. ginseng* (Ali et al. 2006b).

Yeast extract elicited the production of ginsenosides and schisandrin B in adventitious root of *Panax ginseng* (Marsik et al. 2014) and also in microshoot culture of *Schisandra chinensis* (Szopa et al. 2018). Another elicitor chitosan has been used to stimulate the ginsenoside production in *Panax ginseng* (Jeong and Park 2005). The abiotic elicitors such as NO and $AgNO_3$ increased the accumulation of salidroside in *Rhodiola sachalinensis* during cell suspension culture (Ai et al. 2009). Several studies exhibited remarkable increase in antistress compound production through the induction of elicitors as summarized in Table 2.

Table 2 Elicitors used to enhance the production of antistress compounds in plant cell culture system

Compound	Plant species	Elicitor	Fold increase	Reference
Ginsenoside	*Panax ginseng*	Organic geranium (60 mg/L)	1.33	Yu et al. (2005a, b)
Ginsenoside	*Panax ginseng*	Jasmonic acid (2 mg/L)	1.52	Yu et al. (2002)
Ginsenoside	*Panax ginseng*	Vanadate (50 μM)	3.6	Huang and Zhong 2013
Ginsenoside	*Panax ginseng*	Copper (25 μM)	1.53	Ali et al. (2006a)
Ginsenoside	*Panax ginseng*	Dicyclohexylcarbodiimide (10 M)	2.96	Huang et al. (2013)
Ginsenoside	*Panax quinquefolius*	*Alternaria panax*	3.2	Yu et al. (2016)
Syringin	*Saussurea medusa*	Yeast extract (1.5%), chitosan (0.2 g/L), and Ag^{+} (75 μM)	2.16	Xu et al. (2007)
Salidroside	*Rhodiola sachalinensis*	Methyl jasmonate (200 μM)	1.32	Wang et al. (2012)
Salidroside	*Rhodiola sachalinensis*	*Aspergillus niger*	5	Xu et al. (1998)
Eleutherosides B and E	*Eleutherococcus sessiliflorus*	Methyl jasmonate (200 μM)	2.83	Shohael et al. (2008)

3.3 Agrobacterium-*Mediated Genetic Transformation*

Among different tissue culture methods, hairy root culture is frequently used for enhancing secondary metabolite production because of their high degree of cellular differentiation and enriched biochemical or genetic stability. *Agrobacterium rhizogenes*-mediated transformation has been widely used for accomplishing a transformed roots induction. Thousands of plant species have been successfully transformed using *A. rhizogenes* for production of bioactives (Giri et al. 2001; Yan et al. 2006; Lee et al. 2007). In this chapter, various reports on the production of antistress compounds using hairy roots have been covered.

A. rhizogenes have been used to transform several *Rhodiola* spp. for production of adaptogens. In *Rhodiola kirilowii* hairy roots, a high rosavin level (505 ± 106 mg/L) was reported when supplemented with cinnamyl alcohol and sucrose in the 1st and 14th day of culture, respectively (Grech-Baran et al. 2014). Yu et al. (2011) observed overexpression of two UDP-glycosyltransferases (UGT72B14 and UGT74R1) in *R. sachalinensis* hairy root cultures lines which resulted in the highest production of salidroside. The overexpression of UGT74R1, UGT73B6, and UGT72B14 genes in hairy root culture of *R. sachalinensis* produced about 5.72, 8.76, and 19.81 mg/g DW of salidrosides, respectively (Ma et al. 2007).

Salidroside has also been successfully produced in hairy roots of other species like *Rhodiola kirilowii* (Grech-Baran et al. 2013), *Rhodiola crenulata* (Lan et al. 2013), and *Rhodiola sachalinensis* (Zhou et al. 2007). Fu et al. (2005) transformed *Saussurea involucrate* by three *A. rhizogenes* strains like R1000, R1601, and LBA9402. The transformed roots produced 43.5±1.13 (50-fold higher than wild plants), 39.7±1.37, and 32.5±3.08 mg/g DW of syringin (eleutheroside B) in hairy root lines like HR1601-1, HRLBA9402-1, and HR1000-1, respectively. Further, in *Saussurea involucrate* hairy root, 43.5±1.13 mg/g DW of syringin was produced which is about 40 times higher in comparison to that of wild plants (Fu et al. 2006).

Overexpression of *rol*C gene in *Panax ginseng* transgenic root cultures produced >6% ginsenosides on the dry weight basis (Bulgakov 2008). Being the important gene for production of ginsenoside and suppressor of cycloartenol synthase (CAS), overexpression of dammarenediol synthase (DDS) led 50–100% rise of ginsenoside yield in *P. ginseng* hairy roots (Liang et al. 2009). Overexpression of PgFPS (farnesyl pyrophosphate synthase) gene in *P. ginseng* hairy roots produced 36.42 mg/g DW of total ginsenoside, representing a 2.4-fold hike compared to the control (Kim et al. 2014). Gingenosides have also been successfully produced by hairy roots of several *Panax* species such as *P. ginseng* (Yu et al. 2000; Mallol et al. 2001; Jeong et al. 2005a; Kim et al. 2009b; Liang et al. 2015), *P. quinquefolius* (Mathur et al. 2010; Kochan et al. 2012), and *P. japonicas* (Zhang et al. 2011). RNAi-mediated downregulation of β-amyrin synthase gene in *P. ginseng* hairy roots resulted in the reduced level of oleanane and β-amyrin-type ginsenoside and increased level of dammarane-type ginsenoside leading to increase in total ginsenoside content (Zhao et al. 2015).

Supplementation of extracellular precursor coniferaldehyde to *Linum album* hairy root culture enhanced the accumulation of pinoresinol, about 8.7-fold (8.7 μg/g fresh weight) (Ahmadian Chashmi et al. 2016). Sykłowska-Baranek et al. (2018) studied the accumulation of pinoresinol in two different cultures of KT and ATMA hairy root lines of *Taxus*. They reported that in ATMA hairy roots, pinoresinol was present mostly as their aglyca, whereas in KT hairy roots, pinoresinol predominantly identified as glucosides.

3.4 Metabolic Engineering Approach

Metabolic engineering approach provides new perspectives in understanding the expression profiles of secondary metabolite biosynthetic pathway genes via their overexpression, thus in turn leading to the modification of biosynthetic pathways (O'Connor 2015). It is gaining momentum for production of numerous pharmaceutically active molecules outside the plant system, using microorganisms. Microbial biotransformation provides great opportunities for production of the bioactive compounds in comparison to their extraction from natural sources or their chemical synthesis. In the case of biosynthesis of adaptogens, metabolic engineering has been effectively applied for improving the biosynthetic process (Table 3).

Table 3 Selected examples for production of antistress compounds by metabolic engineering approaches

Species	Strategies	Induced gene	Compound	Amount produced	Reference
Escherichia coli	Heterologous expression (aglycone strain+ glycone strain)	–	Salidroside	6.03 g/L	Liu et al. (2018)
Saccharomyces cerevisiae	Heterologous expression	Aromatic aldehyde synthase (AAS) from *P. crispum* and UDP-glycosyltransferases	Tyrosol Salidroside	965.4 mg/L 239.5 mg/L	Jiang et al. (2018)
Escherichia coli	Recombinant strain for production of hydroxytyrosol and hydroxysalidroside	Tyrosine decarboxylase from *P. somniferum*, tyrosine oxidase from *M. luteus*, and 4-hydroxyphenylacetate 3-monooxygenase from *E. coli* and UGT	Hydroxytyrosol Hydroxysalidroside	~ 268.3 mg/L ~134.15mg/L	Choo et al. (2018)
Saccharomyces cerevisiae (S288C yeast strain BG4)	Recombinant strain	*ARO4*^{K229L}, *ARO7*^{T266I}, *TYR1*, and *ARO10* from yeast and OsUGT13 from *Oryza sativa*	Salidroside	640 mg/L	Kallscheuer et al. (2019)
Corynebacterium glutamicum	Recombinant strain	Aro10 from *S. cerevisiae*, YqhD from *E. coli*, MalE-OsUGT13 fusion protein	Salidroside	150 mg/L	Kallscheuer et al. (2019)
Rhodiola crenulata	Transgenic plant (engineered biosynthetic pathway in hairy root)	RcTYDC and RcUDPGT	Tyrosol Salidroside	1.50–2.19-fold 1.27–3.47-fold	Lan et al. (2013)
Nicotiana benthamiana	Transgenic expression of *Rhodiola* salidroside biosynthetic genes	RrT8GT and Rr4HPAAS from *Rhodiola rosea*	Salidroside	2% DW	Torrens-Spence et al. (2018)
Saccharomyces cerevisiae	Transgenic expression of *Rhodiola* salidroside biosynthetic genes	Codon optimized coRr4HPAAS and coRrT8GT from *Rhodiola rosea*, ARO4 K229L and ARO7 G141S of yeast	Salidroside	1.5 mg/L	Torrens-Spence et al. (2018)

Arabidopsis thaliana	Transgenic overexpression	UGT72E3/2 (recombinant plant glycosyltransferase), F5H (sinapyl alcohol synthesis pathway gene), and MYB58 (lignin biosynthesis transcriptional activator)	Syringin	56-fold high	Chu et al. (2014)
Glycine max L.	Modified half-seed method (β-UGT72E3/2 and 35S-UGT72E3/2 transgenic plant)	Chimeric protein UGT72E3/2 from *A. thaliana* under control of CaMV-35S or promotor β-conglycinin	Syringin Coniferin	0.15 μmol/g 0.29 μmol/g	Kwon et al. (2017)
Escherichia coli	Engineering of shikimate pathway genes (feaB deletion)	ARO10, ARO8 Overexpression	Tyrosol	8.71 mM	Xue et al. (2017)
Escherichia coli	Engineering of shikimate pathway genes (aroG, tyrA, ppsA, aroE, aroD, aroB overexpression; tyrR, pykA, pykF, phe A deletion)	Aro10	Tyrosol	6.71 mM	Bai et al. (2014)
Escherichia coli	TyrR, PheA, feaB deletion	AAS overexpression	Tyrosol	3.77 mM	Chung et al. (2017)
Escherichia coli BFPE2	Co-expression of genes in engineered strain	*aroA* and *tyrA*	Tyrosol	5.72 mM	Yang et al. (2019)
Escherichia coli BFPG1	Co-expression of genes in engineered strain	*aorG*, aorB, aorD, aorE, aorK/L. aorA, aorC, tyrA*, aor10, and yahK*	Tyrosol	5.8 mM	Yang et al. (2019)

Heterologous expression of rDNA facilitates quick production of desirable proteins, thus playing a vital role in modern functional genomics. It has been extensively used as a sustainable approach for the production of bioactive compounds having several industrial values with higher efficiency. Heterologous expression of secondary metabolite biosynthetic pathways in cell factories or model systems accelerates product identification, elucidation, as well as production. Several antistress compounds like ginsenosides, salidroside, rosin, rosavin, tyrosol, and syringin have been produced by heterologous expression of the gene of interest in bacteria and yeast as well as in plant systems (Bai et al. 2014; Wang et al. 2015; Ku et al. 2016; Chung et al. 2017; Kallscheuer et al. 2019; Han et al. 2019).

Zhou et al. (2017a) engineered *E. coli* recombinant strain by transferring UGT73C5 and UGT73B6 gene from *Arabidopsis thaliana* and *Rhodiola sachalinensis*, respectively. Optimized phenylalanine pathway of *E. coli* produced 258.5 ± 8.8 mg/L of rosin. Seven rosavin analogues were produced from glucose by engineered *E. coli* (Bi et al. 2019). Fed-batch fermentation of the strain producing rosavin B resulted in 4.7 g/L of rosavin B. Bai et al. (2014) metabolically engineered *E. coli* by introducing endogenous alcohol dehydrogenases, yeast pyruvate decarboxylase ARO10, and glycosyltransferase-UGT73B6 (*Rhodiola*-derived) for efficient production of tyrosol and salidroside (56.9 mg/L). Xue et al. (2016) actively produced salidroside by expressing UGT72B14 gene of *Rhodiola* in *E. coli* and optimizing the codon accordingly. Chung et al. (2017) produced three phenylethanoids like hydroxytyrosol, tyrosol, and salidroside by using engineered *E. coli*. Tyrosol (531 mg/L) was produced by transferring aromatic aldehyde synthase (*AAS*) to *E. coli*. Expression of *HpaBC* gene along with *AAS* in *E. coli* resulted 208 mg/L of hydroxytyrosol. Salidroside was synthesized by the engineered *E. coli* having *AAS* and UGT85A1 gene with an optimum concentration of 288 mg/L. Hydroxytyrosol and hydroxysalidroside have been produced in an engineered *E. coli* which converted 50% of hydroxytyrosol to hydroxysalidroside (Choo et al. 2018). FeaB-knockout mutant *E. coli* with pBbS1a-2 plasmid accumulated 1.22 mM tyrosol by converting tyrosine (1mM) (Satoh et al. 2012). This engineered *E. coli* was able to produce tyrosol by utilizing both supplemented and central metabolic tyrosine. Gou et al. (2019) engineered the *S. cerevisiae* chorismate-related pathways by heterologous expression of *E. coli* mutant EcTyrA$^{M53I/A354V}$. The engineered strain GFT-4 in turn produced 126.74±6.70 mg/g DW tyrosol at 48 h (440 times in comparison to wild type). Ginsenoside Rh2 was produced in *S. cerevisiae* by modifying the mevalonate pathway and optimizing P450 expression level (Wang et al. 2019). Both natural and unnatural ginsenosides were produced by utilizing UGT-SuSy cascade reaction in *Bacillus subtilis* 168 (Dai et al. 2018). Production of ginsenoside by employing metabolic engineering approach has also been reported by several researchers (Kim et al. 2015; Biswas et al. 2017).

Overexpression of secondary metabolite biosynthetic pathway genes can lead to suppressed or increased production of the desired bioactive compounds. Overexpression of early genes of the pathway can divert the metabolic flux toward the enhanced production of metabolites of interest leading to their overproduction.

Various experiments have been accomplished comprising the overexpression of numerous adaptogen biosynthetic pathway genes, aiming to produce adaptogens in inside as well as outside the plant cell. Several antistress compounds like ginsenosides, salidroside, rosin, rosavin, tyrosol, and syringin have been produced by overexpression of the gene of interest in bacteria and yeast as well as in plant systems (Landtag et al. 2002; Ma et al. 2007; Shim et al. 2010; Han et al. 2013).

A. tumefaciens-mediated transformation has already been performed in *R. sachalinensis* by overexpression of salidroside biosynthesis pathway genes (Ma et al. 2007, Ma et al. 2008, Yu et al. 2011, Zhang et al. 2011). Chu et al. (2014) developed a transgenic *Arabidopsis thaliana* plant, in which overexpression of three different genes like UGT72E3/2, F5H, and MYB58 in leaves accumulated 56-fold higher level of syringin (eleutheroside B) than wild plants. Pinoresinol and its diglucoside have been produced by Zhang et al. (2016) by adding substrates like glucose, phenylalanine, cinnamic acid, leucine, tyrosine, and p-coumaric acid to phenyl propanoid pathway of *Phomopsis* sp. XP-8. A transgenic cell line was developed by overexpression of RNAi construct of PLR gene in *Forsythia* (Kim et al. 2009a). Downregulation of the gene led to the production of 20-fold higher pinoresinol glucoside compared to wild type. Co-expression of *PLR*–RNAi and *CYP81Q1* in transgenic *Forsythia* cell revealed production of sesamin along with pinoresinol glucoside.

4 Scale-Up Production Using Bioreactors

The primary aim of biotechnology is large-scale production of active compounds for industrial applications. Most of the published literature deals with the increased production yield of these compounds on a laboratory scale using shake flask culture systems which is not feasible for commercial exploitation of these compounds at industrial scale (Krajewska-Patan et al. 2007; Kosturkova 2010). Advances in scale-up approaches such as the use of bioreactors and immobilization techniques have led to a considerable increase in the biomass concentration of these compounds and have elicited much attention. Scaling up in large-scale bioreactors is not relatively easy as plant cells have relatively unstable productivity, a high shear sensitivity, and a slow cell growth rate, so it is necessary to choose a proper bioreactor which can have adequate oxygen transfer, limited shear, and good mixing rate (Georgiev et al. 2013).

The scaling up comprises use of bioreactors with varying sizes and features, the most popular being the stirred tank, balloon-type bubble, and vertical airlift bioreactors used for the production of antistress compounds such as ginsenosides, eleutherosides, and salidroside (Park et al. 2005; Lee and Paek 2012; Wang et al. 2012; Li et al. 2014). Commercial, temporary immersion systems such as RITA and Plantform bioreactor system were used for production of schisandrin from shoot culture of *Schisandra chinensis* (Szopa et al. 2017). Shohael et al. (2005) also used temporary immersion system (ebb and flood) for production of eleutherosides from

somatic embryos of *Eleutherococcus sessiliflorus*. The scale-up study of *Eleutherococcus senticosus* demonstrated the influence of different light radiation on the production of eleutherosides (Shohael et al. 2006).

Some of the successful application of scale-up process of antistress compounds is the production of ginseng by Nitto Denko Corporation (Japan) (Sharma et al. 2014). Mitsui Petrochemical industry in Japan used submerged fermentation process for commercial production of ginseng in large bioreactor vessels of 4000–20000 liter capacity sizes. CBN Biotech company, South Korea, has scale-up production of ginsenosides using ginseng adventitious roots using 10,000 liter airlift pilot bioreactor vessel (Murthy et al. 2014b). Hopefully, some of the listed antistress compounds in Table 4 used for the pilot-scale production will finally result in commercial implementation.

5 Conclusion and Future Prospective

Growing demand for antistress compounds in medical applications faced with a challenge of low product yields has necessitated their biotechnological production as a viable alternative. The present chapter comprises updated reports on various, efficient methods for biotechnological production of major classes of adaptogenic compounds such as phenylethane derivatives, lignans, and tetracyclic triterpenes. Biotechnological approaches such as optimization of tissue culture conditions, elicitation, *Agrobacterium*-mediated genetic transformation, and engineered microbial cells hold great promise to increase the production of antistress compounds.

Biotechnological production of antistress compounds has been achieved through different methods with varying degree of success. Cell suspension culture method has been optimized for in vitro production of several antistress compounds such as ginsenosides, salidroside, tyrosol, etc. Elicitors such as methyl jasmonate, salicylic acid, yeast extracts, and chitosan have been effectively used for biotechnological production of several antistress compounds. *Agrobacterium*-mediated genetic transformation is a promising alternative for biosynthesis of antistress compounds and needs to be optimized for large-scale production through bioreactors. The scale-up study comprises the use of bioreactors of varying sizes and features, the most popular being the stirred tank, balloon-type bubble, and airlift bioreactors for production of antistress compounds like ginsenosides, eleutherosides, and salidroside. Similarly, biotechnological production of antistress compounds should be explored through metabolic engineering approaches by overexpressing the genes present in biosynthetic pathway in fast-growing microorganism.

In spite of several reports available on in vitro production of antistress compounds, so far, only a few reports have highlighted the practical application of viable biotechnology tools for production of antistress products such as ginsenosides, salidroside, and tyrosol on a commercial scale. Cost-effectiveness of different available methods should be worked out to establish more suitable ones for commercial production. Proper scale-up studies should be carried out by choosing appropriate

Table 4 Enhanced production of antistress compounds in plant cell culture using bioreactor

Compound	Bioreactor	Culture	Fresh weight (g fw L^{-1})	Dry weight (g fw L^{-1})	Maximum yield	Growth ratio[a]	Reference
Ginsenosides	Cone-type bubble bioreactor	Adventitious root culture	366.8	31.92	Ginsenoside yield 106.61 mg/L	30.4	Sivakumar et al. (2005)
Ginsenosides	Internal turbine bioreactors	Embryogenic culture	80	14.5	Total ginsenoside content 4.53 mg/g	1.8	Asaka et al. (1993)
Ginsenosides	Wave bioreactor	Hairy root culture	284.9	46.4	Ginsenoside content of 145.6 mg/L	28	Palazon et al. (2003)
Eleutherosides	Balloon-type bubble bioreactor	Suspension culture of somatic embryos	102.3	11.3	Eleutheroside B content (327 μg/L) and eleutheroside E content (533.9 μg/L)	17	Shohael et al. (2014)
	Bulb-type bioreactor		98.6	11.3	Eleutheroside B content (194 μg/L) and eleutheroside E content (550 μg/L)	16.9	
	Cone-type bioreactor		85	9.8	Eleutheroside B content (223 μg/L) and eleutheroside E content (418 μg/L)	14.5	
	Cylinder-type bioreactor		73.8	8.2	Eleutheroside B content (164 μg/L) and eleutheroside E content (291 μg/L)	12	
Eleutherosides	Airlift bioreactors	Adventitious root culture	40.64	4.70	Eleutheroside B content (2329.67 μg/L)	-	Lee et al. (2015)

(continued)

Table 4 (continued)

Compound	Bioreactor	Culture	Fresh weight (g fw L^{-1})	Dry weight (g fw L^{-1})	Maximum yield	Growth ratio[a]	Reference
Schisandrin	Plantform temporary immersion system	Microshoot culture	118	7	Schisandrin content 186.8 mg/L	-	Szopa et al. (2017)
Salidroside	Balloon-type airlift bioreactor	Callus culture	286.9	14.5	Salidroside content of 7.90 mg/L	27.7	Li et al. (2014)

[a]Growth ratio = [harvested FW (g) - inoculated FW (g)/inoculated FW (g)]

bioreactors for commercial viability of biotechnology-based antistress compounds. To overcome the existing constraints in biotechnological production of antistress compounds, future research should focus on the integration of primary and secondary biosynthetic pathways with more fine-tuning and balancing of gene expression of different antistress compounds. Product scale-up should be emphasized in all methods of biotechnological production for their viable commercial application.

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Elicitors as a Biotechnological Tool for In Vitro Production of Bioactive Phenolic Compounds

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Abstract Phenolic compounds are important secondary metabolites produced in plants and include flavonoids, biflavonoids, phenolic acids, tannins, stilbenes, coumarins, and lignans. Several studies describe the beneficial effects of phenolics on human health, which include antimicrobial, antitumor, antioxidant, anti-inflammatory, and antiallergenic activities. For plants, phenolic compounds are involved in the defense response against pathogen and herbivore attacks. In vitro production of bioactive compounds using plant cell and tissue culture is a real possibility. This biotechnological technique allows the manipulation of biosynthetic routes, which can lead to increases in the production and accumulation of secondary metabolites. Elicitation is an effective and viable tool that is used to induce physiological changes in plants and may stimulate the accumulation endogenous compounds, or the induction of the de novo synthesis of bioactive metabolites, including phenolic compounds. Methyl jasmonate, salicylic acid, chitosan, yeast extracts, salts, and light are some elicitors that regulate a large number of biochemical control points and are capable of inducing a series of signal transduction processes in plants.

Keywords Elicitation · Phenols · In vitro culture · Biological activity · Medicinal plants

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1 Introduction

Plants may produce secondary metabolites that contribute to the adaption of the cell to the surrounding environment and provide defense against bacteria, fungi, and viruses (Baenas et al. 2014). These metabolites also are of particular interest to humans, because they function as health-promoting compounds and are used as sources for pharmaceuticals, food additives, flavors, and other industrial materials (Zhao et al. 2005). However, the availability of many bioactive metabolites is limited by seasonal and environmental variation, soil type, and plant age (Cherdshewasart et al. 2007). Additionally, chemical synthesis is often impractical due to the structural complexity and specific stereochemical requirements of many metabolites (Namdeo 2007; Oksman and Inzé 2004). Plant cell culture has been used as a promising alternative for producing secondary metabolites that are difficult to be obtained by either chemical synthesis or extraction from plant materials (Namdeo 2007; Zhao et al. 2005). However, low yields for the extraction of secondary metabolites have been one of the main biological and biotechnological limitations of this technique (Zhao et al. 2005).

Some biotechnological strategies based mainly on the role of secondary metabolites in plants have been developed to improve in vitro yield. Of these, elicitation is an effective and viable tool designed to induce physiological and morphological changes in plants by the use of elicitors (Baenas et al. 2014; Liu et al. 2019; Namdeo 2007; Zhao et al. 2005). Elicitors can function to increase the accumulation and/or stimulate the induction of the de novo synthesis of several bioactive metabolites, including phenolic compounds, in plant cells, tissues, or even the whole plant (Dias et al. 2016; Gabr et al. 2016; Namdeo 2007; Zhao et al. 2005). Plants respond to elicitors by activating signaling cascades, similar to the defense responses against pathogen infection or environmental stress, that go on to affect plant metabolism and can enhance the synthesis of metabolites (Baenas et al. 2014). Usually elicitors are divided in two groups based on their nature: biotic elicitors (derived from the host plant or plant pathogens) and abiotic elicitors (predominantly inorganic compounds such as salts or physical factors) (Namdeo 2007; Zhao et al. 2005). Methyl jasmonate, salicylic acid, chitosan, salts, and light are some elicitors that regulate a large number of biochemical control points and induce a series of signal transduction process (Dias et al. 2016; Liu et al. 2019; Mendoza et al. 2018; Ramakrishna and Ravishankar 2011; Thakur et al. 2019; Zhao et al. 2005).

Phenolic compounds are one of the most important secondary metabolites produced by plants. These compounds aid in the environmental adaptation of plants to stress under in vivo growth conditions and protect plants against pathogens and herbivores (Dias et al. 2016; Mendoza et al. 2018). The bioactive properties of phenolic compounds for human health has been associated mainly with the protective effect against cancer and cardio- and cerebrovascular diseases caused by oxidative stress and subsequent free radical damage to lipids, proteins, and nucleic acids (Choi and Lee 2009; Matkowski 2008). Other beneficial effects include antimicrobial, anti-inflammatory, antidiarrheal, antiulcer, and antiallergenic activities (Carvalho et al. 2015; Dias et al. 2016; Działo et al. 2018; Lu et al. 2018).

In vitro plant cell culture has previously been used to promote the continuous production of phenolic compounds and other metabolites by providing increases in the growth rates, biomass, and culture stability (Mendoza et al. 2018). Elicitors, signal compounds, and abiotic stress are some tools used to improve the in vitro production of phenolic compounds (Baenas et al. 2014; Mendoza et al. 2018; Namdeo 2007; Thakur et al. 2019; Vogt 2010; Zhao et al. 2005). Several elicitors can be used to increase the in vitro production of phenolic compounds in cell suspension and cell and tissue cultures of diverse plant families (Dias et al. 2016; Mendoza et al. 2018). Among these, both jasmonates and salicylic acid are signaling molecules that induce catalytic reactions by active modulation of specific enzymes such as phenylalanine ammonia-lyase, involved in the biosynthesis of many phenolic compounds through the phenylpropanoid pathway (Dias et al. 2016; Vogt 2010).

This chapter provides a summary of the main bioactive phenolic compounds produced by plant cell and tissue culture, details their biological activities, and provides insights into the effects of elicitation on their production. Additionally, there will be a discussion about the potential mechanisms involved when making changes to the production of bioactive phenolics through elicitor challenge under in vitro conditions.

2 Phenolic Compounds

2.1 Definition, Structure, and Classification

Phenolic compounds (PCs) are organic compounds that represent the second largest group of substances found in plant tissues, behind only cellulose (Quideau et al. 2011). The compounds are defined by the presence of an aromatic ring containing one or more hydroxyl groups (Wang et al. 2018) (Fig. 1). Most naturally occurring PCs contain conjugated mono- and polysaccharides that are linked to one or more of the phenolic groups or are functional derivatives such as esters and methyl esters (Balasundram et al. 2006).

PCs have very diverse chemical structures with some PCs, consisting of 15 carbon atoms, having a low molecular weights, while others have complex structures and high corresponding molecular weights (Bravo 1998; D'Archivio et al. 2007). PCs are classified according to the number of aromatic rings within the structure of the compound, along with the type of structural elements binding rings to each other. According to Balasundram et al. (2006), these compounds can also be classified based on the number of carbons in the molecule (Table 1).

Fig. 1 Phenol (basic structure)

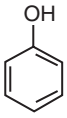


Table 1 Classification of phenolic compounds

Class	Structure	Examples	References
Simple phenolics, benzoquinones	C_6	Embelin	Kim et al. (2017)
Hydroxybenzoic acids	$C_6 - C_1$	Gallic acid, vanillic acid	Działo et al. (2018)
Hydroxycinnamic acids, Phenylpropanoids (coumarins, isocoumarins, chromones, chromenes)	$C_6 - C_3$	Caffeic acid; edgeworoside A; syringin	Działo et al. (2018) and Venditti et al. (2017)
Naphthoquinones	$C_6 - C_4$	Atovaquone	Kim et al. (2017)
Xanthones	$C_6 - C_1 - C_6$	Cratoxanthone	Ito et al. (2017)
Silbenes, anthraquinones	$C_6 - C_2 - C_6$	*t*-resveratrol, obtusin	Kiselev et al. (2017) and Zhu et al. (2008)
Betacyanins	C_{18}	Betanidin	Kumorkiewicz et al. (2018)
Lignans	$(C_6 - C_3)_2$	Pinoresinol	Działo et al. (2018)
Lignin	$(C_6 - C_3)_n$	Phenolic polymer	Vanholme et al. (2010)
Condensed tannins (proanthocyanidins or flavolans)	$(C_6 - C_3 - C_6)_n$	Procyanidin B2	Ropiak et al. (2017)
Flavonoids, isoflavonoids	$C_6 - C_3 - C_6$	Luteolin; kaempferol	Venditti et al. (2017)
Biflavonoids	$(C_6 - C_3 - C_6)_2$	Dulcisbiflavonoid C	Abdullah et al. (2018)

Simple Phenolics

Simple phenolics are substituted phenols. Usually the double substitution pattern on the benzene ring, 1,2-, 1,3-, and 1,4-, are designated by *ortho*, *meta*, and *para* nomenclature, respectively, if one of the functional groups is the hydroxyl group. When the benzene ring has three identical functional groups, the 1,3,5-substitution pattern is called *meta*-tri, while the 1,2,6- substitution pattern is indicated by the prefix *vic* (Del Rio et al. 2013) (Fig. 2).

Phenolics Acids

Phenolic acids contain a single phenyl group substituted by a carboxylic group and one or more hydroxyl group(s). Phenolic acids can be further divided into hydroxybenzoic acids (basic C6-C1 skeleton), hydroxycinnamic acids (basic phenylpropanoid, C6-C3 skeleton), and other hydroxyphenyl acids that differ with respect to the length of the chain containing the carboxylic group (Fig. 3) (Bravo 1998).

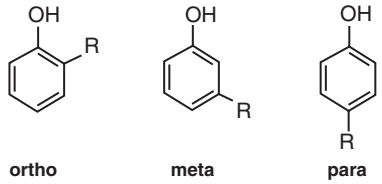


Fig. 2 Substitution patterns of phenolic compounds. R = generic substituents

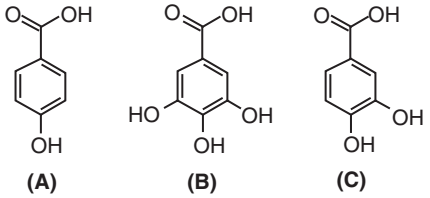


Fig. 3 Phenolic acids. (**a**) *p*-hydroxybenzoic acid; (**b**) gallic acid; (**c**) protocatechuic acid

Naphthoquinones

Structurally, quinones have aromatic rings with two highly reactive ketone substitutions. Among the quinones, the naphthoquinones are divided into 1,2- and 1,4-naphthoquinone groups which can be highlighted and, depending on the presence of an oxygenated heterocyclic ring, can further be classified as prenylnaphtoquinones, furanonaphtoquinones, difuronaphtoquinones, and piranonaphtoquinones (Fig. 4) (Babula et al. 2009).

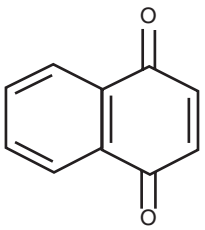
Xanthones, Stilbenes, and Anthraquinones

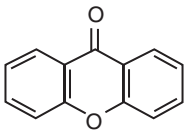
Xanthones have a C6-C1-C6 structure, while the stilbenes have a C6-C2-C6 structure. Xanthones are yellow pigments found in flowers, and stilbenes are usually associated with heartwood (Gutierrez-Orozco and Failla 2013). Anthraquinone is the most widely distributed quinone in higher plants. The chemical structure of anthraquinones includes an anthracene (aromatic tricyclic) ring with two carbonates in the C9 and C10 positions (Tsimogiannis and Oreopoulou 2019) (Fig. 5).

Betacyanins

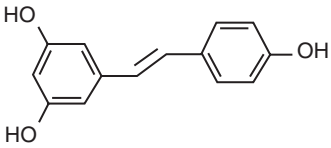
Betacyanins are red pigments and generally exist as glycosides. Betacyanins have an absorption spectrum that resembles anthocyanins; however, betacyanins contain nitrogen, while anthocyanins do not (Sant'Anna et al. 2013). An example of a typical betacyanin is betanidin (Fig. 6).

Fig. 4 Structure of 1,4-naphthoquinone





(A)



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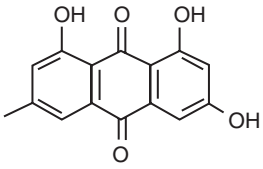
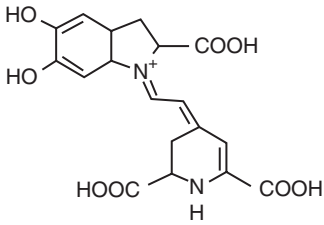


(C)

Fig. 5 Structures of xanthone (**a**), stilbene resveratrol (**b**), and the anthraquinone emodin (**c**)

Fig. 6 Structure of betanidin



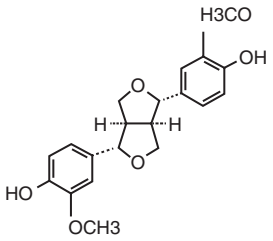
Lignans

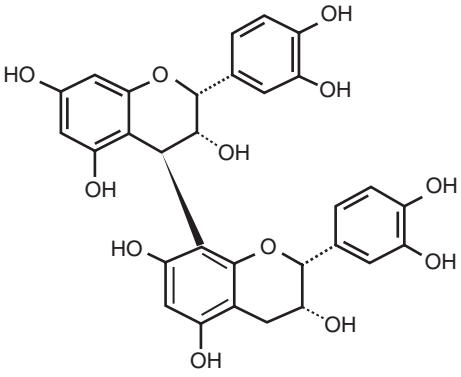
Lignans are formed by two units of phenylpropanoids (C6-C3-C3-C6). Lignans are dimers or oligomers resulting from the coupling of monolignols such as *p*-coumaryl alcohol, coniferyl alcohol, and synapyl alcohol. Most lignans are optically active, and, generally, only one enantiomer is found in a given species (Apers et al. 2003). An example of lignan is (+)-pinoresinol (Fig. 7).

Lignin

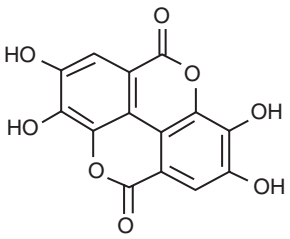
Lignin is a phenolic polymer that plays an important role in providing structural support and mechanical protection for plants. Its hydrophobicity also facilitates the transport of water through vascular tissue (Boerjan et al. 2003). The polymeric structure of lignin is comprised of monolignol (alcohol coumaryl, coniferyl, and sinapyl) and lignan (monolignol dimers) units. It is nonlinear and is synthesized randomly to form a polymer within a complex three-dimensional network (Vanholme et al. 2010). Lignin is formed through a radical-mediated polymerization process,

Fig. 7 Structure of (+)-pinoresinol





(A)



(B)

Fig. 8 Some examples of tannins. (**a**) Condensed tannin – procyanidin B2 (epicatechin-(4β → 8′)-epicatechin. (**b**) Hydrolyzable tannin – ellagic acid

but the resulting lignin polymer is not optically active (Ralph et al. 1998). The lignin polymer increases in size as additional monolignol radicals react with reactive sites within the polymer (Boerjan et al. 2003).

Tannins

Tannins are compounds of relatively high molecular weight and can be classified into three groups: condensed tannins, hydrolyzable tannins, and complex tannins (Khanbabaee and Van Ree 2001). Hydrolyzable tannins are esters of gallic acid (gallo- and ellagi-tannins), while condensed tannins (also known as proanthocyanidins) are polymers of flavan-3-ol monomers. Complex tannins are defined as tannins in which a unit of catechin is glycosidically attached to a unit of gallotannin or ellagitanin (Smeriglio et al. 2017) (Fig. 8).

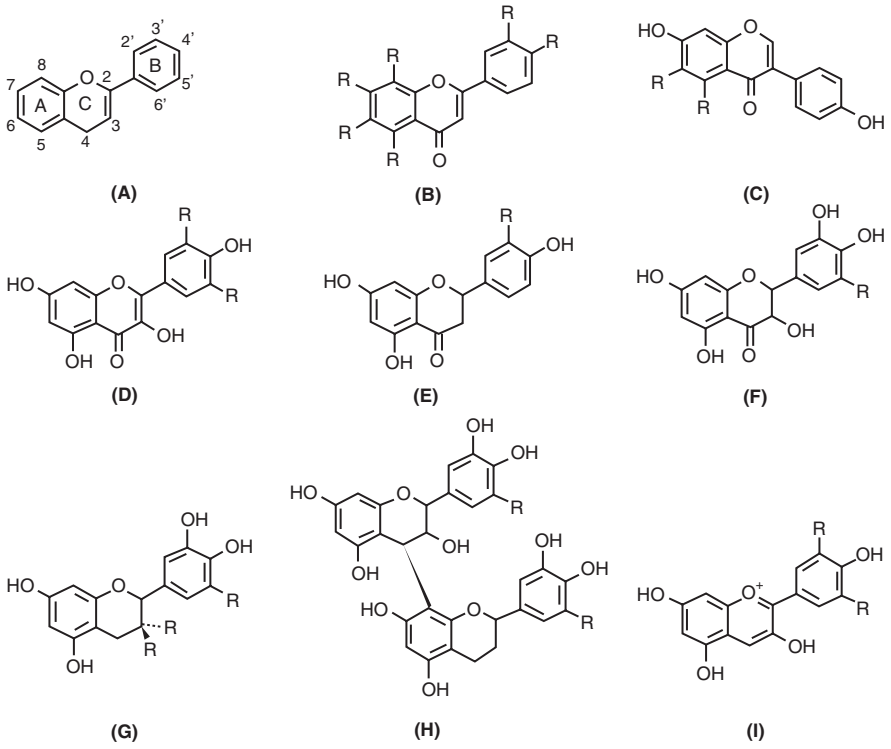


Fig. 9 Structure and some groups of flavonoids. (**a**) Phenylbenzopyrane skeleton (basic structure and numbering of carbon atoms of flavonoids); (**b**) flavones; (**c**) isoflavones; (**d**) flavonols; (**e**) flavanones; (**f**) dihydroflavonols; (**g**) flavan-3-ols; (**h**) proanthocyanidins; (**i**) anthocyanidins; R = site where hydroxylation or methylation may occur

Flavonoids

Flavonoids are a large class of PCs, ubiquitous in plants that usually occur as glycosides (Rice-Evans et al. 1997) (Fig. 9). They contain a phenylbenzopyran skeleton composed of two phenyl rings (A and B) attached through a heterocyclic pyran ring (C) (Fig. 9a). Flavonoids can be classified into several groups, according to differences in pyran ring substitution patterns and differences in oxygenation, alkylation, glycosylation, acylation, sulfation, hydroxylation, and methylation of rings A and B (Coutinho et al. 2009; Hollman and Katan 1999; Winkel-Shirley 2002). The major subgroups of flavonoids include flavones, isoflavones, flavanols, flavanonols (dihydroflavonol), flavanones, flavan-3-ol (catechin), anthocyanins, anthocyanidins, proanthocyanidins, and chalcones (Coutinho et al. 2009).

Flavones

Flavones are the most basic structure of flavonoids (Fig. 9b). They contain a C4 ketone group, a C2 and C3 double bond, and a ring that is attached to C2 (Del Rio et al. 2013).

Isoflavones

Isoflavones have the C6-C3-C6 ring structure, but ring B is at a different position in the oxygen heterocycle (Fig. 9c). This structural feature causes isoflavones to resemble estrogens (female sex hormones), and, as a result, they moderate estrogenic activity and are known as phytoestrogens (Del Rio et al. 2013).

Flavanols

Flavanols are C3 hydroxylated flavones (Fig. 9d). They are some of the most highly antioxidant flavonoids due to their hydroxylation pattern in which the hydroxyl at the C3 position of the carbon skeleton is thought to increase the stability of the flavonoid radical formed once the compound has acted as a radical scavenger (Del Rio et al. 2013).

Flavanones

Typical flavonoids, such as flavanones, have six-membered heterocycles. In the C-ring of flavanones, there is a saturated pyran group (no double bond between C2 and C3) (Fig. 9e). Flavanones also have a C4 ketone group (Birt and Jeffery 2013).

Flavanonols (Dihydroflavonols)

Flavanonols, also known as dihydroflavonols, often occur in association with tannins. As occurs in flavanones, the C-ring of flavanonols has a saturated pyran group (no C2 and C3 double bond) (Fig. 9f). Dihydroflavonols also have a C4 ketone group and contain a hydroxyl attached to C3 (Huang et al. 2009).

Flavan-3-ols (Catechins)

Flavan-3-ols are the most abundant flavonoids in nature (Fig. 9g). The major flavan-3-ols include catechin, epicatechin, gallocatechin, and epigallocatechin. Catechins and epicatechins are C3 stereoisomers (as is the case for gallocatechins and epigallocatechins), while the gallates are esterified on the C3 position with gallic acid. The

oligomeric flavan-3-ols are called proanthocyanidins (Fig. 9h) with polymeric forms termed condensed tannins, although the terms are generally interchangeable. Proanthocyanidins may consist of flavan-3-ols attached by a C-C bond or by monomeric units joined by two bonds (Tsao 2010).

Anthocyanins

Anthocyanins are the water-soluble glycosides of anthocyanidins. Often, ring C contains further 3-hydroxyl substitution (Fig. 9i). About 300 anthocyanidins have been identified in nature, a number that includes the anthocyanins. Anthocyanidins emit almost exclusively the red, violet, and cyan colors observed in flowers, leaves, fruits, and even the roots of plants (Prior and Wu 2006).

Chalcones

Chalcones are considered unconventional flavonoids due their low abundance in nature. Chalcones and dihydrochalcones have a linear C3 chain connecting two aromatic rings. The C3 position on the chalcone chain contains a double bond, while the C3 position of dihydrochalcones is saturated (Bukhari et al. 2012).

2.2 Biosynthetic Pathway

PCs are secondary compounds derived from pentose phosphate, shikimate, and the phenylpropanoid pathway (Fig. 10) (Dias et al. 2016; Randhir et al. 2004). The most important biosynthetic route for the production of phenolic compounds is the shikimic acid pathway. Through this pathway, one molecule of phosphoenolpyruvic acid (PEP), derived from glycolysis, and erythrose-4-phosphate, from the pentose-phosphate pathway, are combined, resulting in the formation of a seven-carbon sugar called DAHP (3-deoxy-O-arabino-heptulosonate phosphate). DAHP is then cyclized and reduced to the form shikimic acid (Boudet 2007; Dias et al. 2016). The shikimic acid pathway is also involved in the formation of proteins, which competes directly with the formation of PCs (Cohen and Kennedy 2010).

Shikimic acid is an intermediate in the formation of phenolic acids and simple phenolics that have a carboxyl group and serve as a precursor for other compounds. Additionally, shikimic acid-derived intermediates may be used in the formation of aromatic amino acids, such as phenylalanine, tyrosine, and tryptophan, compounds that are starting materials for the phenylpropanoid pathway (Ghasemzadeh and Ghasemzadeh 2011). Metabolically this is very interesting, since the production of more complex phenolic compounds begin with the deamination of phenylalanine by phenylalanine ammonia-lyase (PAL) for *trans*-cinnamic acid (directly related to the production of coumarins) and also in the conversion to *p*-coumaric acid (derived from tyrosine). From the synthesis of *p*-coumaric acid, hydroxycinnamic acids such

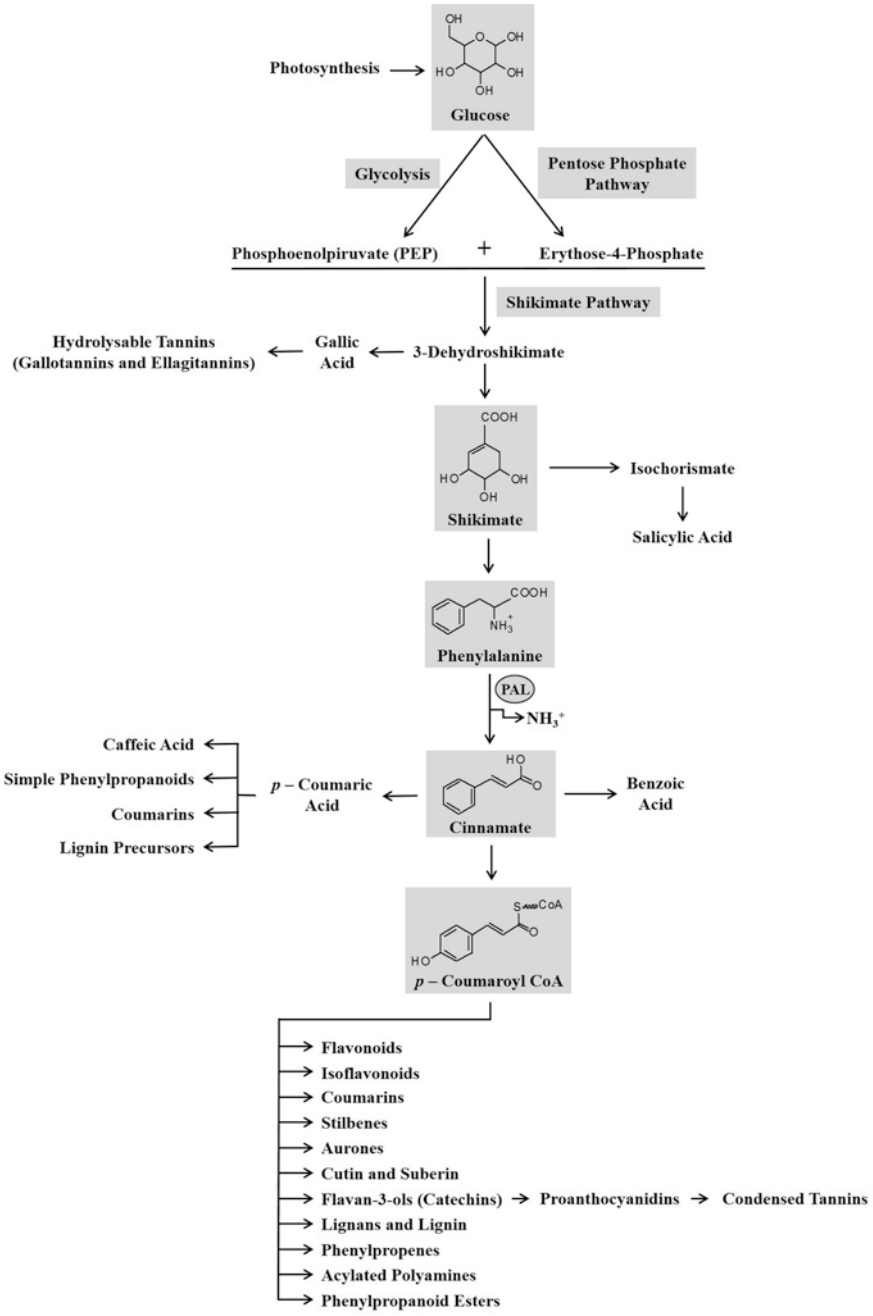


Fig. 10 Summary of the highly complex phenylpropanoid pathway

as caffeic acid are formed, which can be converted either to alcohols or to *p*-coumaric acid, both of which can lead to lignin formation. Alternatively, through the action of the CHS (chalcone synthase), CHI (chalcone isomerase), and F3H (flavanone-3-hydroxylase) enzymes, *p*-coumaric acid can be converted into flavonols (e.g., quercetin) and flavan-3-ols, compounds directly related to production of proanthocyanidins and anthocyanins (Dias et al. 2016; Martens et al. 2010; Winkel-Shirley 2002).

3 Biological Activities of the Phenolic Compounds

PCs perform a wide range of biological functions in the plant (Pollastri and Tattini 2011). For example, they may be structural components of cell walls, participate in growth and development processes by regulating auxin transport (Brown et al. 2001), or may function as plant hormones regulating pollen maturation and pollen tube growth (Napoli et al. 1999). PCs also play an important role in the interaction of plants with the environment (Bautista et al. 2016) and contribute to the color and sensorial characteristics of fruits, vegetables, cereals, olives, dried legumes, chocolate, and beverages such as tea, coffee, and wine (D'Archivio et al. 2007; Quideau et al. 2011). These substances accumulate in different cells and tissues depending upon the stage of ontogenesis of a plant and the influence of various environmental stimuli (Hutzler et al. 1998; Knogge and Weissenböck 1986).

PCs have the ability act as signaling molecules in plant-microorganism interactions (e.g., induction of nodulation in legumes); stimulate defense mechanisms against both herbivory and pathogen – bacterial, viral, or fungal – attack; and can serve as attractants for both pollinators and animals with roles in the dispersion of fruits and seeds (Harborne and Williams 2000). There is much evidence that these secondary metabolites also participate in plant responses to virtually all types of abiotic stress: UV radiation, intense light, extreme temperatures, mineral nutrient imbalance, anoxia, ozone exposure, drought, salinity, heavy metals, and herbicides (Winkel-Shirley 2002; Pollastri and Tattini 2011; Di Ferdinando et al. 2012).

The human health benefits of PCs depend on their uptake and metabolism after consumption, which in turn are determined by their structure, including their conjugation with other phenolic compounds, degree of glycosylation/acylation, molecular size, and solubility (Bravo 1998). Several pharmacological activities are reported for PCs that include antioxidant (Kumar and Pandey 2013; Sharma et al. 2015a,b; Zhang and Tsao 2016), antibacterial (Kachou et al. 2016), antiviral (Fabra et al. 2018), anti-inflammatory (Zhang and Tsao 2016), antilipidemic (Sharma et al. 2015a,b), anticarcinogenic (Huang et al. 2009; Ravishankar et al. 2013), antidiabetic (Coman et al. 2012; Lin et al. 2016; Sharma et al. 2015a,b), neuroprotective (Szwajgier et al. 2017), hepatoprotective (Shehab et al. 2015), and cardioprotective properties (Croft et al. 2018). These properties have been related to the strong antioxidant character of many phenolic compounds and their ability to scavenger "reactive oxygen species" (ROS) (Kumar and Pandey 2013).

4 In Vitro Production of the Phenolic Compounds

Plant cell and tissue culture systems allow for the continuous, high-quality production of secondary metabolites, regardless of geographic or environmental variations and constraints. However, despite evident improvements in culture technologies, cell lines, and bioreactor design, there have been few commercial successes reported (Davies and Delores 2014).

It is clear that biotechnological products, such as bioactive secondary metabolites, produced in vitro for human consumption must have their safety and efficacy studied with intense scrutiny. The KFDA (Drug Administration and Korean Food) approved adventitious roots of *Panax ginseng* cultivated in vitro, after careful evaluation of its biosafety and efficacy, for market. The approval made the cultivation of this tissue one of the few successful examples of a raw material derived from in vitro culture that was approved and subsequently marketed to the public for profit (Murthy et al. 2015).

In theory, in vitro culture can be applied to any plant, since within genetics of every plant cell lies the genetic information necessary to maintain cell function in an artificial medium. This includes the information needed for production of secondary metabolites and maintenance of totipotency. Continuous monitoring of target metabolites is also a prerequisite for the successful development of a production protocol and can be accomplished using several different methods including high-performance liquid chromatography (HPLC) and gas chromatography (GC). However, colorimetric methods that use spectrophotometric principles appropriate for each group of metabolites are rapid and cost-effective techniques that may be of interest, especial for large-scale operations (Matkowski 2008).

The production of PCs by in vitro plant culture is well described in the literature, but there are large differences between the production of individual PCs, disparities that are mainly due to the complexity of the biosynthesis extraction procedures for individual compounds (Dias et al. 2016). The in vitro production of secondary metabolites offers several benefits compared to the usual production methodologies using plants grown ex vitro. As an example, consider the production of flavonoids in callus cultures of *Hypericum perforatum* (Dias et al. 1998), as well as the production of steroids, phenols, flavonoids, and derivatives of gallic, benzoic, ferulic, and *p*-coumaric acid in *Pyrostegia venusta* calli (Braga et al. 2015; Coimbra et al. 2017; Coimbra et al. 2019; Loredo-Carrillo et al. 2013).

However, the in vitro production of PCs is still controversial since there are reports that the synthesis and the accumulation of these compounds can inhibit the growth in vitro cultures (Coimbra et al. 2019). Resultantly, if the production of the target metabolite is linked to the reductions in biomass, this property must be considered.

5 Elicitors

Productions of phenolic compounds are naturally stimulated by in vitro plant tissue and cell culture conditions. However, in general, these compounds are not produced to sufficient levels as a result of the brief nature of the stationary phase of growth (Michael and John 1985; Rao and Ravishankar 2002). Culture medium optimization, cell line selection, cell immobilization, precursor addition, elicitation, and metabolic engineering are some tools used to change biosynthetic pathways in efforts aimed at enhancing the production of secondary compounds (Georgiev et al. 2009; Gupta et al. 2016; Zhao et al. 2005). Elicitation is a good biotechnological tool used to induce physiological changes and stimulate defense or stress-induced responses in plants, such as the production of phenolic compounds (Baenas et al. 2014; Dias et al. 2016). This stimulation by use of elicitors or stress-related compounds that activate the defense mechanisms of the plant ensure plant survival, persistence, and competitiveness (Namdeo 2007; Rea et al. 2011).

An elicitor can be defined as a signaling molecule or biofactor which, whenintroduced in small concentrations to a living cell system, trigger a series of intracellular signals that promote physiological and morphological responses and phytoalexin accumulation (Khan et al. 2017; Thakur et al. 2019; Zhao et al. 2005). As a result of elicitation, plants alter regulatory mechanisms in the same ways that they do when defense responses to pathogen infections or environmental stimuli are activated. These changes affect biosynthetic pathways, enhancing the production or inducing de novo synthesis of secondary compounds in both plant, cell culture conditions, and whole plants (Baenas et al. 2014; Ferrari 2010; Gabr et al. 2016; Khan et al. 2017; Namdeo 2007; Ramirez-Estrada et al. 2016; Zhao et al. 2005).

The general mechanism of action of the elicitor at the cellular level consists, initially, of signal perception by receptors on the plasma membrane, followed by the activation of second messengers that amplify the signal and relay it to downstream mediators of reactions (Baenas et al. 2014; Liu et al. 2019; Ramirez-Estrada et al. 2016; Zhao et al. 2005). In consequence, a cascade of reactions is triggered which include the phosphorylation and dephosphorylation of plasma membrane and cytoplasmatic proteins; regulation of ion fluxes, especially Ca^{2+} influx and Cl^{-} and K^{+} efflux; and the depolarization of the membrane, inactivating H^{+}-ATPase, causing the cytoplasm acidification. In sequence, production of reactive oxygen species (ROS) and the activation of NADPH oxidase promote the activation of transcription factors that regulate the expression of defense genes, increasing the production of signaling molecules like ethylene, salicylic acid, jasmonate, and secondary metabolites (Akula and Ravishankar 2011; Ferrari 2010; Liu et al. 2019; Namdeo 2007; Zhang et al. 2012; Zhao et al. 2005). The expression level of certain genes is associated not only in response to ROS but also with cold and high temperature and salt and in drought stress (Akula and Ravishankar 2011). Increased ROS production may help to combat invasive pathogens but can also be triggered by harmful environmental factors such as UV radiation, xenobiotics, heavy metals, drought, and mechanical damage (Matkowski 2008). The efficacy of the use of elicitors depends mainly on plant genetics, elicitor nature, dose, selectivity, the duration of

elicitor exposure, culture age, cell line, growth regulation, nutrient composition, and quality of cell wall materials (Baenas et al. 2014; Namdeo 2007).

Elicitors can be divided into two main categories based their origin and molecular structure: biotic and abiotic elicitors (Namdeo 2007; Zhao et al. 2005). Table 2 summarizes elicitors used to improve the production of phenolic compounds in vitro over the last 6 years (2014–2019).

5.1 Biotic Elicitors

Biotic elicitors are defined as substances of biological origin that are derived from fungi, bacteria, viruses, or herbivores (exogenous elicitors) or from the plant itself (endogenous elicitors), which enhance biosynthesis of metabolites in a living cell system (Angelova et al. 2006; Patel and Krishnamurthy 2013). Their mechanism of action is coupled to receptors function to activating or inactivating enzymes or ion channels (Thakur et al. 2019)

Biotic elicitors include polysaccharides derived from plant cell walls (pectin or cellulose) as well as chemicals that are released at attack sites by plants upon pathogen or herbivore attack (Namdeo 2007). Some major biotic elicitors are pathogens (fungi, bacteria, and viruses) or pathogen-derived substances, such as yeast extracts, plant cell-like components, and polysaccharides (pectin, chitin, chitosan, dextran) (Gorelick and Bernstein 2014; Namdeo 2007; Świeca 2015; Vasconsuelo and Boland 2007). Multiple active ingredients such as yeast extracts and microbial cell-wall preparations often are composed of biotic elicitors (Vasconsuelo and Boland 2007). Yeast extract contains chitin, N-acetylglucosamine oligomers, β-glucan, glycopeptides, and ergosterol and is capable of eliciting plant defense responses (Baenas et al. 2014). Fungal-derived elicitors activate several responses when used, including the production of ionic fluxes across the plasma membrane, the synthesis of reactive oxygen species (ROS), and the induction of the de novo synthesis of defense compounds (Dewanjee et al. 2014; Janusz et al. 2013; Yuan et al. 2002).

5.2 Abiotic Elicitors

Abiotic elicitors are defined as substances or factors of non-biological origin enhance biosynthesis of metabolites in a living cell system (Namdeo 2007; Rao and Ravishankar 2002; Thakur et al. 2019). Abiotic elicitors can be chemical factors like inorganic salts, metal ions, or others that are able to disturb the membrane integrity or physical factors, such as extreme pH, heavy metals, drought, excess UV-light, high salinity, low and high temperature, wounding, etc. (Baenas et al. 2014; Ramakrishna and Ravishankar 2011). The main abiotic elicitors for phenolic compound production are described below.

Table 2 Some elicitors used in plant tissue culture for the phenolic compounds production by in vitro cell and tissue culture over the last 6 years (2014–2019)

Biotic elicitors					
Elicitor	Compound	Activity	Culture system	Plant species	References
Alternaria alternata (50 mg L^{-1})	Rosmarinic acid	Antioxidant	Shoots	*Solenostemon scutellarioides* (Lamiaceae)	Dewanjee et al. (2014)
Fungal mycelia extracts (50 mg L^{-1})	Phenylpropanoids and naphtodianthrone	Anti-inflammatory, hepatoprotective, antiviral, antimicrobial, antioxidant, antitumoral, wound-healing and antidepressive	Cell suspension	*Hypericum perforatum* (Hypericaceae)	Simic et al. (2015a)
Polysaccharides (chitin, dextran and pectin) (100–200 mg L^{-1})	Phenylpropanoids and naphtodianthrone	Anti-inflammatory, hepatoprotective, antiviral, antimicrobial, antioxidant, antitumoral, wound-healing, and antidepressive	Cell suspension	*Hypericum perforatum* (Hypericaceae)	Simic et al. (2015b)
Chitosan (200–800 mg L^{-1})	Symilarin	Liver protective	Callus	*Silybum marianum* (Asteraceae)	Gabr et al. (2016)
Chitosan (10 mg L^{-1})	Phenolic acids Echinacoside	Anti-inflammatory, anti-cancer, and cardiovascular protective Neuroprotective, vasodilatory, antioxidant, and anti-hepatotoxic activities	Cell suspension	*Scrophularia striata* (Scrophulariaceae)	Kamalipourazad et al. (2016)
Chitosan (150 mg L^{-1})	Flavonoids	Antioxidant	Hairy roots	*Isatis tinctoria* (Brassicaceae)	Jiao et al. (2018)
Coronatine (1 μM)	Hydroxycinnamic acid	Antitumor and immunomodulatory activities	Whole plants	*Lemna paucicostata* (Araceae)	Kim et al. (2017)
Live microbial cultures (bacteria and fungi) (10^4 spores/flask)	Glycyrrhizic acid	Anti-inflammatory, antitumoral and antimicrobial activities	Roots	*Taverniera cuneifolia* (Fabaceae)	Awad et al. (2014)

Streptomyces sp. (200 µL)	Quercetin	Not reported	Roots and shoots	*Eucalyptus grandis* and *Eucalyptus globulus* (Myrtaceae)	Salla et al. (2014)
Yeast extract (500 mg L^{-1})	Rosmarinic acid	Antioxidant, anti-inflammatory, neuroprotective, chemopreventive, cardioprotective, and antidiabetic	Shoots	*Thymus lotocephalus* (Lamiaceae)	Gonçalves et al. (2019)
Yeast extract (50 mg L^{-1})	Eugenol	Antiseptic, analgesic, antioxidant, antimicrobial, antitumor and anesthetic	Hairy roots	*Ocimum tenuiflorum* (Lamiaceae)	Sharan et al. (2019)
Yeast extract (50 ppm)	Rosmarinic acid Salvianolic acid Caffeic acid	Antioxidant, anti-cancer, anti-inflammatory, antimicrobial Antioxidant; Antinociceptive and chemoprotective	Shoots	*Salvia virgata* (Lamiaceae)	Dowom et al. (2017)
Yeast extract (50 mg L^{-1})	Phenolic compounds and flavonoids	Antitumoral, antidiabetic, and anti-hyperlipidemic	Adventitious roots	*Polygonum multiflorum* (Polygonaceae)	Ho et al. (2018)
Yeast extract (1.5 g L^{-1})	Vinblastine and vincristine	Anti-cancer and antioxidant	Seeds	*Catharanthus roseus* (Apocynaceae)	Maqsood and Abdul (2017)
Yeast extract (200 mg L^{-1})	Tanshione II A	Antibacterial, antioxidant, antidiabetes, anti-cancer, and anti-inflammatory	Adventitious roots	*Perovskia abrotanoides* (Lamiaceae)	Zaker et al. (2015)
Yeast extract (200 mg L^{-1})	Tanshione II A	Anti-inflammatory and antioxidant	Hairy roots	*Salvia castanea* Diels f. *tomentosa* (Lamiaceae)	Li et al. (2015)

(continued)

Table 2 (continued)

Biotic elicitors					
Elicitor	Compound	Activity	Culture system	Plant species	References
Ag^+ ions (15 µM) Ag^+ ions (2.5 ppm)	Tanshione II A Rosmarinic acid	Anti-inflammatory and antioxidant Antioxidant, anti-cancer, anti-inflammatory, and antimicrobial	Hairy roots Shoots	*Salvia castanea* Diels f. *tomentosa* (Lamiaceae) *Salvia virgata* (Lamiaceae)	Li et al. (2015) and Dowom et al. (2017)
$AgNO_3$ (25 µM) $AgNO_3$ (100 µM)	Tanshione II A Vinblastine and vincristine	Anti-cancer and anti-inflammatory Anti-cancer and antioxidant	Roots Shoots	*Perovskia abrotanoides* (Lamiaceae) *Catharanthus roseus* (Apocynaceae)	Zaker et al. (2015) and Paeizi et al. (2018)
Benzyladenine (8.8 µM) + Glutamine (40 µM)	Flavonoid	Antimicrobial	Shoots	*Coleonema pulchellum* (Rutaceae)	Baskaran et al. (2014)
$CuSO_4$ (25 µM)	Eugenol and methyl eugenol	Antiseptic and antimicrobial	Seeds	*Ocimum basilicum* (Lamiaceae)	Trettel et al. (2018)
Jasmonic acid (50 µM)	Plumbagin	Anti-cancer and neuroprotective	Adventitious roots	*Plumbago rosea* (Plumbaginaceae)	Silja and Satheeshkumar (2015)
Jasmonic acid (100 µM)	Emodin and physcion	Antimicrobial, anti-cancer and antioxidant	Cell suspension	*Polygonum multiflorum* (Polygonaceae)	Thiruvengadam et al. (2016)
Light (white)	Phthalides (3-Butylidenephthalide)	Against brain tumor, angina pain, and atherosclerosis	Callus	*Cnidium officinale* (Apiaceae)	Adil et al. (2019)
Light quality	Polyphenols	Antioxidant	Cell suspension	*Thevetia peruviana* (Apocynaceae)	Arias et al. (2016)

Light (continuous) + 2βmethyl-cyclodextrin (15 mM)	Baicalein and wogonin	Anti-anxiolytic, anti-cancer, anti-inflammatory, and antioxidant	Hairy roots	*Scutellaria lateriflora* (Lamiaceae)	Marsh et al. (2014)
Methanol (1.5% v/v)	Phenolic compounds	Antioxidant	Shoots	*Stevia rebaudiana* (Asteraceae)	Alvarez-Robles et al. (2016)
MeJA (60 mg L^{-1})	Eugenol	Antiseptic, analgesic, antioxidant, antimicrobial, antitumor, and anesthetic	Hairy roots	*Ocimum tenuiflorum* (Lamiaceae)	Sharan et al. (2019)
MeJA (0.1 mM)	Salicylic acid, kaempferol, and quercetin	Not reported	Seeds	*Solanum lycopersicon* (Solanaceae)	Król et al. (2015)
MeJA (200 μM)	Tanshione II A	Anti-inflammatory and antioxidant	Hairy root	*Salvia castanea* Diels f. *tomentosa* (Lamiaceae)	Li et al. (2015)
MeJA (5 μM)	Andrographolide	Not reported	Cell suspension	*Andrographis paniculata* (Acanthaceae)	Sharma et al. (2015)
MeJA (20–80 mg L^{-1})	Symilarin	Liver protective	Callus	*Silybum marianum* (Asteraceae)	Gabr et al. (2016)
MeJA (100 μM)	Phenolic compounds	Not reported	Cell suspension	*Dendrobium candidum* (Orchidaceae)	Wang et al. (2016)
MeJA (0.5 mg L^{-1})	Phenolic compounds	Anti-cancer	Callus and adventitious roots	*Fagonia indica* (Zygophyllaceae)	Khan et al. (2017)
MeJA (11.2 ppm) + Ag^{+} (2.5 ppm)	Rosmarinic acid	Antioxidant, anti-cancer, anti-inflammatory, and antimicrobial;	Shoots culture	*Salvia virgata* (Lamiaceae)	Dowom et al. (2017)

(continued)

Table 2 (continued)

Biotic elicitors					
Elicitor	Compound	Activity	Culture system	Plant species	References
MeJA (50 µM)	Phenolic compounds and flavonoids	Antitumoral, antidiabetic and anti-hyperlipidemic	Adventitious roots	*Polygonum multiflorum* (Polygonaceae)	Ho et al. (2018)
MeJA (100 µM)	Anthraquinone	Photosensitizing properties and photodynamic therapy against cancer cells	Hairy root	*Rubia tinctorum* (Rubiaceae)	Perassolo et al. (2017)
MeJA (3 µM)	Phenolic compounds and flavonoids	Antimicrobial and antineoplastic	Cell suspension	*Thevetia peruviana* (Apocynaceae)	Mendoza et al. (2018)
MeJA (100 µM) + 2βMethyl-cyclodextrin (50 mM)	Bergapten	Not reported	Cell suspension	*Pimpinella anisum* (Apiaceae)	Soto-Argel et al. (2017)
2βMethyl-cyclodextrin (15 mM) + light	Wogonin and baicalein	Anxiolytic conditions, anti-cancer, anti-inflammatory, anti-tumor, and antioxidant activities	Hairy roots	*Scutellaria lateriflora* (Lamiaceae)	Marsh et al. (2014)
2βMethyl-cyclodextrin (50 mM)	Eugenin	Not reported	Cell suspension	*Pimpinella anisum* (Apiaceae)	Soto-Argel et al. (2017)
Naphthalene acetic acid (10 µM)	Phenolic compoundsAnthocyanins	Antioxidant	Shoots	*Vaccinium corymbosum* (Ericaceae)	Lucioli et al. (2018)
Ozone (200 ppb – 3 h)	Rosmarinic acid	Antioxidant	Shoots	*Melissa officinalis* (Lamiaceae)	Tonelli et al. (2015)
Polyethylene glycol (30%)	Phenolic acids and flavonols	Antioxidant	Seedlings	*Agave salmiana* (Asparagaceae)	Puente et al. (2017)
Salicylic acid (50 µM)	Phenolic compounds and flavonoids	Antitumoral, antidiabetic, and anti-hyperlipidemic	Adventitious roots	*Polygonum multiflorum* (Polygonaceae)	Ho et al. (2018)

Salicylic acid (100 μM)	Emodin and physcion	Antimicrobial, anti-cancer, and antioxidant	Cell suspension	*Polygonum multiflorum* (Polygonaceae)	Thiruvengadam et al. (2016)
Salicylic acid (20–40 mg L^{-1})	Symilarin	Liver protective	Callus	*Silybum marianum* (Asteraceae)	Gabr et al. (2016)
Salicylic acid (50 mg L^{-1})	Phenolic compounds	Rheumatic disorders, cold symptoms, fevers, gastrointestinal complications, motion sickness, bronchitis, diabetes, and anti-cancer	Callus	*Ajuga bracteosa* (Lamiaceae)	Ali et al. (2018)
Salicylic acid (120 μM)	Phenolic compounds	Not reported	Cell suspension	*Dendrobium candidum* (Orchidaceae)	Wang et al. (2016)
SiO_2 (100 mg L^{-1})	Rosmarinic acid and methoxilated flavonoids (xanthomicrol and cirsimaritin)	Anti-cancer, anti-spasm, and anti-platelet properties	Hairy roots	*Dracocephalum kotschyi* (Lamiaceae)	Nourozi et al. (2019)
Sucrose (30 g L^{-1}) + 2,4 D (5 mg L^{-1}) + Dark	Amentoflavone and agathisflavone	Epilepsy control, antiviral, antineoplastic, and neuroprotection potential	Callus	*Poincianella pyramidalis* (Fabaceae)	Gomes et al. (2018)
2,3,5-triiodobenzoic acid (0.5 mg L^{-1}) + kinetin (5 mg L^{-1})	Isoflavones	Anti-cancer, antioxidant	Callus	*Genista tinctoria* (Fabaceae)	Luczkiewicz et al. (2014)
Thidiazuron (1 mg L^{-1})	Gallic acid, myricetin, caffeic acid, catechin and apigenin	Anti-cancer	Callus	*Fagonia indica* (Zygophyllaceae)	Khan et al. (2016)

Light

Light is a physical factor that can affect the production of metabolites. A positive correlation exists between increased light intensity and level of phenolic compounds. In particular, light intensity has been linked to increases in the production of anthocyanins and other flavonoids in previous reports (Dixon and Paiva 1995; Ramakrishna and Ravishankar 2011). Light and UV radiation promote binding of transcription factors (TF) to promoter regions of genes involved in the biosynthesis of phenolic compounds such as chalcone synthase (CHS) and PAL and lead to increases in the production of flavonoids, anthocyanins, and other pigments (Dias et al. 2016; Ramakrishna and Ravishankar 2011). However, Castro et al. (2016) reported that in callus cultures of *Byrsonima verbascifolia*, phenol, and flavonoid content ranged according to a combination of the presence or absence of light and type and concentration of regulators used. In *Poincianella pyramidalis*, the addition of 30 g L^{-1} of sucrose +5 mg L^{-1} of 2,4-D resulted in a higher accumulation of amentoflavone (16.44 mg L^{-1}) and agathisflavone (0.58 mg L^{-1}), and the absence of light was ideal for the growth of callus biomass and biflavonoid production.

Salinity

Altering salinity has been reported as a strategy for increasing polyphenol and anthocyanin content, due to cellular dehydration and corresponding reductions in cytosolic and vacuolar volume, causing osmotic and ionic stress (Parida and Das 2005; Ramakrishna and Ravishankar 2011). Gupta et al. (2016) reported that salinity is one of the most hazardous factors used to induce oxidative stress.

Metal Ions

The induction of oxidative stress and ROS and, consequently, the accumulation of phenolic compounds have also been reported using with metals ions like Co, Ni, Fe, and Ag as elicitors. However, the mechanism of elicitation with metals is unclear (Ramakrishna and Ravishankar 2011; Zhao et al. 2005). Heavy metals induce changes in the metabolic activities of plants and affect the production of photosynthetic pigments, sugars, proteins, and non-protein thiols (Thakur et al. 2019). According to Krupa et al. (1996), traces of nickel inhibited accumulation of anthocyanin by inhibiting the activity of PAL. Dowom et al. (2017) described that, as an effective abiotic elicitor, Ag^+ ions could improve production of phenolic acids in the *Salvia virgata* shoot cultures, while the highest levels (26 mg g^{-1} dry weight) of rosmarinic acid (RA) were reported on the fifth day after exposure of regenerated shoots to 2.5 ppm of Ag^+ ions.

Drought

In order to study the effects of drought on plant responses under controlled, in vitro conditions, simulation of drought stress can be accomplished using polyethylene glycol (PEG) (Khayatnezhad et al. 2010). According to Jaleel et al. (2009), the effect of drought on various physiological and biochemical processes, such as photosynthesis, respiration, carbohydrate and nutrient metabolism, and secondary metabolism, especially, reduces plant growth. However, the plant response to drought seems to depend upon the species in question. The in vitro culture of *Agave salmiana* resulted in the lowest observed levels of flavonols in response to drought stress that was induced via PEG (Puente et al. 2017). Sánchez-Rodríguez et al. (2011) reported that this result could be explained by the fact that phenylpropanoid and shikimate pathway enzymes exhibited reductions in activity under drought stress, as has been demonstrated in other plants.

Plant Growth Regulators

Auxins and Cytokinins

Auxins and cytokinins are usually employed to induce callus formation, as they promote cell growth by stimulating cell division and elongation through synergistic, antagonistic, and additive interactions (Coenen and Lomax 1997). Auxin and its mimic, 2,4-dichlorophenoxyacetic acid (2,4-D), has been widely used both alone and in combination with cytokinins, especially 6-benzylaminopurine (BAP), to stimulate callus induction and synthesis of bioactive compounds in vitro. However, the concentration and combination of these regulators must be determined for each species (Loredo-Carrillo et al. 2013). Examples of this were reported by Castro et al. (2016) and Coimbra et al. (2019) for *Byrsonima verbascifolia* and *Pyrostegia venusta*, respectively. All callus cultures using *Byrsonima verbascifolia*, in general, produced significant levels of phenols and flavonoids, regardless of the presence or absence of light, or type and concentration of regulators. Bioactive phenolic compounds derived from benzoic, *p*-coumaric, cinnamic, and gallic acids, as well as catechins, were detected in the culture. For *Pyrostegia venusta* callus culture, Coimbra et al. (2017) reported that the interaction between 4.52 μM 2,4-D and 8.88 μM BAP in the absence of light provided the best friable calli, with high levels of total phenols and flavonoids. HPLC-DAD analyses showed that bioactive phenolic compounds included gallic, caffeic, and benzoic acid derivatives within the hydromethanolic extracts of calli. A preferential synthesis of caffeic acid derivatives was observed after 60 and 120 days of culture (Coimbra et al. 2019).

Jasmonates and Salicylic Acid

Jasmonates are cyclopentanone compounds represented by jasmonic acid (JA), methyl jasmonate (MeJA), and other derivatives. They are considered signaling molecules in both biotic and abiotic stress pathways (Creelman and Mullet 1997; Mendoza et al. 2018; Portu et al. 2016), acting as elicitors of a wide spectrum of signaling pathways (Ramakrishna and Ravishankar 2011). MeJA is the methyl ester of JA, a plant growth regulator that plays an important role in the plant growth and development, the plan defense response to pathogens, wounding, and insect feeding (Portu et al. 2017; Złotek et al. 2014). MeJA can be used as an elicitor inducing defense responses in plants through activating enzymes involved in the biosynthesis of phenolic compounds (e.g., phenylalanine ammonia lyase, stilbene synthase, chalcone synthase, or anthocyanin synthase) (Belhadj et al. 2008; Namdeo 2007).

JA and MeJA elicit a wide range of compounds by inducing the expression of plant genes for various biosynthetic pathways and are also defined as hormones because they induce widespread cellular responses at low concentrations that are distant from their site of synthesis (Baenas et al. 2014). Jasmonates are the best studied oxylipin compounds. They are generated by the oxidative catabolism of polyunsaturated fatty acids, through the action of lipases, lipoxygenase, and a group of cytochrome P450 enzymes (Ramakrishna and Ravishankar 2011).

Exogenous JA and MeJA are able to enhance the production of phenolic phytoalexins, coumarins, and anthocyanins and are widely used as an elicitor in plant cell cultures (Baenas et al. 2014) For example, the content phenolic acids in *Exacum affine* shoot cultures increased from 0.712% to 1.160% when jasmonates were added to the culture medium (Skrzypczak-Pietraszek et al. 2014). Chung et al. (2017) reported a significant increase in the production of phenolic compounds such as hydroxybenzoic acid, hydroxycinnamic acid, flavonols, vanilin, and others in cell suspension cultures of spine gourd (*Momordica dioica*) when using JA as an elicitor. MeJA activates phenolic synthesis pathways by activation of transcription factors resulting in the upregulation of biosynthetic genes (Khan et al. 2017). Zhao et al. (2005) reported all of the known mechanisms involved in signal transduction mediated by elicitors and provided an excellent schematic illustration of biosynthetic pathway of JA.

Salicylic acid (SA) plays an important role in plant growth, development, and plant-pathogen interactions, inducing plant systematic acquired resistance and enhancing the accumulation of phenolic compounds (Chun et al. 2012; Lee et al. 2013). It is involved in some signal transduction pathways that induce and regulate enzymes, mainly PAL (Zhao et al. 2005).

SA and JA are signaling molecules that confer abiotic or biotic stress tolerance through modulation of highly branched metabolic pathways (Horváth et al. 2007; Khan et al. 2015). However, SA and JA are two different signaling molecules that regulate different pathways. SA induces resistance to fungal, bacterial, and viral pathogens through phenylpropanoid biosynthesis (Ryals et al. 1996), and JA is involved in the production of various proteins by activation of the octadecanoid signaling pathway (Farmer et al. 1992). According to Zhao et al. 2005, both MeJA

and SA can strongly stimulate membrane peroxidases, preventing oxidative stress and providing defense against pathogens. Cold stress, salinity, and the induction of oxidative stress and ROS upon elicitation with heavy metals also induce the production of signaling molecules like JA and SA (Ramakrishna and Ravishankar 2011; Zhao et al. 2005).

6 Conclusion

Phenolic compounds obtained from plants have considerable commercial importance, because many of these compounds are difficult or virtually impossible to synthesize economically. The in vitro culture system allows the synthesis of bioactive phenolic compounds, independently of environmental and soil conditions. Elicitation is an effective tool that improves production of phenolic compounds in vitro, within plant cell culture systems. However, the exact mechanism of elicitor action is not completely understood. This provides researchers with excellent opportunity to increase knowledge about the optimal conditions for the use of each kind of elicitor and the potential mechanisms of elicitor action used to increase production of bioactive phenolic compounds.

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Immobilization and Application of Industrial Enzymes on Plant-Based New Generation Polymers

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Abstract Biological catalysts, which are synthesized by living cells and that operate in both in vivo and in vitro conditions, are called enzymes. Industrial enzymes are widely used in medicine, pharmacy, detergent, food industry (starch, dairy and dairy products, juice, meat, bread production, etc.), and textile industries. Enzymes are fixed to suitable materials to prevent reaction medium contamination from conditions affecting enzyme activity due to lower environmental costs and different environmental conditions (pH, temperature, metal ions, etc.) that negatively affect enzyme activity. Enzymes are applied in various fields, including technical use, food manufacturing, animal nutrition, cosmetics, and medication, and as tools for research and development. Immobilization of enzymes with hydrolytic and oxidative activity with a wide market share provides more industrial use. This chapter includes the immobilization of enzymes used in some different industrial areas to new generation polymers based on plants.

Keywords Immobilization · Biopolymer · Pectin lyase · Mannanase · Phytase · Lipase · Chitinase · Protease · Industrial enzymes

1 Introduction

Enzymes are defined as biocatalysts synthesized by living cells. Enzymes are synthesized in the cell, but they also show high activity in extracellular environments. Enzymes have a protein structure, colloidal character and are very specific in their functions. In this context, the use of enzymes in many fields has become widespread, and the opportunity to be used in many industrial processes has emerged.

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Enzymes that perform very important functions in the cell are used for different purposes in the industry (Sheldon and van Pelt 2013).

In enzyme immobilization, enzyme is affixed to organic and inorganic carriers in order to maintain continuous enzyme activity by chemical or physical methods (Bilal et al. 2019). When choosing the enzyme immobilization method, the method is selected considering the reliability, enzyme activity, cost, and stability of the enzyme (Wu et al. 2015).

According to a research report:

- 158 enzymes were used in food industry.
- 64 enzymes in technical application.
- 57 enzymes in feedstuff.

Of which:

- 24 enzymes are used in three industrial sectors.

Almost 75% of all industrial enzymes are hydrolytic enzymes. Carbohydrases, proteases, and lipases dominate the enzyme market, accounting for more than 70% of all enzyme sales.

As illustrated in Fig. 1, the technical industries, dominated by the detergent, starch, textile, and fuel alcohol industries, account for the major consumption of industrial enzymes.

The enzymes can be attained from animals (chymosin, pepsin, trypsin, etc.), plants (ficin, papain, and bromelain), and microorganisms (pectinases, glucoamylase, α-amylase, etc.).

As the dimensions of the materials are reduced to nanoscale, they acquire unique physical and chemical properties (optical, magnetic, fluorescent, electrical conductivity, etc.). The modification of the support materials with nanoparticles during the immobilization of enzymes provides antimicrobial properties and facilitates easy control of different reaction mechanisms in the reaction medium. Interactions between the enzyme and the carrier contribute to precise judgments about the physicochemical and kinetic properties of the enzyme that must be determined for practical applications of the immobilized enzyme.

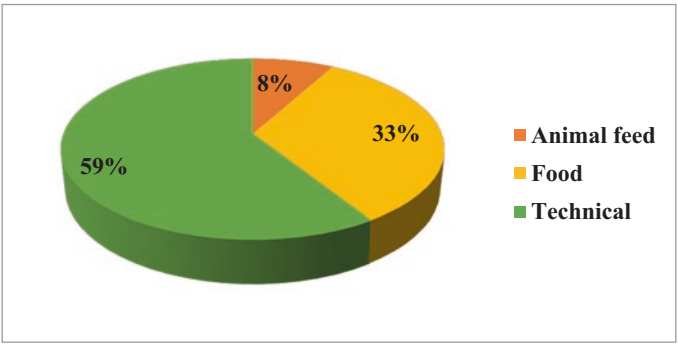


Fig. 1 Distribution graph of industrial enzymes

Purification, characterization, and usability of some hydrolytic enzymes such as pectin lyase (polymethylgalacturonate lyase; EC 4.2.2.10, PMGL), endo-β-D-mannanase (EC 3.2.1.78, mannan endo-1,4-β-D-mannosidase), phytase (myo-inositol hexakisphosphate phosphohydrolases, EC 3.1.3.8), lipase (EC 3.1.1.3), chitinase (EC 3.2.1.14), and protease (EC 2.3.1.1) in industrial areas are described.

2 Enzyme Immobilization

The carrier, which can significantly improve the operational performance of the immobilized enzyme, should be chosen very wisely. Although there is no general carrier for all enzymes and enzyme applications, there should be many properties sought in substances to be used as carriers (Çelenkli et al. 2019; Onem and Nadaroglu 2018; Soleymeni et al. 2017).

These are as follows:

- High interest in proteins.
- Chemical modifications and the presence of functional groups necessary to react directly with the enzyme.
- Hydrophilicity.
- Renewability.
- Mechanical stability and rigidity.
- Ease of preparation in different geometric shapes and desired surface areas for selected biotransformation.
- Large surface area.
- High chemical stability.
- Suitable particle diameter/size (0.2–1 mm/30–60 nm).
- High ion-exchange capacity.
- Low swelling ability.
- Microbial stability is high.
- It does not harm human and environmental health.
- Adequate elasticity.
- Low cost.
- Can be reduced to substances that will not harm natural environments.

Enzymes' ability to show desired activity and characteristics after immobilization is the most important issue in immobilization of biocatalysts. For immobilization, it is necessary to choose a carrier suitable for the enzyme and the immobilization method. There is no systematic method for immobilization methods designed for different catalysts. Since the methods have advantages and disadvantages in themselves, the choice of carrier and method is made according to the intended purpose, the type of enzyme, and the reaction. Carriers should contain functional groups with properties necessary for the immobilization of biocatalysts, as well as adequate mechanical strength; it should have properties such as physical, chemical, and biological stability and nontoxicity.

2.1 The immobilization methods of enzymes are classified as follows:

A. Immobilization into the carrier

1. Cross-linking
2. Connecting to the carrier:

   (a) Physical adsorption
   (b) Ionic bonding
   (c) Covalent attachment
   (d) Complex binding

B. Entrapment:

1. Microencapsulation
2. Fiber entrapment

Immobilization methods of enzymes can be listed as below (Zucca et al. 2016).

The structure of the enzyme molecule, a protein in binding to the carrier, is used. Functional groups, ionic groups, and hydrophobic regions on the molecular surface are involved in this binding. Covalent binding of enzymes to reactive carriers is carried out by methods known in protein chemistry and generally in an aqueous medium. The enzyme is bound to the fixed matrix by covalent methods with the reactions listed below:

A. **Acylation reactions**: The group of lysine is the most commonly used functional group for immobilization by covalent binding on the enzyme protein. Such as anhydride-bearing polymers easily react with carboxyl-bearing polymers and carboxylate polymers (Badgujar et al. 2013).
B. **Alkylation and arylation reactions**: Especially halogen-substituted aromatic rings bind the enzyme molecule over the free-NH_2 group. Nitronization of the benzene ring increases reactivity. In polymers containing monohaloacetyl functional group, it can bind enzymes by alkylation of the free-NH_2 this group on protein (Klunder and Posner 1991).
C. **Diazolation reactions**: Polymeric carriers carrying aromatic primary amine groups can form protein diazonium salt and bind protein molecules with diazolation reactions via phenol, imidazole, or free-NH_2 groups (Nadaroglu et al. 2019).
D. **Carbamylation and thiocarbamylation reactions**: Many polymers carrying isocyanate and isothiocyanate groups can be used as carriers in immobilization of enzymes. Such polymers react only with the free -NH_2 group of proteins, and urea or thiourea derivatives are formed. Since they are more stable, isothiocyanate derivatives and immobilization are preferred over isocyanate derivatives (Goldstein and Manecke 1976).
E. **Linking with CN-Br:** Cyanogen bromide-activated polyhydroxy-polymers are extremely important for both enzyme immobilization and preparation of affinity

resins. Especially water-insoluble polysaccharides such as cellulose, cross-linked dextran, and agarose can be easily activated with CN-Br. While the activation takes place at very high pH, the free $–NH_2$ group on the protein reacts in a weak alkaline environment and binds to the protein carrier (Araripe et al. 2017).

F. **Reactions with imidoesters**: It is used in polymers carrying imidoester functional group and protein immobilization. These reagents are formed by the treatment of polymeric nitriles with alcohol and HCl. The imidoester selectively reacts with the free $–NH_2$ groups on the protein. (Handa et al. 1984).
G. **Reactions with polymeric aldehydes**: Polymers containing aldehyde function, poly-dialdehyde derivatives formed as a result of oxidation of polysaccharides by periodate, can bind proteins by forming Schiff base. Besides the free $–NH_2$ group on the protein, the -SH and imidazole groups give similar reactions. The implementation of this method is very limited. Schiff base formation is an equilibrium reaction. And the formation of aldimine is less in aqueous solution and especially at low pHs (Cejudo-Sanches et al. 2020).
H. **SH-disulfide exchange reactions**: The substances used in the quantitative determination of sulfhydryl groups in proteins such as 2,2-dipyridyldisulfide act as mediators in SH-disulfide exchange reactions (Simons et al. 2013).
I. **Ugi reaction**: Ugi reaction, also known as four-component condensation, occurs between carboxyl, primary amine, aldehyde, and isonitrile functional groups. With the help of this reaction, there are many different options for carrier selection in enzyme immobilization; the carrier can carry any of the four functional groups. Enzyme must be sensitive to aldehyde for enzyme immobilization by Ugi reaction (Vrbová and Marek 1990).
J. **Other binding reactions**: Salts of transition elements are used in the activation of carriers such as cellulose and glass. The most used salt for this purpose is $TiCl_4$. Attachment takes place over serine amino acid (Kennedy et al. 1984).

Nano-Hybrid Structures In the 1960s, with the development of enzyme immobilization technology, it inspired chemists to design new biomaterials containing stabilized enzymes. The immobilization of enzymes using conventional immobilization techniques often led to a loss of enzyme activity to a certain extent. By the 1990s, the activity of immobilized enzymes could be achieved at the level of free enzyme or higher, since they were on nanoscale materials. Interest in this area was reawakened in 2012 with the emergence of the first organic-inorganic hybrid nanoflowers and focused on new flowerlike hybrid nanomaterials (Lee et al. 2015).

The organic-inorganic synthesis mechanism of hybrid nanoflower structure takes place in three steps.

- The first step is the development step; in this step, the crystallization of the phosphate metal $[M_3(PO_4)_2$, (M:Cu, Ca)] is primarily shaped. At this stage, amide groups in the structure of organic molecules (protein, DNA) are combined and coordinated with metal ions (Cu^{2+}, Ca^{2+}) in a complex. These complexes provide a location for nucleation of primary crystals.

- The second stage is the growth stage; in this step, metal-protein crystals are clustered together with protein molecules, and the first flower leaf appears. Kinetically, the development/growth of the surface-borne metal phosphate crystal is controlled kinetically; the leaves appearing initially cause the formation of the flower shape. The cause of this agglomeration is the Cu^{2+} binding sites.
- At the last stage, growth causes the formation of a flowerlike structure. During this growth, the protein forms a skeleton for the leaves and acts as an adhesive glue to bond the leaves (Babagil 2018).

When there is no protein in the environment, large crystals are formed, but nanocrystal structures are not formed (Lee et al. 2015). Since the three-dimensional structures of nano-hybrid structures, which are one of the nanomaterials, have a floral appearance, these structures are generally called nanoculture. With this method, it provides high-efficiency enzyme immobilization and increases the stability of the enzyme. The main types of nano-hybrid structures are copper, calcium, manganese, protein, and capsule hybrid structures. Enzymes have a very strong affinity for metal ions such as protein. Enzymes are generally immobilized on the metal surface (Polaina and MacCabe 2007).

Chitosan is used in making nano-hybrid structure; due to being biocompatible, biodegradable, and nontoxic, it is widely used in medicine, pharmacy, medical, wastewater treatment, food, biotechnology, cosmetics, paper, textile and agriculture sectors, and academic studies. In addition, chitosan is also used as a drug release tool (Çabuk et al. 2011; Synowiecki et al. 2003).

The **advantages** *advantages* of using immobilized enzymes in the reactions are listed below:

1. Enzymes are used repeatedly. This is especially important for difficult and expensive enzymes to manufacture.
2. The product is not contaminated with the enzyme because the enzyme is retained in the matrix.
3. Since the matrix protects the enzyme as a physical barrier, the enzyme becomes resistant to extreme pH and temperature.
4. It makes the enzyme more useful for continuous fermentations.
5. Immobilized enzymes are controlled much more accurately.
6. Since many enzymes will be immobilized with immobilized cells, more than one reaction can take place at the same time (Basso and Serban 2019).

The most important *disadvantage* of the covalent bonding method is that the active structures of enzyme molecules tend to deteriorate due to partial modification (Basso and Serban 2019).

3 Some Hydrolytic Enzymes and Their Immobilization to Plant-Based Materials

As part of this chapter, hydrolytic enzymes pectin lyase (polymethylgalacturonate lyase; EC 4.2.2.10, PMGL), endo-β-D-mannanase (EC 3.2.1.78, mannan endo-1,4-β-D-mannosidase), phytase (myo-inositol hexaphosphophatos phohydrolase 3.1.3.8), lipase (EC 3.1.1.3), chitinase (EC 3.2.1.14), and protease ((EC: 2.3.1.1) enzymes are defined, and their immobilization to new generation plant-based materials is described.

3.1 Pectinases and Pectin Lyase

Pectin is one of the most widely available polysaccharides in nature. The basic unit of pectin is a-D-galacturonate units linked through α-1,4-glycosidic linkages (Fig. 2). The major component in the middle lamella and primary cell wall of higher plants has a great impact on the viscosity and turbidity of fruit juices.

Pectin does not have a certain molecular weight. The molecular weights of pectic substances range from 25 to 360 kDa. It is a fine or rough-looking, odorless, yellowish, or white powder and dissolves in water to form a colloidal, negatively charged, viscous, and opaque solution. The side chains of pectin molecule consist of rhamnose, galactose, arabinose, and xylose (Atalay et al. 2018).

Pectinases or pectin lyase enzymes show activity by hydrolyzing pectic substances. Pectinases are one of the enzymes that have a wide source of bacteria, fungi, and plant origin. Pectinolytic enzymes have an important share, accounting for approximately 25% of enzyme sales (Jayani et al. 2005). Pectinases are biotechnologically important because they have potential applications in clarification of fruit juices, retting of natural fibers (ramie, hemp, flax, bast), treatment of pectic wastewater, coffee and tea leaf fermentation, oil extraction, virus purification, etc. (Jayani et al. 2005).

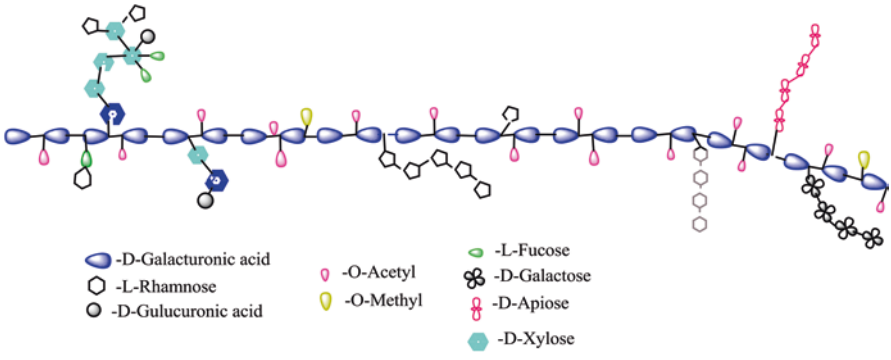


Fig. 2 Pectin molecules chemical content

3.2 Industrial uses of pectinases are as follows:

a) Applications in food industry:

- Jelly, marmalade, jam, etc. It plays a role in providing gelling in products.
- It increases the taste density and consistency of products such as tomato paste, cream, cream cheese, sauce, mayonnaise.
- It is used to increase the fruit flavor of the fruit yogurt.
- It acts as a stabilizer in ice cream.
- It is used to delay stale in bakery and bakery products.
- It is used to provide homogeneous and beautiful appearance in fruit juice production.
- It plays a role in obtaining olive oil from olive pulp.
- It is used in coffee and tea fermentation.
- It plays a big role in wine production.

b) Other sectors:

- Medicine.
- Cosmetics.
- Feed industry.
- It is used in textile industry to prevent glue formation of fibrous plants.

Extracellular pectin lyase (EC 4.2.2.10) was produced by *Bacillus pumilus* (P9), *Geobacillus stearothermophilus* (Ah22), *Brevibacillus borstelensis* (P35), etc. in solid-state fermentation. Pectin lyase enzyme was purified using DEAE cellulose anion-exchange column chromatography and gel filtration chromatography techniques, and then it was characterized. The purified pectin lyase enzyme was used for getting fruit juices. It was determined that yields of fruit juices significantly improved according to the control group (Demir et al. 2014; Nadaroglu et al. 2010).

Some research done by our group is listed below:

- Extracellular pectin lyase (EC 4.2.2.10) was produced by *Bacillus pumilus* (P9), *Geobacillus stearothermophilus* (Ah22), *Brevibacillus borstelensis* (P35), ext. in solid-state fermentation.
- Pectin lyase enzyme was purified using DEAE cellulose anion-exchange column chromatography and gel filtration chromatography techniques, and then it was characterized.
- The purified pectin lyase enzyme was used for getting fruit juices.
- It was determined that yields of fruit juices significantly improved when it was compared with control (Demir et al. 2011a, 2014; Nadaroglu et al. 2010).

Also from *Neisseria gonorrhoeae***,** *Lactobacillus plantarum***,** *etc***.:** Pectin lyase was purified using “three-phase partitioning (TPP)” batch method.

- Three-phase partitioning (TPP) is a relatively new, three-stage, batch method for rapidly and effectively purifying protein.

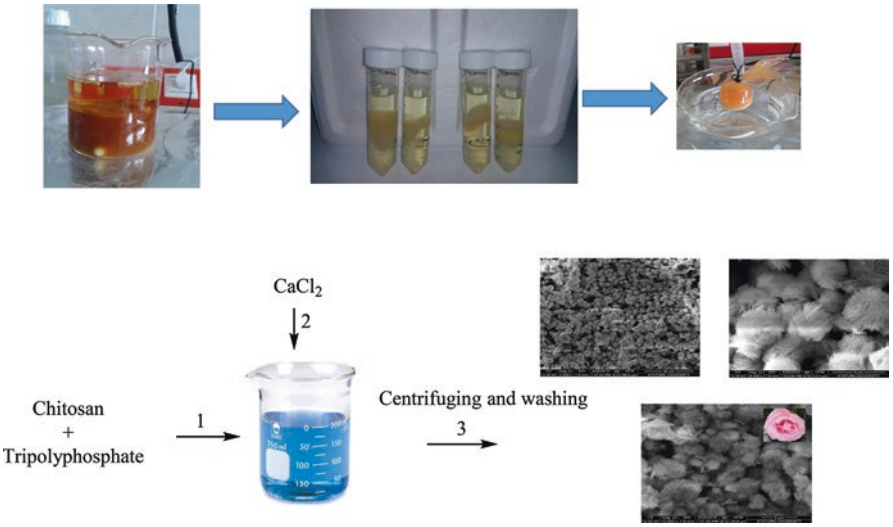


Fig. 3 Purification of pectin lyase enzyme by TPP method and immobilization procedure in nanoflower structure (Babagil 2018; Nadaroglu and Ghebleh, 2019; Ghebleh 2017)

- TPP uses t-butanol and ammonium sulfate to precipitate enzymes and proteins from aqueous solution.
- The simplicity of the method combined with its rapid result makes this a popular choice in large-scale protein purification (Fig. 3).

Also, we used these nanoflowers for clarification of fruit juices: It was determined that yields of fruit juices were more significantly effective according to control.

- It was also observed that pectin lyase activity of hybrid nanostructure is more stable than the free enzyme in the immobilization techniques (Nadaroglu et al. 2016).

Recombinant non-glycosylated and glycosylated polygalacturonase enzymes expressed by *E. coli* and *P. pastoris* bacteria were prepared and immobilized to chitosan. The use of the prepared immobilized enzyme system in the hydrolysis of pectin-rich biomass has been demonstrated (Xu et al. 2018). Polygalacturonase (PG) and pectin lyase (PL) enzymes from *Neurospora crassa*-fermented citrus peel waste were purified using column chromatography technique and immobilized to the chitosan structure by using L glutaraldehyde as a cross-linking agent. It has been determined that chitosan PG and chitosan PL immobilized enzymes have retained more than 75% of their initial activities even after seven consecutive cycles (Bibi et al. 2017). In addition, it has been synthesized in magnetic nanoparticles, and the support materials have been made magnetic so that the enzyme immobilized PL enzyme can be used more efficiently and efficiently (Dal Magro et al. 2019). Agarose is a carbohydrate material and is a good support for enzyme

immobilization. Because there is no modification in its chemical structure, it is inert, and the groups that are desired to be bound with enzyme are connected by modification (Zucca et al. 2016).

The pectin lyase (from Rohapect 10 L) was immobilized on the monoaminoethyl-N-aminoethyl (MANAE) agarose particles modified with ethylenediamine with the enzyme L glutaraldehyde. The resulting immobilized PL increased the enzyme activity almost exponentially when the free enzyme was almost ineffective until it reached the maximum temperature (90 °C). However, immobilization at pH 8 was completed after a few minutes, and the enzyme was bound to the support (L. Dal Magro et al. 2020).

In some studies, it has been immobilized by physical adsorption or covalent bonds of PL enzymes, which have also been modified to some support materials such as plant-derived cellulose, sulfides, XAD amber, and alginates. Improvements in immobilization efficiency and enzyme activity of the immobilized enzyme obtained were observed (Spagna et al. 1995)**.**

3.3 Mannanases

Mannanese Mannanase and heteromannanes contain a backbone of β-1,4-linked mannose and/or D-glucose units that can be acetylated from C-2 or C-3 linked by α-1,6-linked D-galactose units. These polysaccharides are commonly found in fungi, tree structures, beans, and legumes (Dhawan and Kaur 2007). They are also available in plants of vegetable origin, such as coconut, clover seeds, konjac roots, and ivory nuts (Chauhan et al. 2012; Singh et al. 2018).

Mannanases (EC 3.2.1.78, endo-1,4-β-mannanases or mannan endo-1,4-β-mannosidases) are very important enzymes for the biodegradation of hemicellulosic mannans and heteromannans (Chauhan et al. 2012; Dhawan and Kaur 2007; Yamabhai et al. 2016). β-Mannanases hydrolyze 1,4-n-β-D-mannosidic bonds, including extracellular mannan, galactomannan, glucomannan, and galactoglucomannan. (Van Zyl et al. 2010) (Fig. 4).

Mannanases are the enzymes produced in animals, plants, and microorganisms. We purified endo-β-(1,4)-mannanase (EC 3.2.1.78) enzyme from some bacteria and mushrooms (especially thermophilic bacteria and edible, nontoxic mushrooms).

Mannanases are used in many industrial areas. Mannanases can be used in the biobleaching of pulps, in the detergent industry, in the conversion of biomass waste to fermented sugars, in the process of increasing the nutritional value of animal feeds, and in reducing the viscosity of coffee extracts. It can be used in the synthesis of manno-oligosaccharides, which are effective in the development of beneficial bacteria in our intestines, expressed as the second brain (Jørgensen et al. 2010; Lv et al. 2013; Titapoka et al. 2008; Van Zyl et al. 2010).

In some researches conducted by our group, mannanase enzyme was purified and characterized from some microorganisms and nontoxic fungi. Some of them have been covalently immobilized to plant-based materials, and their use has been

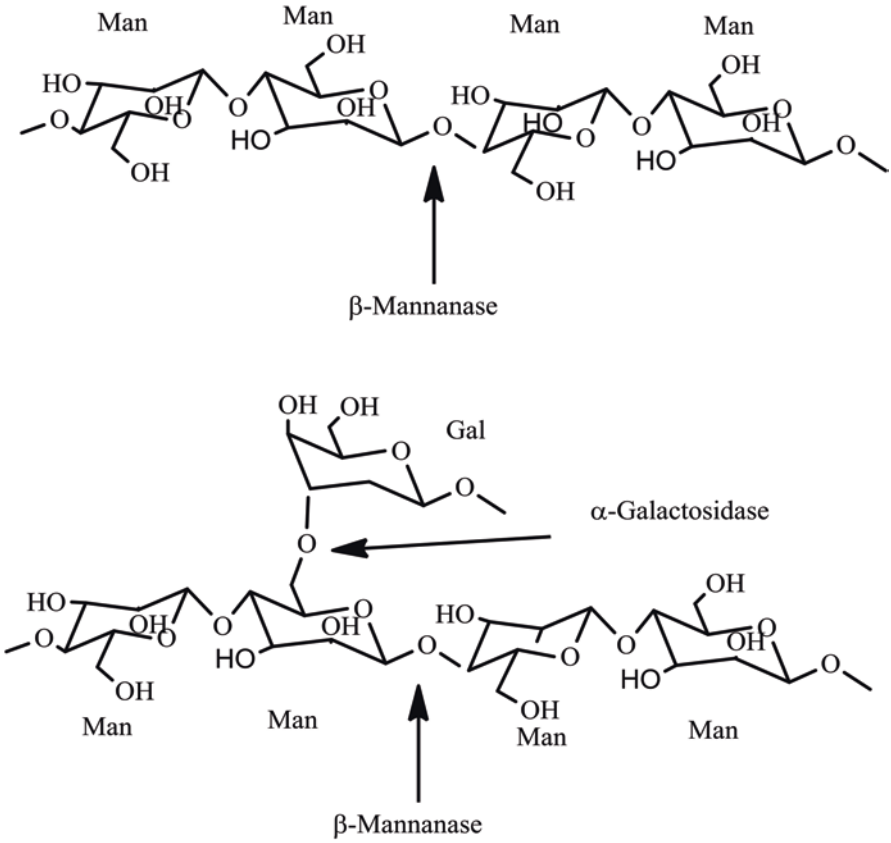


Fig. 4 Hydrolysis mechanisms of mannanase and galactosidase enzymes

tested for industrial purposes. Enzymes are purified with high yield using three-phase precipitation (TPP) technique and are listed below:

- **Thermophilic bacteria**: *Bacillus pumilus* (M27), *Lactobacillus plantarum* (M24), *Lactobacillus plantarum* (ATCC® 14917TM), *Lactobacillus acidophilus*, *Pediococcus acidilactici* (M17), *Weissella viridescens* LB37, *Brevibacillus brevis* (P14), and *Brevibacillus borstelensis* (Adiguzel et al. 2015, 2016; Nadaroğlu and Dikbaş 2018; Nadaroglu et al. 2017; Sadigh et al. 2019).
- **Mushroom**: *Clitocybe geotropa*, *ext.* (Nadaroglu and Sonmez 2016).

In one study, the mannanase enzyme obtained from the *Penicillium occitanie* bacteria was covalently immobilized to the chitin via the L glutaraldehyde intermediate arm using a cross-linking reaction. The yield and recovery of mannanase activity of the immobilized mannanase enzyme were 94.81% and 72.17%, respectively. The immobilized enzyme showed very high stable pH, temperature, and storage stability. It was determined that the initial activity was 70% behind the storage

for 120 days (Blibech et al. 2011). The main hydrolysis products obtained from locust bean gum are obtained from mannotriosis and mannotetraose. After all, it has been concluded that the resulting manno-oligosaccharides can be used as a special nutrient for lactic bacteria (Blibech et al. 2011).

The mannanase enzyme partially purified from the bacterium *E. asburiae* SD26 (GenBank accession number MH718845) by Dhiman et al. (2019) was covalently bound to the surface of sodium alginate-grafted-β-cyclodextrin (Dhiman et al. 2019). It was determined that the obtained immobilized mannanase structure was quite stable against temperature, pH changes, and storage time.

As seen in Fig. 5, sodium alginate and β-dextrin structure have many –OH groups. After aminating the cyclodextrin and sodium alginate structure by using intermediate arm, covalent immobilization will be carried out by creating a Schiff bond over the carbonyl groups in the mannanase enzyme structure.

3.4 Phytase

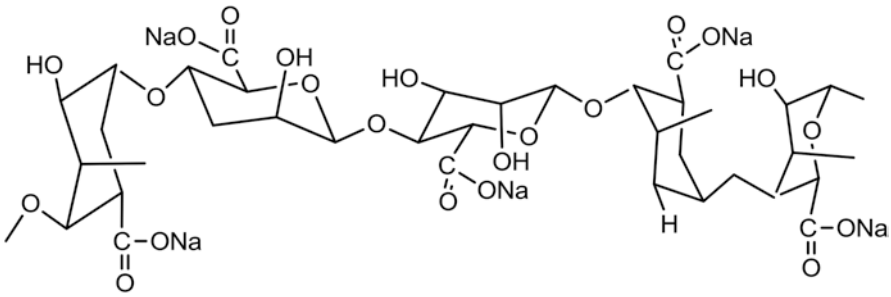
It is an enzyme that plays a catalytic role in the hydrolysis of phytic acid inorganic monophosphate, myo-inositol phosphate, and free myo-inositol (Fig. 6).

Phytate, which is especially present in cereal-containing foods, negatively affects mineral intake in living organisms. e. Phytate binds with cofactors or metal ions such as zinc, iron, calcium, magnesium, manganese, copper which are taken with nutrients forming insoluble complexes and preventing them from being metabolized. The phytase enzyme provides the hydrolysis of phytate to ensure adequate and balanced absorption of minerals into living things (Fig. 7).

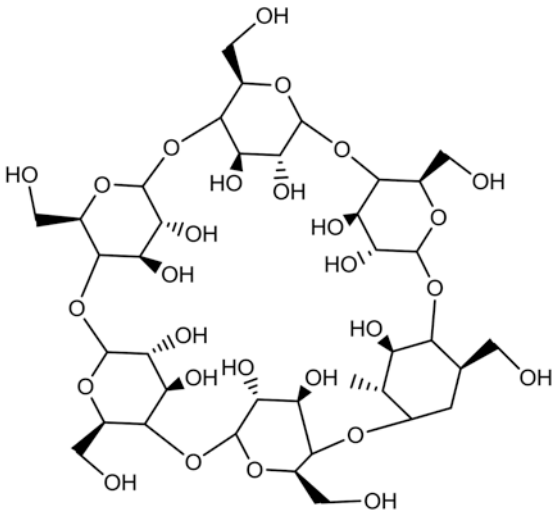
In our research, phytase enzyme was purified from some edible, nontoxic mushrooms [Tirmit (*Lactarius volemus*), Pinar melkior (*Lactarius piperatus*), milkcap (*Lactarius quietus*) mushroom, ext.] (Nadaroglu and Onem 2014; Onem and Nadaroglu 2018; Onem et al. 2016) and some lactic acid bacteria (LAB) [*Lactobacillus plantarum* and *Lactobacillus acidophilus*] (Sarıbuga et al. 2014a, b) using ammonium precipitation and DEAE-Sephadex ion-exchange chromatograph and gel filtration chromatography techniques, and then it was immobilized on surface of magnetite-CTS NPs to ensure prolonged use and its industrial use of pure enzyme (Fig. 6) (Onem and Nadaroglu 2018).

To make comparison and optimization methods, values of optimum pH, optimum temperature, stable pH, and stable temperature of soluble and immobilized phytase enzyme were determined. F Finally, the effectiveness of phytate degradation in cereal based foodstuffs was investigated using free and immobilized phytase enzyme.

It was observed that phytase enzyme which was purified and immobilized onto magnetic chitosan NPs could have a huge potential for use in the field of food, feed, and fertilizer (Nadaroglu and Onem 2014; Sarıbuga et al. 2014a, b).



Sodium alginate structure



β- cyclodextrin structure

Fig. 5 Sodium alginate and β-dextrin structure

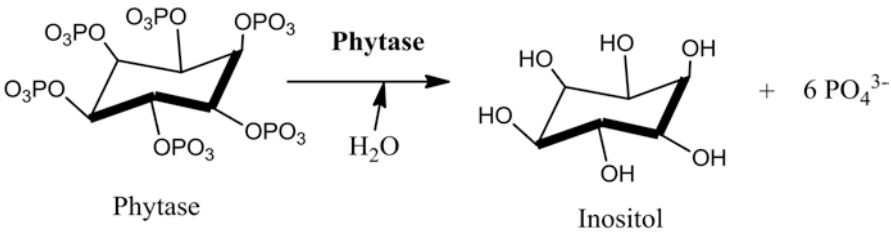


Fig. 6 Phytate hydrolysis mechanism by phytase

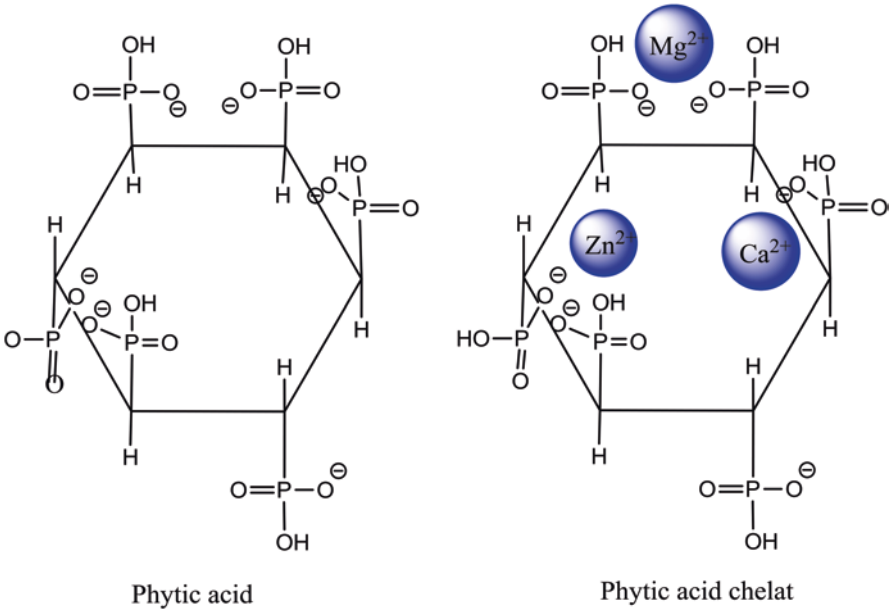


Fig. 7 Phytic acid and phytic acid chelate structure

3.5 Lipase

Lipase (E.C.3.1.1.3; triglycerol acylhydrolases) enzymes can catalyze hydrolysis, esterification, alcoholysis, acidolysis, and aminolysis reactions. Lipases are enzymes used for many applications such as detergents, foods, and synthesis of pharmaceutics, paper industry, cosmetics, leather, and many other industrial fields. Constituting approximately 75% of industrial enzymes, lipases are hydrolytic enzymes. The largest commercial application of lipases is laundry detergents. Approximately 1,000 tons of lipase have been added to 13 billion tons of detergent produced every year (Onem and Nadaroglu 2018; Zaitsev et al. 2019).

Lipase enzyme is also used for aromatization of dietary (Longo and Sanromán 2006; Verger 1997) hydrolysis of cellulose materials in paper production, improvement of skin quality (Hasan et al. 2006), oil waste removal (Kowalski et al. 2004; Shekarchizadeha et al. 2009), biodiesel production (Fan et al. 2012; Luna et al. 2010; Paula et al. 2007; Ribeiro et al. 2011; Tan et al. 2010), margarine production (Carrin and Crapiste 2008; Sellami et al. 2012), food, cosmetics, and detergents (Chang et al. 2014; Huang et al. 2013; Martinalle and Hult 1995; Paula et al. 2007).

Lipase enzyme purified from different sources has been immobilized by using different techniques (adsorption, covalent, crosslinking, etc.) on many biopolymers such as alginate beads, chitosan, cellulose, agarose, Irish moss, guar gum, agar, gelatin, dextran, xanthan. In addition, the purified lipase enzyme carboxymethyl Sephadex, carboxyl agarose, carboxymethylcellulose, p-aminobenzylcellulose, and

Sepharose 4B were immobilized using combine immobilization methods (Kroll et al. 1980).

The lipase enzyme from *Candida antarctica* (CAL), *Thermomyces lanuginosus* lipase (TLL), and the recombinant *Geobacillus thermocatenulatus* (BTL2) was covalently immobilized on glyoxylated agarose beads (Godoy 2017). *Burkholderia cepacia* lipase enzyme was immobilized by cross-linking method to cellulosic materials (Valdés et al. 2011).

Lipase enzyme obtained from *Rhizopus arrhizus* was immobilized on O-pentynyl dextran (PyD) carrier matrix using adsorption method. Lewatit VP OC 1600, Amberlite XAD761, and Duolite A568 ion-exchange resins were used as fixed matrix material (Tahir et al. 2012).

Lipase enzymes from *Thermomyces lanuginosus* lipase (TLL), *Rhizomucor miehei* (RML), and *Candida antarctica* lipase B (CALB) were also covalently immobilized by cross-linking to dextran derivatives via aldehyde derivatives (Orrego et al. 2018).

A thermotolerant and alkaline lipase enzyme was purified from *Lactobacillus brevis* and immobilized onto modified α-Fe_3O_4 florisil nanoparticles (α-Fe_3O_4 MF NPs), and the usability of free lipase (FL) and immobilized lipases (IML) as detergent additives was investigated. Lipase enzyme was purified by fractional precipitation using 20% ammonium sulfate, DEAE-Sephadex ion-exchange chromatographic column, and Sephacryl S-200 gel filtration chromatographic techniques (Soleymeni et al. 2017).

3.6 Chitinase

Chitin ((1,4)-2-acetamido-2-deoxy-beta-D-glucan) is a polysaccharide created by beta, 1–4 bonding of N-acetyl glucosamine residues. Functional properties and physiological activities of chitin oligomers depend on molecular weight and chain length mostly (Fig. 8).

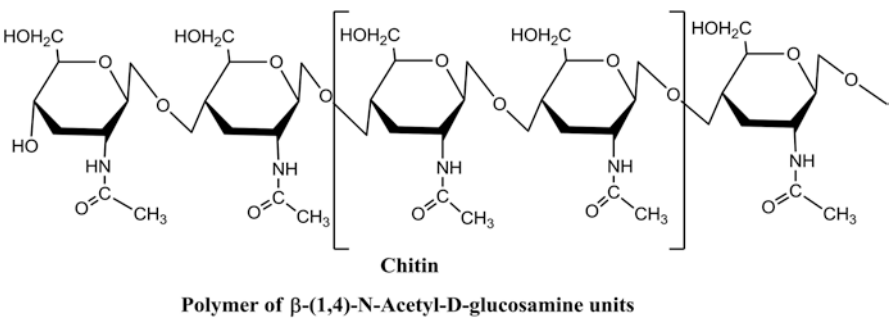


Fig. 8 Chitin chemical structure

Chitin is hydrolyzed by two types of enzymes, namely, chitinase (E.C. 3.2.1.14) and beta-N-acetylhexosaminidase (E.C. 3.2.1.52). In our study, first it was aimed to determine antifungal activities of 100 different microorganisms against *F. culmorum* isolated from cucumber that causes root rot of vegetables as a pathogenic fungus, and also characterization of chitinase enzyme was intended by selecting the strains that have the highest chitinase production capability. In the second phase of the study, purification and characterization of the chitinase enzyme produced extracellularly by using *Bacillus subtilis* TV-125a bacteria, which was found to have the highest chitinase activity, were aimed; and in the last phase of the study, its antifungal activity was investigated against *F. culmorum* (Senol et al. 2014).

As outlined above, enzymes are currently used in several different industrial products and processes, and new areas of application are constantly being added. Thanks to advances in modern biotechnology, enzymes can be developed today for processes where no one would have expected an enzyme to be applicable just a decade ago. Common to most applications, the introduction of enzymes as effective catalysts working under mild conditions results in significant savings in resources such as energy and water for the benefit of both the industry in question and the environment.

Enzymes that have two main chitinolytic activities are chitinase and N-acetyl glucosaminidase (NAGase). Chitinase enzyme; N-acetyl glucosaminidase catalyzes the hydrolysis reaction of the N-acetyl glucosamine in the structure while catalyzing the hydrolysis of the α-1,4-glycosidic bond in the chitin (Dahiya et al. 2006).

Enzymes with chitinolytic activity have high reusability, high pH, and temperature stability by immobilizing; moreover, they can be easily removed from the reaction medium, which provides great advantages (Sakai et al. 1991).

Chitinase and N-acetyl glucosaminidase enzymes are CM-Sephadex, AH-Sepharose, CNBr-Sepharose, AS-Alumina, tannin-sepharose, and tannin-chitosan (Sakai et al. 1991) They were immobilized to different support materials such as chitosan beads (Seoa et al. 2012) and cashew gum polysaccharide/polyvinyl alcohol, etc. and used for biopesticide and biosensor applications.

Chitinase and N-acetyl glucosaminidase enzymes had been immobilized on herbal materials such as CM-Sephadex, AH-Sepharose, CNBr-Sepharose, AS-Alumina, tannin-sepharose and tannin-chitosan, chitosan beads and cashew gum polysaccharide / polyvinyl alcohol (Sakaiark 1991), chitosan beads (Seoa et al. 2012). Immobilized enzymes have been used for biopesticide and biosensor applications (Silva et al. 2012).

3.7 Proteases

Proteases (EC: 2.3.1.1) are members to the main group of hydrolase, and they catalyze the cleavage of peptide bonds with the water inlet. Proteases are the most important industrial enzymes, accounting for approximately 75% of the total hydrolytic industrial enzyme market. They have multiple applications, for example, in

detergent formulations and textile, food, and pharmaceutical industries. Therefore, the industrial demand of proteolytic enzymes, with appropriate specificity and stability to pH, temperature, and chemical agents, continues to motivate the search for new sources.

It was intended to explore an alternative protease of vegetal origin for use in the production of cheese, which could be cheaper and more useful than rennin and the other sources of protease enzyme. In our research, it was aimed to purify and characterize proteases from some plants, and its substrate specify, pH, and thermal stability and the influence of surfactants, organic solvents, metal ions, and inhibitors on the protease's activity were determined. So the other aim of this is to determine whether this characterized enzyme can be used for congealing the milk. Congealing the milk is the main step in producing cheese. As result of our research, it was determined that the enzyme activity was stable even in the presence of detergents and organic solvents, the purified proteases could congeal milk, and it would be used for cheese production. The purified proteases had potential application in food processing (Demir et al. 2008, 2011b; Nadaroglu and Demir 2012).

Alkaline protease was produced from *Bacillus licheniformis* NCIM-2042 bacteria, purified and immobilized on calcium alginate. In the immobilization, optimization was made by taking into account four different factors: sodium alginate concentration, calcium chloride concentration, grafting amount, and agitation rate. Ca-alginate hydrogels, one of the most used materials and polysaccharide gel matrices, have been successfully used in immobilization of the protease enzyme. From the findings, it was determined that immobilization was performed by 24 full factorial central composite design and subsequent analysis and model validation by a second-order regression equation (Potumarthi et al. 2008).

Proteases were immobilized onto some polysaccharide derivatives such as cellulose, cellulose acetate, AE-cellulose, DEAE-cellulose, TEAE-cellulose, CM-cellulose, xylan, pullulan, chitosan, sodium arginate, Sephadex G-25, Sephadex LH-20, DEAE-Sephadex, QAE-Sephadex, and CM-Sephadex support materials with physical adsorption, covalent binding, and ionic binding (Kise et al. 1990).

In a study by Mehdi et al., the enzyme of protease and lipase purified from sunflower was purified and immobilized on chitin + starch fixed material, and the change in the dirt removal effect of detergents was investigated. In the immobilization of purified protease and lipase enzymes, chitin and starch have been used in combination, and the IMB-enzyme obtained has been used as a highly active biocatalyst in many biotechnological applications (Mehdi et al. 2018).

In the study of Elchinger et al., the protease enzyme from *Bacillus licheniformis* was purified and immobilized to the chitosan surface in approximately 94% yield. The chitosan immobilized protease enzyme has been converted into a biofilm layer, and its effectiveness has been investigated against toxic bacteria, also known as hospital microbes such as *Staphylococcus aureus*, *Listeria monocytogenes*, and *Pseudomonas aeruginosa*. The biofilm created from the results of the study was found to be highly effective against *Bacillus licheniformis* and *Bacillus amyloliquefaciens* bacteria (Elchinger et al. 2015).

4 Conclusion

In recent studies, natural biopolymers such as alginate, agarose, gelatin, chitin/chitosan, cellulose, guar gum, agar, carrageenan, dextran and hydrolyzates, xanthan, and pectins have been involved in the immobilization of enzymes of hydrolytic origin. The common features of the selected support materials are very abundant in nature, they are biocompatible and biodegradable, and they provide a lot of functionality in multipurpose applications of immobilized enzymes because they are nontoxic. The use of new generation matrices provides safe use in the medical, pharmaceutical and food, biomedical, environmental, and biofuel/energy industries. Thus, the usage of the catalytic properties of immobilized and fixed enzymes increases in the industrial environment by protecting pH, temperature, and storage stability. The performance of immobilized enzymes obtained by immobilization with new methods to the new biopolymer matrices developed can be increased on a larger scale.

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Recent Advances Toward Development of Plant Cell Culture Process for Sustainable Production of Lignans and Their Health Benefits

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Abstract Cancer has been recognized as a global health burden in rates of morbidity and mortality. Much effort has been devoted to discover promising cancer therapeutic agents from natural sources, and there has been some progress in introducing new anticancer drugs into the pharmaceutical market. Plant-derived natural products have played a very important role as cancer chemotherapeutic agents, either in their unmodified (naturally occurring) or synthetically modified forms. In this respect, lignans which are presented in a wide range of plants such as flaxseed, sesame, and other seeds, as well as vegetables, fruits, and beverages such as coffee and tea, show diverse spectrum of health-promoting effects, such as anticancer, antioxidant, antiviral, antidiabetic, and protective against cardiovascular diseases. A number of lignans (arctigenin, matairesinol and its glycosides pinoresinol and phillygenin) have come to the fore in research. The production of lignans from plant by the conventional methods is met with several problems. The seasonal production, agricultural practices, handling, and poor storage of plant materials impede offering such demand compounds to pharmaceutical factories. The biotechnological methods, particularly plant cell cultures, are an attractive alternative source to whole plant for the production of such compounds with significant amounts which are not

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always easily available. The aim of this review is to focus on lignans: biosynthesis in plant, sources, health benefit, and production of flaxseed lignans by plant biotechnological methods.

Keywords Lignans · Anticancer · Flax and biotechnology production

Abbreviations

AC	Aberrant crypts
ACF	Aberrant crypt foci
BA	Benzyladenine
CHD	Coronary heart disease
CVD	Cardiovascular disease
DP	Dirigent protein
DW	Dry weight
ED	Enterodiol
EL	Enterolactone
ER	Estrogen receptor
FW	Fresh weight
HDL	High-density lipoprotein
IAA	Indole acetic acid
LAR	Lariciresinol
LDL	Low-density lipoprotein
LS	Linsmaier & Skoog *medium*
MAT	Matairesinol
MeJA	Methyl jasmonate
MS	*Murashige and Skoog medium*
NAA	*Naphthaleneacetic acid*
NO synthase	Nitric oxide synthases
PAL	Phenylalanine ammonia lyase
PINO	Pinoresinol
SDG	Secoisolariciresinol diglucoside
SECO	Secoisolariciresinol
TAG	Triacylglycerol

1 Introduction

Plants have been an important source of medicine for thousands of years. Since the early days of mankind, plants and their secondary metabolites have been used by humans to treat infections, health disorders, and illness (van Wyk and Wink 2004).

Plants are also the source of many modern medicines. According to recent estimates, 25% of all prescribed medicines in the developed world contain ingredients derived from plants, and roughly 80% of the world's population living in the developing world relies on herbal remedies for their primary healthcare needs (Tripathi and Tripathi 2003). Some of the most effective cancer treatments to date are natural products or compounds derived from plant products. Isolation of anticancer pharmaceuticals from plants is difficult due to their extremely low concentrations. The industry currently lacks enough methods for producing all the desired plant-derived pharmaceutical molecules. Some substances can only be isolated from extremely rare plants. Plant cell cultures are an attractive alternative source to whole plant to produce high-value secondary metabolites. The biotechnological method offers a quick and efficient method for producing these high-value medical compounds in cultivated cells.

As a folk medicine, lignans from phenolic compounds have been used owing to anticancer and laxative effect for centuries. Lignans are naturally occurring phenylpropanoid dimers (C6-C3 unit; e.g., coniferyl alcohol), in which the phenylpropane units are linked by the central carbons of the side chains (Ayres and Loike 1990; Hearon and MacGregor 1995). They are derived from the phenylpropanoid pathway (Imai et al. 2006). Numerous structurally different forms of lignans exist, even if their molecular backbone consists only of two phenylpropane units (Mazur and Adlercreutz 1998; Imai et al. 2006).

Lignans are commonly included in the human diet, as they are widespread in the plant kingdom (Umezawa 2003; Milder et al. 2005a, b; Penalvo et al. 2008). Nowadays, with the growing interest toward nutraceuticals and pharmaceuticals, plant lignans are becoming important therapeutically active class of compounds because of their putative beneficial health effects, such as antioxidant, antiviral, anticancer (Hano et al. 2017; Garros et al. 2018; Ayres and Loike 1990; Saarinen et al. 2007; Willfor et al. 2003), antidiabetic, and antiobesity (Bhathena and Velasquez 2002) effects, and they may protect against cardiovascular diseases (Vanharanta et al. 1999). Secoisolariciresinol and matairesinol were among the first lignan precursors identified in the human diet and are therefore the most extensively studied, especially as anticancer agents (Fuss 2003).

The highest lignan concentrations have been found in flaxseeds and sesame seeds (Milder et al. 2005a; Smeds et al. 2007, 2012; Thompson et al. 2006), and lower concentrations are present in, e.g., *Brassica* vegetables (Milder et al. 2005a), nuts, and cereals (Mazur and Adlercreutz 1998; Milder et al. 2005a; Penalvo et al. 2008; Smeds et al. 2007). Flaxseed is the richest source of plant lignans due to its high content of secoisolariciresinol diglucoside (SDG) and secoisolariciresinol (SECO) and relatively high matairesinol (MAT). SDG and MAT are metabolized by the intestinal microflora into their biologically active forms enterodiol (ED) and enterolactone (EL), respectively (Adlercreutz and Mazur 1997). Lignans (SDG, SECO, and MAT) are conceded as natural cancer chemopreventive substances, effective against the onset of breast, prostate, and colon cancer, and display a wide range of health-promoting activities for humans in indirect manner (Laine et al. 2009). The beneficial effects of these compounds on human health are well

recognized (Westcott and Muir 2003; McCann et al. 2005), and the in vivo studies confirm the observed in vitro effect (Bylund et al. 2005).

In an effort to study the biosynthesis and the accumulation of secondary metabolites, cell cultures have been established as a very useful tool because this system allows uniformity, accessibility, and reduced complexity (Facchini 2001; Mesnard et al. 2002; Verpoorte et al. 2002). Many studies were aimed to accumulate the different lignans (podophyllotoxin, justicidin B, SDG, SECO, or MAT) in cell culture of different *Linum* species (Mohagheghzadeh et al. 2002; Schmidt et al. 2006; Hemmati et al. 2007; Hano et al. 2006; Attoumbre et al. 2006; Renouard et al. 2012; Ionkova et al. 2013). Additionally, *Agrobacterium rhizogenes*-mediated transformation system was found to be very useful for hairy root induction and production of phytochemicals (Choi et al. 2000; Veena and Taylor 2007). Successful induction of hairy root has been reported in some *Linum* spp. such as *L. flavum* (Oostdam et al. 1993; Lin et al. 2003), *L. austriacum* (Mohagheghzadeh et al. 2002), *L. leonii* (Vasilev et al. 2006), *L. tauricum* (Ionkova and Fuss 2009), *L. usitatissimum* (Gabr et al. 2016, 2018), and *L. album* (Tashackori et al. 2016), and main metabolites (lignans) from resulted hairy roots have been shown to have biological activity.

The aim of the present chapter is to highlight on lignans: plant biosynthesis, sources, health benefits, and the effort for lignan production in plant cell cultures.

2 Lignans

Lignans are widely distributed in the plant kingdom. They can be already found in mosses and ferns (Stafford 2000). Most lignans are dimers of phenylpropanoid units which are linked via their β-carbon atoms (Fig. 1). This general structure leads to a broad variety of derivatives (Fig. 1). Also, most lignans contain chiral C-atoms. Other natural compounds with chiral centers usually occur only in one enantiomeric form. In contrast, lignans can be found in both forms in different plant species or organs of the same species (Umezawa 2003).

3 Lignan Biosynthesis in Plants

Lignans are di-phenolic compounds derived from the oxidative dimerization of two phenylpropanoid (C6-C3) units (Moss 2000). They are defined by the link of the C3 side chains between their two central carbon atoms (Harmatha 2005). The deamination of phenylalanine acid by phenylalanine ammonia lyase is the first step in phenylpropanoid pathway to form cinnamic acid. After the oxidation steps, the hydroxycinnamic acids are formed. These reactions are activated by coenzyme A (CoA) to their aldehydic forms and into alcohols including the coniferyl alcohols (Davin and Lewis 1992; Boerjan et al. 2003; Costa et al. 2003).

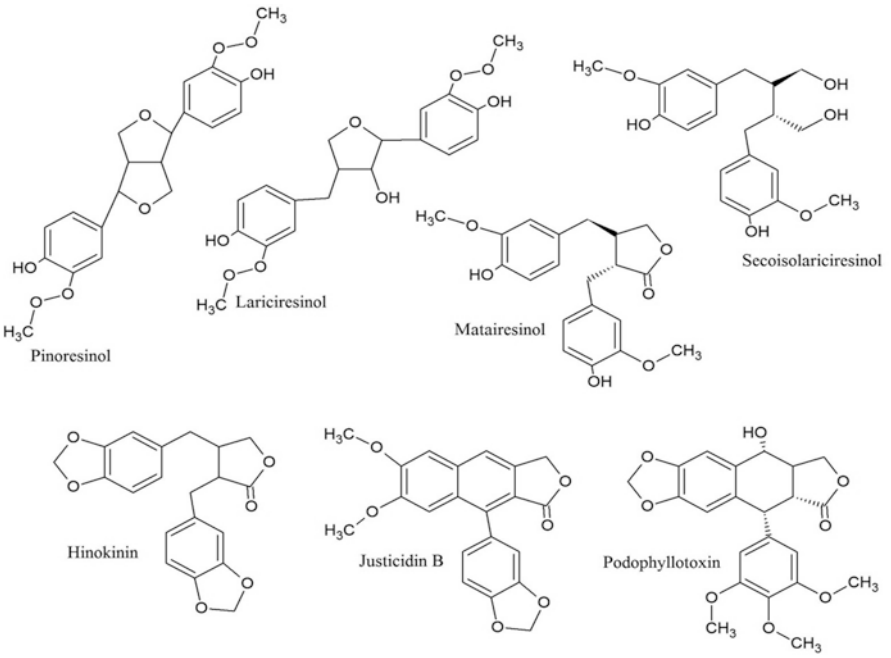

Fig. 1 Basic chemical structure of lignans and their derivatives

The formation of lignans starts via stereoselective coupling of two coniferyl alcohols. The dirigent protein (DP), which catalyzes the dimerization of trans-coniferyl alcohol, entraps the coniferyl alcohol radicals such that the C6-C3 moieties are aligned to produce only one stereoisomer (pinoresinol, PINO) as shown in Fig. 2 (Umezawa et al. 1990; Davin et al. 1997; Halls et al. 2004).

The lignans can be further converted into tetrahydrofurans (lariciresinol, LAR) and dibenzylbutanes (secoisolariciresinol, SECO), via the action of pinoresinol-lariciresinol reductase (PLR; so-called LuPLR1 LuPLR1), and to dibenzylbutyrolactones (matairesinol, MAT) by secoisolariciresinol dehydrogenase (SDH) or to secoisolariciresinol diglucoside (SDG) by secoisolariciresinol diglucosyl transferase (SDT) as shown in Fig. 3 (Ayres and Loike 1990; Lewis and Davin 1999; Ford et al. 2001).

SDG is hardly present in an unbound form (Hano et al. 2006), but is linked in a macromolecular structure, called the lignan macromolecule or lignan complex (Kamal-Eldin et al. 2001), as represented in Fig. 4. The SDG moieties are esterified to each other via the linker molecule 3-hydroxy-3-methyl glutaric acid (HMGA) (Klosterman and Smith 1954). CoA-activated HMGA links to the C-6 of the glucosyl moieties of SDG resulting in dimer and higher oligomer formation (Ford et al. 2001).

An average chain length of 5 SDG units has been suggested based on NMR analysis of the lignan macromolecule, resulting in an average molecular mass of

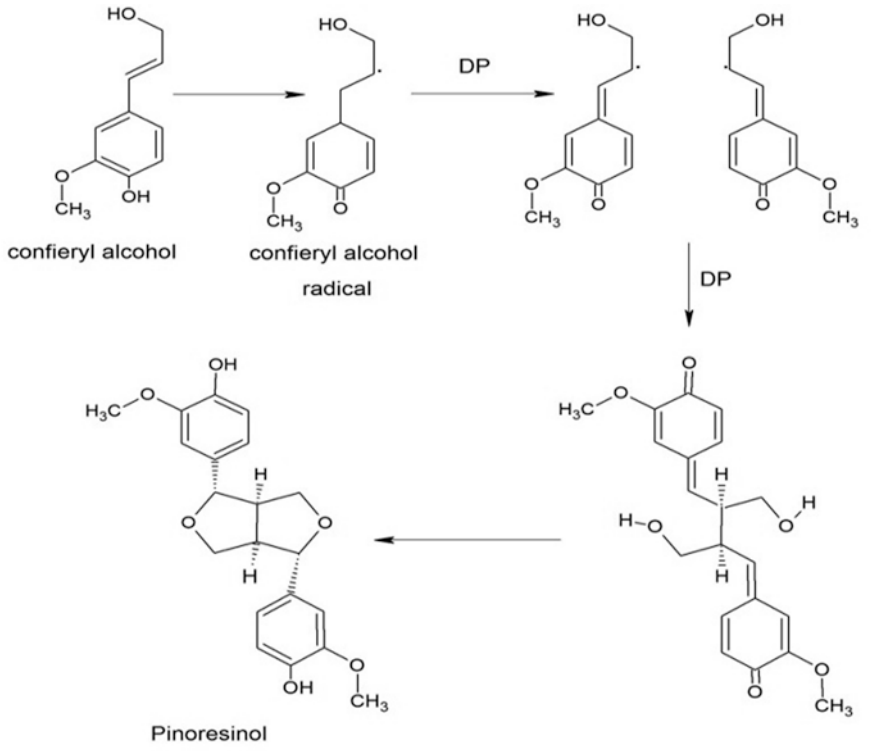

Fig. 2 Dirigent protein-mediated stereoselective radical coupling of coniferyl radicals, DP: dirigent protein. (Modified and adapted from Davin and Lewis 2005)

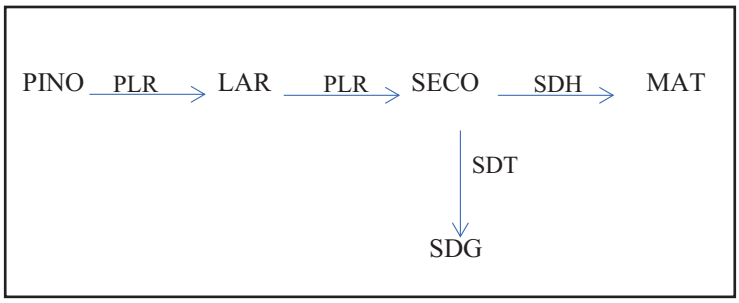

Fig. 3 Biosynthetic pathways of lignans, where PLR, pinoresinol-lariciresinol reductase; SDH, secoisolariciresinol dehydrogenase; and SDT, secoisolariciresinol diglucosyl transferase (modified and adapted from Gabr et al. 2016)

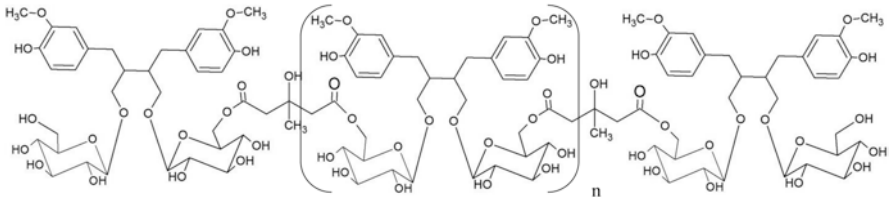

Fig. 4 Chemical structure of the lignan macromolecule (modified and adapted from Kamal-Eldin et al. 2001)

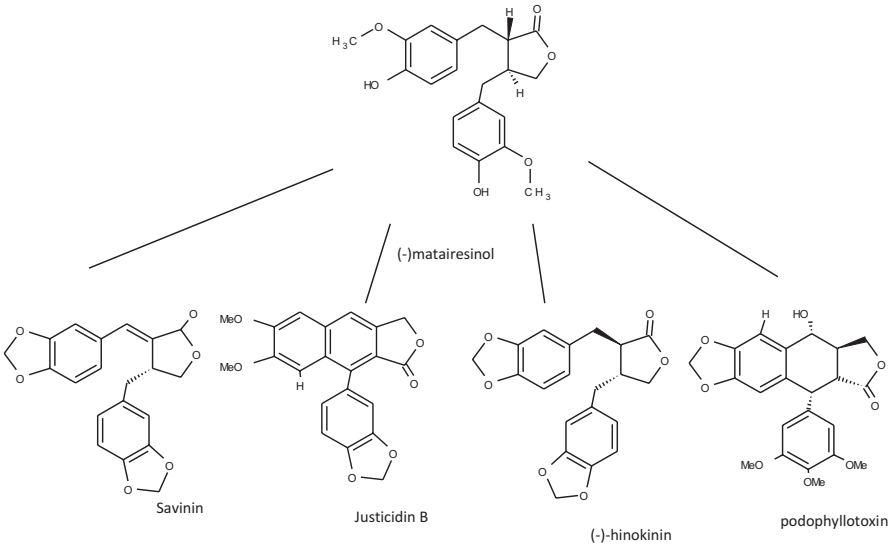

Fig. 5 Possibly from matairesinol-derived lignans (modified and adapted from Fuss 2007)

4000 (Kamal-Eldin et al. 2001). Besides SDG and HMGA, coumaric acid glucoside (CouAG) and ferulic acid glucoside (FeAG) have been identified as constituents of the lignan macromolecule (Ford et al. 2001; Johnsson et al. 2002).

The conversion from coniferyl alcohol to matairesinol is believed to be the general biosynthetic pathway (Umezawa 2003; Suzuki and Umezawa 2007; Davin and Lewis 2003). Matairesinol is believed to be a central intermediate leading to all diverse lignan structures (Fig. 5), i.e., podophyllotoxin, justicidin B, savinin, and (−)–hinokinin.

Podophyllotoxin: During the last decades, most work was performed to understand the biosynthesis of podophyllotoxin and the related 6-methoxypodophyllotoxin. The steps from matairesinol to deoxypodophyllotoxin are most hypothetical since they were figured out by feeding of possible intermediates to *Anthriscus sylvestris* which accumulates mainly yatein (Sakakibara et al. 2003). In addition, Marques et al. (2013) reported that the podophyllum biosynthetic pathway though remains largely unknown, with the last unequivocally demonstrated intermediate

being (−)–matairesinol. Justicidin B: The biosynthesis of justicidin B is not investigated to the present (Hemmati et al. 2007). But, it can be assumed that the first steps are similar to the lignan biosynthesis in *Forsythia intermedia* shown by Davin and Lewis (2003). But, one can presume that the biosynthesis starts with the dimerization of coniferyl alcohol to pinoresinol which is further converted to matairesinol. Several unknown steps can lead to the biosynthesis of justicidin B. (−)–Hinokinin: (−)–Hinokinin biosynthesis has not been investigated to date (Bayindir et al. 2008). Takaku et al. (2001) suggested two possible hypothetical pathways for the further biosynthesis of (−)–hinokinin according to the lignan composition found in *Chamaecyparis obtusa*. In the first pathway, (+)-pinoresinol is reduced via (+)-lariciresinol to (−)–secoisolariciresinol by a pinoresinol-lariciresinol reductase (PLR). Subsequently, (−)–matairesinol is formed by secoisolariciresinol dehydrogenase. In the last two steps, (−)–hinokinin may be synthesized by the formation of two methylenedioxy bridges, either via (−)–pluviatolide or via (−)–haplomyrfolin, depending on the benzene ring on which the first methylenedioxy bridge is formed. In the second hypothetical pathway, the two methylenedioxy bridges are formed directly on (+)–pinoresinol by piperitol-sesamin synthase to give (+)–sesamin (Jiao et al. 1998; Kato et al. 1998). (+)–sesamin may be converted to (−)–dihydrocubebin by a predicted sesamin-dihydrosesamin reductase (SDR), with a two-step reduction reaction similar to the reductions catalyzed by PLR. Finally, (−)–hinokinin could be formed by a secoisolariciresinol dehydrogenase-like enzyme.

Cytotoxic lignans derived from podophyllotoxin are currently used in cancer chemotherapy. Podophyllotoxin for semisynthetic derivatization is isolated from the rhizomes of *Podophyllum* plants growing wild, some of which are counted as endangered species (Petersen and Alfermann 2001). An alternative source for podophyllotoxin or related lignans may in future be cell cultures derived from different plant species, such as *Podophyllum* spp. or *Linum* spp. These cell cultures were shown to accumulate considerable amounts of podophyllotoxin or 5-methoxypodophyllotoxin. Etoposide, teniposide, and etopophos are semisynthetic derivatives of podophyllotoxin and are used in the treatment of cancer. Biotechnological approaches, including the use of cell cultures, biotransformation, or metabolic engineering techniques to manipulate the biosynthetic pathway, represent an alternative to produce podophyllotoxin. As chemical synthesis of podophyllotoxin is not yet economic, it still must be isolated from wild growing *Podophyllum* species, some of which are endangered species. Therefore plant in vitro cultures may serve as alternative sources for podophyllotoxin and for other types of lignans as well (Fuss 2003). Hairy root system with growth medium D (HRGM-D) containing hormone-free MS basal medium with an extra 1-day preincubation period at 35 °C can be a suitable platform for optimization and production of satisfactory level of aryltetralin lignans like podophyllotoxin and its derivatives from *L. mucronatum* (Samadi et al. 2012).

4 Lignan Sources

When comparing results of the lignan content of foods obtained in different studies, it is important to consider that there are several factors affecting results. The lignan content and composition is greatly dependent on natural variations (both genetic and environmental); on the sample pre-treatment applied, including sampling, storage, drying, and extraction methods; and on the analytical method used (Smeds et al. 2012; Durazzo et al. 2018).

Lignans are detected at relatively high concentrations in coniferous trees, especially in knotwood of *Norway spruce* (Willfor et al. 2004). Also, lignans occur in plants included in our food, while the concentrations are significantly lower than in coniferous trees. The highest concentrations have been found in flaxseeds and sesame seeds (Milder et al. 2005a; Smeds et al. 2007, 2012; Thompson et al. 2006), and lower concentrations are present in, e.g., *Brassica* vegetables (Milder et al. 2005a), nuts, and cereals (Mazur and Adlercreutz 1998; Milder et al. 2005a; Smeds

Table 1 Lignans in selected food

Food and foods group		SDG content (μg/100 g)
	Flaxseed	369,900
Seeds	Pumpkin	21,370
	Sunflower	610
	Soybean	273
	Caraway	221
Legumes	Peanut	333
	Urad dal bean	240
	Pigeon pea	50
Nuts	Blackberry	3710
	Lingberry	1510
	Walnut	163
	Almond	107
Berries	Cranberry	1510
	Strawberry	1210
	Red currant	160
	Oat bran	24
	Oatmeal	10
Cereals	Rye bran	132
	Rye meal, whole grain	50
Vegetables	Broccoli	414
	Garlic	379
	Carrots	192
Coffee	Green tea	2460
Tea	Black tea	1590
	Arabica coffee (instant)	716

Modified and adapted from Patel et al. (2012)

et al. 2007; Penalvo et al. 2008). Plant SDG is known to be part of a macromolecule in which they are connected through the linker-molecule HMGA. Hence, SDG has a different extraction method including saponification step to be degraded to its monomeric constituents. The food contents of SDG were presented in Table 1 as analyzed by Adlercreutz and Mazur (1997) and Mazur (1998) and reviewed by Patel et al. (2012)

Thompson et al. (2006) developed a lignan database and reported in the following order: nuts and oilseeds (25–379,012 μg per 100 g by serving weight (g)), cereals and breads (2.0–7239.3), legumes (1.8–979.4), fruits (0.3–61.8), vegetables (1.2–583.2), soy products (2.2–269.2), meat products and other processed foods (0.2–415.1), alcoholic beverages (1.1–37.3), and nonalcoholic beverages (0.9–12). Matairesinol was the least-concentrated lignan in most studied foods, whereas secoisolariciresinol reached the highest concentration in 63 foods, lariciresinol in 44 foods, and pinoresinol in 14 foods among 120 investigated foods (Thompson et al. 2006).

4.1 Seeds and Nuts

Flaxseed is the richest source of plant lignans (Mazur and Adlercreutz 1998; Smeds et al. 2012). Defatted flaxseed is containing high amounts of SDG (9–30 mg/g dry weight, DW) which is considered 800 times higher than any other food (Dobbins and Wiley 2004). SECO concentration in flaxseed (294,210 μg/g fresh weight, FW) is higher than in other food as well. PINO, LARI, and MAT are also present in substantial amounts (Milder et al. 2005a). Other authors have also analyzed lignan concentrations in flaxseed (Johnsson et al. 2000; Kraushofer and Sontag 2002; Mazur and Adlercreutz 1998; Obermeyer et al. 1995; Thompson et al. 2006; Smeds et al. 2012). The second highest lignan concentration was detected in sesame seeds, with PINO and LARI as the main constituents, 29,331 μg/100 g FW and 9470 μg/100 g FW, respectively (Milder et al. 2005a). However, the major lignans in sesame seeds are the oil-soluble sesamin and sesamolin which was not included in these analyses. After lignan-rich food sources (flaxseeds and whole sesame seeds), cloudberry seeds are considered the third richest lignan food source, with total lignan content 43,876 μg/100 g DW and LARI and medioresinol (MED) as the main constituents, 11,581 and 13,595 μg/100 g DW, respectively, and total lignan content 43,876 μg/100 g DW (Smeds et al. 2012). Also, as a first report, Smeds et al. (2012) found that camelina seeds (whole) seem to have a relatively high lignan content, with LAR and SECO dominating. Several other members of the oilseeds, i.e., cashew nuts and peanut, are also found to be relatively high in their content of total lignans (629 and 94 μg/100 g FW), respectively (Milder et al. 2005a). Lignan contents in several food sources are presented in Table 2.

Table 2 Lignan contents in different food sources (μg/100 g)

Food	SECO	PINO	LARI	MAT	References
Seeds					
Flaxseed	294,210	3324	3041	553	Milder et al. (2005a)
	369,900			1087	Mazur and Adlercreutz (1998)
	385,000–670,000				Johnsson et al. (2000)
	1,261,700				Liggins et al. (2000)
	495,700–1,006,200			700–2850	Kraushofer and Sontag (2002)
	81,700				Obermeyer et al. (1995)
	379012.3	729.6	2807.5	153.3	Thompson et al. (2006)
Sesame seed	66	29,331	9470	481	Milder et al. (2005a)
	7.3	6814.5	1052.4	123.1	Thompson et al. (2006)
Sunflower seeds	53	167	671	0	Milder et al. (2005a)
	26.2	33.9	149.7	0.5	Thompson et al. (2006)
	127.8			0	Horn-Ross et al. (2000)
Nuts					
Cashew	133	0	496	0	Milder et al. (2005a)
Peanut	53	0	41	0	Milder et al. (2005a)
Cereals					
Rye	462	1547	1505	729	Smeds et al. (2007)
Wheat	868	138	672	410	Smeds et al. (2007)
Oat	90	567	766	440	Smeds et al. (2007)
Corn	125	33	69	21	Smeds et al. (2007)
Vegetables					
Asparagus	743	122	92	14	Penalvo et al. (2008)
	68.4			–	Horn-Ross et al. (2000)
Brassica vegetables (*Brassica oleracea* L.)	19	1691	599	12	Milder et al. (2005a)
Broccoli	5.8	6.1	82.0	0.1	Thompson et al. (2006)
Cabbage	2.6	44.2	32.3	0.1	Thompson et al. (2006)
Carrot	93	19	60	0	Milder et al. (2005a)
Courgette	18	37	64	0	Milder et al. (2005a)
Corn	7	–	14	–	Penalvo et al. (2008)
French beans	29	24	220	0	Milder et al. (2005a, b)

(continued)

Table 2 (continued)

Food	SECO	PINO	LARI	MAT	References
Garlic	42.0	481.9	54.4	4.8	Thompson et al. (2006)
Glover sprouts	0			0	Horn-Ross et al. (2000)
Mushroom	0	0	0	0	Penalvo et al. (2008)
Myoga spike	7	7	–	1	Penalvo et al. (2008)
Salad green, rucola	78.3			3.4	Valsta et al. (2003)
Seri	449	491	746	13	Penalvo et al. (2008)
Spinach	0.2	0.3	3.1	0.1	Thompson et al. (2006)
Cucumber	8	1	59	0	Milder et al. (2005a)
Sweet peppers (green)	7	1	164	0	Milder et al. (2005a)
Sweet peppers (red)	7	1	106		Milder et al. (2005a)
Tomatoes	1.2	1.9	6.0	0.0	Thompson et al. (2006)
Tomatoes (cherry)	16	19	38	–	Penalvo et al. (2008)
Wheat sprout juice	1.6			0	Valsta et al. (2003)
White potatoes	0.4	0.2	0.6	0	Thompson et al. (2006)
Fruits					
Apricot	328.3			Tr	Horn-Ross et al. (2000)
	31	105	314	0	Milder et al. (2005a)
Brussels sprouts	21			0.4	Valsta et al. (2003)
Dried apricots	147.6	190.1	62.1	0.6	Thompson et al. (2006)
	328.3			Tr	Horn-Ross et al. (2000)
Dried dates	106.2	100.2	116.9	0.3	Thompson et al. (2006)
Dried prunes	1.8	103.8	71.5	2.1	Thompson et al. (2006)
	75.8			Tr	Horn-Ross et al. (2000)
Dried raisins	9.2	0.8	11.5	0.2	Thompson et al. (2006)
	Tr			52.2	Horn-Ross et al. (2000)
Grape, green	0.2			1.3	Valsta et al. (2003)
Grapefruit	26.3			0	Valsta et al. (2003)
	0			Tr	Horn-Ross et al. (2000)
Kiwi	174.6			1.2	Valsta et al. (2003)

(continued)

Table 2 (continued)

Food	SECO	PINO	LARI	MAT	References
Nashi (Japanese pear)	7	0	21	–	Penalvo et al. (2008).
Olive	55.9			2.7	Valsta et al. (2003)
Pear	9.9			0.7	Valsta et al. (2003)
Raspberries	11.6	17.7	8.2	0.3	Thompson et al. (2006)
Rosehip	78.8			2.2	Valsta et al. (2003)
Strawberries	5.1	20.8	22.9	0.1	Thompson et al. (2006)
	5	212	117	0	Milder et al. (2005a)
Valencia orange	56	51	193	–	Penalvo et al. (2008)
Yuzu	26	654	192	–	Penalvo et al. (2008)
Nonalcoholic beverage					
Coffee	Tr			0	Horn-Ross et al. (2000)
	9.4–16.1	0.4–1.5	9.0–13.1	0.0–0.7	Milder et al. (2005a)
	4.7	0.2	0.9	0.1	Thompson et al. (2006)
Orange juice	8.0	0.1	0.2	0.0	Thompson et al. (2006)
Pomegranate juice	0	2.1	0	0	Bonzanini et al. (2009)
Milk, cow	0.4	0.3	0.3	0.1	Thompson et al. (2006)
Milk, soya	1.1	30.0	6.6	0.0	Milder et al. (2005a)
Tea, black	5.0–6.2	27.0–40.6	28.9–30.8	1.1–1.5	Milder et al. (2005a)
Tea, green	12.9	5.7	18.7	2.0	Milder et al. (2005a)
Alcoholic beverage					
Beer	0.0–1.0	12.6–22.2	5.9–9.2	0.0	Milder et al. (2005a,)
Wine, red	29.4	0.4	7.4	0.0	Thompson et al. (2006)
	41.7–61.3	6.3–11.9	8.6–15.9	5.9–7.8	Milder et al. (2005a)
Wine, white	5.9	0.1	0.4	1.6	Thompson et al. (2006)
	5.2–7.6	1.7–3.0	4.6–11.9	2.7–3.1	Milder et al. (2005a)

4.2 Cereals

In grains, the plant lignans are localized in the outer fiber-containing layers of the kernel, with high concentrations in the aleurone layer. Mazur et al. (1998a, b) reported that lignan concentrations in whole grain were 48–112 μg/100 g while in grain brans the concentration is higher than in whole grain (63–299 μg/100 g DW). On the other hand, lignan concentrations in the flour (8–32 μg/100 g DW) are low. Determination of plant lignans in different cereal species were listed them from the highest total lignans concentration to the lowest as follow: rye, wheat, triticale, oat, spelt wheat, Japanese rice, wild rice, buckwheat, barley, amaranth, corn, millet, quinoa, red rice, brown rice, dhurra were confirmed by Smeds et al. (2007) and Durazzo et al. (2013).

4.3 Vegetables and Fruits

It is worth noting that vegetables contain higher concentration of lignans than fruits. The total lignan concentrations in vegetables are 16–3874 μg/100 g DW while in fruits are 5.0–1510 μg/100 g DW (Mazur et al. 1998a, b). *Brassica* vegetables (cabbages, brussels sprouts, kale), asparagus, seri, and garlic contain high levels of total lignans in the selected vegetables for analysis by Horn-Ross et al. (2000), Milder et al. (2005a), Thompson et al. (2006), and Penalvo et al. (2008) with values 2321, 1034, 1724, and 583.2 μg/100 g FW, respectively. French beans (273 μg/100 g FW), sweet peppers (green, 172 μg/ 100 g; red, 113 μg/100 g FW), carrots (171 μg/100 g FW), and courgettes (119 μg/100 g FW) also have relatively high lignan concentrations.

The richest source among fruits analyzed by Penalvo et al. (2008) was yuzu, a citrus fruit originating in East Asia. Mazur et al. (2000) found relatively high levels of SECO in berries. Horn-Ross et al. (2000) were able to detect SECO and MAT in a few dried fruits (apricots, prunes, raisins) and in peaches, while a higher concentration of MAT was reported in raisins (52 μg/100 g FW) and only trace amounts of SECO.

Moreover, Penalvo et al. (2008) showed for avocado a profile of decreasing concentration of lignans, syringaresinol > pinoresinol > medioresinol > secoisolariciresinol > lariciresinol > matairesinol, and for pineapple, syringaresinol > lariciresinol > matairesinol > secoisolariciresinol > pinoresinol > medioresinol, whereas the most representative lignan for navel orange was lariciresinol and secoisolariciresinol for kiwifruit. In berries, as reported by Smeds et al. (2009), the most representative lignans among those studied were lariciresinol for cloudberries (5008 μg/100 g dry weight) and secoisolariciresinol for blackberries (2902 μg/100 g dry weight) (Smeds et al. 2009).

4.4 Beverage

According to Mazur et al. (1998a, b), lignans are also found in beverages including tea, coffee, and wine. The lignan concentration in tea (770–3050 μg/100 g dry tea leaves) is higher than that in coffee powder (393–716 μg/100 g DW). Total lignan concentrations in red wine are 69.1–91.3 μg/100 ml with SECO being the superior one (Milder et al. 2005a). In 2008, Peñalvo et al. reported that plant lignan concentration in Japanese food varies from 0 to 1724 μg/100 g wet weight. Within the coffee samples from different countries, secoisolariciresinol varies from 27.9 to 52.0 μg L^{-1} and lariciresinol from 5.3 to 27.8 μg L^{-1}, respectively, contrary to matairesinol that was not possible to detect in each type of coffee (Angeloni et al. 2018).

5 Daily Intake

Daily intake of SECO and MAT ranges from 0.18 to 0.65 mg/d in studied populations in the Netherlands, the United States, and Finland (Horn-Ross et al. 2000; de Kleijn et al. 2001; Keinan-Boker et al. 2002; McCann et al. 2003; Valsta et al. 2003). Milder et al. (2005b) reported that the intake of the total plant lignan MAT, SECO, PINO, and LARI was 1.2 mg/day in Dutch adults. Similar results were obtained by Nurmi et al. (2010) in a study population of 100 Finnish men. In 2010, Pellegrini et al. conducted a cross-sectional study on 242 men and postmenopausal women in Northern Italy in which the daily intake of individual lignans including the daily intake of MAT, SECO, PINO, and LARI were 20.9, 335.3, 96.7, and 175.7 μg/d, respectively, and the total lignans were 665.5 μg/d. The major dietary sources for lignan intake in the Dutch cohorts were coffee and tea (Nurmi et al. 2010). In contrast, in an Italy cohort, red wine accounts for about one-third of total lignan intake (Pellegrini et al. 2010).

6 Lignans for Human Health

Since lignans can show, e.g., antiviral, fungicidal, antibacterial, and cytotoxic activities, they are thought to be involved in the plant defense against pathogens and are important for human health benefits against diseases such as breast cancer, prostate cancer, colon cancer, cardiovascular disease, obesity, and diabetes (Landete 2012). Experimental evidence in animals has shown clear anticarcinogenic effects of flaxseed or pure lignans in many types of cancer. Most of the plant lignans in human foods are converted by the intestinal microflora to enterolactone (EL) and enterodiol (ED), called mammalian lignans or enterolignans (Adlercreutz 2007). Intestinal bacteria catalyze the transformation of plant lignans to enterodiol and enterolactone

by a series of metabolic steps. ED and EL are thought to be responsible for the health benefits for lignan (Mabrok et al. 2012).

6.1 Effect of Lignans on Breast Cancer

Breast cancer is one of the most common cancers in the world. It accounts for ~30% of all diagnosed cancer cases in women each year (Bange et al. 2001) and is considered a major public health issue with over 1 million newly diagnosed cases per year. More than 400,000 annual cases of death and 4.4 million women living with breast cancer are reported (Malik et al. 2010). Plant lignans (SDG, SECO, and MAT) are metabolized by human intestinal bacteria to the enterolignans, ED and EL (Borriello et al. 1985), as shown in Fig. 6. ED and EL exhibit structural similarity to estradiol and have therefore been hypothesized to modulate hormone-related cancers such as breast cancer (Adlercreutz et al. 1982). Studies revealed that the enterolignans are mostly associated with the reduction of breast cancer (Serraino and Thompson 1992; Thompson et al. 1996; Wang et al. 2005; Fabian et al. 2010). The chemopreventive effect of lignans is well established in human cancer cell lines and animal cancer models (Boccardo et al. 2006; Saarinen et al. 2007, 2008). In Mousavi and Adlercreutz (1992) reported that the combination of EL (0.5–2.0 μM) and estradiol (1.0 nM) resulted in a lower cell proliferation of human estrogen receptor-positive (ER+) breast cancer cell (MCF-7 cell). This anti-proliferative effect of lignan in MCF-7 cells depends on the dose and the estradiol status. Flaxseed consumption reduces the incidence, number, and growth of tumors in carcinogen-treated rats at the pre-initiation, promotion, or progression stages of carcinogenesis (Serraino and Thompson 1992; Thompson et al. 1996). Moreover, consumption of rye decreased tumor number and size in breast cancer model (Davies et al. 1999). Tumor growth and incidence of metastases are also reduced by dietary flaxseed in athymic mice injected with human ER-negative (Dabrosin et al. 2002; Chen et al. 2002) or ER-positive (Chen et al. 2004) breast cancer cells. The tumor size and number of breast tumors as well as breast cancer biomarkers were reduced in breast cancer rats fed with SDG and/or flaxseed diet (Saggar et al. 2010a, b; Mabrok et al. 2012). Dietary flaxseed and its equivalent amount of pure SDG reduced tumor size and cell proliferation in ovariectomized-athymic mice with an increase in apoptosis in this model. This effect may be mediated through the inhibition of ER- and growth factor-mediated signaling pathways (Chen et al. 2002, 2009a; Dabrosin et al. 2002; Saggar et al. 2010a, b). One of the main components of flaxseed is the lignans, of which 95% are made of the predominant secoisolariciresinol diglucoside (SDG). SDG is converted into enterolactone and enterodiol, both with antiestrogen activity and structurally similar to estrogen; they can bind to cell receptors with farther decreasing cell growth. Some clinical trials showed that flaxseed can have an important role in decreasing breast cancer risk, mainly in postmenopausal women (Velentzis et al. 2009; Calado et al. 2018).

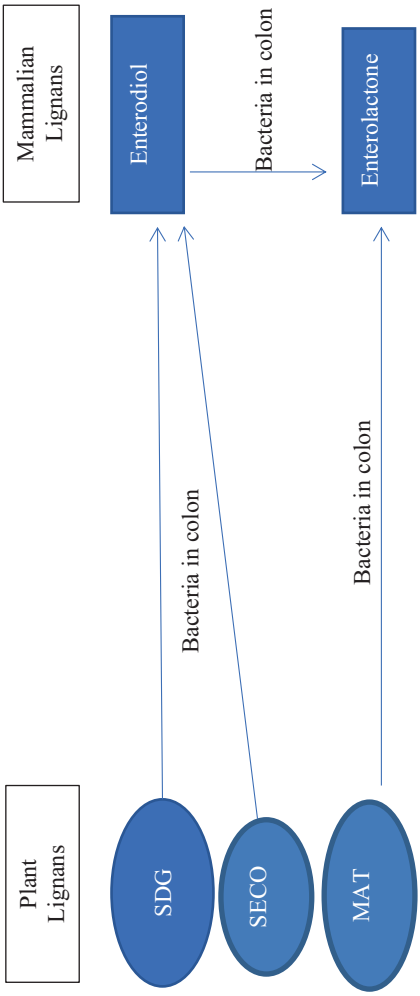

Fig. 6 Metabolism of plant lignans

The urinary excretion of EL is lower in breast cancer patients than those in controls (Adlercreutz et al. 1982). In Stuedal et al. (2005) investigated possible associations between circulating EL and normal breast tissue morphology. They reported that EL concentration in plasma is negatively correlated with breast mammographic density in postmenopausal Norwegian women. In pilot study, the observed modulation of breast cancer biomarkers in premenopausal women showed a significant decrease in Ki-67 index (80% of the studied subjects) with 9- and 16-fold increase in plasma of EL and total lignans, respectively (Fabian et al. 2010). A very recent study showed that lignan intakes are associated with lower risks of breast cancer in premenopausal women with more positive prognostic characteristics (McCann et al. 2012).

6.2 Effect of Lignans on Prostate Cancer

Prostate cancer is the second most frequently diagnosed cancer of men and the sixth leading cause of cancer death among men. About 899,000 new cases of prostate cancer are diagnosed every year worldwide and cause of over 200,000 deaths. The incidence of the disease is increasing with a rise of 1.7% over 15 years (Ferlay et al. 2008). There are numerous reports on the potential tumor-suppressive influence of lignans. Previous studies have shown that Asian men have much lower incidences of prostate cancer and possibly of benign prostatic hyperplasia (BPH) than their Western counterparts. Vegetarian men also have a lower incidence of prostate cancer than omnivorous males (Serraino and Thompson 1992). Lignans and their derived metabolites (ED and EL) are believed to be partly responsible for growth inhibition of human prostate cancer cell lines (Lin et al. 2001). In prostate cancer cell lines (PC-3, DU-145, and LNCaP), 10–100 μM ED and EL significantly inhibit growth of all cell lines (Lin et al. 2001). The density of LNCaP human prostate cancer cells was reduced by 57.5%, the metabolic activity of the tumor cells by 55%, and the cut secretion of prostate specific antigen by 48.50% after treatment of prostate cancer cells with a subcytotoxic concentration of EL (60 μM for 72 h) compared to untreated cells (McCann et al. 2008). Additionally, several key genes such as MCMs, survivin, and CDKs, which produce important effects on programmed cell death of prostate cancer cells, were beneficially regulated by EL treatment (McCann et al. 2008). Activation of insulin-like growth factor-1 (IGF-1) receptor signaling is critical for prostate cancer cell growth and progression (Chen et al. 2009b). EL suppresses proliferation and migration of prostate cancer cells, at least partially, through inhibition of IGF-1/IGF-1R signaling (Chen et al. 2009b). High local levels of estrogen can initiate prostate cancer, and then aromatase activity must be involved, as aromatase converts testosterone to estrogen (Friedman 2009). The ability of lignans to inhibit aromatase can decrease excess estrogen while simultaneously increasing beneficial free testosterone (Griffiths et al. 1998), which might protect against *prostate cancer.*

In a human pilot study, rye whole grain and bran intake has shown beneficial effects on prostate cancer progression as assessed by prostate-specific antigen concentrations (Landberg et al. 2010). Morton and others (Morton et al. 1997) reported that higher EL levels in prostatic fluid were associated with populations (from Portugal, Hong Kong, and the United Kingdom) with a low risk of prostate cancer, indicating a potential prostate cancer protective effect of lignans. A case-control study conducted in Scotland on 433 prostate cancer cases found that higher serum EL concentrations were associated with a lower risk of prostate cancer (Heald et al. 2007). In a small clinical study, prostate cancer cell proliferation decreased and apoptosis increased in men fed 30 g of flaxseed per day (Demark-Wahnefried et al. 2001). A significant factor which may have influenced their study was that the subjects were on a low-fat diet. A subsequent study by those authors further supported the role of flaxseed in combination with a low-fat diet to control prostate growth (Demark-Wahnefried et al. 2004). In Azrad et al. (2013) investigated possible associations between urinary ED and EL with Ki-67 (cell proliferation biomarker) among 147 patients with prostate cancer who participated in a presurgical trial of flaxseed supplementation (30 g/day) for ~30 days. They reported that total urinary enterolignans and EL were inversely correlated with Ki67 in the tumor tissue.

6.3 Effect of Lignans on Colon Cancer

Colorectal cancer is the third most common cancer (9.4%) worldwide after lung and breast cancers. It ranks fourth in mortality (7.9%), after lung, stomach, and liver cancers (Stewart and Kleihues 2003). ED and EL at 10–100 μM levels have been shown to significantly decrease the cell proliferation of four cancer cell lines (LS174T, T84, Caco-2, and HCT-15) (Sung et al. 1998; Cos et al. 2003). These cell lines are not estrogen-dependent, because estradiol at 0.5, 1, and 10 μM did not enhance their cell proliferation. Hence the reduction in colon cancer cell proliferation by ED and EL may be due to non-hormone-related mechanisms (Sung et al. 1998). Ayella and Wang (2007) assessed the effect of different doses of commercially available plant lignans (SDG) on human colon cancer cell growth. It was found that cancer cell growth was significantly inhibited by SDG treatment. Increasing SDG dosage (0–40 μM) did not kill the cancer cells as cell viability was not affected but instead resulted in S-phase cell cycle arrest as measured by DNA flow cytometry analysis (Ayella and Wang 2007). ED and EL both alone and in combination at different concentrations caused a significant increase in apoptotic cells and decrease in cell proliferation of human colon adenocarcinoma Caco-2 cell line (Bommareddy et al. 2010). EL and ED significantly inhibited cell proliferation of Caco-2 cells starting at 50 μM and 150 μM, respectively. Both EL and ED at the concentration of 100 μM significantly induced apoptosis in Caco-2 cells. However, EL-treated cells showed higher increase of apoptotic cells when compared to ED (Bommareddy et al. 2010). A new tetrahydrofuran lignan called (+)-camerarialdehyde extract of

the stems of *Cameraria latifolia* was found to show cytotoxicity toward HT-29 human colon cancer cell line (Ren et al. 2013).

The impact of lignans on colon carcinogenesis has been studied mainly with carcinogen-treated rats (Serraino and Thompson 1992; Jenab and Thompson 1996; Davies et al. 1999). Flaxseed and defatted flaxseed meal (at a level of 5% or 10%) decreased the number of aberrant crypts (AC) and aberrant crypt foci (ACF) in the descending colon of carcinogen-treated rats by 41–53% and 48–57%, respectively (Serraino and Thompson 1992). In a subsequent study, where carcinogen-treated rats were fed either a control diet or a diet supplemented with flaxseed and defatted flaxseed meal (at level of 2.5% or 5%) or pure SDG, the number of AC per focus (AC multiplicity) was significantly reduced in the distal colon of flaxseed and SDG groups (Jenab and Thompson 1996). Feeding 30% rye bran diet, which contains lignans, to AOM-treated rats for 31 weeks lowered the number of large ACF (>6 AC per ACF) and the number of tumors compared with the control (Davies et al. 1999).

In a case-cohort study of Danish middle-aged men and women, Kuijsten et al. (2006) investigated the association between plasma enterolignans and the incidence of colon cancer. The authors observed a substantial reduction in colorectal adenoma risk among subjects with high plasma concentrations of enterolignans, in particular ED (Kuijsten et al. 2006). In an Ontario-based colon cancer population case-control study involving 1095 cases and 1890 control subjects, the dietary lignan intake is associated with a significant reduction in colorectal cancer risk (Thompson et al. 2006). Recently, Johnsen et al. (2010) examined the association between plasma EL concentration and incidence of colon and rectal cancer in 57,053 participants aged 50–64. They concluded that higher EL levels are associated with lower risk of colon cancer among women and higher risk of rectal cancer among men.

6.4 Effect of Lignans on Cardiovascular Disease (CVD)

Oxidative stress, inflammation, obesity, diabetes, dyslipidemia, and hypertension are interrelated and contribute to an atherogenic environment that promotes the development of CVD (O'Keefe et al. 2009; Mathieu et al. 2009). Psychosocial stress may increase cardiovascular risk by activating the sympathetic nervous system and increasing cortisol, blood glucose, and lipid levels as well as elevating blood pressure (O'Keefe et al. 2009). Lignan intake may have an important protective effect against vascular disease. Dupasquier et al. (2007) demonstrated how dietary flaxseed can inhibit the atherogenic effects of a high-cholesterol diet in the LDLrKO mouse through a reduction of circulating cholesterol levels and, at a cellular level, via anti-proliferative and anti-inflammatory actions. A series of studies from the same laboratory investigated the effects of flax lignan complex and pure SDG on atherosclerosis in rabbits (Prasad et al. 1998; Prasad 1999, 2005, 2007, 2008, 2009). They used similar methods for each study; however, they included important variations such as the level of cholesterol in the atherogenic diet (0.25–1.0%), the dosage, and duration of the treatment (2–4 months). Despite these

differences, all of these studies found that consumption of flax lignan complex and SDG resulted in improvements in the lipid profile and was also shown to be effective in reducing the development of aortic atherosclerosis. Hypercholesterolemia induced endothelial cell dysfunction, and decreased endothelial nitric oxide formation results in impaired angiogenesis and subsequent cardiovascular disorders. SDG (20 mg SDG/kg per d) increased expression of vascular endothelial function, endothelial NO synthase, and heme oxygenase-1-mediated myocardial angiogenesis in a hypercholesterolemic myocardial infarction model indicating its cardioprotective effect (Penumathsa et al. 2008).

The intake of lariciresinol was linked by 30% to the reduced odds for hypercholesterolemia. The data on lignan content in food, i.e., lariciresinol, matairesinol, pinoresinol, and secoisolariciresinol, were collected from the available lignan databases. It was shown the existing concept that dietary total lignans are linked to CVD risk factors such as hypertension, hypercholesterolemia, and central obesity (Witkowska et al. 2018).

Similar to most animal studies, several human studies have shown cardiovascular benefits from lignans. A study consisted of the prospective follow-up for an average of 12·2 years of 1889 men free of CVD at baseline showed a significant association between elevated serum EL concentrations and reduced risk of CHD-related and CVD-related mortality (Vanharanta et al. 2003). Since flaxseed is rich sources of plant lignan precursors for EL, the results of these studies suggest that flaxseed might provide cardiovascular benefits. Hypercholesterolemic participants receiving 600 mg SDG/d had decreased plasma total cholesterol and LDL-cholesterol. Plasma concentrations of SECO, ED, and EL were also measured, and the observed cholesterol-lowering values were correlated with SECO and ED concentrations (Zhang et al. 2008). In 6-week-long, randomized, double-blinded, placebo-controlled study, the effects of flaxseed lignans on cardiovascular risk factors were investigated. High-lignan flaxseed (0.41 g lignans) decreased total cholesterol by 12%, LDL-cholesterol by 15%, and oxidized-LDL by 25% (Almario and Karakas 2013).

6.5 Effect of Lignans on Diabetes

Diabetes and the metabolic syndrome are risk factors for CVD. Thus, dietary interventions that lower the risk of diabetes and the metabolic syndrome (increases in central adiposity, serum TAG, serum glucose, blood pressure, and inflammation; decreases in HDL-cholesterol) will help to decrease the incidence of CVD (Cornish et al. 2009). SDG from flaxseed has been shown effective in preventing/delaying the development of type 2 diabetes through suppressing the expression of phosphoenolpyruvate carboxykinase (PEPCK) gene, a rate-limited enzyme in the gluconeogenesis in the liver (Prasad 2002). Lignan-rich fraction improves glucose homeostasis by increasing glucose disposal rates and enhancing hepatic insulin sensitivity by working as a peroxisome proliferator-activated receptor (PPAR)-γ

agonist in type 2 diabetic rats (Kwon et al. 2011). Dietary flaxseed supplementation in diabetes mellitus may have beneficial effects on diabetic nephropathy evolution by reducing the levels of oxidative stress and increasing the antioxidant defense systems (Haliga et al. 2009).

SDG is effective in retarding the development of diabetic complications. In a multidose 14-day study, lower doses of SDG (5 and 10 mg/kg B.W.) exhibited moderate reduction in glucose levels and lipid profile, restoration of antioxidant enzymes, and improvement of the insulin and c-peptide levels which shows the regeneration of β-cell which secretes insulin. The synthetic SDG exerts anti-hyperglycemic effect by preventing the liver from peroxidation damage through inhibition of ROS level-mediated increased level of enzymatic and nonenzymatic antioxidants (Moree et al. 2013).

Lignans have been shown to provide benefits among type 2 diabetic patients. Supplementation of lignan capsules (360 mg/d) reduced glycosylated hemoglobin (HbA1C) compared with placebo in participants with type 2 diabetes, and there was no effect on fasting glucose and insulin concentrations, homeostasis model assessment of insulin resistance (HOMA-IR), and blood lipid profiles (Pan et al. 2007). Blood glucose and cholesterol, particularly low-density lipoprotein cholesterol of type 2 diabetes patients (n = 60), were decreased after consumption of wheat flour chapatis containing flaxseed gum (5 g) (Thakur et al. 2009). In a randomized, crossover study of overweight or obese men and postmenopausal women (n = 25) with pre-diabetes, flaxseed intake decreased glucose and insulin and improved insulin sensitivity as part of a habitual diet in overweight or obese individuals with pre-diabetes (Hutchins et al. 2013).

7 Biotechnological Approaches for Lignan Production

7.1 Plant Cell Cultures

Today, the potential of plant cells to produce secondary metabolites in dedifferentiated cultures is used extensively to produce plant-derived drugs. Screening by selection of high-yielding cell lines is the most common method to enhance productivity.

Biotechnological approaches for producing aryltetralin lignans from *Linum* species have been studied (Malik et al. 2014). The *Linum* sp. contains lignans such as podophyllotoxin (PTOX), deoxypodophyllotoxin (a precursor of both PTOX and 6-methoxypodophyllotoxin, the latter via β-peltatin and β-peltatin-A-methyl ether), and various derivatives. Lignans are natural compounds derived from two 8,8′-linked C_6C_3 (propylbenzene) units. PTOX is an aryltetralin lignan with strong cytotoxic and antiviral activities. Thus, it is used as a starting material for producing various semisynthetic derivatives that are widely used in chemotherapy, such as etoposide, teniposide, and etopophos. It is currently produced largely from *Podophyllum*

hexandrum and *P. peltatum*, slow-growing endangered species of the Berberidaceae (Malik et al. 2014).

Biotechnological production of PTOX by in vitro plant cell and hairy root cultures from genera such as *Podophyllum*, *Linum*, *Callistris*, or *Juniperus* has been reported but far from an industrial level (Ionkova 2008). Tissue cultures of *Podophyllum peltatum* to produce podophyllotoxin were initiated from various explants such as rhizomes, roots, and leaf segments of field-grown plants. The efficiency of various factors such as the source of explant, growth regulators, and light conditions on callus growth and podophyllotoxin production was studied (Kadkade 1981, 1982). Callus was also induced from *P. hexandrum* in vitro-grown seedlings (van Uden et al. 1989). When the callus derived from *P. hexandrum* was incubated in B5 medium containing 2,4-D, gibberellic acid (GA3), and 6-benzylaminopurine (BA), podophyllotoxin, 4′-demethyl-podophyllotoxin, and podophyllotoxin-4-*O*-glucoside were produced, and the levels of podophyllotoxin and its derivatives were similar to those in the parent plant (0.3% DW) (Heyenga et al. 1990). Different plant parts such as roots, shoots, and embryo have been cultured and produce compounds similar to that from the whole plant. Embryogenic roots from a *P. peltatum* callus were induced in liquid MS medium supplemented with NAA, kinetin, and casein hydrolyzate. The roots were then transferred to the medium without growth regulators, whereupon 1.6% of podophyllotoxin was detected in the dried tissues, which was sixfold higher than in the mother plant (Sakata et al. 1990). Rapidly producing and high-yielding *P. peltatum* plants were developed from in vitro propagation protocol by growing rhizome tips on the basal MS medium containing sucrose, supplemented with benzyladenine and activated carbon. The podophyllotoxin contents of in vitro-rooted bud and plantlet cultures were similar to the contents found in the wild (Moraes-Cerdeira et al. 1998). As the levels found in these cultures were quite low, the interest in podophyllotoxins was revived when root cultures of *L. flavum* were found to contain high levels (1% DW) of 5-methylpodophyllotoxin and its glucosides (Berlin et al. 1988). Other podophyllotoxin-producing callus cultures are those from sterile leaves in *Juniperus chinensis*, needles of *Callitris drummondii*, and the stem and leaves of young seedlings of *L. album* on different media (van Uden et al. 1990a; Smollny et al. 1992; Wichers et al. 1991; Muranaka et al. 1998). One of the cell lines produced 0.3% podophyllotoxin of dried cells, together with small amounts of 5-methyl podophyllotoxin, lariciresinol, and pinoresinol after 3 weeks of cultivation (Smollny et al. 1992). Uden et al. (1990a) studied the accumulation of the phenylpropanoid-derived lignan of 5-methoxyl podophyllotoxin in cell suspension cultures of *L. flavum*; cultures that were routinely grown on a NAA-containing medium accumulated low levels of 5-methylpodophyllotoxin. In cell culture of *L. album*, *L. flavum*, *L. nodiflorum*, *L. mucronatum*, and *L. tauricum*, 6-methyl podophyllotoxin is the main lignan accumulated in cell lines (Fuss 2003; Mohagheghzadeha et al. 2007).

Mohagheghzadeh et al. (2002) established cell cultures of *L. austriacum*, which accumulates the arylnaphthalene lignan justicidin B. The identification of justicidin B or other arylnaphthalene lignans in *L. glaucum* in vitro cultures agrees with the

morphological data. *L. glaucum* has many morphological similarities with *L. austriacum* (Mohagheghzadeh et al. 2003). However, arylnaphthalene-type lignans were not found in *L. bienne* and *L. tenuifolium* in vitro cultures (Mohagheghzadeh et al. 2009). In this respect, Hemmati et al. (2007) accumulated justicidin B as the main lignan in *Linum* species before in cell suspension culture of *L. perenne* Himmelszelt. The accumulation of justicidin B was with 23 mg/g DW 2–3 times higher than that observed in cell cultures of *L. austriacum*. Cell cultures of *L. austriacum* accumulate 6.7 mg/g DW justicidin B (Mohagheghzadeh et al. 2002). The diarylbutyrolactone hinokinin was detected for the first time in the genus *Linu*m in in vitro cultures of *L. strictum* ssp. *corymbulosum* (Mohagheghzadeh et al. 2006).

Flax lignans (SECO and SDG) were succeeded to accumulate in plant cell culture as reported by Attoumbre et al. (2006) accumulated lignans in *L. usitatissimum* cell suspension on LS medium supplemented with NAA alone or in combination with BA. However, SDG was barely detected, and MAT was not detected. Anjum et al. (2017) developed a protocol for SDG production in in vitro callus derived from leaf and stem explants of *L. usitatissimum* inoculated with different concentration of plant growth regulators (α-naphthalene acetic acid (NAA), thidiazuron (TDZ), and 6-benzyl adenine (BA)). They reported that the stem-derived calli (1.0 mg/l NAA) accumulated optimum concentrations of SDG (2.7 ± 0.021 mg/g DW) compared with other growth regulators. Nadeem et al. (2018) found that *L. usitatissimum* were grown in in vitro culture on 200 mg/l yeast extract enhanced the accumulation of SDG 3.36-fold or 10.1 mg/g DW than untreated culture.

7.2 *Optimization of Culture Media Composition and Culture Conditions*

Medium and culture condition optimization can increase the productivity in suspension culture by a factor of 20–30 and is very important in order to obtain an efficient system for the production of high levels of secondary metabolites from plant cells.

Podophyllotoxin accumulation was strongly affected by light, with red light stimulating its production in *P. peltatum* cell cultures (Kadkade 1982). Illumination stimulated the endogenous production of podophyllotoxin-β-D-glucoside, to a concentration of 0.11% (DW), in *L. flavum* cultures (Uden 1993). Dark-grown suspension cultures of *P. hexandrum* accumulated 0.1% podophyllotoxin, which was three- to fourfold that in light-grown cultures (Uden 1993). Similar results were also observed in the authors' laboratory (Chattopadhyay et al. 2002a). *P. hexandrum* cells growing in shake flasks were found to be sensitive to hydrodynamic stresses generated by changing rotational speeds. A rotational speed of 150 rpm showed more than 80% viability of *P. hexandrum* cells. A medium pH of 6.0 was favorable for high biomass production and podophyllotoxin accumulation in *P. hexandrum* cell cultures (Chattopadhyay et al. 2002b). MS medium with 60 mM nitrogen

having an ammonium to nitrate salt ratio of 1:2, 60 g/l glucose, and 1.25 mM phosphate was found to be optimum for podophyllotoxin production (Chattopadhyay et al. 2003a). Statistical optimization methodology, such as Plackett-Burman design and response surface methodology, has been employed to optimize the media and culture conditions for growth and podophyllotoxin production in *P. hexandrum* cultures. The optimized values of important nutritional parameters were found to be medium pH 6.0, 1.25 mg/l IAA concentration, 72 g/l glucose concentration, and an inoculum level of 8 g/l. When *P. hexandrum* was cultivated under statistically optimized culture conditions, a maximum of 20.2 g/l (DW) of biomass and 48.8 mg/l podophyllotoxin were obtained (Chattopadhyay et al. 2002b).

Optimization of cell/tissue culture of *Linum persicum* for production of lignan derivatives including podophyllotoxin was established. The farther evaluation for anticarcinogenic activity on MCF7 cell line found that shoot extract of the in vitro plantlets has greater cytotoxic effect on breast cancer cells and leads to significant raise in ratio of Bax/Bcl-2 value = 1.42 and Rbl1 gene expression that are well-known as apoptosis-inducing agents (Esfandiari et al. 2018).

7.3 *Plant Transformation by* Agrobacterium rhizogenes

Various species of bacteria are capable of transferring genes to higher plant species (Chung et al. 2006). Among them, *Agrobacterium tumefaciens* is most widely studied. The soil bacterium *A. rhizogenes* infects the plant tissues and leads to the formation of adventitious roots called "hairy roots." Fast and hormone-independent growth and high genetic stability make hairy root cultures superior in comparison to cell cultures. Hairy roots are known to produce the same or even higher amounts of the metabolites found in normal roots (Christensen and Muller 2009; Mukundan et al. 1998). Integration of new genes into hairy roots has opened another way for metabolic engineering (Guillon et al. 2006) which is based on the ability to transform plant cells with the root-inducing (Ri) plasmid of *A. rhizogenes*. Two important regions in the Ri plasmid are essential for transformation by transferred DNA (T-DNA). These are the T-DNA itself as a mobile element and the virulence region (*vir*) (Klee et al. 1987). The T-DNA is flanked by 25 bp directly repeated sequences. Any DNA between these sequences will be transferred to the plant cell. The Ri plasmids are grouped into two main classes according to the opines synthesized by hairy roots: agropine-type (e.g., A4, 15834, LBA9402, 1855) and mannopine-type (e.g., 8196, TR7, TR101) strains (Sevón and Oksman-Caldentey 2002). The T-DNA of the agropine-type Ri plasmid consists of two separate T-DNA regions: the TL-DNA and TR-DNA. The genes encoding auxin (*tms1* and *tms2*) have been localized on the TR-DNA of the agropine-type Ri plasmid. The mannopine-type Ri plasmids contain only one T-DNA that shares considerable DNA sequence homology with the TL-DNA of the agropine-type plasmids. The TL-DNA contains four genetic loci, *rol*A, *rol*B, *rol*C, and *rol*D, the first three of which mainly contribute to

the root initiation and maintenance. A plant cell becomes susceptible to *Agrobacterium* when it is wounded.

A threefold increase in podophyllotoxin content in comparison to controls was obtained in transformed calli of *P. hexandrum* developed by transformation of embryo using different strains of *A. rhizogenes*, viz., A4, 15834, and K599 (Giri et al. 2001). Hairy root cultures of *L. flavum* were also reported to produce up to 1% of 5-methoxypodophyllotoxin and its glucoside derivative on a dry cell weight basis. 5-Methoxypodophyllotoxin, isolated from the root cultures of *L. flavum* grown on vitamin-free MS basal medium supplemented with sucrose, appeared to have about the same cytotoxic potency as podophyllotoxins and its semisynthetic derivatives (Uden et al. 1992). The levels of podophyllotoxins varied from 0.05% to 0.3% DW, depending on the culture conditions and the tendency to differentiate. Thus, hairy root cultures of *L. flavum*, producing 1.5–3.5% podophyllotoxins in DW, seem to be most suitable for future research efforts (Oostdam et al. 1993). Hairy root cultures were induced from leaf explants of *L. tauricum* by infection with *A. rhizogenes* strains TR 105 and ATCC 15834. The cultures produced 2.6% of the lignan 4′-demethyl-6-methoxypodophylotoxin and 3.5% of the lignan 6-methoxypodophyllotoxin on a DW basis, which was 10–12 times higher than in *L. tauricum* cell suspensions (Ionkova and Fuss 2009). Whereas cell cultures of *L. austriacum* accumulate 6.7 mg/g DW justicidin B, hairy roots of the same species can accumulate 16.9 mg/g DW justicidin B (Mohagheghzadeh et al. 2002). More recently, Ionkova et al. (2013) reported that the content of justicidin B in the *L. narbonense* intact roots is about 0.5 mg/g DW and in callus cultures is 1.57 mg/g DW. Suspensions of *L. narbonense* contain much smaller amounts of justicidin B, 0.09 mg/g DW. Hairy roots produced fivefold higher justicidin B (7.78 mg/g DW) compared to callus. As a first report for lignans SECO, SDG and MAT accumulation in *L. usitatissimum* transformed with *A. rhizogenes* A4 strain with fourfold lignan higher than in untransformed root culture (Gabr et al. 2018).

The study with determination of lignans, phenolic acids, and antioxidant capacity in transformed hairy root culture of *Linum usitatissimum* showed that total lignin content (secoisolariciresinol diglucoside, secoisolariciresinol, and matairesinol) was 55.5% higher in transformed root cultures than in the non-transformed root culture. In the transformed root culture were detected secoisolariciresinol diglucoside and matairesinol, but they were not found in the non-transformed root

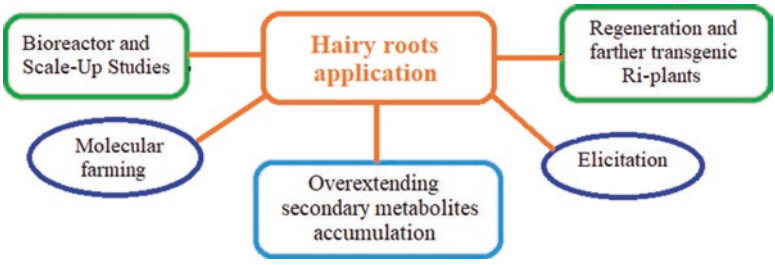

Fig. 7 Schematic model of hairy root application

culture. Secoisolariciresinol content and overall production of phenolic acids in transformed roots were approximately 3.5 times higher than that of the corresponding non-transformed culture (Gabr et al. 2018).

The hairy root application can be used in the different directions (Fig. 7). Different aspects and applications of hairy root cultures include recombinant protein production, phytochemicals, introduction of desirable foreign genes, phytoremediation, molecular breeding and crop improvement, rhizosphere physiology and biochemistry, metabolic engineering, bioreactor design, and general overviews of the system (Ono and Tian 2011). Elicitation, bioreactor, and scale-up studies plus overextending accumulation of secondary metabolites of lignans have been developed during last years. The hairy root culture gets more attention as biological matrices for producing valuable metabolites as they have several attractive features, including high genetic stability and relatively fast growth rates (Panda et al. 2017).

7.4 Feeding of Precursors

The addition of precursors to the media in order to direct metabolic flux toward enhanced production of the desired products represents an interesting approach to exploit the biosynthetic potential of the enzymes present in plant cell cultures.

The addition of coniferyl alcohol complexed with β-cyclodextrin to *P. hexandrum* cell suspension cultures increased the concentration of podophyllotoxin fourfold, to 0.013% on a DW basis. Noncomplexed coniferyl alcohol, suspended in the medium, also enhanced podophyllotoxin production, albeit to a lower degree (Woerdenbag et al. 1990). Coniferin, the β-d-glucoside of coniferyl alcohol, was found to be a more potent precursor in terms of yield of the anticancer compound (0.055%) but is not available commercially (Woerdenbag et al. 1990). Various phenylpropanoid precursors (phenylalanine, tyrosine, cinnamic acid, caffeic acid, coumaric acid, ferulic acid, coniferyl alcohol, coniferin, etc.) were utilized in cell cultivation for the improvement of podophyllotoxin levels in *P. hexandrum* cell cultures. Of these, only coniferin at a concentration of 2.1 mM was able significantly to increase podophyllotoxin accumulation on the tenth day of cultivation, by a factor of 12.8. However, most of the coniferin was transformed into unknown products (Woerdenbag et al. 1990; van Uden et al. 1990b). Feeding cultures with the precursor L-phenylalanine resulted in a three- to fivefold increase in 5-methoxyl podophyllotoxin levels but caused the levels of phenylalanine ammonia lyase (PAL) activity to fall. Treatment of the cultures with the elicitor nigeran, either alone or in combination with phenylalanine, caused the 5-methoxyl podophyllotoxin production to cease, even though PAL activity was rapidly enhanced by these treatments. Transfer of the cultures to NAA-free medium resulted in a 40–50-fold higher level of 5-methoxyl podophyllotoxin accumulation, the PAL activity levels being lowered compared to the routinely grown cells. With these more differentiated cultures, phenylalanine feeding and elicitor treatment, both on its own and in combination

with the precursor, had no effect on 5-methoxyl podophyllotoxin production, even though the PAL activity levels were higher than in the untreated cells (van Uden et al. 1990c). Co-cultured hairy roots of *L. flavum* and *P. hexandrum* suspensions resulted in increased podophyllotoxin concentrations by 240% in shake flasks and by 72% in a dual bioreactor, as compared to *P. hexandrum* suspension cultured alone. The coniferin provided by *L. flavum* hairy roots acted as a precursor for the production of podophyllotoxin by *P. hexandrum* suspension cultures (Lin et al. 2003).

7.5 Elicitors

Elicitation is the induction of secondary metabolite production by molecules or treatments known as "elicitors." Elicitation is used to induce the expression of genes often associated with the enzymes responsible for synthesis of secondary metabolites by mimicking the pathogen defense or wound response in plants (Zhao et al. 2005). Therefore, the treatment of plant cells with biotic and/or abiotic elicitors has been a useful strategy to enhance secondary metabolite production in cell cultures. The most frequently used elicitors in previous studies were fungal carbohydrates, yeast extract, methyl jasmonate (MeJA), and chitosan (Ionkova 2009).

Attempts have been made to increase the accumulation of aryltetralin lignans in *Linum* spp. cultures by inducing a hypersensitive defense reaction with a variety of elicitors. However, nigeran, *Phytophthora megasperma* cell wall fractions, methyl jasmonate, hydrogen peroxide, and salicylic acid were not found significantly to increase lignan accumulation in *Linum* spp. (Aapro 1998). Bhattacharyya et al. (2012) showed approximately seven- to eightfold change in accumulation of podophyllotoxin on *P. hexandrum*, after 9 days of elicitation, elicited with 100 μM methyl jasmonate as compared to the control. The simulative effect of addition of autoclaved and filter-sterilized culture filtrate of *Piriformospora indica* (a root endophytic fungus) to the growing *Linum album* hairy root cultures on growth and lignan production was observed as well. The podophyllotoxin and 6-methoxypodophyllotoxin (the lignans) concentrations were maximally improved by 3.8 times and 4.4 times in comparison to control cultures together with increasing of phenylalanine ammonia lyase activity at 3.1 times (Kumar et al. 2012).

The addition of methyl jasmonate to cell cultures of *Forsythia intermedia* resulted in three- and sevenfold accumulations of the lignans, pinoresinol and matairesinol, predominantly as glucosides (Schmitt and Petersen 2002). The effect of adding methyl jasmonate to various cell lines in suspension culture of *L. album* has also been studied, with a twofold increase in podophyllotoxin (7.69 ± 1.45 mg/g DW) and 6-methoxypodophyllotoxin (1.11 ± 0.09 mg/g DW) being achieved as maximum in one of the cell lines (Furden et al. 2005). The effect of yeast extract and abiotic elicitors (Ag2+, Pb2+, and Cd2+) was also studied on podophyllotoxin production in *L. album* suspension cultures, though only Ag2+ at 1 mM concentration was found to enhance production up to 0.24 mg/g cell DW (Shams-Ardakani et al. 2005). Ionkova (2009) concentrated on the induction of production of ariltetralin

lignans in hairy root cultures of *L. tauricum*, transformed by *Agrobacterium rhizogenes*, *ATCC* 15834, by exposing them to different concentrations of methyl jasmonate during the culture period. The content of 4′-demethyl-6-methoxypodophylotoxin and 6-methoxypodophyllotoxin, the main constituents in hairy roots of *L. tauricum*, increased about 1.2-folds by elicitation of MeJA; however, the fresh weight, DW, and growth ratio were inhibited by increasing methyl jasmonate concentrations. The highest total lignan yield was obtained with 150 μM methyl jasmonate treatment. Hano et al. (2006) studied the effects of fungal elicitors on lignan metabolism in *L. usitatissimum* cell suspensions. They found that flax (*L. usitatissimum*) cell cultures accumulated SECO and no MAT could be detected either in control or elicited cells.

7.6 Bioreactor and Scale-Up Studies

The environment in which plant cells grow usually changes when cultures are scaled up from shake flasks to bioreactors, and this may result in reduced productivity. With the ultimate aim of implementing an industrial-scale process, the behavior of cell cultures in bioreactors has received much attention.

Submerged batch and fed-batch cultivation of *P. hexandrum* cells for podophyllotoxin production was studied in a 3 L stirred-tank bioreactor. A 36% increase in volumetric productivity of podophyllotoxin was achieved in fed-batch cultivation of *P. hexandrum* cells over batch cultivation in a stirred-tank bioreactor (Chattopadhyay et al. 2002b, 2003b, c). Continuous cultivation of *P. hexandrum* with cell retention was also carried out in a 3 L bioreactor equipped with a spin filter mounted on the agitator shaft of the bioreactor. The result was an accumulation of 53 g/l biomass and 48.8 mg/l podophyllotoxin, with volumetric productivity of 0.8 mg/l per day) (Chattopadhyay et al. 2003b). A maximal product yield of podophyllotoxin, up to 0.2% DW, was achieved when *Linum album* cell suspensions were cultured in a 20 L bioreactor (Arroo et al. 2002). Ionkova et al. (2013) found that on *L. narbonense* maximum biomass of 22.5 g/l DW was harvested from the bioreactor culture vessel (recording about eight times increase over initial inoculum), with 1.42% ± 0.12 justicidin B, greater than contents obtained from flasks.

8 Conclusion

Some of the most effective cancer treatments to date are natural products or compounds derived from plant products. Recently, the interest of international pharmaceutical industries has been directed more and more to plant-based anticancer compounds. Isolation of anticancer pharmaceuticals from plants is difficult due to their extremely low concentrations. The industry currently lacks sufficient methods for producing all of the desired plant-derived pharmaceutical molecules. Some

substances can only be isolated from extremely rare plants. Plant cell cultures are an attractive alternative source to whole plant for the production of high-value secondary metabolites. From the previous data in this review, it's very clear the importance of lignans as anticancer agents, and lignan production through hairy root transformation is the optimum biotechnological method for high productivity than cell suspension culture or elicited culture. Bioreactor studies represent the final step leading to commercial production of economically important lignans from plant cell cultures.

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Physiology of Camptothecin Synthesis in Plants and Root Organ Cultures of *Ophiorrhiza mungos* L. and Its Production in Root Fermenters

Bernhard Wetterauer, Eric Hummel, Steffen Walczak, Melanie Distl, Markus Langhans, Pille Wetterauer, Frank Sporer, Eckhart Wildi, and Michael Wink

Abstract Production of the anticancer drug camptothecin (CPT) was examined in *Ophiorrhiza mungos* with regard to concentration and localization of CPT and CPT derivatives within all plant parts. First, morphology and histology studies of all organs were examined by light and confocal laser scanning microscopy to localize the accumulation of CPTs down to the subcellular level. In addition, CPTs were analysed qualitatively and quantitatively in all parts of a young and adult plant. A reverse distribution of CPT and 9-methoxycamptothecin (9-methoxy CPT) in the aerial parts became obvious,

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localized predominantly in the vacuoles of evenly distributed cells of the outer parenchymal tissues. On an average, the young plant contained 795 µg CPTs/g DW and the old plant 1182 µg CPTs/g DW. The plant roots contain only CPT, mainly localized in the rhizodermis and exodermis (866–1853 µg/g DW or 180–433 µg/g FW). The highest contents of CPTs were detected in the youngest leaves of the young plant (about 1664 µg/g DW or 304 µg/g FW; 60% 9-methoxy CPT), in the ripening seed capsules of the adult plant (1688 µg/g DW), and its oldest leaves (2000 µg/g DW). The average 9-methoxy CPT content in the aerial plant parts of the young and old one was about 490–600 µg/g DW and 68–81 µg/g FW, respectively. The storage in aerial parts shows a classical protection and defence pattern against herbivores. The epidermally localized CPT of the roots is partly released in the surroundings for an allelopathic purpose.

CPT localization and storage differed in root organ cultures from those in plants. CPT distribution within the root tissues of the untransformed and the transformed root organ culture line 607 and the occurrence of CPT in root tip exudates were investigated. Surprisingly, CPT was dispersed equally in both root culture lines, independent of age and part, but in relation to the dry biomass. The root tip exudates showed calcium oxalate crystals with embedded CPT. Additional elicitor studies on root cultures with many simple and complex elicitors, as well as precursors and physical/chemical stressors, did not stimulate CPT production. Therefore, the CPT biosynthesis in *O. mungos* seems to be constitutive and developmentally regulated.

Finally, preliminary scale-up experiments with transformed and untransformed root organ cultures in a mist fermenter were conducted, showing that a CPT production on a larger scale by root organ cultures of *O. mungos* is feasible.

Keywords 9-Methoxycamptothecin · Anticancer drug · Camptothecin · Elicitation · Fermenter · Localization · *Ophiorrhiza mungos* · Regulation · Root organ cultures · Storage

Abbreviations

2,4-D	2,4-Dichlorophenoxyacetic acid
9-Methoxy CPT	9-Methoxycamptothecin
CPT	Camptothecin
DMSO	Dimethyl sulfoxide
DW	Dry weight
ER	Endoplasmic reticulum
FAA	Formalin-acetic acid-alcohol
FW	Fresh weight
MeJA	Methyl jasmonic acid
PDA	Photodiode array
rpm	Revolutions per minute
SA	Salicylic acid
STR	Strictosidine synthase
TDC	Tryptophan decarboxylase
TIC	Total ion current

1 Introduction

Since the discovery of camptothecin (CPT) by Wall and Wani (Wall et al. 1966), CPT has led to medicinal research with many ups and downs resulting in chemotherapeutical agents of high efficacy like irinotecan, topotecan, and others (e.g. Oberlies and Kroll 2004). Additionally, CPT and its derivatives show other interesting applications with antiviral, antiparasitic, pesticidal, and antipsoriatic activities (reviewed in Liu et al. 2015), as a plant growth regulator (e.g. Buta and Worley 1976; Wang et al. 1980), or in traditional medicines against bites by snakes and rabid dogs, for treatment of stomatitis and ulcers, and for wound healing (Anaswara Krishnan et al. 2014). This broad spectrum of use is attributed to the two modes of action hither to known for CPT. Firstly, CPT inhibits the DNA topoisomerase I and thus replication, which was elucidated by Hsiang et al. (1985), and secondly, it binds to tubulin and therefore inhibits the microtubule formation in dividing cells, as shown recently by Wang et al. (2016) in our lab.

CPT is biosynthesized by different plant species of diverse plant families in the Asterids (APG III; Wink 2003). As already described by Li and Adair (1994), CPT is obtained as a raw material for the chemical industry mainly by cultivation of *Camptotheca acuminata* Decaisne and related species in plantations in China, India, further southeastern Asian countries, and North America (see also Liu and Adams 1996; Liu et al. 1999; Sirikantaramas et al. 2007a). But also natural sources are exploited by collections of *C. acuminata*, *Nothapodytes* spec., and other plants. In the producing countries, no environmental conservation legislation exists for the most part and the labour costs are minimal; otherwise, the actual low market prices of CPT would not be possible (≥3500 €/kg). At the moment, it seems that the supply of the pharmaceutical industry with CPT and CPT derivatives as raw material poses no problem, especially on examination of the sinking market prices in the last two decades and the CPT bulk (Wetterauer et al. 2018). However, this might be an illusion in a long-term view (Nagesha et al. 2018). In the last few decades, many investigations have been done in the field of chemistry and biotechnology to establish sustainable and ecologically compatible ways of CPT production and to become more independent from the classical sources (Wetterauer et al. 2018). However, until now, no breakthrough has taken place, mostly for economic reasons. In our case, we described a running and cost-effective system of root organ cultures of *Ophiorrhiza mungos* L. (Rubiaceae) that was developed together with the ROOTec company in the early 2000s (Wink et al. 2005; Wink and Wetterauer 2019). But in the end, the pharmaceutical industry showed no interest to use this technology, so expectations should be realistic.

Nevertheless, there are two other gaps of knowledge in this field. On the one hand, the anabolic pathway of the biosynthesis of CPT and its derivatives has not been completely elucidated until now. In this connection, the so-called ‘poststrictosamide events’ are mainly affected (e.g. Asano et al. 2013). Therefore, the biotechnological possibilities, like genetically engineered bacterial or yeast cultures, cannot be fully exploited at present for the bio-production of the highly

valuable CPT. On the other hand, many studies have been carried out for CPT content and derivate analyses in CPT-producing plants (comp. overview in Wetterauer et al. 2018), but very little is known about the regulation of their CPT biosynthesis, the cellular and subcellular localization of CPT, or its manner of storage and content regulation. To obtain more information about these questions, deep transcriptome analysis coupled to untargeted metabolomic profiling was performed by Yamazaki et al. (2013) in *O. pumila*, which did lead to new hints, but unfortunately not to clearer findings.

In the present study, we compiled the results of our investigations on *O. mungos* plant and root organ cultures over many years, focussing on the content of CPTs; their storage at the organ, tissue, and subcellular level; and regulation in relation to function and potential productivity for CPT bio-production. In doing this, we performed intensive confocal laser scanning microscopy studies and CPT content determinations by LC-MS/MS for root organ cultures and on intact plants. To stimulate the CPT production in transformed and untransformed root organ cultures, extensive elicitor studies were conducted. Finally, the results of the first fermenter studies of transformed and untransformed root organ cultures with respect to the CPT production, nutrition, and growth parameters are shown. The challenges of scaling up a root fermenter system are discussed as well.

2 Material and Methods

2.1 Plant Material and Culture Conditions

Ophiorrhiza mungos seeds were obtained from the Old Botanical Garden of Göttingen, Germany. The primary roots of germinating seeds were taken in a sterile cultivation regime of plates and shaking cultures. By transformations of primary roots, root organ shaking cultures, and stem axis parts by means of underpressure infiltration with different *Agrobacterium rhizogenes* strains (LBA 9402, Atcc 15834, and TR 105), ROOTec GmbH (Heidelberg, Germany) cultivated 23 lines of root organ cultures with individual characteristics with regard to vitality, growth behaviour, and CPT biosynthesis (see also Wetterauer et al. 2018). The mainly investigated root culture line 607 was transformed with the *A. rhizogenes* strain LBA 9402 by underpressure infiltration of a primary root. Transformation controls were carried out as described in Wetterauer et al. (2018).

The cultivation of the transformed and untransformed root organ cultures was conducted on plates by using a modified Gamborg B5 agar (0.4% Gelrite®, Carl Roth GmbH & Co., Karlsruhe, Germany) with 3% sucrose at 25 °C in the dark. The root organ shaking cultures of *O. mungos* were cultivated in modified Gamborg B5 medium (Gamborg et al. 1968) with 3% sucrose and ½ MS vitamins at a pH of 5.7 without hormone addition at 25 °C and 60 rpm on a rotary shaker in the dark.

Two adult plants of *O. mungos* were made available to us from the Old Botanical Garden of Göttingen, Germany, for first content studies. Identification of the plant species was performed by C. E. B. Bremekamp in September 1958 (personal communication with E. J. Gouda, curator of the Botanical Garden of Utrecht, Netherlands). These plants of *O. mungos* were integrated into the inventory of the Botanical Garden of Heidelberg under the accession number 108108 in October 2004. Herbarium vouchers were deposited at the Institute of Pharmacy and Molecular Biotechnology (IPMB) in November 2006 under the accession numbers P6984 and P6985.

Plant clones, for direct comparative studies, were grown out of young sprouts of the untransformed root plate cultures and cultivated on general soil substrate in the greenhouse at 20–25 °C and 80% air humidity. These plants were integrated in the Botanical Garden of Heidelberg, Germany, under the accession number 108181.

2.2 Histological Preparations

Fresh shoot cross sections of *O. mungos* were fixed in FAA, stepwise dehydrated in an ethanol and tertiary butanol series, embedded in Peel-A-Way® (Micro-cut paraffin, Polyscience Inc., Warrington PA, USA; melting point 56 °C) at 58 °C, cut with a Jung slide microtome at 10–12 μm, and stained with safranin O and fast green FCF (both obtained from Merck KGaA, Darmstadt, Germany). Leaves of *O. mungos* were not rigid enough and broke during the cutting by that method. For this reason, histological studies of the leaves were done in situ by ordinary microscopy and confocal microscopy. Roots were not treated in this way, because the histological structure became obvious in the confocal microscopy studies without any treatment.

2.3 Microscopy

All histological light microscopical examinations were done by an Axiovert inverted oil immersion microscope (Zeiss, Jena, Germany). The in situ localization studies of CPTs were performed by a confocal laser scanning microscope LSM 510 META (Zeiss, Jena, Germany).

2.4 CPT Localization by Confocal Laser Scanning Microscopy

All visual localizations of CPTs in situ were done by confocal laser scanning microscopy at 405 nm excitation wave length and 470 nm emission wave length. For excitation, a laser diode 405 nm was employed. Lambda scans were done from

417 to 748 nm in 10–11 nm steps (cf. Fig. 1). The evaluation of the measurements was done by LSM Image Browser Software (Zeiss, Jena, Germany).

For CPT, reference pure (*S*)-(+)-CPT (Sigma-Aldrich Chemie GmbH, Steinheim, Germany) was used, and for comparison, lambda scans were done continuously for all in situ tested tissues like exemplarily shown in Figs. 1 and 2. The emission minimum at 513 nm was a technical effect due to the main beamsplitter 405/514 of the used confocal laser scanning microscope.

Because of the suboptimal excitation wavelength of 405 nm, determined through the available laser diode, (371 nm for CPT and 9-methoxy CPT would be the best), the results are not perfect but a localization of the CPTs in the living tissues was possible. A differentiation of CPT and 9-methoxy CPT in spite of different emission maxima (for pure dissolved CPT at 435 nm and for 9-methoxy CPT at 502 nm in spectroscopic measurements) was also not possible. Therefore, only mixed results can be shown in the present work for the aerial parts of *O. mungos*, so that in the following, they are called simply CPTs.

2.5 Extraction Methods and Analytic Procedures

Quantitative Determination of CPT in Non-photosynthetic Tissues

For high-throughput screening, an extraction and spectroscopic method for tissues without chlorophyll (e.g. root, callus) was developed for quantitative determination of CPT based on its absorption maximum at 366 nm and emission wave length at 465 nm (96-well-plate reader GENIOS; Tecan Austria GmbH, Grödig/Salzburg, Austria). For this purpose, 10–12 mg of lyophilized and ground tissue samples were extracted with 1 ml MeOH twice. Purification was followed by addition of 500 µl KH_2PO_4 buffer (50 mM, pH 2.6) and two times shaking out with 500 µl CH_2Cl_2. After the combined organic layers were reduced to dryness, the remaining residues were dissolved in 1 ml MeOH. 100 µl was used for spectroscopic measurement in 96-well plates (FluoroNunc, black, F96, CC; Nalge Nunc International, Wiesbaden, Germany). For reader calibration, an external CPT solution series from 0 to 10 µg CPT/ml in MeOH was used. Qualitative and quantitative validations of this method were carried out by HPLC.

The CPT content in liquid medium samples was measured directly with the same spectroscopic method as described. As calibration standard, an external CPT solution series from 0 to 5 µg CPT/ml in Gamborg B5 culture medium was used. For more details, see Wetterauer et al. (2018).

Quantitative and Qualitative Determination of CPT and 9-Methoxy CPT Contents in Photosynthetic Tissues by LC-MS/MS

In the case of chlorophyll-containing tissues or the qualitative determination of CPTs in tissues not previously investigated, a determination of the CPT contents was carried out by LC-MS/MS. The extraction method for tissue samples was

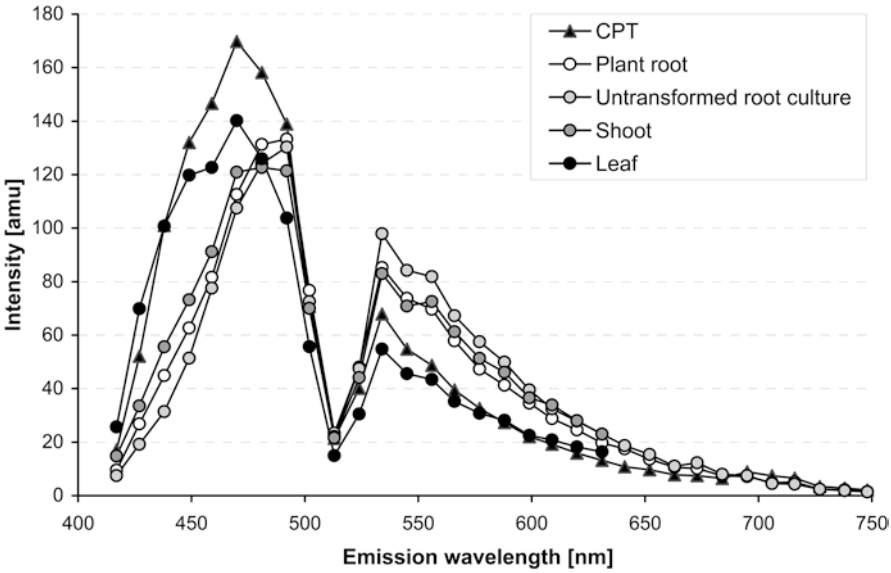

Fig. 1 Lambda scans of selected fluorescent vacuoles in different living tissues of *Ophiorrhiza mungos* plant and root organ cultures in comparison to pure CPT (cf. Fig. 2) at 405 nm excitation wavelength using emission filters 417, 427, 438, 449, 459, 470, 481, 492, 502, 513, 524, 534, 545, 556, 566, 577, 588, 599, 609, 620, 631, 641, 652, 663, 673, 684, 695, 706, 716, 727, 738, and 748 nm

conducted in the same way as described before for the non-photosynthetic tissues. Medium samples were analysed directly without any treatment.

LC-MS/MS analysis was carried out on a Finnigan LCQ-Duo ion trap mass spectrometer with an ESI source (ThermoQuest, San Jose, CA, USA) connected to a Finnigan Surveyor HPLC system (MS pump plus, autosampler, and PDA detector plus) (Thermo, San Jose, CA, USA) with an EC 150/3 Nucleodur 100-3 C18ec column (Macherey-Nagel, Düren, Germany). A gradient of water and acetonitrile (ACN) (with 1% formic acid each) was applied from 10 to 50% ACN in 20 min at 30 °C. The flow rate was 0.5 ml/min. The injection volume was 20 µl. All samples were measured in the positive mode. The MS was operated with a capillary voltage of 10 V, source temperature of 240 °C, and high purity nitrogen as a sheath and auxiliary gas at a flow rate of 80 and 40 (arbitrary units), respectively. The ions were detected in a mass range of 50–2000 m/z. The collision energy of 35% was used in MS2 for fragmentation. Data acquisitions and analyses were carried out by Xcalibur™ 2.0.7 software (Thermo Scientific, Karlsruhe, Germany).

As external calibration, a combined standard of CPT (Sigma-Aldrich Chemie GmbH, Steinheim, Germany) and 9-methoxy CPT (Chengdu Biopurify Phytochemicals Ltd., Chengdu, Sichuan, China) dissolved in MeOH was used (concentrations of CPT/9-methoxy CPT: 0.47/0.50, 4.65/5.05, 23.25/25.23, and 46.50/50.45 µg/ml). An example of an *O. mungos* leaf sample is given in Fig. 3.

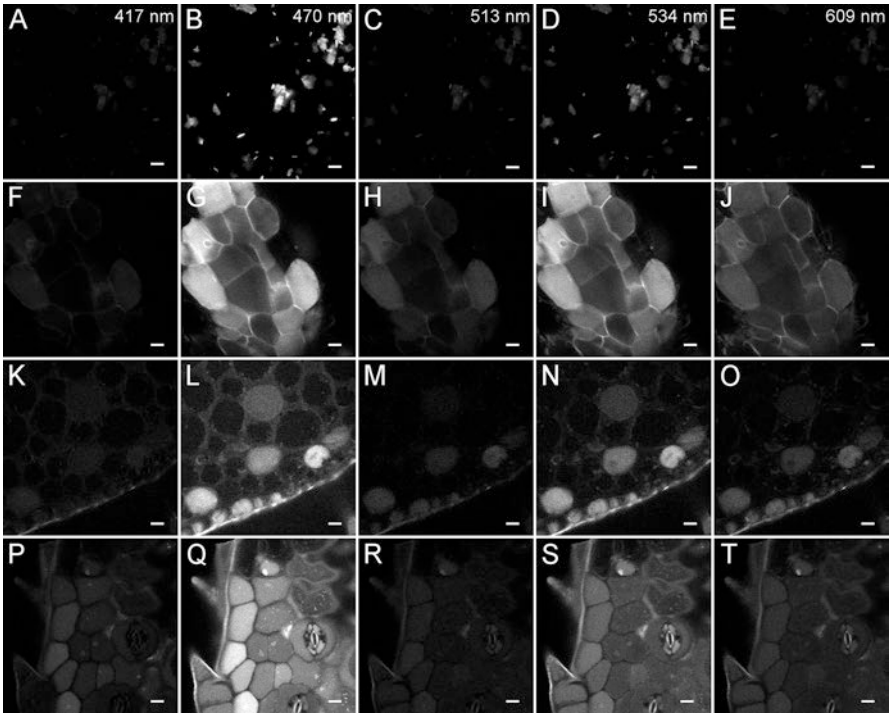

Fig. 2 Confocal laser scanning microscopy fluorescence images of selected wavelengths (minima and maxima) of lambda scans (cf. Fig. 1) of CPT standard (**a–e**) (CPT crystals measured in 1 µl slideVI flow chamber for cell microscopy, ibidi GmbH, Munich, Germany), differentiated rhizodermis of the plant root of *Ophiorrhiza mungos* (**f–j**), epidermis and cortex parenchyma of a shoot cross section of *O. mungos* plant (**k–o**), and epidermis of the abaxial leaf surface with hairs of *O. mungos* (**p–t**). All samples were measured in Gamborg B5 medium at 405 nm excitation wavelength; all scale bars correspond to 10 µm

2.6 *Further Analyses of Root Organ Cultures of* Ophiorrhiza mungos

Studies on Root Tip Exudates

As known, root organ cultures of *O. mungos* release CPT in the culture medium (e.g. Wetterauer et al. 2018). On the culture plates, the transformed root cultures of *O. mungos* (Fig. 4a) grew in a unipolar fashion, and droplets of root exudate were observed hanging from the tips of roots growing in the air. Under light exposure of 366 nm, these droplets showed the same fluorescence as CPT. Therefore, we tried to investigate the CPT content in the root exudates (Fig. 4b).

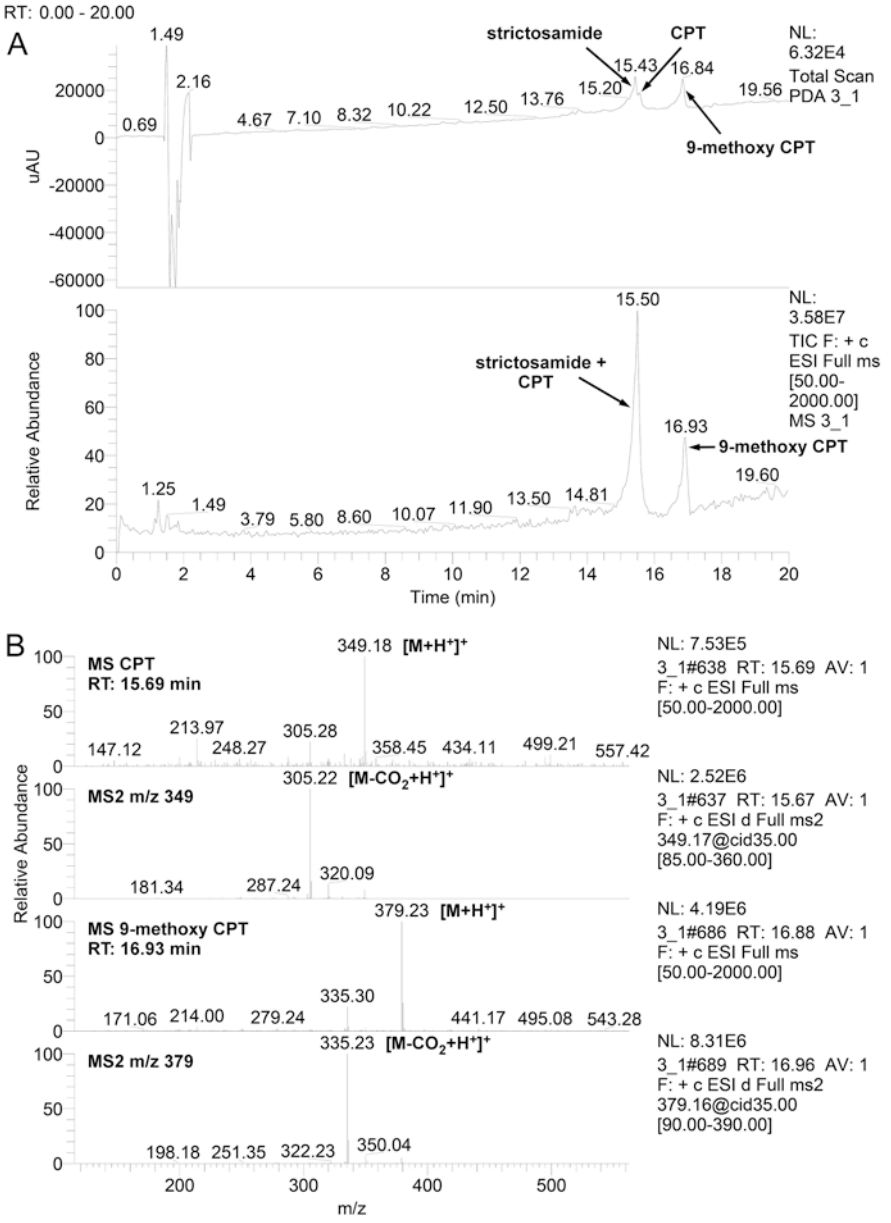

Fig. 3 LC-MS/MS analysis of an *Ophiorrhiza mungos* leaf sample. (**a**) PDA (200–600 nm) and TIC chromatograms of strictosamide, CPT, and 9-methoxy CPT; (**b**) MS and MS2 mass spectra of CPT and 9-methoxy CPT from the TIC of part A. For strictosamide, no mass spectra were shown (MS m/z 499.15 $[M + H^+]^+$, MS2 m/z 337.07 $[M\text{-}Glc + H^+]^+$) (compare Yamazaki et al. 2003b)

CPT Distribution in Transformed and Untransformed Root Organ Cultures

Four 7-week-old transformed (line 607) and untransformed root organ cultures each were cut into five equally thick parts from the youngest to the oldest tissue (Fig. 5) and each analysed for CPT content in relation to FW, DW, and DW density. After FW determination, all samples were frozen at − -80 °C, lyophilized, the DWs measured, and the CPT contents defined by the described standard method. The dry weight density was determined by measuring the volume of a defined amount of dry root powder with modified insulin syringes connected to a vacuum pump with maximum vacuum.

2.7 Elicitor Studies

Extensive elicitor studies on *O. mungos* root cultures [transformed (line 607) and untransformed] were performed to investigate whether a stimulation of CPT biosynthesis is possible or not. To this end, many different single elicitors, both simple (defined) and complex (defined and undefined), as well as precursors and abiotic physical and chemical stress conditions, were tested (Table 1). More detailed experimental conditions are described in Wetterauer (2008). Additionally, a combination of 0.1 mg/flask indole acetic acid (IAA) and 0.01 mg/flask kinetin was tested on untransformed root organ cultures with an inoculum size of 3.7 g ± 0.7 g (n = 3). Medium sampling for CPT content determination was done after 0, 5.5, 18.5, 26, 52, 75, 96.5, 145, and 193 h. At the end of the testing period, the roots were harvested, and the CPT content of the tissues was determined in different relations.

2.8 Fermenter

For our own studies and the optimization, or rather determination, of root growth and culture conditions for a stimulation of CPT formation during upscale, we built our own mist bioreactor (cf. Bastian et al. 2008). This test mist fermenter, called WEWA I, was based functionally on the Low Cost Mist Bioreactor (LCMB) of the ROOTec GmbH company (patents: US 2003/0129743A1; EP 1268743B1) (cf. Wink et al. 2005; Wink and Wetterauer 2019).

For scaling up a root culture system in the WEWA I, many factors had to be considered first. Of particular importance were the inoculum size and its dispersion on the gauze carrier; the nutrition concentrations in the culture medium (to avoid an osmotic shock or over-fertilization); the balance between spray volume, pressure, and cycle time; and, of course, the aspects of sampling and sterility.

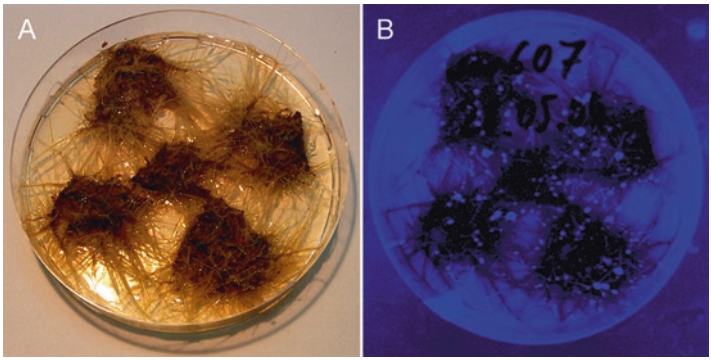

Fig. 4 Plate of the transformed root organ culture line 607 of *Ophiorrhiza mungos*: (**a**) Plate at daylight, (**b**) fluorescence exposure at an excitation wavelength of 366 nm. The culture agar and the root exudate droplets on the air growing root tips show the blue CPT fluorescence

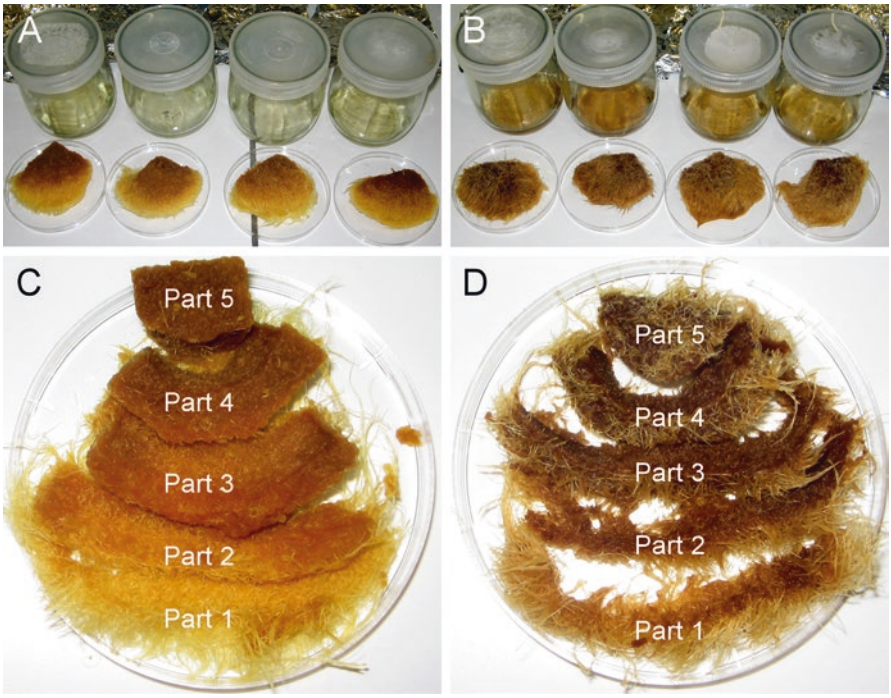

Fig. 5 Seven-week-old untransformed (**a**, **c**) and transformed (line 607, **b**, **d**) root organ cultures of *Ophiorrhiza mungos*. Cultures were cut into five equally thick parts from the root tips to the oldest tissues (**c**, **d**) for CPT analyses

The sterile even dispersion of the inoculum roots on the culture carrier (nylon gauze 250 µm; surface area approx. 185 cm^2) was the first challenge. As shown in the scheme of Fig. 6a, the assembly of the preparation device looks quite complex. A longer period of testing was necessary to obtain fairly good results. The root tips used for 6-week-old transformed or untransformed root cultures were harvested (length 1–1.5 cm) (Fig. 7a), dispersed in 3 litres of sterile B5 medium without sucrose by stirring (Fig. 7b), filled at once in the brass cylinder of the assembly, and placed on the gauze carrier by underpressure suction of the medium. In the testing phase, when the assembly was not sophisticated, the roots agglomerated quickly, as shown in Fig. 7c. The relevant factors here are pumping speed, dispersion volume, and linearity of the siphoning over the whole gauze area. After many modifications, the dispersion of the roots became better (Fig. 7d, e). Regarding the inoculum size at the beginning, 4 g FW of root tips were used (Fig. 7d). Over time we recognized that an inoculum of >20 g FW led to better vitality in the fermentation process (Fig. 7e).

Table 1 Elicitors, precursors, and abiotic stressors tested for amplification of the CPT biosynthesis in root organ cultures of *Ophiorrhiza mungos*

Simple elicitors

- Methyl jasmonic acid (1–1000 µM)
- Salicylic acid (0.01–1 µM)
- Methanol (0.05–5%)
- Ethanol (0.05–5%)
- Indole acetic acid (auxin) (0.1 mg) + kinetin (0.01 mg)
- Hydrogen peroxide (0.001–0.1%)
- Coniine (3 mM)
- Diethyldithiocarbamate (5 mM)
- Caffeine (1 mM)
- Quercetin (1 mM)
- Papaverine (1 mM)
- Tannin (1.7 mg)
- Spermine (1 mM)
- Spermidine (1 mM)
- cAMP (0.5 mM)

Complex elicitors

Defined

- Ionophore A23187 (0.025–2.5 µM)
- Nystatin (1–20 mg)
- Chitosan 86/30/A1 (10 mg)
- Chitosan lactate (10 mg)
- Carboxymethyl chitosan (10 mg)

Undefined

- Mycelium *Pythium aphanidermatum* CBS 101589 (10–100 mg DW)
- Fungal cell wall extract *Pythium aphanidermatum* CBS 101589 (100–1000 µl)
- Mycelium *Phytophthora cinnamomi* DSM 62654 (20 mg DW)
- Mycelium *Phytophthora drechsleri* DSM 62680 (20 mg DW)
- Mycelium *Pythium ultimum* DSM 62987 (20 mg DW)
- Mycelium *Ascochyta hordei* CBS 112524 (20 mg DW)
- Mycelium *Ascochyta hordei* var. *hordei* CBS 504.71 (20 mg DW)
- Self-elicitation: root organ cultures were elicited by fresh root cultures ground with a pestle (0.14–1.3 g FW)
- Orange juice (2% (v/v))
- Tomato puree (0.58 ± 0.11 g)

Precursors

- Tryptophan (1 mM)
- Tryptamine (1 mM)

Abiotic stressors

- pH value (3–9)
- Shaking conditions (60–110 rpm)

Also, the nutrient concentrations of the culture medium were important during scaling up, because of a different biomass-to-medium volume ratio. In all procedure, we had to avoid osmotic shock and a supply of excess nutrients. After many test series with root tips in 12-well plates for optimization (not shown), a half concentrated B5 medium with 0.5% sucrose was used in the first fermenter tests. For the prevention of bacterial or fungal contaminations, 0.5 g/l Claforan® (bacteriostatic activity) and 40 mg/l nystatin (fungicidal activity) were added to the culture medium.

The fermenter vessel (volume approx. 3 litres) and the medium reservoir (volume approx. 1.8 litres) were made out of stainless steel (Fig. 6b, d), further attachment parts out of brass. All gas and medium connections were PTFE tubes. The medium filter filled with glass wool, necessary to avoid solid clogging of the spray nozzle, was a special element made of glass, as was the resin column. In the gas inlet and outlet of the fermenter system, safety sterile filters (0.45 µm, ∅ 50 mm; neoLab®, Laborbedarf-Vertriebs GmbH, Heidelberg, Germany) were integrated. The spray nozzle was a modified brazen replica of the two-component nozzle Hago GSC long nose (DIVA Sprühtechnik GmbH/DELAVAN® Industriedüsen, Hamburg, Germany). The piloting of the four regulation magnetic valves (Typ D118V03-Z610A-2.7-230V AC, SIRAI Magnetventile Deutschland GmbH, Steinhöring, Germany) for the control of gas and medium flow were done by a home-made central processing unit (Fig. 6d bottom right).

The function principle of the WEWA I is based on gas pressure that can be divided into three phases (Fig. 8). The carrier gas was synthetic air (20% O_2, 80% N_2) with a pressure of 2 bars, which was responsible for all processes by control of the four valves. In phase I (spraying phase), valves 1 (medium inlet) and 2 (gas inlet) are opened and valves 3 (gas/pressure outlet) and 4 (medium outlet) are closed. The carrier gas drives the nozzle, and through the suction effect and the pressure being raised to that of the medium reservoir, the medium is pulled into the nozzle and sprayed out. In phase 2 (backflow phase), valves 1 and 2 are closed and valves 3 and 4 are opened, while an overpressure is in the fermenter, so that the redundant medium from the bottom of the fermenter vessel is led back to the medium reservoir. During the process, excess gas vents out to the atmosphere through valve 4. Finally, in phase III (resting phase), all valves are closed, and the roots get the opportunity for nutrition uptake under normal atmospheric pressure.

The main challenge in the regulation is to adjust the system so that the optimal medium volume will be sprayed per cycle and the excess medium will be carried back almost completely by the overpressure to the medium reservoir. Furthermore, the pressure should not become too high, to avoid damage in the system and stress to the roots. In the previous function tests, we defined the possible spray volume range by time and backpressure and set the following parameters by the three timers of the central processing unit. For a spray volume of 20 ml, we fixed a spray time of 3.5 sec. In this 3.5 sec, the system pressure rises up to 0.3 bars. This was enough backpressure to lead back the redundant medium to the reservoir (phase II takes 16 sec). The whole system was laid out to stand a pressure of max. 1 bar, but we restricted the max. pressure at 0.5 bars by a pneumatic overpressure valve on the

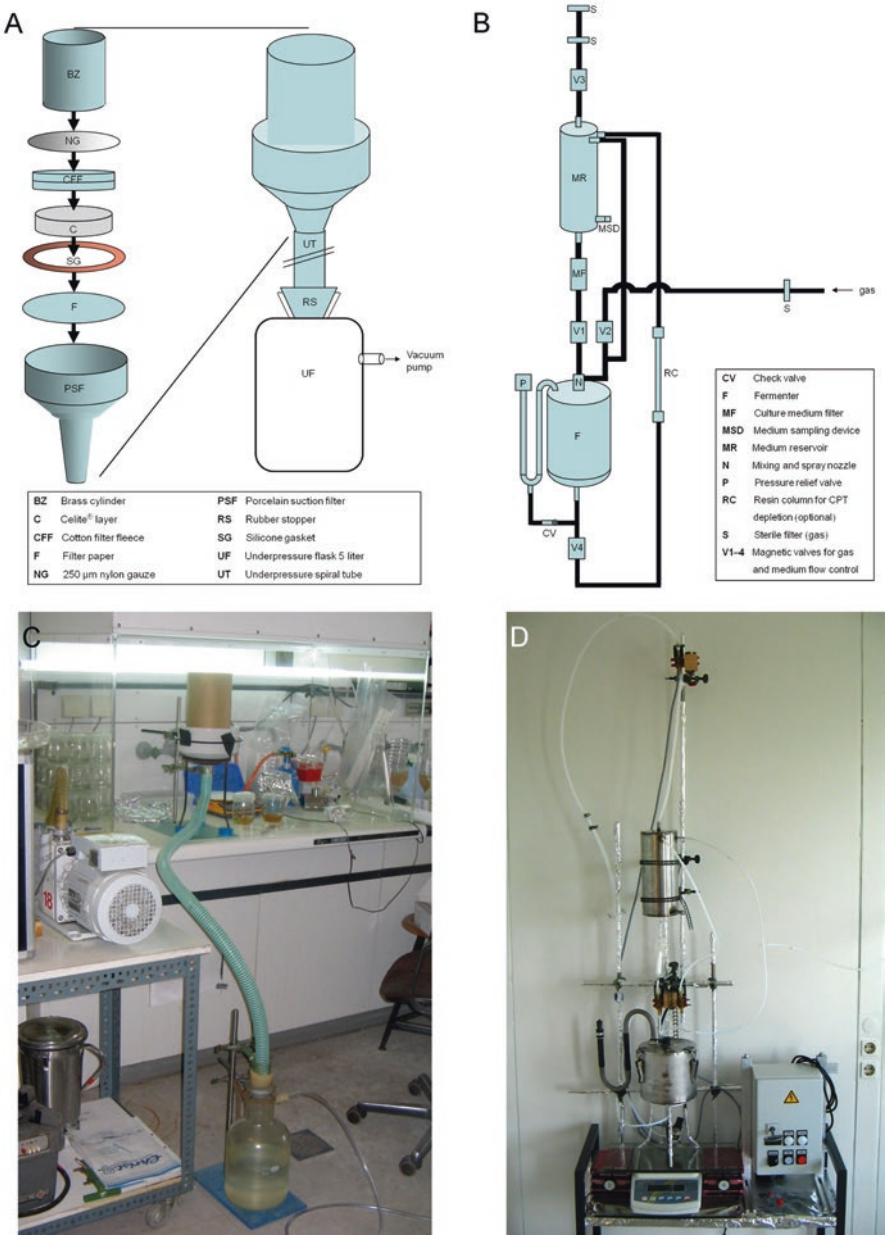

Fig. 6 Schematic and real assembly of the root carrier preparation device (**a**, **c**) and the test mist fermenter WEWA I (**b**, **d**)

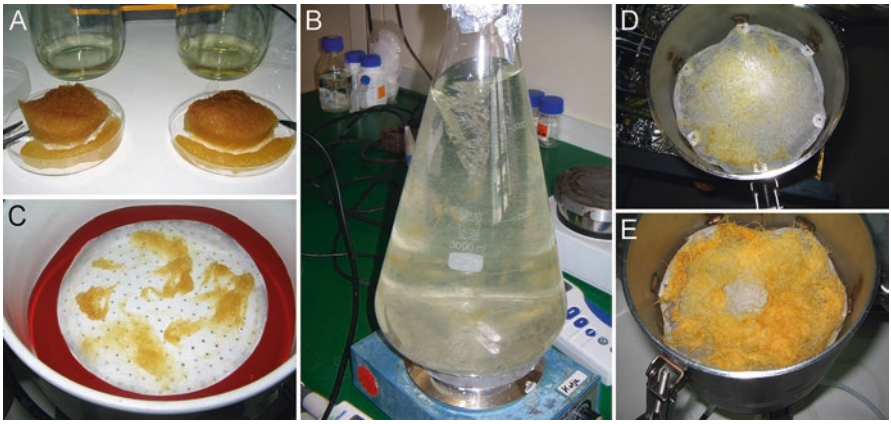

Fig. 7 (**a**) Harvest of untransformed root organ culture tips of *Ophiorrhiza mungos*; (**b**) Dispersion of the inoculum by stirring; (**c**) Agglomerated inoculum on nylon gauze carrier at the beginning of the dispersion tests; (**d**) 4 g inoculum nearly equally dispersed on nylon gauze carrier fixed in fermenter; (**e**) 25.5 g inoculum of transformed root tips (line 607) after 44 h of fermentation with central spray gap

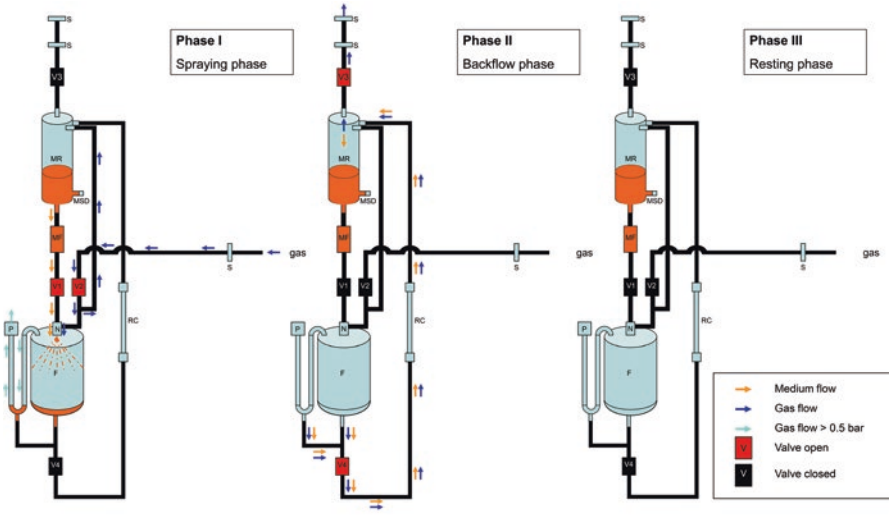

Fig. 8 Functional scheme of the WEWA I with the three-phase cycle. Abbreviations see Fig. 6b

fermenter vessel (Figs. 6b and 8). The medium volume of 20°ml was enough to ensure a good medium exchange in the root inoculum, especially in relation to the interstitial capacity of the roots. Root cultures were able to store up to three times more medium in relation to their own weight. One cycle takes at least 15 min, including the resting phase.

Finally, the problem of sterility was important, on the one hand fundamentally and on the other hand with regard to sample-taking. As mentioned, bacteriostatic and fungicidal substances were added to the culture medium, because it was nearly impossible to get the whole system totally sterile by autoclaving and other kinds of sterilization with our available facilities. Especially the integration of the fermenter vessel with the inoculum and the fixture of the sterile valves were critical points. Also, the sampling of the medium has to be considered under the aspects of sterility and pressure. Therefore, a special medium sampling device was integrated into the medium reservoir (Figs. 6b and 8), so that it was possible to take medium samples by sterile syringes during fermentation.

In this section, the first results of a fermentation of transformed root tips (line 607, 25.5 ± 0.5 g inoculum) over 19 days (with a CPT depletion trail by a XAD PAD II resin column between day 12 and 13) and a fermentation of untransformed root tips (25.7 ± 0.5 g inoculum) over 22 days with a culture medium exchange at day 10 are illustrated.

3 Results

3.1 Basic Morphological and Histological Studies of Ophiorrhiza mungos

Ophiorrhiza mungos is a crude perennial tropical dicotyledonous plant (Fig. 9a). The cross section of the stem axis showed in the centre a more or less unstructured parenchymatous pith surrounded by the typically mostly closed xylem cylinder of the Rubiaceae (Figs. 10a, c and 11a, b). Further outwards, the related phloem elements follow in regular intervals and create open collateral bundles (Fig. 10b–d). After a cortical parenchyma, on average eight to nine cell layers thick, and a four cell-layer thick angular collenchyma, an epidermal monolayer with wax coating formed the final tissue. The first three cell layers of the cortical parenchyma next to the phloem contain crystal granular bodies, as well as raphides and scattered druses, most likely consisting of kinds of oxalate.

The leaves showed the anatomy of a dicotyledonous, zygomorphic, bifacial leaf with upper and lower monolayer epidermis with cuticle, palisade parenchyma, and spongy parenchyma. The stomata were localized on the abaxial leaf surface (e.g. Fig. 2q). The thickness of 300 to 400 μm of the leaves was quite low.

The stem and leaves were covered with unevenly distributed hairs. Here, two types of hairs were present. The first type consists of two cells, a basal one and a second pointed one (Fig. 12c, g). The second type was multicellular, with an additional basal cell (Figs. 10b and 12e, f).

The structure of the inflorescence looks similar to a corymbose cyme, mostly with 7–11, sometimes more, branches (Fig. 9b). The flowering sequence takes place from the centre to the outside. Flower buds, flowers, and seed capsules have stalks.

The seeds of *O. mungos* are minute (1 gram of *O. mungos* seeds contains approx. 60,000 single seeds of angled habitus, Fig. 9c). The whole surface of the inflorescences was covered with a third type of hairs, which only exists there. They seem to be glandular hairs, because the surface becomes sticky when touched. They look like little strawberries, because of a partly thinner cuticle. For that, we named them 'strawberry hairs' (Fig. 11f).

The plant root of *O. mungos* showed homorhizy, even though it is a dicotyledonous plant. The young plant root showed a longitudinal habitus and the root of an old plant a spherical one (Fig. 9a down right). The branching factor was mainly of the second, rarely of the third, order. Morphologically, the plant root showed the typical anatomy of a plant root with stele and cortex without any special modifications.

Fig. 9 (**a**) Flowering, adult plants of *Ophiorrhiza mungos* in the greenhouse and one of their homorhizous, spherical roots; (**b**) Single inflorescence of *O. mungos* (reprinted from Wetterauer et al. (2018), Copyright (2018) Springer Nature Singapore, with permission from Springer); (**c**) Comparison of the seed sizes between *Camptotheca acuminata* (on the left) and *O. mungos* (on the right)

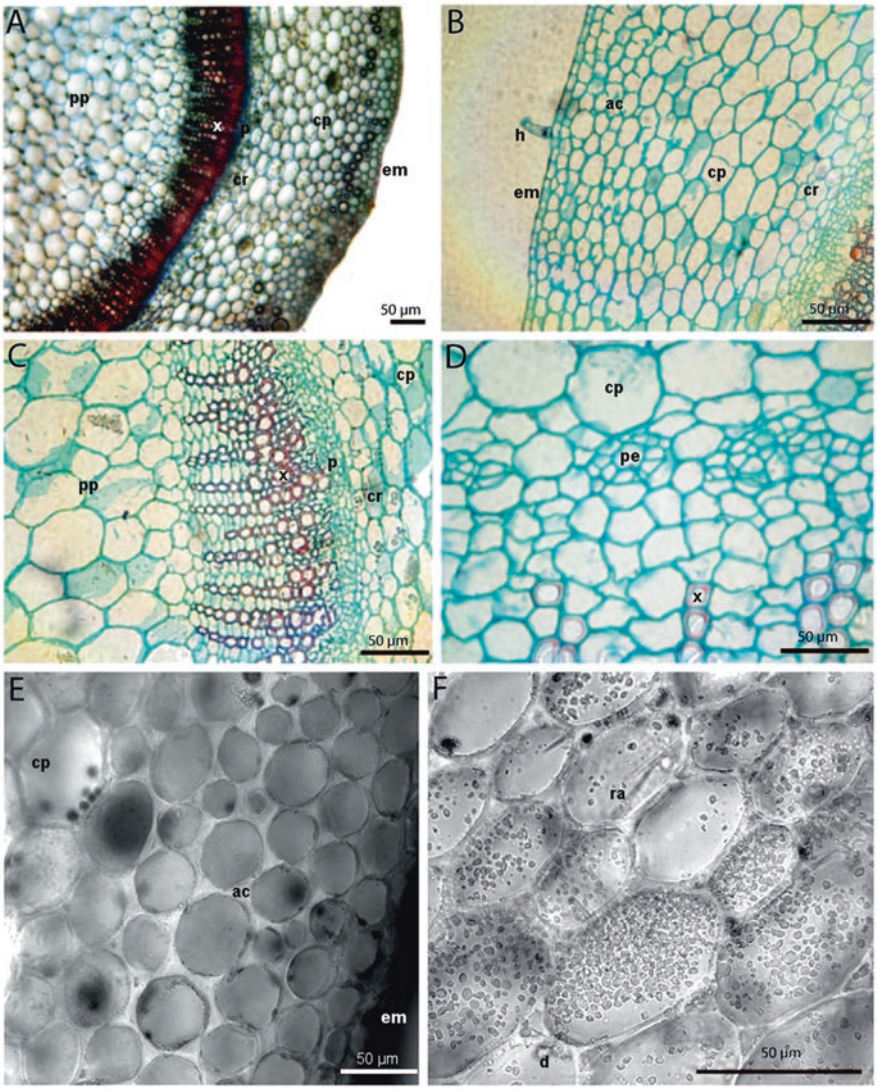

Fig. 10 **a**–**d**: Histological images of a cross section of the stem axis of *Ophiorrhiza mungos* with safranin-fast green stain taken by light microscopy: (**a**) Overview of the cross section of the stem axis; (**b**) Hair, epidermal monolayer, angular collenchymas, and cortical parenchyma; (**c**) Vascular cylinder with adjacent parenchymatous pith and cortical parenchyma; (**d**) Region of cambium with phloem elements of the open collateral bundles. **e** + **f**: Transillumination images of the stem axis of *O. mungos* in situ taken by confocal laser scanning microscopy: (**e**) Angular collenchymas; (**f**) Crystal layer with raphides, druses, and crystal granulous bodies. *ac* angular collenchymas, *cg* crystal granulous bodies, *cp* cortical parenchyma, *cr* crystal layer, *d* druses, *em* epidermal monolayer, *h* hair, *p* phloem, *pe* phloem element, *pp* parenchymatous pith, *ra* raphides, *x* xylem (red)

3.2 *CPT Localization in the Aerial Parts of* Ophiorrhiza mungos

In the aerial parts of *O. mungos*, the storage of CPTs takes place in the vacuoles of idioblastic storage cells. In the stem axis and the side shoots, these storage cells were evenly distributed in the angular collenchyma. The epidermis and the parenchymatous pith did not contain CPT storage cells (Figs. 2k–o and 11b, d, e).

In the leaves, single CPT storage cells or groups of them appeared in the upper and lower epidermis (Figs. 2p–t and 12a, b, d). The spongy and the palisade parenchyma were CPT-free. The leaf stalks showed evenly distributed CPT storage cells in the parenchyma (Fig. 12d).

Additionally, most of the stem and leaf hairs of the two cell types contained CPTs in their vacuoles too (Fig. 12c, g). If the multicellular type contained CPT, it was localized in the cytosol (Fig. 12e, f).

The inflorescences of *O. mungos* showed a high content of 9-methoxy CPT (cf. Figs. 19 and 20). We could thus detect high fluorescence in the receptacles and the flower buds. However, we were not able to differentiate the subcellular localization of CPTs in those parts, because of the high fluorescence of all compartments. Only the ovules seemed to be CPT-free (Fig. 12h, i).

3.3 *CPT Localization in the Plant Root and Transformed as well as Untransformed Root Organ Cultures*

In all studied roots, only pure CPT could be detected, but no derivatives. In principle, the cortex and the stele were CPT-free and the CPT was localized in the border tissues. The cellular and subcellular localization of the CPT in the plant root and the transformed and untransformed root organ cultures differs in the calyptra, the exodermis, and the rhizodermis, as shown in the scheme of Fig. 13.

In the plant root, we were able to detect CPT in the rhizodermis between the root-hair zone and the calyptra localized vacuolarly. The calyptra was CPT-free in principle (Figs. 13 and 14c–e). In the rhizodermis of the root-hair zone and the exodermis of the elderly root parts, the subcellular compartmentation of CPT was vacuolar as well as cytosolic (Figs. 2f–j, 13 and 15f–i).

In untransformed and transformed root organ cultures, the occurrence of CPT in the exodermis of the elderly root parts was strictly vacuolar (Figs. 13 and 15d). In the region of elongation and the zone of differentiation in both root types, the CPT was localized in the cytosol (Figs. 13, 14a, b and 15a, c). In the calyptra of both root

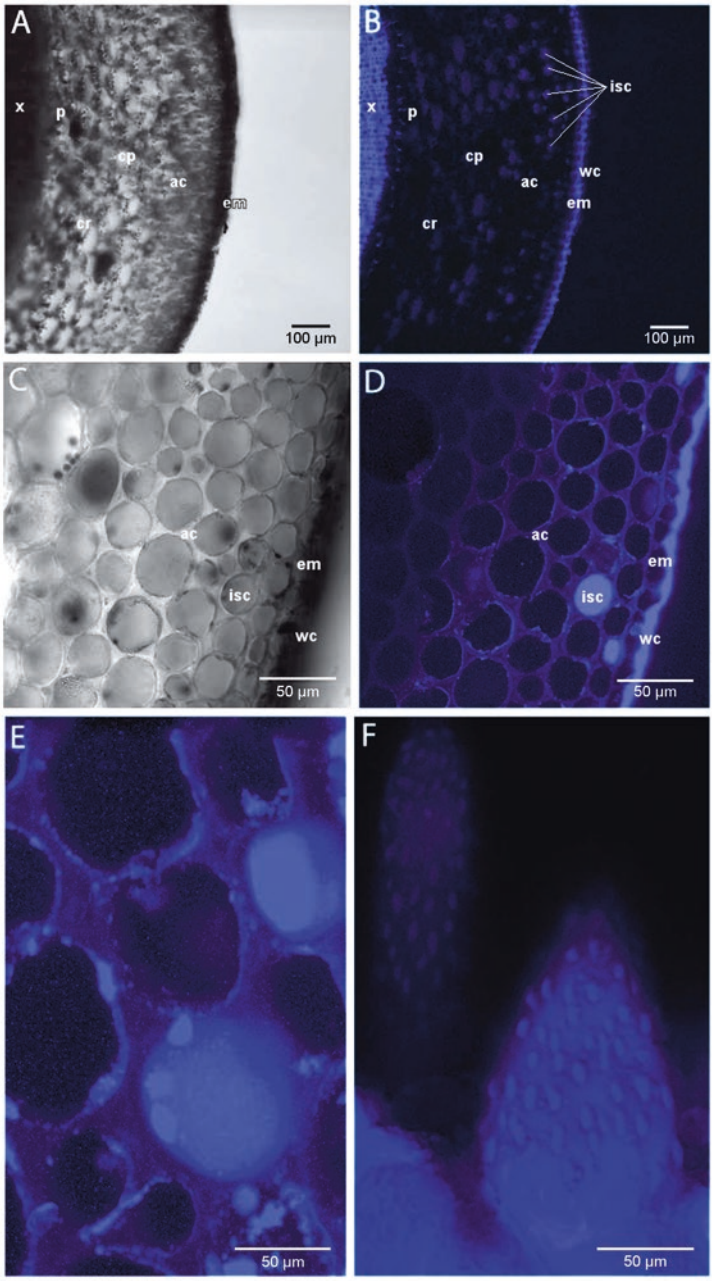

Fig. 11 Transillumination and fluorescence images in situ taken by confocal laser scanning microscopy. **a**–**e**: Selected cross section of the stem axis of *Ophiorrhiza mungos*. (**a**) Overview of stem axis cross section, transillumination image; (**b**) Overview of stem axis cross section (like A), fluorescence image at 405 nm excitation wavelength; (**c**) Selected section image of the angular

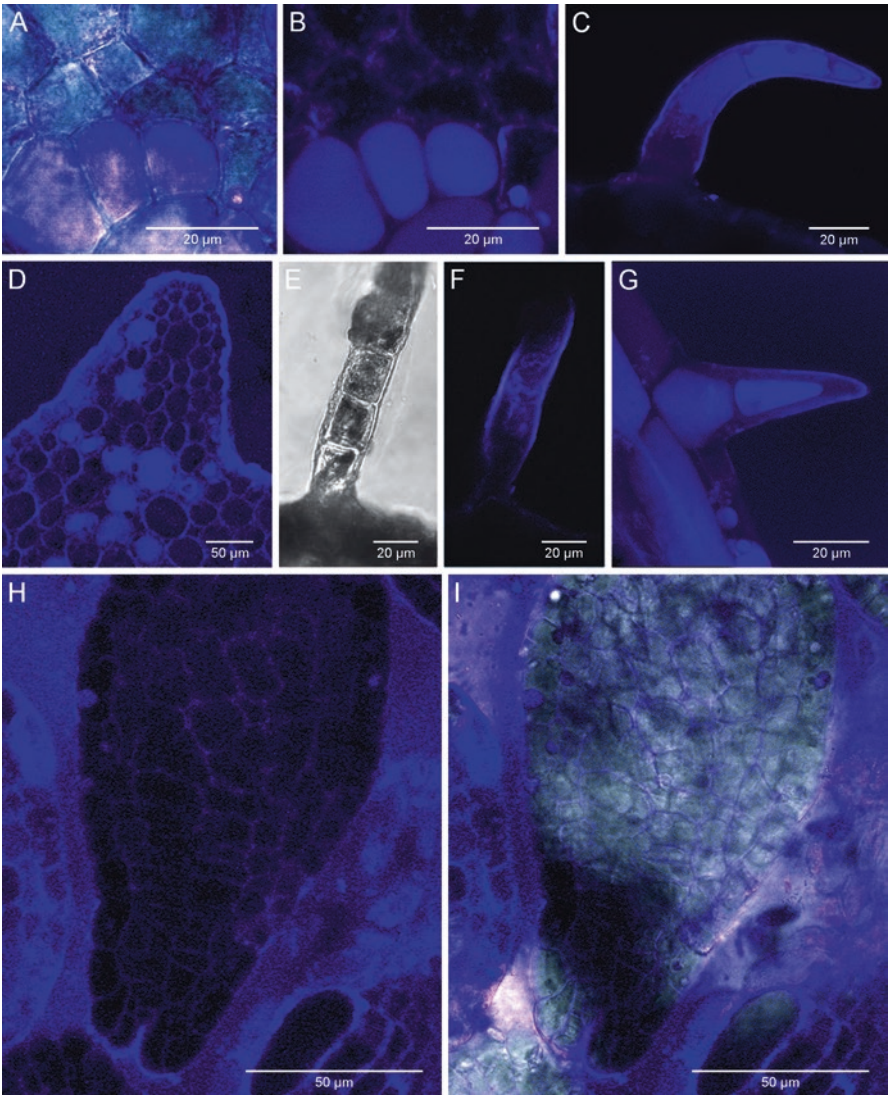

Fig. 12 Transillumination and fluorescence (405 nm excitation wavelength and 470 nm emission wavelength) images and overlays of both in situ taken by confocal laser scanning microscopy of *Ophiorrhiza mungos*. **a** + **b**: Overlay image (**a**) and fluorescence image (**b**) of a group of idioblastic storage cells of the adaxial leaf epidermis; **c**, **e**, **f**, **g**: Two- and multicellular hairs with basic cells of the leaf and stem surface with and without vacuolar CPT storage; (**d**) Leaf stalk parenchyma with evenly distributed CPT storage cells; **h** + **i**: Fluorescence image (**h**) and overlay image (**i**) of CPT-free ovule

Fig. 11 (continued) collenchymas and the epidermis of the stem axis cross section, transillumination image; (**d**) Selected section image of the angular collenchymas and the epidermis of the stem axis cross section (like C) with fluorescent vacuoles in the idioblastic storage cells and fluorescent epidermal wax coating, fluorescence image; (**e**) Reconstructed 3D fluorescence image from a z-stack of CPT containing vacuoles of the idioblastic storage cells in the angular collenchymas of the stem axis cross section; (**f**) Glandular hairs (named 'strawberry hair') of the inflorescence regions of *O. mungos* in situ, fluorescence image at 405 nm excitation wavelength. *ac* angular collenchymas, *cp* cortical parenchyma, *cr* crystal layer, *d* druses, *em* epidermal monolayer, *isc* idioblastic storage cells, *p* phloem, *wc* epidermal wax coating, *x* xylem

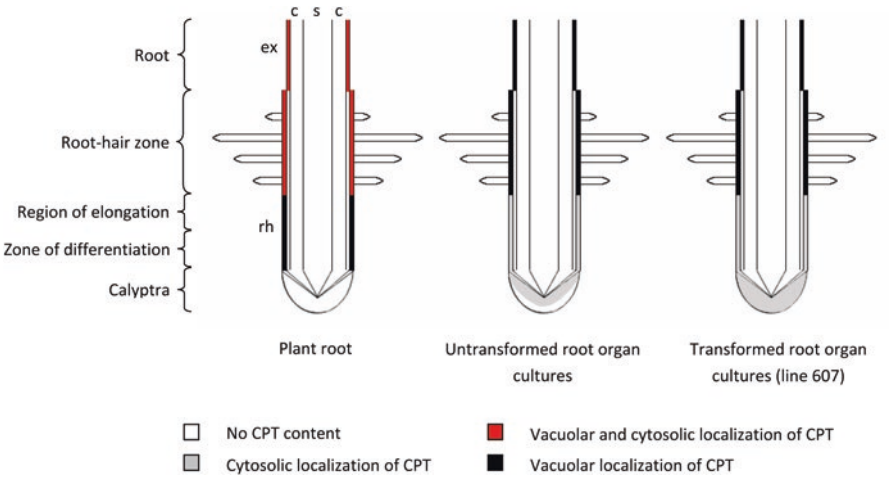

Fig. 13 Comparative scheme of CPT localization on the cellular and subcellular level in root tips of the plant, as well as untransformed and transformed root organ cultures of *Ophiorrhiza mungos*. *c* cortex, *ex* exodermis, *rh* rhizodermis, *s* stele

culture lines, a cytosolic localization was observed. But in the untransformed roots, the front part of the calyptra was generally CPT-free (Figs. 13 and 14a). On the other hand, the transformed roots showed cytosolic CPT storage in all parts of the calyptra (Figs. 13, 14b and 15a, b). This general scheme of localization is not absolute and can vary a little in the individual roots, especially in the rhizodermis and calyptra of the root organ cultures.

The root hairs seem in general to contain no CPT (Fig. 15e). Sometimes, there is the appearance of a slightly cytosolic condition of CPT in some root hairs, but we were not able to confirm this.

3.4 CPT in Root Exudates of Transformed Root Organ Cultures

To our surprise, we could not detect any CPT in the liquid phase of the harvested root exudates. Instead, we found fluorescent crystals in the root exudates by fluorescence microscopy (Fig. 16a). No quantitative studies could thus be performed here. Based on the histological studies of the cross section of the stem axis (Fig. 10f), we proposed that these crystals probably consist of calcium oxalate with embedded CPT. In order to verify this hypothesis, a precipitation of calcium oxalate in a

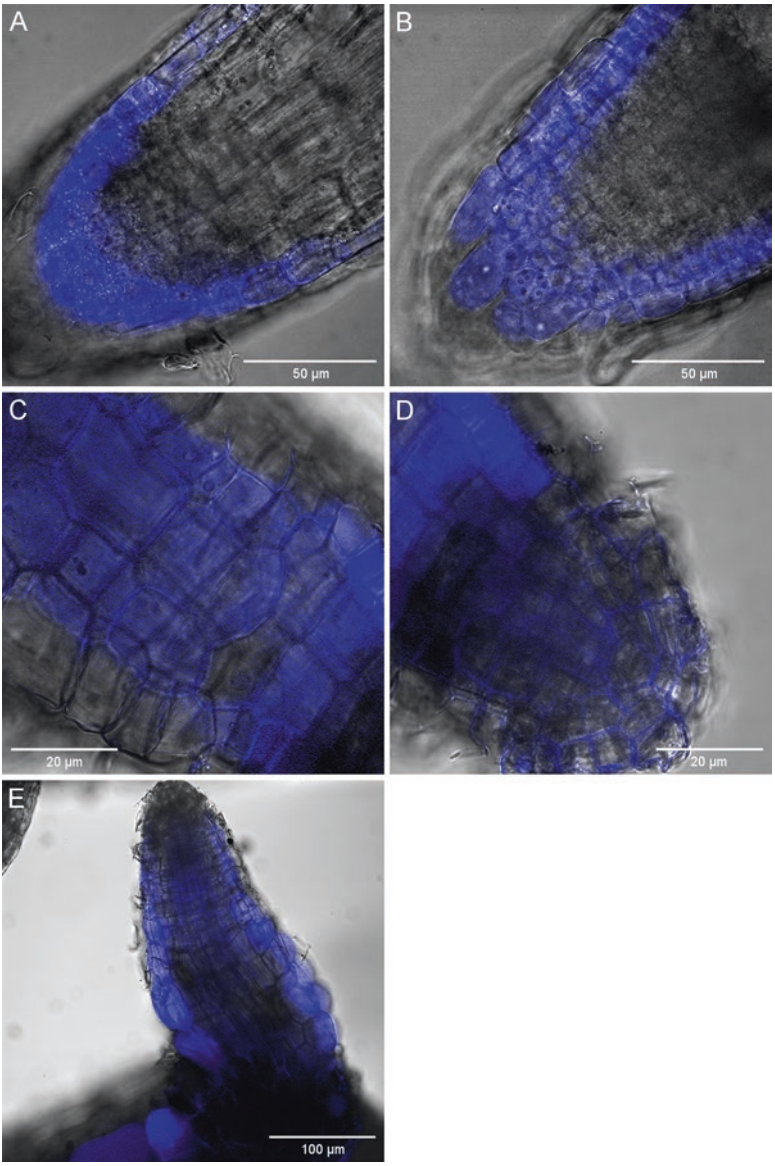

Fig. 14 CPT distribution in root tips in plant root and root organ cultures in situ of *Ophiorrhiza mungos*. Combined fluorescence and transillumination images taken by confocal laser scanning microscopy (405 nm excitation wavelength and 470 nm emission wavelength). (**a**) Root tip of untransformed root organ culture; (**b**) Root tip of transformed root organ culture (line 607); (**c**) + (**d**) Root tip of plant root [zone of elongation and differentiation (**c**), calyptra (**d**)]; (**e**) Young lateral root of plant root

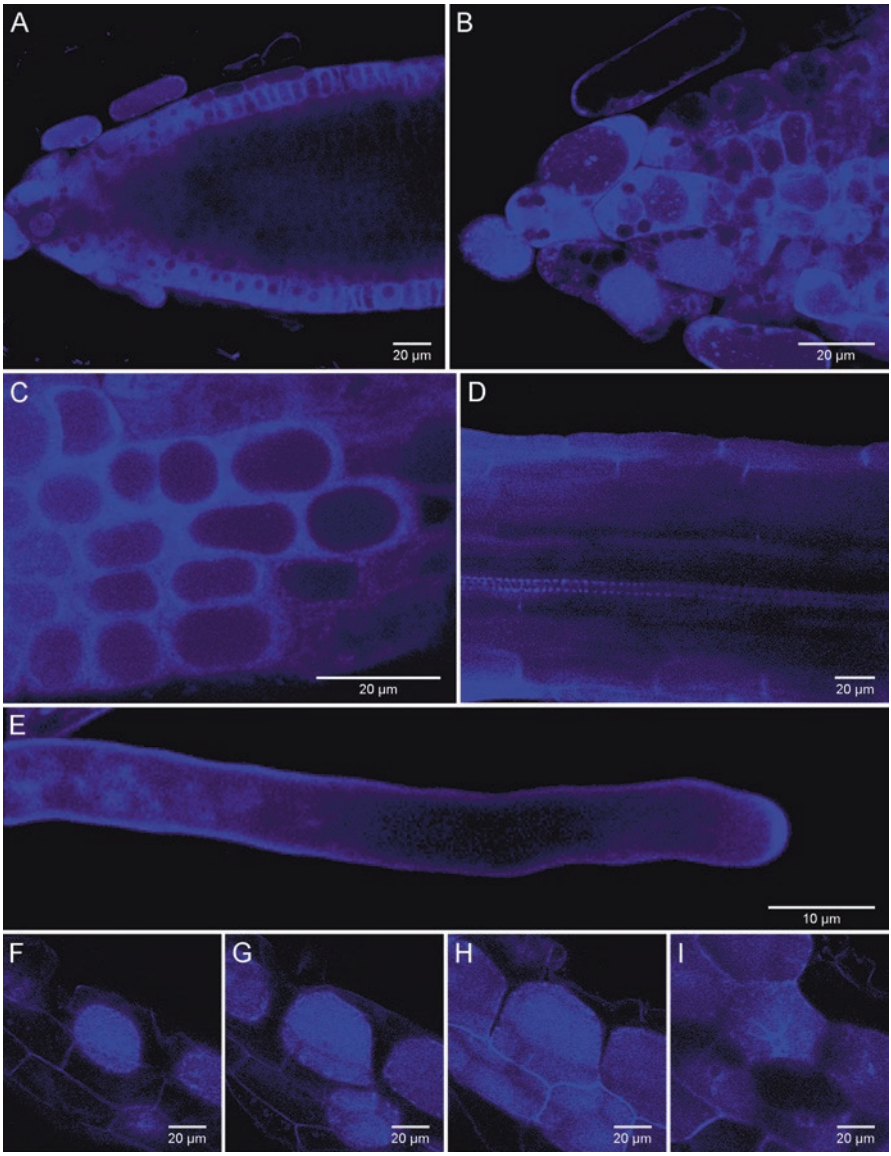

Fig. 15 Fluorescence images in situ taken by confocal laser scanning microscopy (405 nm excitation wavelength and 470 nm emission wavelength) of selected regions of transformed root organ cultures (line 607) of *Ophiorrhiza mungos*. **a–e**: (**a**) Overview of longitudinal section of root tip; (**b**) Calyptra; (**c**) Rhizodermis in the region of elongation; (**d**) Longitudinal section in the old root with exodermis and xylem elements of the stele; (**e**) Growing root hair; **f–i**: Vacuolar and cytosolic CPT fluorescence in situ in the rhizodermis of the plant root of *O. mungos*: (**f**) Cut vacuoles; (**g**) Central layer through vacuoles; (**h**) Surface of the rhizodermis; (**i**) Cytosolic fluorescence and nucleus in negative contrast

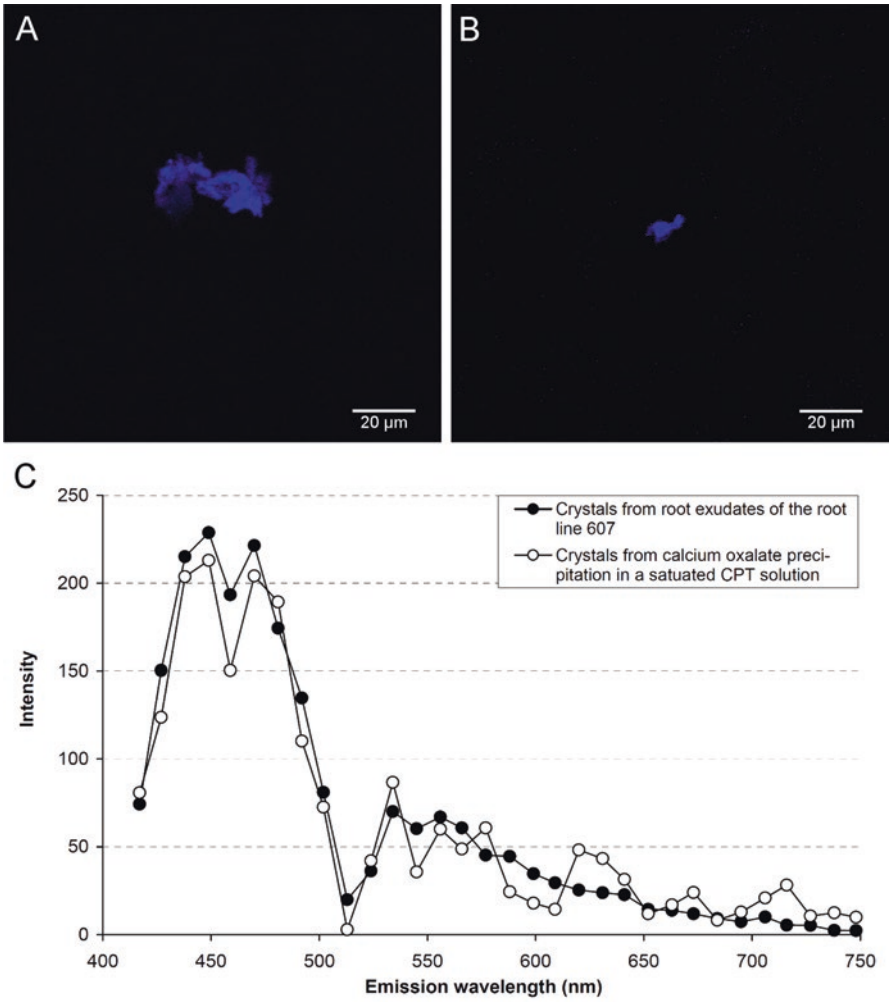

Fig. 16 Confocal laser scanning microscopy images of root exudate crystals of the root organ culture line 607 of *Ophiorrhiza mungos* (**a**) and calcium oxalate crystals precipitated in a saturated CPT solution (**b**). (**c**) Lambda scans of crystals of A and B measured by confocal laser scanning microscopy. Excitation wavelength at 405 nm; measurements ensue in 50 mM KH_2PO_4 solution

saturated and filtered CPT solution was executed. After repeatedly cleaning the precipitate, we could detect some fluorescent oxalate crystals (Fig. 16b) and thus verify that CPT can be included in calcium oxalate crystals. A comparison of the fluorescent root exudate crystals with the self-prepared calcium oxalate crystals by lambda scans made this obvious (Fig. 16c). The other calcium oxalate crystals without CPT did not show any fluorescence.

3.5 *CPT Contents and Distribution in Young and Adult Plants of* Ophiorrhiza mungos

In the next step, the qualitative and quantitative distributions of CPTs in the total plant were investigated. To this end, a young 10-week-old *O. mungos* plant clone and an approx. 6-month-old flowering plant were harvested and the contents of CPT and 9-methoxy CPT determined in every plant part.

In the young plant (Figs. 17 and 18), the highest relative yields of CPTs were found in the youngest leaves (about 1664 µg/g DW or 304 µg/g FW) with an approx. 60% proportion for 9-methoxy CPT. All other plant parts varied between 400–1000 µg/g DW and 50–150 µg/g FW, respectively, in the relative contents of the CPTs. In this context, the proportion of 9-methoxy CPT was 60–90% in the leaves. The proportional content of CPT increased down the stem axis by up to 50–70% and in the root by up to 99%. The highest absolute CPT yield in the plant was found in the root with 70 µg CPT, which correlated with 52.6% of the complete CPT in the whole plant; the root biomass was about 20% of the total plant. In the stem axis, the absolute CPT content was about 35 µg CPT (26.3%) and in the leaves 28 µg CPT (21.1%). A different picture was shown for 9-methoxy CPT. Here, the root contained only 1 µg (0.4%), the stem axis 18 µg (8.5%), and the leaves 194 µg 9-methoxy CPT (91.1%). The whole young plant contained 106 µg CPTs/g FW and 795 µg CPTs/g DW, or in total 212 µg 9-methoxy CPT and 134°µg CPT (total FW, 3.27 g; total DW, 0.44 g; DW/FW ratio, 13.46%).

In contrast to the young plant, the adult plant of *O. mungos* (Figs. 19 and 20) shows a more complex picture of absolute and relative CPT yields. Basically, again the predominant alkaloid in the aerial parts was 9-methoxy CPT and CPT in the root. The absolute CPT content in the root was about 3163 µg CPT, which correlated with 87.1% of the complete CPT in the whole plant; the root biomass was about 41.3% DW or 29.5% FW of the total plant, respectively. Therefore, the root contained 66% of the total CPTs. The aerial parts (58% of DW or 70.5% of FW) showed a total content of CPTs of 34% 460 µg CPT and 1164 µg 9-methoxy CPT of the whole plant, but contained 94.75% of the total 9-methoxy CPT.

On closer examination of the aerial parts, a reduction of the CPT yields from the roots to the main inflorescence of the stem axis from approx. 500 to 100 µg/g DW became obvious. Inversely, the 9-methoxy CPT content rose in the same concentration range. The young side shoots and their leaves as well as the oldest leaves contained 100–200 µg/g CPT and the other leaves about 0–100 µg CPT/g DW. In the case of 9-methoxy CPT, the leaves contained 200–1500 µg/g DW. The oldest leaves in the lower third showed the highest concentrations of 800–1500 µg/g DW, the leaves of the oldest side shoot contained about 200–800 µg/g DW, the leaves of the next upper node about 400–600 µg/g DW, and the leaves of the supreme node about 200–600 µg/g DW. Therefore, the 9-methoxy CPT tends to decrease in the leaves from bottom up, but on a high level.

The main inflorescence exhibited CPT levels of 100–400 µg/g DW. In this context, the seed capsules were in the lowest range, but showed the highest content of

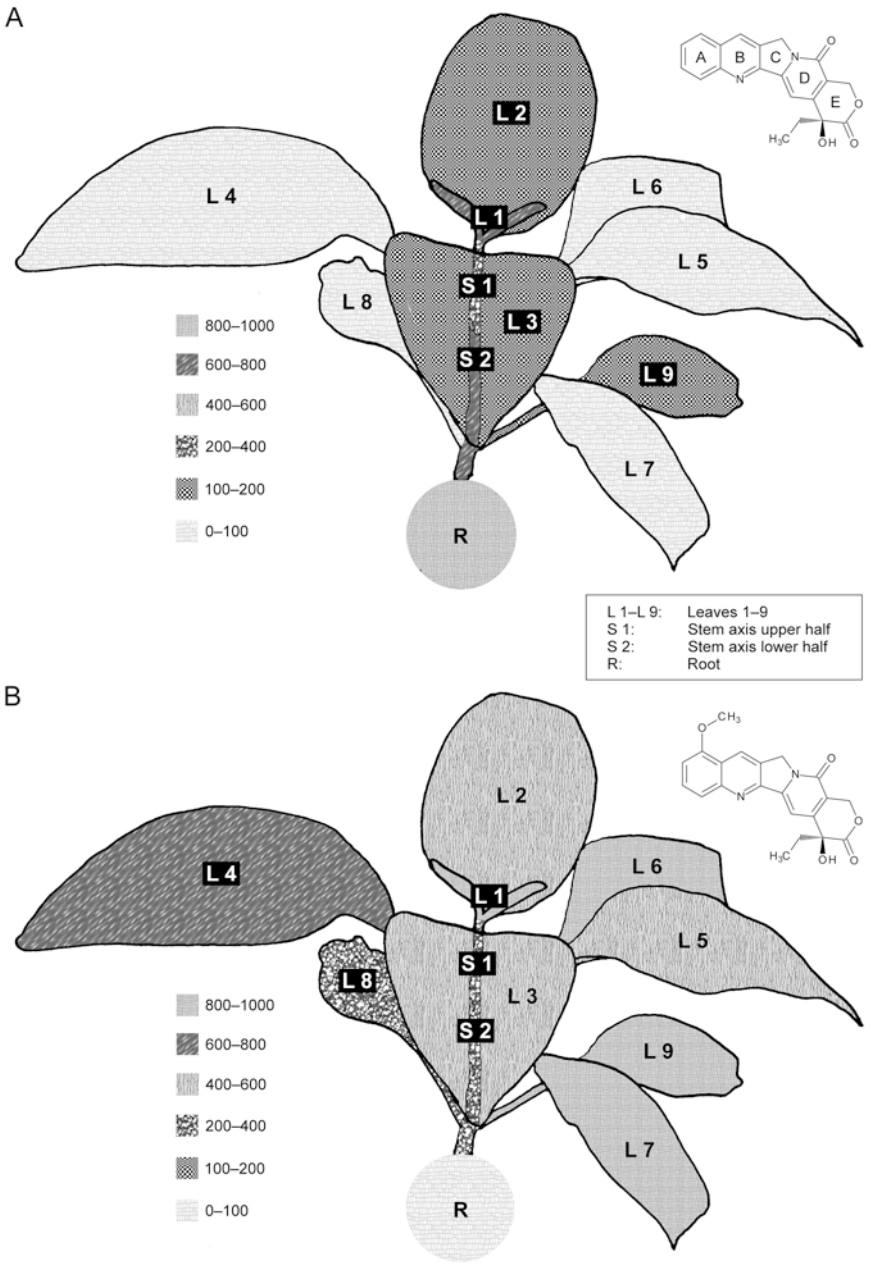

Fig. 17 CPT (**a**) and 9-methoxy CPT (**b**) contents in a young plant clone of *O. mungos* (age about 10 weeks). All values are given in μg/g DW. The graphic representation of the root is simplified and the stem axis has been made visible behind the leaves

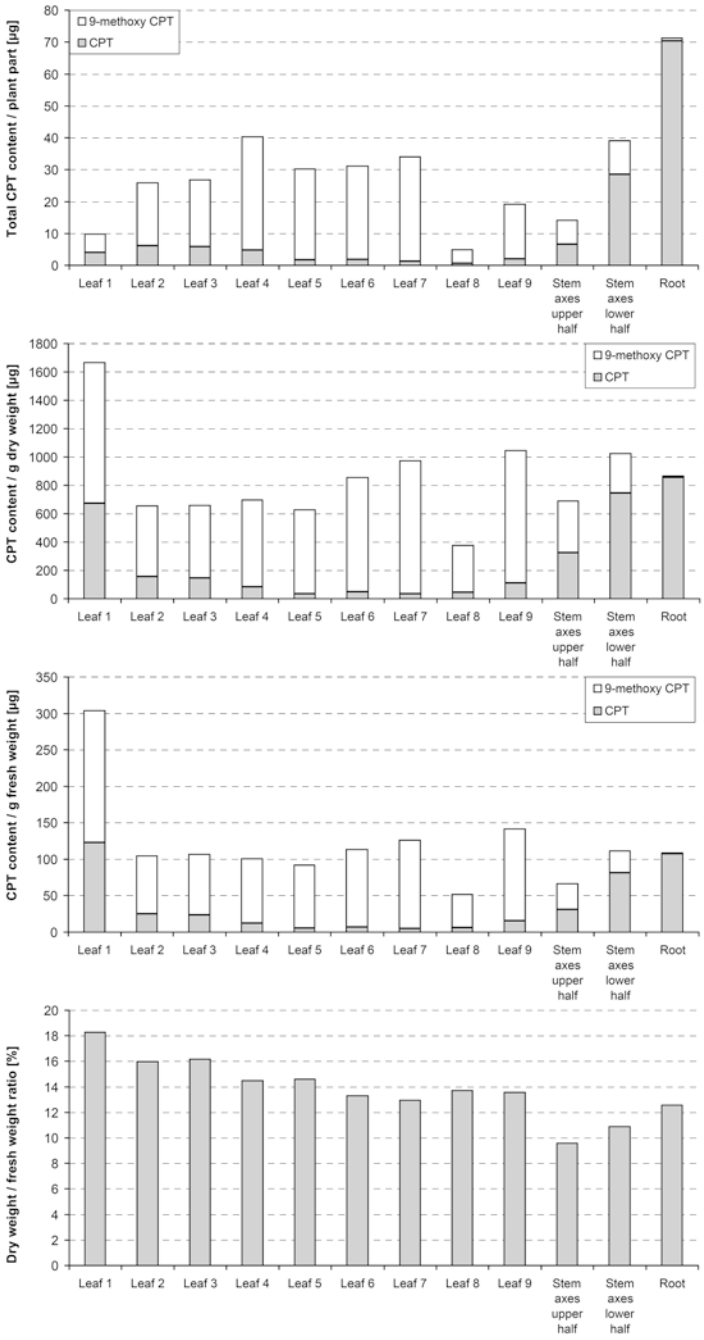

Fig. 18 Contents of CPT and 9-methoxy CPT in different relations and the DW/FW ratio of leaves, stem axis, and root of a plant clone of *Ophiorrhiza mungos* (cf. Fig. 17), about 10 weeks old

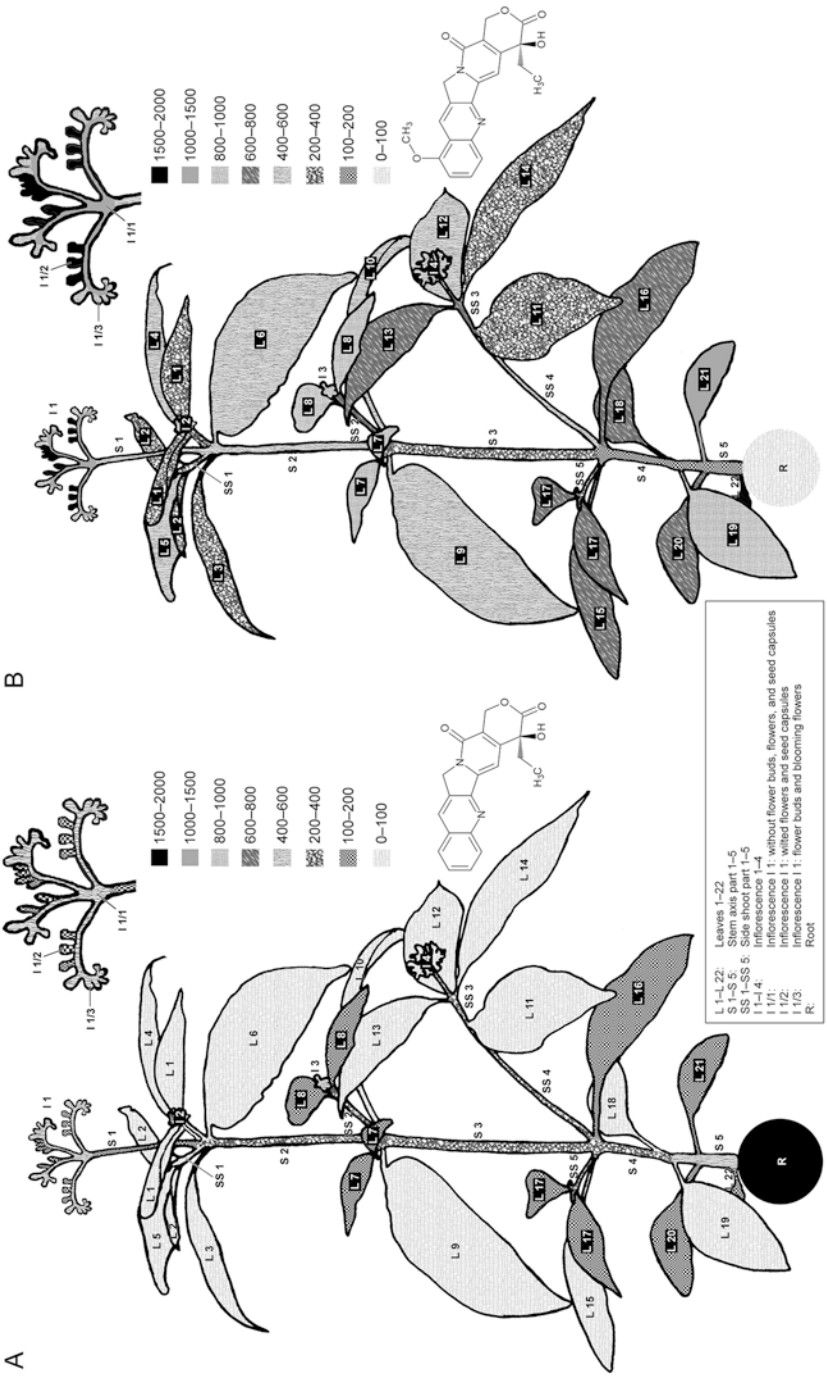

Fig. 19 Contents of CPT (**a**) and 9-methoxy CPT (**b**) in an adult plant of *Ophiorrhiza mungos* (older than half a year). All values are given in µg/g DW. The graphical representation of the root (R) and the main inflorescence (I 1) has been simplified

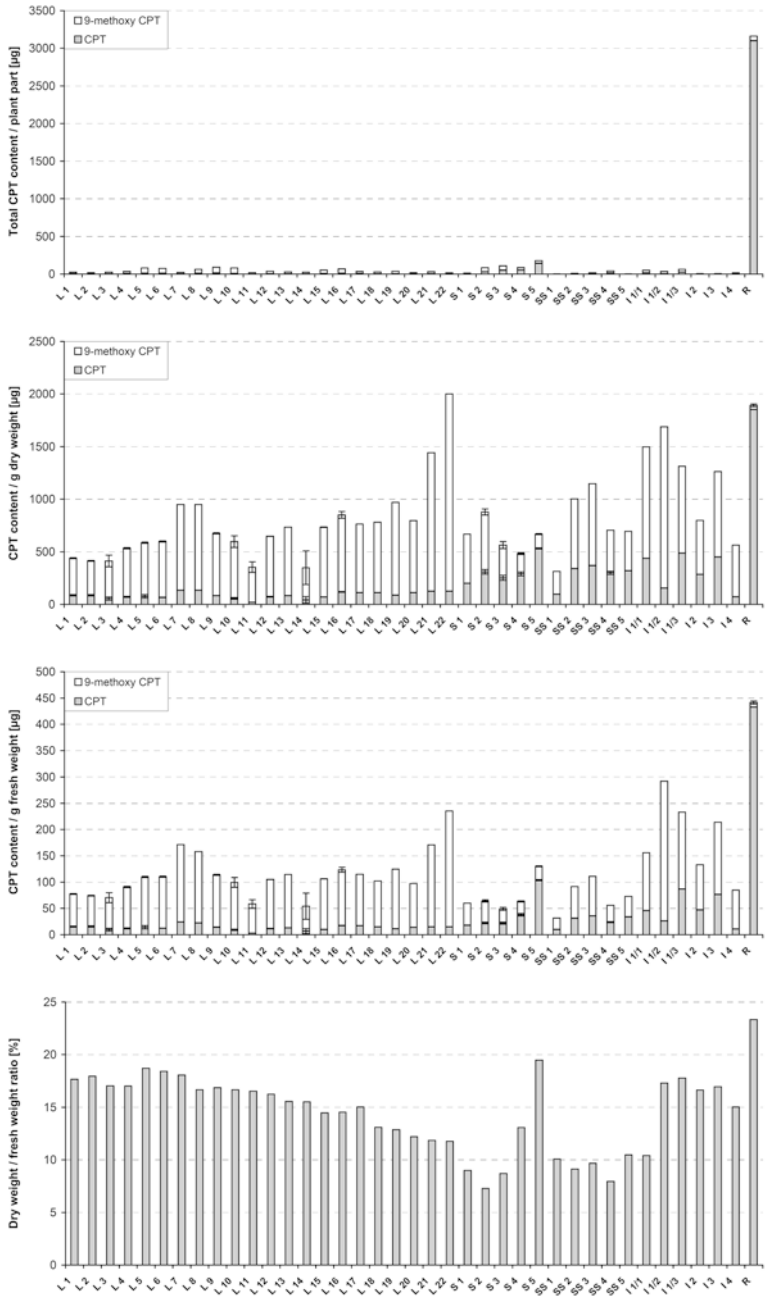

Fig. 20 Contents of CPT and 9-methoxy CPT in different relations and the DW/FW ratio of leaves (L), inflorescence (I), stem axis (S), side shoots (SS), and root (R) of a plant clone of *Ophiorrhiza mungos* (cf. Fig. 19), older than 6 months

9-methoxy CPT of approx. 1800 µg/g DW. The buds and flowers contained about 800–1000 µg/g DW and the rest of the inflorescence between 1000 and 1500 µg/g DW. Consequently, the main inflorescence showed the highest 9-methoxy CPT content and the highest content of CPTs in the aerial parts of the adult plant. In contrast, the minor inflorescences showed a CPT content of 80–450 µg/g DW and a 9-methoxy CPT content of 400–800 µg/g DW.

The whole adult plant contained about 197 µg CPTs/g FW and 1182 µg CPTs/g DW, or in total 1229 µg 9-methoxy CPT and 3559 µg CPT (total FW, 24.29 g; total DW, 4.05 g; DW/FW ratio, 16.68%).

3.6 CPT Distribution Inside Transformed and Untransformed Root Organ Cultures

The CPT content in the root organ tissues is quite stable in relation to FW and DW for each line (cf. Wetterauer et al. 2018). After examination of the distribution and storage of the CPTs in the total plant and the differences in CPT deposition in the plant root in comparison to transformed and untransformed root organ cultures (secs. 3.4 + 3.5), we wanted to know the distribution pattern of CPT in the transformed and untransformed root organ cultures of *O. mungos*. The results are shown in Fig. 21.

Of course, the root parts did not have the same weights because of their different lengths and the manual cutting, but the corresponding parts were in a comparable range. The same was the case in the CPT content per root part. As this implies, the CPT content in both root lines showed the same level for all parts calculated per gram FW; the transformed line is four times less in relation to the untransformed one. The DW to FW ratio rose a little from the younger tissues (part 1) to the older tissues. That means that the younger tissue contains more water than the older ones. In view of this, it is clear that the CPT content per gram DW decreased a little, because the corresponding biomass increased a little. In summary, the CPT content in the total roots of both lines is evenly distributed and related to the primary dry biomass in the oldest parts, because here a swlling of the root exodermis or a secondary growth takes place (browner tissue), or both. The dry weight density emphasizes this change, interestingly by a continuous decrease by age.

3.7 Elicitor Studies

In extensive elicitor studies on *O. mungos* root cultures, we were able to confirm that no amplification of CPT biosynthesis took place by single elicitor treatments. In these studies, many different elicitors, both simple (defined) and complex (defined and undefined), as well as precursors and abiotic stress conditions, were tested (Table 1).

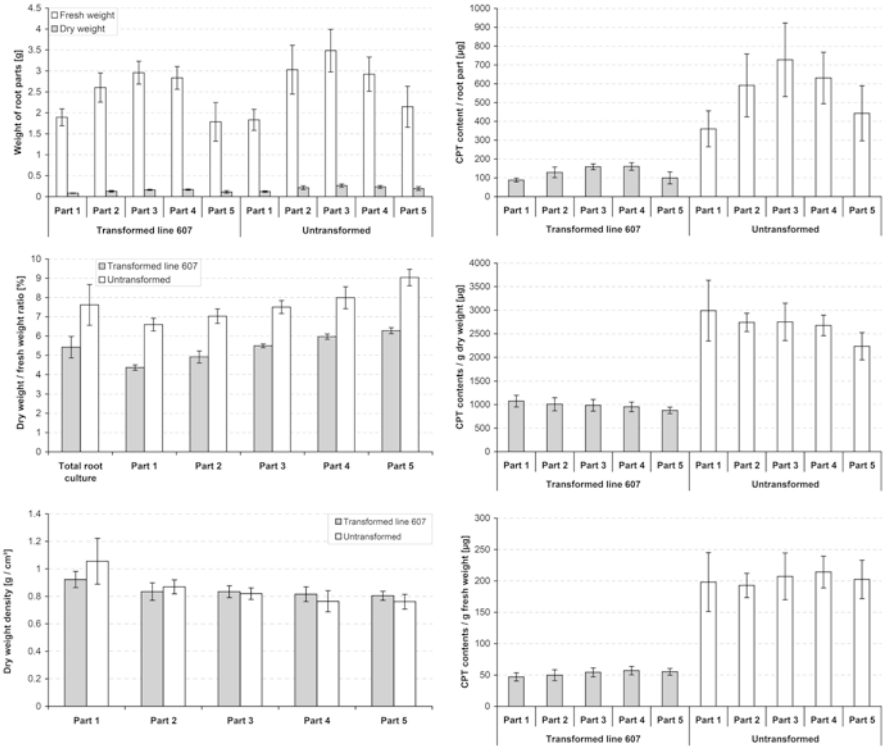

Fig. 21 Weights and CPT content in different relations of 7-week-old transformed and untransformed root organ cultures of *Ophiorrhiza mungos* cut into five parts each (young to old tissue, cf. Fig. 5), as well as their dry weight density (n = 4)

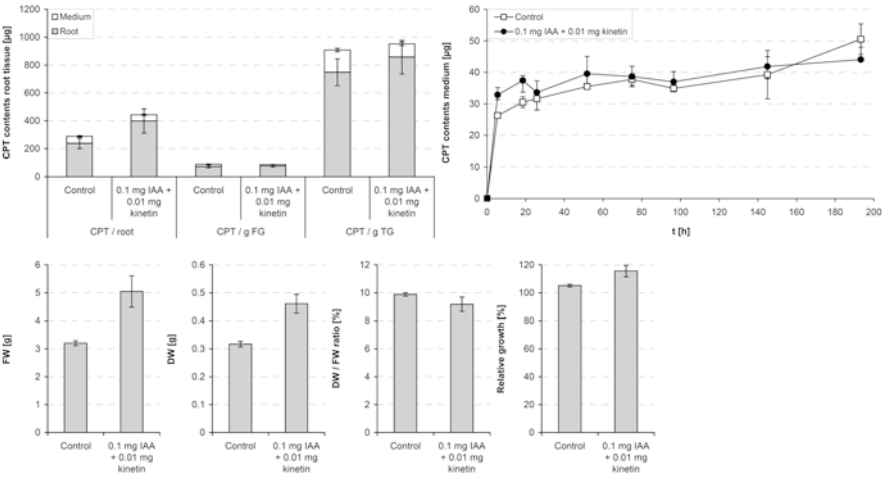

Fig. 22 CPT content in media and root tissues and growth parameters of untransformed root organ cultures of *Ophiorrhiza mungos* by combined treatment with 0.1 mg (≙11.4 µM) IAA and 0.01 mg (≙0.9 µM) kinetin per flask (n = 3)

Only a higher total CPT content, combined with higher relative and absolute growth through indole acetic acid (IAA) and kinetin induction, was detected (Fig. 22). With a higher growth rate, a higher absolute CPT content is realistic, of course. But the relative CPT content in relation to DW did not show the increase in the CPT tissue content as did the absolute ones and in relation to the FW is seen to be even lower in relation to the control. The explanation is quite simple. A brief look at the DW/FW ratio shows that it went down through the hormone treatment. A part of the difference in the absolute growth is based on higher water content in the treated roots. In the end, an increase in CPT tissue content in the treated root tissues can be noticed, but only related to the DW of a higher relative growth rate. This confirms again our hypothesis that CPT biosynthesis seems to be constitutive and growth regulated.

3.8 First Fermentations of Transformed and Untransformed Root Cultures of Ophiorrhiza mungos *in the Test Mist Fermenter WEWA I*

On closer examination of the medium contents during fermentation of the transformed line 607, a good agreement to the course of the corresponding root shaking cultures became obvious (Fig. 23; cf. Wetterauer et al. 2018). As in the root organ cultures, ammonium as a source of nitrogen was preferred, before the nitrate could be taken up. The ammonium content declined in the medium from approx. 0.45 mM to 0.03 mM from 70 to 290 h. In the fermentation time shown, the nitrate was not taken up by the roots and remained at around 15 mM. For longer fermentation, this would be more relevant with regard to growth and the pH value, because during ammonium uptake, the pH became more acidic and during nitrate uptake more alkaline. Here, the pH value became first a little more acidic, up to max pH 5.3 at 216 h during ammonium uptake and afterwards more alkaline nearly pH 6.2 at the end. The reason can be the start of nitrate uptake or other unknown factors. The phosphate content shows nearly the same course as the ammonium content, and the phosphate was taken up nearly completely until the end of the test period at 432 h. The sucrose content went down continuously and was inverted into glucose and fructose in concentrations of max. 1 g/l. At the end of the fermentation period, the sugar was not completely taken up.

The CPT content in the medium rose in the first 48 h up to 0.2 mg/l and increased continuously, at 0.04 mg per day. At the end of the test period, the CPT reached a concentration of 0.7 mg/l (0.2 µM). Only one break during the downstream test by a XAD PAD II column existed at day 13. This test was aborted quickly, because the pressure of the system was too low for the return flow of the medium to the medium reservoir through the column.

During fermentation, the relative growth rate was about 30%, in total 8 g gain. This was relatively low in relation to the root organ cultures, which tripled their fresh weights in the same incubation time. The roots on the carrier show a browning

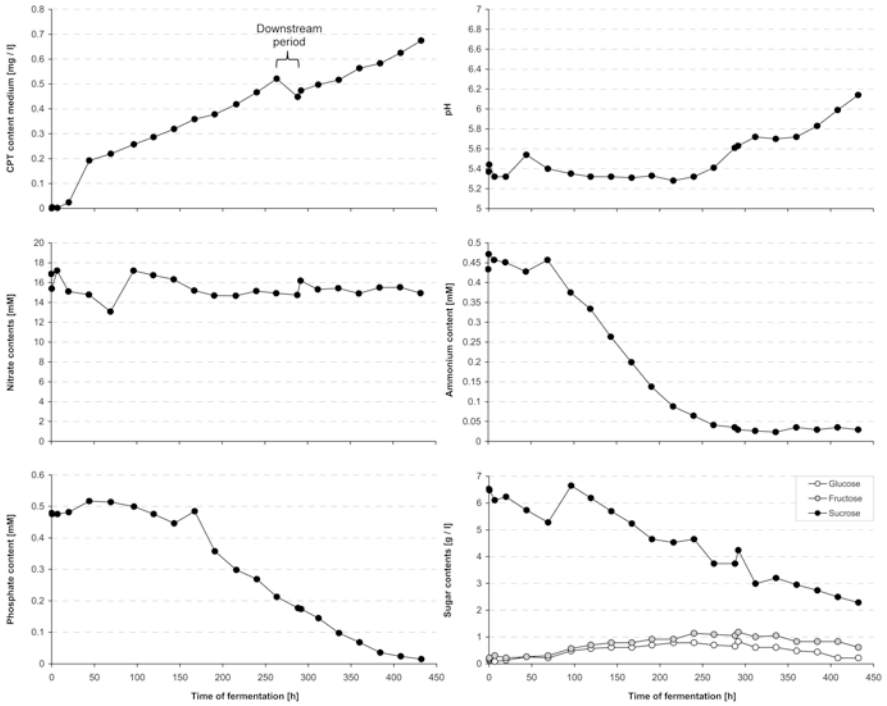

Fig. 23 Medium parameter courses during fermentation of transformed *Ophiorrhiza mungos* root tips (line 607) over 19 days. On day 13, a CPT downstream test by a XAD PAD II column was performed

at the end of the test period (Fig. 24) that implies stress or over-fertilization or both, especially with carbohydrates. In the centre, a spray gap with partially extinct tissue existed (circles in Fig. 24a, b; cf. Fig. 7e). The remaining tissue was vital and robust. Towards the outer half, young root tips were growing on the top and on the bottom side of the carrier. It appears that the medium spray was too strong. To correct this, the distance between the spay nozzle and the root carrier has to be enlarged or the spray pressure has to be reduced by reconstruction of the nozzle or a more indirect spraying method has to be developed for less irritation of the root tissue.

In another fermentation run with untransformed root tips of *O. mungos* over 22 days, a culture medium exchange was conducted after 10 days. In Fig. 25, the CPT medium content, the fermenter vessel weights, and the pH values over the test period are shown. This time, to obtain a better view of the roots' growing behaviour, the fermenter vessel was disconnected briefly at every reading point, and the acquisitions of the root weight course were done by weighing the complete fermenter vessel.

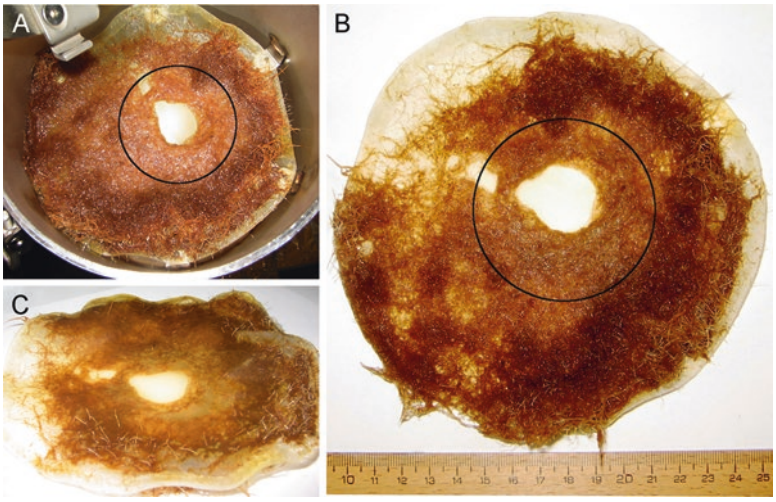

Fig. 24 Root carrier with transformed root tips of *Ophiorrhiza mungos* (line 607) after 19 days of incubation in the test mist fermenter WEWA I. (**a**) Opened fermenter vessel; (**b**) Top side of the carrier; (**c**) Bottom side of the carrier; Circles: central spray gap with partially extinct tissue

The CPT content increased in the first 2 days up to nearly 0.6 mg/l (1.7 µM). This was three times higher than in our experiments with the transformed line and showed the same difference in the medium content between the transformed and untransformed lines as in the root organ cultures (cf. Wetterauer et al. 2018 Fig. 14.5). Afterwards, the CPT content continued to rise up to nearly 0.9 mg/l (2.6 µM), parallel to the root growth, until the medium exchange. Afterwards, the CPT increased in the first 2 days up to 0.3 mg/l and afterwards by about 0.1 mg/l every 3 days. Unfortunately, a bacterial contamination occurred on day 32 (Fig. 25, grey area). Thus, no concrete statements about final CPT contents and absolute and relative growth without bacterial contamination can be given. The growth rate of the untransformed roots seemed to be in the same range as that of the transformed ones. The pH values showed also nearly the same course, until the bacterial contamination. During the contamination, the CPT contents continued to rise without reaching a plateau. The growth data are semi-quantitative and the pH values became more and more alkaline.

In summary:

For improvement, a longer period of optimization of the culture conditions and technical changes are necessary. Also, more effort has to be put to the sterile handling. But, in principle, a CPT fermentation of roots of *O. mungos* is feasible. Additionally, continuous or interval downstream processing for CPT out of the culture medium has to be developed.

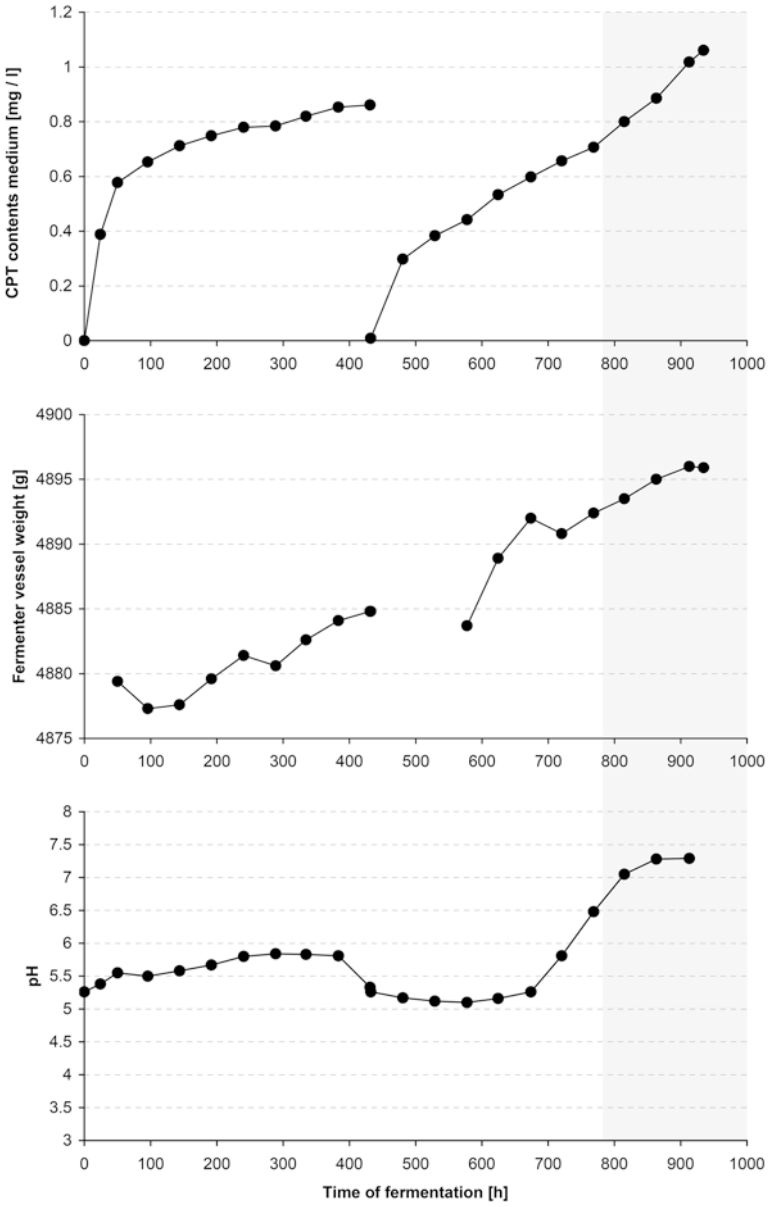

Fig. 25 CPT content, pH values, and approx. fermenter vessel weight during fermentation of untransformed root tips over 22 days. At day 10, a culture medium exchange was conducted

4 Discussion

Initially, only places of findings, basic morphological descriptions, and the traditional usages of *O. mungos* existed in the available literature (i.a. Hooker 1882; Kirtikar and Basu 1975; Nadkarni 1927; Quisumbing 1978; Sanyal 1984; Thwaites 1864; von Linne' 1753). The rest of the literature covered phylogenetic aspects of the Rubiaceae. Even nowadays, the genus *Ophiorrhiza* has not been thoroughly analysed. This becomes obvious when looking at the number of species, a number varying from 50 (Li and Adair 1994) up to 150 (Beegum et al. 2007; Darwin 1976). Additionally, this genus is problematic owing to double or multiple species descriptions, and thus synonyms are common (cf. nomenclature database of the Botanical Garden of Missouri).

Interestingly, Darwin (1976) discussed, besides other taxonomic characters, the occurrence or absence of crystals and raphides in *Ophiorrhiza* species as a marker for the position within the Rubiaceae system. However, the crystals were not a continuously present character either in the different *Ophiorrhiza* species or in the studied tissues. In addition, exactly *O. mungos* was not part of the species considered. In the present study, we describe the occurrence of crystals (raphides, druses, and crystal granular bodies) in this plant for the first time, at least in the border region of the cortical parenchyma near the phloem of the stem axis (cf. Fig. 10, Wetterauer 2008). Later, we will hypothesize why these crystals can be found there. At first, other parts of the plant were not investigated in relation to the occurrence of crystals, because we placed the main focus on the CPT distribution. But we were able to determine indirectly, by our studies of the root tip exudates of the root organ cultures (q.v. Flores et al. 1999), that the crystals consist of calcium oxalate (cf. Fig. 16). Afterwards, we prepared some handmade sections of *O. mungos* leaves and were able to detect microscopically that needles are present in the leaves (data not shown). In all the investigated root tissues, crystals, in whatever form, were never observed. Some years ago, Madhavan et al. (2013) did a pharmacognostical study on the leaves of *O. mungos*, but they did not describe any crystals or needles in the investigated tissues apart from the macerated trichomes. We can confirm a granulose appearance within the trichomes, but during our confocal laser scanning microscopy studies, we were not able to detect any real crystals in the living trichomes (Fig. 12c + E–G). However, the presence of calcium oxalate was verified by the histochemical tests of Madhavan et al. (2013).

All of our further findings in relation to the morphology of *O. mungos* were in agreement with the existing literature, apart from one new discovery. We were able to detect for the first time a new type of glandular hair on the surface of the inflorescence of *O. mungos*. We have called them 'strawberry hairs' because of their appearance (cf. sec. 3.1 + Fig. 11f).

When focussing on the CPT content and other ingredients in *O. mungos*, the historical view is quite interesting. Nadkarni (1927) first described that *O. mungos* contains an amorphous alkaloid (the method of detection was not given) besides starch, resin, and fat. Agarwal and Dhar (1959) isolated β-sitosterol and two

ergosterol derivatives out of the roots, and Tafur et al. (1976) determined for the first time CPT (12 μg/g DW) and 10-methoxy CPT (10.4 μg/g DW) in the leaves of *O. mungos*. In contrast, Sanyal (1984) remarked that no alkaloids were detected in *O. mungos*, but starch, chlorophyll, and a light-brown resin soluble in chloroform. In 2005, we published for the first time the CPT concentrations in our transformed and untransformed root organ shaking cultures of *O. mungos* (890–3200 μg/g DW, Wink et al. 2005), and Roja (2006) gave CPT concentrations for plant roots (176 μg/g DW) and shoots (96 μg/g DW) and for 9-methoxy CPT (only traces in both). Therefore, the question occurs why the content of CPTs in *O. mungos*, as determined by Tafur et al. and Roja in plants from natural habitats, should be so low in relation to the content levels in our data from root cultures or the concentrations in the plant roots with 860–1850 μg CPT/g DW and the aerial parts with 179–193 μg CPT/g DW and 490–600 μg 9-methoxy CPT/g DW, or total CPT contents with 795–1182 μg/g DW. First, we had a suspicion that the common method of defatting with petroleum ether may be a factor. In this regard, we tested the defatting of herb material with petroleum ether, followed by our normal extraction procedure, and saw no differences in the contents of CPTs. A degradation of the very stable CPTs by common handling of the drug is nearly impossible. Thus, we assume that the plant material of *O. mungos* from the natural sources in comparison to our greenhouse plants, or other factors of the declared methods, may be responsible for the low concentrations of CPTs given in these publications. Renjith et al. (2013, 2016) extracted the entire plants of *O. mungos* from a total of 17 different collection locations and obtained CPT contents between 150 and 540 μg CPT/g DW, but unfortunately analyses of other CPT derivatives were not described. We determined 300–880 μg CPT/g DW in our plants in total and of this 52.6–87.1% was localized in the roots. This confirms our hypotheses, because, on the one hand, Renjith et al. had lower CPT values, but in the same range. That indicates a slightly lower content in natural collected plant material (cf. Pasqua et al. 2004). On the other hand, even if the natural plant material has lower CPT values, the really low CPT and 9-/10-methoxy CPT values in Tafur et al. and Roja can only be explained by a big loss during the extraction procedure or a not fully developed quantitative analytic method. Next, Deepthi and Satheeshkumar (2016, 2017a, b) published studies on cell suspension and adventitious root cultures and Nagesha et al. (2018) on in vitro cultures of *O. mungos*; we will discuss these later in comparison to our elicitor studies. Finally, we gave an overview of nearly all published CPT content data in plants, a comprehensive look at the CPT content of 1 untransformed and 23 transformed root organ culture lines of *O. mungos*, a comparison of their culturing behaviour, and an overview of the CPT content in different plant tissues and cell culture systems of *O. mungos* (Wetterauer et al. 2018).

We mainly focussed on the distribution and storage of the CPTs within the tissues of the plant and the transformed and untransformed root organ cultures of *O. mungos*, especially in relation to the ecological function of CPTs as defence chemicals. At the time, nothing was known about CPT or its function; the usage in the traditional medicines gave first hints. First of all, the bitterness of the drug was described (Kirtikar and Basu 1975; Nadkarni 1927; Quisumbing 1978; Sanyal 1984), very probably due to the CPTs. Leaves, roots, and bark were used in the

forms of decoction, paste, or pressed juice (out of fresh roots, stem, and leaves), as described in the literature just mentioned. Looking in more detail at the parts of *O. mungos* used as a drug, we observed that these correspond to the localization of the CPTs we found in our in situ confocal laser scanning microscopy studies (cf. secs. 3.2 + 3.3). In general, the CPTs can be detected in the outer tissues of *O. mungos*. The highest concentrations are included in the most sensitive tissues as young leaves of young plants or the main inflorescence. This parallels the findings of Yamazaki et al. (2003b) in *O. pumila* or of López-Meyer et al. (1994) in *C. acuminata*. In *O. mungos*, CPT was stored vacuolarly and cytosolically in the root dermata. In the aerial parts, the CPTs are localized vacuolarly in evenly distributed idioblastic storage cells of the outer parenchymatic tissues, in the epidermis of the abaxial and adaxial leaf surface, and in the surface hairs of the two- and multicellular type (cf. Shitan and Yazaki 2007). Thus, a classical protection and defence pattern against herbivores becomes obvious in all parts of the plant (e.g. De Luca and St Pierre 2000; Wink 1988, 2003). The parenchymatous piths and epidermis of the stem axis and side shoots, the spongy and palisade parenchyma of the leaves, the vascular bundles, and the ovules as well as the calyptras of the root and the root hairs were free of CPTs.

Very interesting are the differences in localization and storage of CPT of the plant root and the transformed and untransformed root organ cultures besides one common aspect, that the stele and the cortex of all of them were CPT-free (Figs. 13, 14, and 15a–d, f–i). In the plant root, the calyptras are CPT-free and the storage is vacuolar in the rhizodermis, initial from the zone of differentiation. With the beginning of the root-hair zone, in addition a cytosolic storage can be noticed upwards. In contrast to the plant root, the untransformed root organ cultures show a cytosolic localization starting in the middle of the calyptras and go up in the cone to the rhizodermis. In the root-hair zone, the storage switches to a vacuolar one. Finally, the root organ cultures of the transformed line 607 showed the same storage and localization as the untransformed ones, with one difference that the calyptras show in total a cytosolic CPT fluorescence (comp. Fig. 13). Why the storage is different belongs to the field of speculation at the moment and needs to be investigated more closely. Maybe the big differences in storage and distribution of CPT between the plant root and the root organ cultures can be attributed to another mode of auxin metabolism, because of the non-existent aerial parts. But here, many further questions will come up in relation to CPT biosynthesis, vacuolar storage, transport, self-protection against the plant's own CPT, and the release in the surrounding medium, as well as its content regulation there, which will be discussed below.

The CPT in the roots and root organ cultures of *O. mungos* is localized in the border tissues, beginning with the zone of differentiation. Apart from the different modes of location and storage in the transformed and untransformed root organ cultures, the CPT appears also in the calyptras, first within the differentiated tissues or the tissues that are in the process of differentiation. Next, the distribution of the CPT within the root organ cultures is equal in relation to the dry biomass. That means all root sections contain the same relative CPT content (cf. sec. 3.6). This

suggests, together with the absolute stable CPT contents in our root organ lines and the clear vacuolar storage of CPTs in the evenly distributed cells of the aerial parts, a developmentally regulated CPT biosynthesis for a constitutive purpose. In a study by Asano et al. (2004), three plant species (aseptic culture) were investigated, and two interesting aspects become obvious in this context. Firstly, the interspecies hybrid *O. kuroiwai* (*O. pumila* × *O. liukiuensis*) showed an intermediate CPT production in the roots in respect to *O. pumila* and *O. liukiuensis* that speaks for a genetically determined constitutive CPT production. Secondly, *O. kuroiwai* showed through its higher CPT levels per flask with a higher growth rate the growth-related biosynthesis of CPT. López-Meyer and Nessler (1997) were able to show that in *C. acuminata*, two autonomously regulated genes for the tryptophan decarboxylase (TDC), one of the key enzymes in the CPT biosynthesis, are present. One copy (*tdc1*) is expressed developmentally in the young tissues, and the other one (*tdc2*) is part of a defence system induced by pathogens. Yamazaki et al. (2003a) found that in *O. pumila*, two encoding genes are presumably also present. This may be the case in *O. mungos* too. In this context, our own intensive elicitor studies on transformed and untransformed root organ cultures show no induction of a significantly higher CPT biosynthesis besides a small one induced by a combination of IAA and kinetin based on a slightly higher growth rate (cf. secs. 2.7 + 3.7). In this, a path of induction as described by the *tdc2*, as in *C. acuminata*, thus neither exists nor is active in *O. mungos*. Some elicitation tests performed in other studies showed partial inductions of the CPT biosynthesis in *Ophiorrhiza* species. An increase of the STR (strictosidine synthase) and TDC mRNA expression was shown for DMSO treatment of root cultures of *O. pumila* by Yamazaki et al. (2003a), but no significant rise in CPT biosynthesis in relation to water controls was detected. Asano et al. (2004) described an enhanced alkaloid production of about 1.3-fold by treatment of the hairy roots of *O. liukiuensis* with 100 μM methyl jasmonic acid (MeJA) based on a higher growth rate. Salicylic acid (SA) showed no effects. Deepthi and Satheeshkumar (2016, 2017a, b) investigated the CPT production in cell suspension and adventitious roots cultures of *O. mungos*. The nutrients ($AgNO_3$; yeast extract) and the MeJA affected the CPT yields in the cell suspension cultures, again proportionally to the growth rates. Plant growth regulators and nutrition tests in adventitious root cultures yielded the same results. Nagesha et al. (2018) reported studies on shoot cultures of *O. mungos*, again with MeJA and SA. The highest CPT yields were attained with 0.23% by treatment with 150 μM MeJA compared to untreated plants with 0.11%. SA failed again. Consequently, the available studies verify our assumption of a growth-related CPT biosynthesis in *O. mungos*.

To come back to the CPT localization and distribution in the plant root and root cultures of *O. mungos*, it can be speculated that some patterns can also be a result of transport phenomena. A systemic transport of CPTs in *O. mungos* was ruled out, because we never detected any CPT fluorescence in the vascular bundles. Additionally, the plants had to fight over years against mealy bugs and aphids in the greenhouse, and these pests loved our *O. mungos* cultures (Fig. 9b). Even the analysis of the mealy bugs from *O. mungos* showed no detectable content of CPTs. A short-distance transport of CPT can be considered in a closer inspection of the

cytosolic distribution in the calyptra and the rhizodermis of the root cultures, the partly cytosolic localization in the root-hair zone and the exodermis of the plant root, the shift of the CPT between the cell layers at the border of rhizodermis and exodermis, and of course of the release in the outer medium. Sirikantaramas et al. (2007b) carried out studies on the questions of inter- and intracellular transport mechanisms and vacuolar storage of CPT in *O. pumila*. Here, an active transport was ruled out by inhibition tests of transporters and vesicle transport. A simple diffusion process was postulated with the finding that the CPT release was increased by blocking the vesicle transport. But, as we saw in our downstream experiments with resin, 60% of the CPT was still retained in the root tissue. As shown in the literature, hydrophilic secondary metabolites are usually stored in idioblasts and vacuoles (e.g. Shitan and Yazaki 2007) by protonation at lower pH. As Fassberger and Stella (1992) described in the case of CPT, the transformation from the lactone to the carboxylate form takes place at a pH ≥ 7 and in reverse at pH ≤ 7. At the cytosolic pH ($\approx$7), then, the CPT can be in both forms. The lactone form should be able to pass the tonoplast because it is highly lipophilic. But within the plant vacuole, the normal pH is around 5, and we know from our own studies that the vacuoles in the storage cells of the plant root of *O. mungos* are at a pH of 3.2 ± 0.2 (unpublished). At this pH, the lactone form will be formed and the CPT can leave the vacuole freely. A protonation of the nitrogen in the B-ring (cf. structure Fig. 17a) does not take place until pH 1–2 has been reached (cf. Fig. 14.2a in Wetterauer et al. 2018). Furthermore, the bottleneck enzymes of the CPT biosynthesis are located in different compartments. The TDC is described as a cytosolic enzyme and the STR as a vacuolar one (Contin et al. 1999). In contrast, based on the data of Yamazaki et al. (2003a), who defined a vacuolar STR, a STR in the endoplasmic reticulum (ER) was proposed by Sirikantaramas et al. (2007b). The following description perhaps can be given. The CPT biosynthesis ends, in whatever way, in the ER and the CPT is transferred by vesicles into the vacuole. In the vacuole, the CPT is stored (partly?), in some way ever, or it disperses passively in respect to its physical and chemical properties (partly?). In sum, we cannot explain all the findings, but we conclude again that a combination of diffusion, as described by Sirikantaramas et al., and a kind of unknown storage process exists for the vacuolar storage, such as an attached pacing of several CPT molecules or non-covalent clustering with other vacuolarly stored molecules or something else. In the case of the cytosolic localizations, we have to mention that the emission spectrum of NADPH shows similar maxima as CPT. Therefore, the fluorescence in these regions should not be attributed to the CPT in total. A third mode of CTP localization and storage, which has not been discussed so far, can take place in the appropriate intercellular matrices in the roots. This can lead to additional effects on the physical and chemical equilibria in respect to a passive diffusion process. As we can see, e.g. in Fig. 15c, fluorescence in these parts becomes obvious too. This can originate from cell wall components as well as from CPT. So CPT localization and also storage cannot be ruled out in these areas. Based on the known lower pH in the apoplast, an attached packing, as contemplated for the vacuoles, can happen. A non-covalent association with, e.g. polyphenols can also not be ruled out (comp. Roberts and Wink 1998).

A very important aspect is the function of the CPTs in *O. mungos* and its self-protection against them. In general, as mentioned above, the CPTs in the aerial parts seem to have a protective function against herbivores in relation to their localization. Additionally, the plant itself may have self-protection against the CPTs by a mutation of the topoisomerase I, as found for *C. acuminata*, *O. pumila*, and *O. liukiuensis* by Sirikantaramas et al. (2008). And for the case of a CPT release by wounding, we hypothesize that the calcium oxalate has a border function by inclusion of the CPTs to avoid a systemic dispersal (cf. Pasqua et al. 2004). Thus, the localization of the calcium oxalate next to the vascular bundles would make sense, apart from the calcium oxalate itself having a protective function against herbivores (Franceschi and Nakata 2005). In the root, the matter is different. Firstly, the root exhibits no calcium oxalate crystals. Secondly, the CPT is only localized in the dermata in the plant root, and therefore only a protection against small herbivores seems possible. Here, the release of CPT into the rhizosphere seems to be the important protection mode, something known from many root organ cultures of CPT-producing plants. This can have an allelopathic reason, such as the defence of resources in relation to other plants (growth regulator) or to keep insects and other vermin away (e.g. Saito et al. 2001). In the case of *O. mungos*, we did not investigate the soil of the plant root for its CPT content, but in sterile plant cultures with roots, we saw a clear CPT fluorescence in the culture agar, so that it has to be assumed that the plant root releases CPT into the soil too. The mode of the CPT release from the plant root is still unclear. Also, for the root organ cultures, the modes of storage and release, or better of outer content regulation, are still not known. Sirikantaramas et al. (2007b) showed for *O. pumila* that the release probably is mainly mediated by passive diffusion. With respect to our aim of large-scale production of CPT with root organ cultures in biofermenters, the item of CPT medium content regulation is so important that we had a closer look for *O. mungos* root cultures. First, we recognized over the years that the different root culture lines exhibit quite strict CPT contents in the culture medium, depending on the line. For example, the untransformed root organ culture kept a CPT content of approx. 12.6 µM and the transformed line 607 of approx. 5.2 µM (cf. Wetterauer et al. 2018). That should be a hint that the outer concentration is actively regulated at first sight. The release can happen by the loss of CPT-containing cells of the calyptras through normal growth and, as shown in the case of the root exudates, by leaking out. However, after refreshing the root culture medium, the growth of the roots takes some time to continue, because of the stress through the changed surrounding. However, the CPT contents in the medium are at the same level after the first 48 h, and, for a short time, even higher, followed by a quick downregulation to the line-dependent level. At the same time, the relative and absolute CPT contents in the root tissues show an inverse behaviour. Again, the one observation speaks for a passive content regulation and the other for an active one. We thus continued to research in two directions (none of the following results are shown here). Firstly, we did downstream experiments by resins to remove all CPT from the culture medium (comp. Saito et al. 2001), with the result that slight growth induction in the roots was observed to compensate the loss of CPT, but in the end, the root tissue contents became adjusted to a lower but

constant level of about 60% of the normal one. This is parallel to the results of Saito et al. (2001) for similar tests on root cultures of *O. pumila*. Secondly, we added CPT in quantities up to 5 mg per flask. All CPT was taken up by the roots until the natural medium concentration was reached again. Under UV light (366 nm), these root cultures showed an amplified CPT fluorescence. To rule out that the CPT only attached to the root surface, we did confocal microscopy studies and saw that the CPT fluorescence was stronger in the same regions in which the CPT was stored normally. After that we added resin in turn, again all the added CPT was removed, and the CPT content went down anew to the same constant level as in the first downstream experiment. On the one hand, the results show again a partly passive regulation; on the other hand, we see the stable, albeit lesser, remnant of the tissue CPT content that was not depleted by the resin. Further on, we performed a competitive uptake experiment with the structurally related ajmalicine. Here, we added 3 mg of CPT and the same amount of CPT combined with the equivalent dose of ajmalicine. As a result, the relative uptake of CPT was reduced by a sixth in the combination with ajmalicine, giving no further hint of a differentiation between active and passive regulations. Last of all, we tested an addition of 9-methoxy CPT, which is not synthesized in the roots. As mentioned, both metabolites, CPT and 9-methoxy CPT, have the same excitation wavelength, but different emission wavelengths (sec. 2.4). By two-photon excitation microscopy, we tried to determine the disposition of the 9-methoxy CPT within the living root tissue. As a result, we were able to verify uptake of 9-methoxy CPT, but failed in determining the localization due to the resolution. In sum, we have not been able to understand the mode of CPT content regulation so far, but we assume that here a combination of passive diffusion and another type of storage (physically or chemically) takes place.

To clarify the complex regulation of CPT biosynthesis, storage, and ecological function a little more, perhaps a closer look at the photosynthetic tissues will give further hints. In our basic studies on green roots (untransformed roots under light explosion, q.v. Flores et al. 1993), we noticed that the biosynthesis of CPTs is comparable to the aerial parts. Not only does the biosynthesis of 9-methoxy CPT start (cf. Fig. 14.1 in Wetterauer et al. 2018 and Fig. 3), but also suddenly a high content of strictosamide occurs in the extracts of the green roots, which is not present in the normal untransformed root culture cultured in the dark. Hence, on the one hand, the green roots show that the biosynthesis of 9-methoxy CPT is related in some way to the photosynthetic metabolism (cf. Chang et al. 2019) and, on the other hand, that maybe another regulation of the biosynthesis or reaction mode in relation to environmental factors will take part due to the occurrence or storage of precursor metabolites in the green tissues at a high level.

As mentioned before, the biosynthetic pathways of the CPT and derivative biosynthesis in the producing plants have so far not been fully elucidated, and so the biotechnological possibilities for an in vitro production are minimal at the moment. Another interesting new field in finding alternative ways for CPT production in the future maybe are endophytic fungi and bacteria from CPT-producing plants that could biosynthesize CPT as well (comp. Wetterauer et al. 2018).

Cell culture systems have failed until now due to their low biosynthetic performance. Therefore, root organ culture systems are the most realistic and economically possible way to mass-produce CPT at the moment, besides the classical plantation. Robust data on the worldwide demand for CPT are hard to obtain. As listed in Wetterauer et al. (2018), Asano et al. (2004) give 1000 kg CPT, based on derivative demands for chemotherapy. Since 2014, the demand for CPT has been quoted at 3000 kg, but without any given sources (Cui et al. 2015; Kai et al. 2014; Raveendran 2015). In contrast, Kai et al. (2014) give an annual worldwide CPT production of 600 kg, whereas our own enquiry in 2013 resulted in a delivery capacity of 1000 kg/month (only one supplier!). Even if the data given are inconsistent, in respect of greater independence from the producing countries and for a more sustainable bio-production, the fermenter technology of root organ cultures represents a good alternative. To confirm this, we want to do a little estimation. As Li and Adair (1994) wrote, a three-year-old tree of *C. acuminata* contains 50 mg of CPTs; on their assumption that 30% of the cancer patients in the United States are treated with CPT and each patient needs about 1–3 g CPT, they calculated that 7–21 million young trees are needed annually on 4200–12,600 hectares of plantations. That corresponds to approx. 350–1000 kg CPT. In our untransformed root organ cultures, 50 mg CPT corresponds to 20–25 g tissue produced in 2 weeks by 10–15 cultures. To produce 1 kg of CPT, 500 kg of root tissue is necessary. This seems a great deal, but in a suitable large-scale fermenter, a cultivation of 25–50 kg of roots/fermenter should be possible within a few weeks. When using 100 fermenters, a production of 10 kg CPT should be possible in 2 months (≥35,000 € value) and in 1 year about 60 kg with a value of over 200,000 €. But a single batch production can be increased by online downstream processing of the CPT out of the culture medium without harvesting the tissue, because up to 40% of the synthesized CPT can be released by the roots into the culture medium, as mentioned above. In this connection, the downstream processing and purification of the CPT can take place by means of resin or semipermeable membranes with little labour and expense. Additionally, by light exposure, the untransformed roots of *O. mungos* turn green and produce 9-methoxy CPT that is twice as expensive as normal CPT. The real productivity of such a fermenter system cannot be specified exactly without more experience. In the present study, we demonstrate the important factors of the upscaling process. Here especially the composition of the culture medium, the inoculum size and its dispersion on the carrier system, and the sterility, besides the technical challenges, are pointed out (sec. 2.8). In two fermenter runs with transformed and untransformed root organ cultures of *O. mungos*, we highlighted the behaviour of the root system, which was close to the root shaking cultures, and we were able to show the CPT post-production of the root batches after a little downstream with resin and after medium exchange (sec. 3.8). Of course, we reached no enormous CPT medium content (≈1 mg/l) in this little fermentation scale here, but firstly, we did not use the most productive root lines for our basic studies, and secondly, on a larger scale, the CPT production will come into a more commercial range, as the former ROOTec company was able to show. Nevertheless, every kg of CPT produced by fermenter technology saves at least 12 hectares of plantations or natural resources. The

purification of the CPTs is quite simple and cheap and does not contaminate the environment. Consequently, it would be a feasible and sustainable method of CPT production as raw material for the chemical industry that can fill supply shortages and save environmental resources in the actual producing countries.

5 Conclusions

The pathway of the CPT biosynthesis in the producing plants has as yet not been completely elucidated. The storage and release of CPT in the different plant organs still pose basic questions too. For *O. mungos*, we were able to show a constitutive and developmentally regulated CPT biosynthesis. Extensive elicitation studies on root organ cultures did not lead to an amplification of the CPT biosynthesis. The vacuolar storage in the aerial parts of the plant exhibits a distribution pattern of CPTs against herbivores with the highest concentrations in the inflorescences and the young leaves. Here, mainly 9-methoxy CPT is biosynthesized, probably with the participation of enzymes related to the photosynthesis apparatus. In the roots, only CPT is biosynthesized and partly released in the surroundings, probably for allelopathic reasons, such as plant growth regulation. Here, the CPT is vacuolarly and cytosolically localized in the epidermis. In the root organ cultures, CPT is dispersed equally in relation to the dry weight, which confirms again a constitutive and growth-regulated CPT biosynthesis in *O. mungos*. In this context, the different CPT content levels in the culture lines are probably genetically determined in a previously unknown way.

Focussing on the biotechnological CPT production, root organ cultures seem to be the only feasible way to produce CPT economically in relation to the classical plantations at the moment. In root organ cultures of *O. mungos*, the CPT contents are at the same or even higher levels as in the mother plant; CPT is partly released in the culture medium, and the downstream processing and purification is quite easy. Therefore, the root fermenter technology is a sustainable method of CPT production to save natural and environmental resources. Also, a higher independence from the CPT-producing countries will be achieved and CPT supply shortages in the demand of the chemical industry for this high-value and medicinally important secondary metabolite can be filled.

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In Vitro Culture of *Haloxylon recurvum* and *Haloxylon salicornicum*: Valuable Source of Food Additives and Pharmaceutical and Nutritional Components from Extreme Arid Zone

Deepmala Goswami and Harchand R. Dagla

Abstract Nutritional and hormonal requirements of seedlings and shoots of *Haloxylon recurvum* and *Haloxylon salicornicum* were analyzed in vitro in present investigation. It occurs naturally in saline and sandy arid areas and belongs to family Amaranthaceae. Dry seeds were pretreated with 70% (v/v) alcohol for 10–20 sec and then sterilized with 0.1% (w/v) $HgCl_2$ for 2–3 min followed by 4–5 washings with autoclaved distilled water and inoculated on hormone-free MS medium for germination. Cultures were maintained at 28 ± 2 °C under 35–40 μmol m^{-2} s^{-1} irradiance and 12hd^{-1} photoperiod by cool white fluorescent tubes. Seventy and 80% seeds of *H. recurvum* and *H. salicornicum* were germinated on hormone-free MS medium. Axillary buds of cotyledonary nodes of *H. recurvum* and *H. salicornicum* were activated on 6 μM BAP and 8 μM BAP respectively. Five and 3% (w/v) sucrose was found to be more appropriate for the growth and development of cotyledonary nodes of *H. recurvum* and *H. salicornicum*. In vitro cultured seedlings can be used as an efficient source of explants for establishment of cell culture and mass multiplication of shoots for industrial production of plant products and micropropagation of these plants.

Keywords Arid areas · Cotyledonary node · *Haloxylon recurvum* · *Haloxylon salicornicum* · In vitro culture

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1 Introduction

Plants of extreme arid and saline habitats of Rajasthan, India, are important source of food supplements, fodder, fiber, and fuel for the local inhabitants. The existences of some of these species are at the verge of extinction and need special attention. Characterization, conservation, and mass propagation of these plants are needed for the maintenance of biodiversity and sustainable development in the region. Plant cell, tissue, and organ culture is an important technique for understanding the nutritional requirements, developmental biology, and regeneration of plantlets in vitro (Dagla 2012). Technique is also useful in production of secondary metabolites from in vitro cultured cells or organs. Molecular and biotechnological approaches have also been made to understand the molecular and in vitro developmental biology of these plants (Dagla and Shekhawat 2005a, b, 2006; Dagla et al. 2007, 2012, 2014; Gehlot et al. 2014; Nair and Dagla 2016; Upendra and Dagla 2016; Goswami and Dagla 2017; Upendra et al. 2017; Nair et al. 2018; Paliwal et al. 2018; Vyas and Dagla 2018). Although micropropagation of *Haloxylon recurvum* and *Haloxylon salicornicum* using mature shoots has been reported respectively by Dagla and Shekhawat (2005b) and Vyas and Dagla (2018), but comparative study related to in vitro seed germination and seedling growth of these plants was lacking. So, in vitro seed germination, growth, and development of seedlings and mass multiplication of shoots of *H. recurvum* and *H. salicornicum* were analyzed in present investigation. *Haloxylon recurvum* and *Haloxylon salicornicum* are perennial under shrub of family Amaranthaceae and are distributed in saline areas of Pachpadra and Phalodi (Fig. 12.1a) and sandy arid region of Jaisalmer (Fig. 12.1b), India.

Haloxylon recurvum (Moq.) Bunge ex Boiss is locally known as Sajji or Khar (source of crude sodium carbonate – barilla or Sajji-Khar). The ash of the plant was used earlier as a substitute of soap for cleansing clothes, and aqueous solution was used against internal ulcers (Anonymous 1959). Ash extract of the plant is also used as a special gradient in famous food additives like Bikaneri Bhujia and Papad of India. *Haloxylon salicornicum* (Moq.) Bunge in Boiss is locally known as "Lana." It is also found in Egypt, Palestine, Jordan, Iraq, Iran, Pakistan, and Kuwait (Ali and Qaiser 2001). It survives well in sandy arid regions and helps in stabilization of shifting sand dunes. It is a good source of food supplements, fuel, and bio-fertilizer (Dagla and Shekhawat 2006). Mature stems are burned with little smoke, and its ash is used as a tooth powder by the local inhabitants. Mixed flour of *Haloxylon salicornicum* and pearl millet (*Pennisetum glaucum* R. Br.) seeds are being used for preparation of local dish "Dhokla." Plant contains alkaloids (Anonymous 1992). It is also used as a source of anti-inflammatory activities (Verma et al. 2014). The present study was aimed at assessment of (i) nutritional requirement of seedlings and (ii) hormonal requirement for the activation of axillary shoot buds of cotyledonary nodes of seedlings. Cotyledons, cotyledonary nodes, hypocotyls, and roots of in vitro cultured seedlings can be a source of explants for induction of callus, somatic embryos, and mass propagation of the plants for prospects. In vitro cultured cells and shoots can also be a source of industrial production of pharmaceutical and nutritional components they contain.

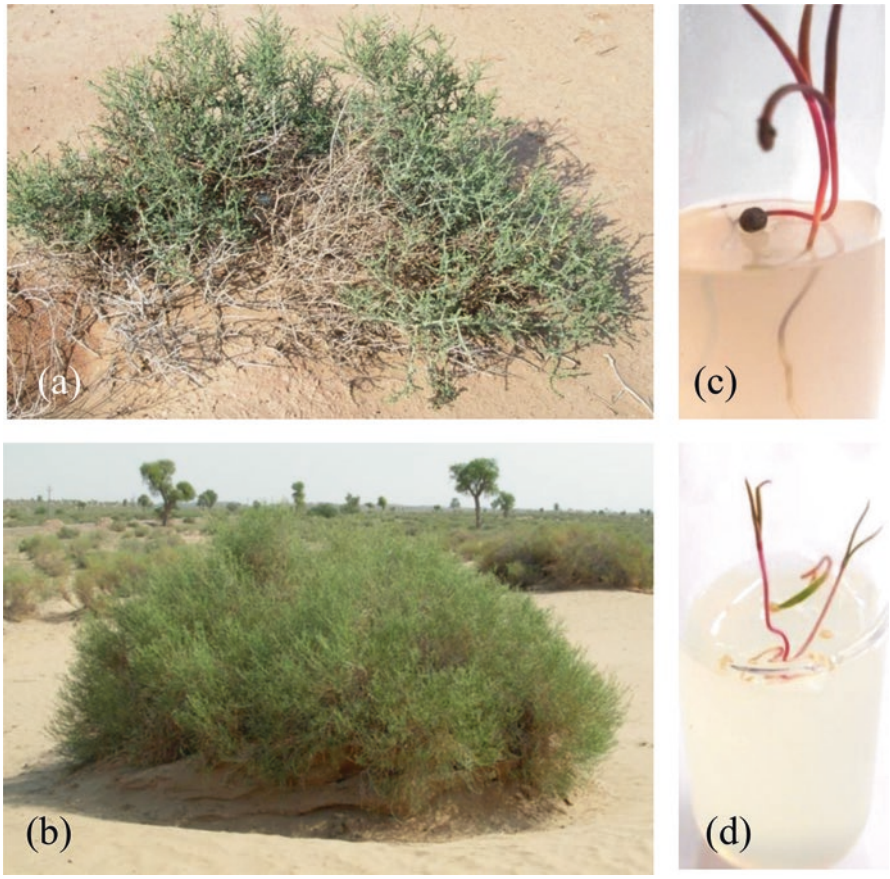

Fig. 12.1 Plant in natural habitat (**a**) *Haloxylon recurvum* and (**b**) *Haloxylon salicornicum*. Seedlings cultured on hormone-free MS (Murashige and Skoog 1962) medium: (**c**) *Haloxylon recurvum* and (**d**) *Haloxylon salicornicum*

2 Materials and Methods

Dry seeds of *H. salicornicum* and *H. recurvum* were pretreated first with 70% (v/v) alcohol for 10–20 sec and then sterilized with 0.1% (w/v) $HgCl_2$ for 2–3 min followed by 4–5 washings with autoclaved distilled water. The surface sterilized seeds were inoculated on hormone-free MS (Murashige and Skoog 1962) medium for germination. The cotyledonary nodes of in vitro cultured seedlings of *H. recurvum* and *H. salicornicum* were inoculated on MS medium supplemented with different concentrations (0, 2, 4, 6, 8, 10 μM) of BAP. The cotyledonary nodes were also cultured on (0, 1, 3, 5, 7, and 10% (w/v)) of sucrose. Cultures were maintained at 28 ± 2 °C under 35–45 μM m^{-2} s^{-1} irradiance and 10–12 h/d photoperiod by cool white fluorescent tubes.

3 Statistical Analysis

SPSS v.17 (SPSS, Chicago, IL) were used for statistical analysis of observed data. Duncan's multiple range tests at $p < 0.05$ were carried out for significance of differences among the means (Duncan 1955).

4 Results and Discussion

Seventy and 80% seeds of *H. recurvum* and *H. salicornicum* were germinated on hormone-free MS medium (Fig. 12.1c and d). Among various concentrations of BAP used for activation of axillary shoot buds, 6 µM and 8 µM BAP were found to be suitable, respectively, for *H. recurvum* (Fig. 12.2a and c) and *H. salicornicum* (Fig. 12.2b and d) from 1-week-old germinated seedlings. Axillary shoot buds of *Salvadora oleoides* and *S. persica* the haloxeric species of arid and saline areas of Rajasthan, India, were also activated on 6 and 4 µM BAP (Upendra and Dagla 2016). Cotyledonary nodes can be an efficient explants for micropropagation of rare and threatened plants which have very low viable seed settings and germination ability in natural habitats. Cotyledonary nodes have also been used as explants for

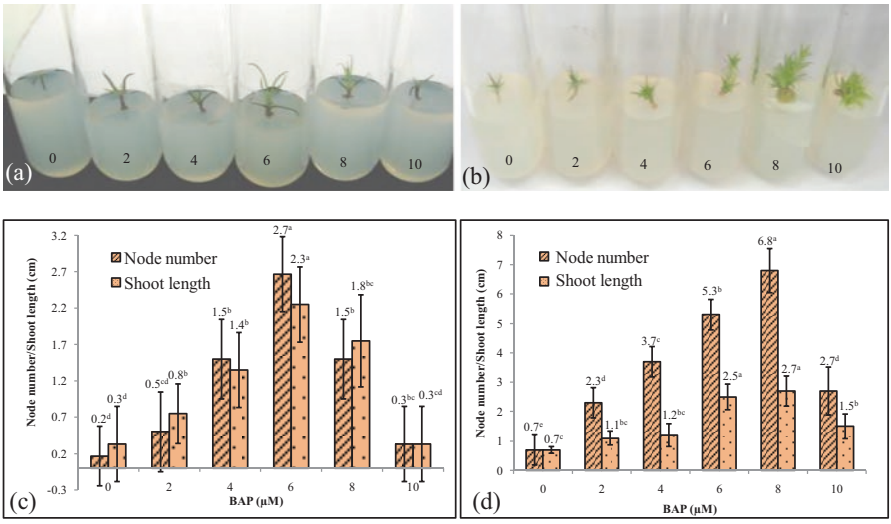

Fig. 12.2 Cotyledonary nodes cultured on MS medium containing different concentrations of 6-benzylaminopurine (BAP) in µM: (**a**) *Haloxylon recurvum* and (**b**) *Haloxylon salicornicum*. Effect of different concentration of BAP (µM) on node number and shoot length of (**c**) *Haloxylon recurvum* and (**d**) *Haloxylon salicornicum*. The observed data were analyzed by one-way ANOVA and Duncan's multiple range test ($p < 0.05$), and each value represents an average number of 6 replicates using 36 explants. Different letters represent statistically significant differences with standard deviation error bars

in vitro multiplication of shoots of *Prosopis laevigata* (Buendia-Gonzalez et al. 2007), *Cassia sophera* (Parveen and Shahzad 2010), *Stereospermum suaveolens* (Darshini et al. 2014), *Aegle marmelos* (Nayak et al. 2007), *Leptadenia pyrotechnica* (Dagla et al. 2012), *Lawsonia inermis* (Moharana et al. 2017), and *Glossonema varians* (Paliwal et al. 2018).

Five and 3% (w/v) sucrose was found to be more appropriate, respectively, for the growth and development of cotyledonary nodes of *H. recurvum* (Fig. 12.3a and c) and *H. salicornicum* (Fig. 12.3b and d) in present investigation. Sucrose is the most common carbon source used in plant cell, tissue, and organ culture (Gago et al. 2014). It acts as fuel ensuring optimal development and other roles as well (Coupe et al. 2006; Fila et al. 2006; Buendia-Gonzalez et al. 2007; Muller et al. 2011). Optimal sucrose concentration for growth may depend on genotype or species (Chen et al. 2003). However, sucrose concentration higher than 5% (w/v) retarded growth in both plant species. This could be due to osmotic stress created and restriction of photosynthesis efficiency of cultured plants (Hazarika 2006). Protocol standardized during present investigation can be a basis of cell culture for industrial production of components of nutritional and pharmaceutical value and micropropagation of these plants for prospects.

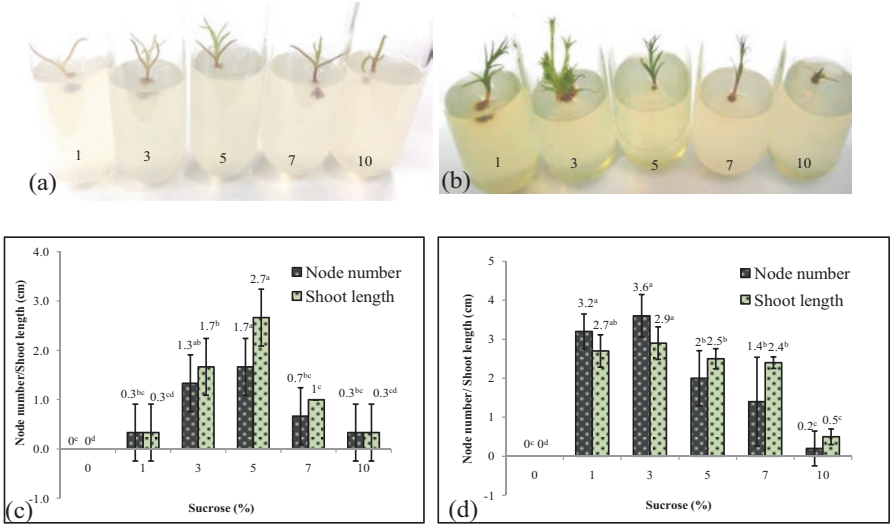

Fig. 12.3 Cotyledonary nodes cultured on MS medium containing various percentages (w/v) of sucrose: (**a**) *Haloxylon recurvum* and (**b**) *Haloxylon salicornicum*. Effect of different percentage (w/v) of sucrose on node number and shoot length of (**c**) *Haloxylon recurvum* and (**d**) *Haloxylon salicornicum*. The observed data were analyzed by one-way ANOVA and Duncan's multiple range test ($p < 0.05$), and each value represents an average number of 6 replicates using 36 explants. Different letters represent statistically significant differences with standard deviation error bars

5 Conclusions

Seventy and 80% seeds of *H. recurvum* and *H. salicornicum* were germinated on hormone-free MS medium. Axillary buds of cotyledonary nodes of *H. recurvum* and *H. salicornicum* were activated on 6 μM BAP and 8 μM BAP. Five and 3% (w/v) sucrose was found to be more appropriate for the growth and development of cotyledonary nodes of *H. recurvum* and *H. salicornicum*.

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