Food Bioactive Ingredients

Nitish Kumar
Ravi S. Singh *Editors*

Biosynthesis of Bioactive Compounds in Medicinal and Aromatic Plants

Manipulation by Conventional and Biotechnological Approaches

Springer

Food Bioactive Ingredients

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The Food Bioactive Ingredients Series covers recent advances and research on the science, properties, functions, technology, engineering and applications of food bioactive ingredients and their relevant products. The series also covers health-related aspects of these bioactive components, which have been shown to play a critical role in preventing or delaying different diseases and to have many health-improving properties. The books in this series target professional scientists, academics, researchers, students, industry professionals, governmental organizations, producing industries and all experts performing research on functional food development, pharmaceuticals, cosmetics and agricultural crops.

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ISSN 2661-8958 ISSN 2661-8966 (electronic)
Food Bioactive Ingredients
ISBN 978-3-031-35220-1 ISBN 978-3-031-35221-8 (eBook)
https://doi.org/10.1007/978-3-031-35221-8

This Springer imprint is published by the registered company Springer Nature Switzerland AG
The registered company address is: Gewerbestrasse 11, 6330 Cham, Switzerland

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About the Editor

Nitish Kumar is Senior Assistant Professor at the Department of Biotechnology, Central University of South Bihar, Gaya, Bihar, India. Dr. Kumar completed his doctoral research at the Council of Scientific & Industrial Research – Central Salt & Marine Chemicals Research Institute, Bhavnagar, Gujarat, India. He has published more than 70 research articles in leading international and national journals, more than 20 book chapters and 7 books with Springer and Taylor & Francis. He has a wide area of research experience in the field of metabolic engineering of bioactive compounds in medicinal and aromatic plants and crop improvement, particularly industrial and medicinal crop plants. He has received many awards/fellowships/projects from various organizations, for example, the CSIR, DBT, ICAR and SERB-DST, BRNS-BARC, among others. He is an active reviewer for journals, including *Biotechnology Reports*, *Aquatic Botany*, *Industrial Crops and Products*, *PLoS One*, *Plant Biochemistry and Biotechnology* and *3 Biotech*. He also serves as an Associate Editor of the journal *Gene* (Elsevier).

Ravi S. Singh is working as an Assistant Professor-cum-Jr. Scientist at the Department of Plant Breeding and Genetics, Bihar Agricultural University, Sabour, Bihar, India. He was awarded the Ph.D. degree in Biotechnology from GNDU, Amritsar/CSIR-IHBT, Palampur. He received CSIR-UGC JRF/SRF during Ph.D., and also qualified National Eligibility Test conducted by ASRB, ICAR, New Delhi. His area of research works is plant biotechnology and molecular plant breeding. Earlier, he also worked as Research Associate at ICAR-NRC on Plant Biotechnology, New Delhi. He received grants from BRNS-DAE and SERB-DST (for young scientist) to carry out research projects on medicinal plants. So far he has supervised four externally funded projects (DST, DBT and BRNS) and eight in-house projects. To his credit there are many research papers in the journals of repute, book chapters and books/manual (36 research papers, 16 book chapters, 04 book/manual), besides one Indian patent as inventor and one international patent as co-inventor. His major achievements are as follows: (1) Development of *Agrobacterium rhizogenes*-mediated hairy root culture in *Selaginella bryopteris* (This is the first report on any pteridophyte.). (2) For the first time, identified candidate genes involved in flavonoid

biosynthesis in *S. bryopteris*. (3) For the first time, studied molecular basis of shikonin biosynthesis in *Arnebia euchroma*. (4) A method for cloning functional gene of copper/zinc superoxide dismutases using oligonucleotide primers. (5) A composition for removal of colours and inhibitors from plant tissues to isolate RNA. (6) Genomic resources developed and released in public domain (NCBI GenBank) from various plants/microbes. (7) Developed a reproducible protocol for direct shoot organogenesis in *S. bryopteris* developed using Thidiazuron. (8) Spatial metabolite profiling and gene expression analysis of *S. bryopteris*. (9) Reported antibacterial activity of *S. bryopteris* fronds extract on bacteria isolated from mastitic milk. He is actively involved in teaching and guiding undergraduate/postgraduate students. He has guided 40 postgraduate students for their research (Advisor: 08; Co-advisor: 04; member: 28). For his achievements in the discipline of Biotechnology, the Venus International Foundation, Chennai, awarded him "Outstanding Scientist Award" in 2016.

Biotechnological Approaches for Medicinal and Aromatic Plant-Based Products

Amar A. Sakure, Amarjeet Singh Thounaojam, Sushil Kumar, and Dipak A. Patel

Abstract Medicinal and aromatic plants (MAPs) are the reservoirs of numerous life-saving drugs called secondary metabolites including terpenoids, essential oil, steroids, saponins, alkaloids, phenolics, etc. These secondary metabolites are the group of a variety of chemical compounds produced by the plant cell in different metabolic pathways that branch off from primary metabolic pathways. The quality and quantity of secondary metabolite in MAP are completely dependent on environmental conditions; moreover, the commercial production of secondary metabolites is also dependent on the area of cultivation of MAPs. The systematic secondary metabolite production can be enhanced through biotechnological intervention with minimal downstream processing. The tissue culture and transgenic technologies available in the current era of agriculture science have been advocated as effective tools for increasing the synthesis of these metabolites at an industrial scale. This chapter focuses on the recent advances made in the production of various secondary metabolites by deploying tissue culture and transgenic technologies.

Keywords Secondary metabolite · Transgenic · Hairy root · Medicinal plant · Biotechnology · Tissue culture

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N. Kumar, R. S. Singh (eds.), *Biosynthesis of Bioactive Compounds in Medicinal and Aromatic Plants*, Food Bioactive Ingredients,
https://doi.org/10.1007/978-3-031-35221-8_1

1 Introduction

Medicinal and aromatic plants (MAPs) are enriched with life-saving preparations, and we are using plant-based medicines from an ancient time. As per report of the World Health Organization (WHO), 88% of the world countries are projected to use herbal medicines in different forms and stated 170 member states are using traditional medicine. Considering the importance of traditional medicines, WHO declared the establishment first-of-its-kind WHO Global Centre for Traditional Medicine (GCTM) in India of fifth Ayurveda Day in November 2020. The traditional herbal-based medicines are known and famous by their different styles like traditional Chinese and Korean medicine, Indian Ayurveda, Japanese Kampo, etc. (Cha et al. 2007; Kobayashi et al. 2010; Hye-Lim et al. 2012). The latest estimate of plant diversity in India stands at 55048 taxa including 21,984 angiosperms (Anon 2022). About 20,000 plant species are assessed as medicinal plants, but only 800 species are used for treating diseases phytochemically (Kamboj 2000). In most of the developing counties for primary health care, 80% of medicine is herbal based because it is locally available and cheap and believed to have no side effects (Gupta and Raina 1998) as well as their strong faith in traditional herbal medicine cultures (Kamboj 2000). Moreover, in India, herbs are major share of all recognized medicine systems like Ayurveda, Yoga and Naturopathy, Unani, Siddha, and Homeopathy (AYUSH). Herbal-based medicine or its industry is potentially expanding worldwide; the annual turnover is Rs. 2300 crores with reference to Indian herbal-based medicinal industry. However, there is a huge gap between the supplier and the demand at national and international markets at present scenario. It was recorded that 1,34,500 MT of herbal raw drugs including extracts were exports while 1,95,000 MT were consumed by local herbal industry in India (Chowti et al. 2018).

Meanwhile, forest is the area where maximum amount of MAP raw material collection has been taken place, and about 95% of the plants consumed by the industries are collected from forests (Chowti et al. 2018). Due to the overexploitation and unsustainable collection/harvesting practices turn into an alarming rate on loss of biodiversity of such good plant genetic resources and great impact on natural biodiversity. Overall, this often influences on supply chain management in this domain. Therefore, it is the time to make a strategy more precisely in a sustainable way to fulfil the present requirement without affecting the tomorrow ecosystem. An all-encompassing solution lies by practicing recommended Good Agricultural Practices to each and every MAP starting from the collection and other necessary cultivation practices until the final target products reach to the consumers. Expansion of area cover under MAPs outside the forest zone is also an option and over the year is also increasing. It was estimated that 262,000 hectares was the total cultivation area of MAPs in the year 2005–2006, but it has jumped to 633,900 hectares in 2015–2016. But we are all aware that land is the main constraint in the cultivation and production technology line. Here, modern and advanced technology will definitely work and will play a significant role to meet the present and future demands of required raw materials as well as identification new bioactive compounds that can be used to treat various illnesses.

2 Secondary Metabolites

Plants produce secondary metabolites (SMs) to increase their competitiveness within their respective ecosystems. These secondary metabolites are amalgamation of a variety of phytochemicals produced by the cell during the course of metabolism in different pathways that branch off from primary metabolic pathways. Albrecht Kossel, a Nobel Laureate in Physiology or Medicine in 1910, first proposed the concept of secondary metabolite (Jones and Kossel 1953). Thirty years later after the discovery of secondary metabolites, Czapek reported these products as derivatives of nitrogen metabolism such as amino acid deamination. With advancement of the chromatographic techniques, recovery of these compounds was possible. SMs have revealed numerous biological effects consequently providing a scientific base for the deployment of herbs as medicine by many ancient communities.

These micromolecules have a wide range of effects on plants and other living things. They either indicate perennial growth or deciduous behaviour. They are also responsible for flowering, fruit set, and abscission; they act as petal-transporting agents, act as agents of symbiosis between plant and microbes, and act as sexual hormones (Demain and Fang 2000). They function as attractants or repellents, as well as antimicrobials.

The metabolites that are required primarily for growth and development of plant and participated directly in metabolic processes are termed as primary metabolites, while SMs are derivations of these primary metabolites. Medicinal and aromatic plants are rich sources of SMs including terpenoids, essential oil, steroids, saponins, alkaloids, glycosides, phenolics and other flavonoids, anthocyanin, lignin and tannin, etc. SMs are broadly classified based on the properties, structure, function, as well as biosynthetic pathway in plants. Some of the SMs are very prominently biosynthesized in MAPs, and compounds are accumulated in a very specific tissue, organ, structure, or part of the plants. However, plant secondary metabolite production is limited under the normal plant growth conditions (Yue et al. 2016), and therefore, tress take years to store the desired quantity of such metabolites in conventional methods.

These secondary metabolites are classified into different chemical compounds, namely, phenolics, alkaloids, saponins, terpenes, lipids, and carbohydrates (Fig. 1). The great resource of all these secondary metabolites is the medicinal and aromatic plants. Medicinal herbs and plants have long been recognized as a valuable source of therapeutic or curative aids in preventing chronic diseases in humans.

2.1 Phenolics

The majority of plant SMs comprising one or more aromatic rings and one or more OH groups are likely phenolics. These are the most prevalent secondary metabolites and are found all over the plant world. It can range from simple molecules to highly

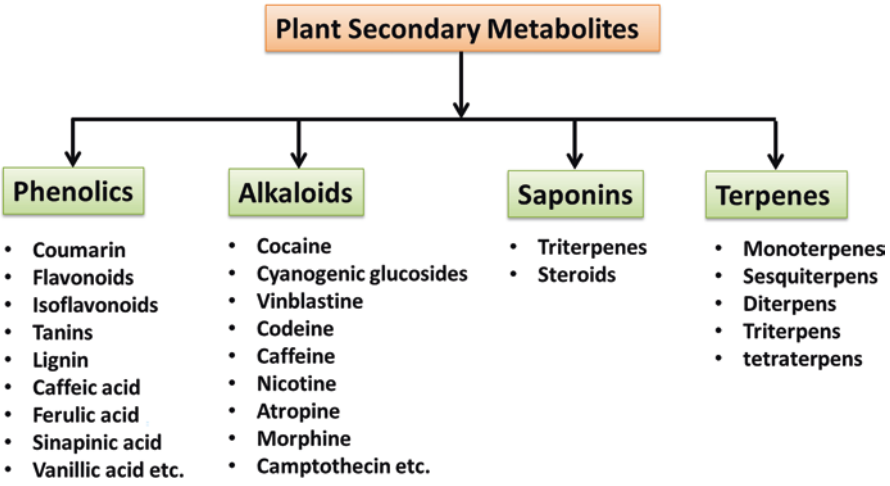

Fig. 1 Broad classification of secondary metabolites

polymeric compounds like tannins. They significantly add to the colour, taste, and flavour in foods and beverages. Selected phenolics, like quercetin, are valued for pharmacology for their anti-inflammatory or anti-hepatotoxic properties. Similarly, genistein and daidzein have phytoestrogenic properties, and naringenin has insecticidal property (Goławska et al. 2014). Among the phenolics, some are active antioxidants and free radical scavengers known as flavonoids. These phenolics are categorized as per their structure and functions. These phenolic compounds are playing very essential role in human physiological defence responses such as antiaging, anti-inflammatory, anti-carcinogenic, antioxidant, and anti-proliferative activities (Huang et al. 2009).

2.2 Alkaloids

Alkaloids are complicated chemical compounds encompassing a heterocyclic nitrogen ring, which have been intensively researched due to their numerous pharmacological properties. Such compounds are manufactured by a variety of entities, including mammals and microbes, but plants produce a particularly diverse range of alkaloids. Although alkaloids can be provided as crude extracts, they are frequently extracted from plants and used as pure compounds. Because of the intricacy of alkaloid compounds, chemical synthesis is practically impossible; hence, extraction from a basic plant mixture remains the most cost-effective method. Plants, on the other hand, synthesize very complex combinations of alkaloids in tiny amounts, resulting in the high cost of commercially manufactured alkaloids.

Glucosinolates are the compounds having sulphur and nitrogen and are derived from glucose and several amino acids (Geu-Flores et al. 2009). Glucosinolates

exhibit a variety of bioactivities and are found in member of family Cruciferae (Brassicaceae). By attracting pollinating insects and deterring predatory herbivores, glucosinolates play an important role in the chemical ecology of their host organisms (Ratzka et al. 2002). The restoration of whole metabolic pathways into heterologous plant hosts necessitates the employment of "gene stacking" approaches that are both efficient and simple. The engineering of benzylglucosinolate biosynthesis into tobacco is a phenomenally successful example. A transient expression method was used to re-establish benzylglucosinolate in *Nicotiana benthamiana*.

2.3 Saponins

Saponins, members of triterpenoid family, are a varied group of naturally derived phytoconstituents, which provides defence to pathogenic microorganisms and herbivores. This group of phytochemicals can be used for a variety of purposes other than medicine, owing to their numerous beneficial properties for mankind. Three main enzymes are essential in saponin biosynthetic pathway: *Oxidosqualene cyclases* form the basic skeleton of triterpenoids, *cytochrome P450 monooxygenases* facilitate oxidations, and uridine diphosphate-dependent *glycosyltransferases* catalyse the glycosylations.

The identification of genes involved in saponin production is crucial for the long-term production of these chemicals through biotechnological applications (Sawai and Saito 2011). Plant saponins are thought to be defence chemicals against harmful microorganisms and herbivores (Osbourn 2010; Kuzina et al. 2009). These saponins also have a vital beneficial effect on human health. Medicinal plants such as *Panax* and *Glycyrrhiza* are known to have surplus amount of saponin, ginsenosides, and glycyrrhizin, with numerous pharmacological properties (Shibata 2001). As the Latin word "sapo", which means soap, indicates, saponin also has the potential to foam when paired with water. Common soapwort (*Saponaria* spp.) and soap bark tree (*Quillaja* spp.) have been successfully used as soap. The saponins extracted from soap bark tree can be used as emulsifiers to prepare cosmetics and food items. Furthermore, glycyrrhizin is reported to be 150 times sweet as sugar and can be used as natural sweetener in many food preparations.

Saponins are commonly stored in particular cell type and organs. Glycyrrhizin and ginsenosides are accumulated in xylems of roots of licorice and ginseng, respectively (Shan et al. 2001). At the cellular level, it has been proven that saponins are accumulated most specifically in vacuoles (Mylona et al. 2008), and hence, it is suggested that there is the presence of vacuolar transporter. These transporters are also targeted to engineer the accumulation of saponins. Thus far, an ATP-binding cassette transporter (NpPDR1) has been reported as a plant terpenoid transporter contributing in the secretion of sclareol, a diterpenoid, with antifungal activities, in the tobacco plant (Jasiński et al. 2001).

2.4 Terpenes

Terpenes are aromatic chemicals, responsible for the aroma in flowers, fruits, seeds, leaves, and roots in various plant species. This aroma is crucial in the development of herb- and fruit-flavoured wines, like vermouth. They help to discriminate the scents of various grape types. Wine has yielded approximately 50 monoterpenic chemicals (Strauss et al. 1987).

Terpenes are chemically classified as a group due to their unusual carbon structure. Fundamentally, it is a 5C isoprene unit. Terpenes are also often made up of 2, 3, 4, and 6 isoprene units, and therefore, they are also known as monoterpenes, sesquiterpenes, diterpenes, and triterpenes, respectively. Terpenes may also encompass diverse functional groups. Several important terpenes contain OH groups that make it terpene alcohols. Other terpenes are called ketones (Strauss et al. 1987).

3 In Vitro SM Production

Various cell cultures can be followed to produce SMs *in vitro* (Fig. 2).

3.1 Callus and Cell Suspension Culture

For SM production, the selection of high metabolites generating cell lines is performed through callus tissues of either small aggregate or single-cell origin. Suspension cultures are made by placing callus tissue in liquid medium of the same composition as callus tissue with continuous shaking. Suspension culture comprises more homogenous cells and less differentiated cell population. The production of secondary metabolite through suspension culture is easily manageable by feeding

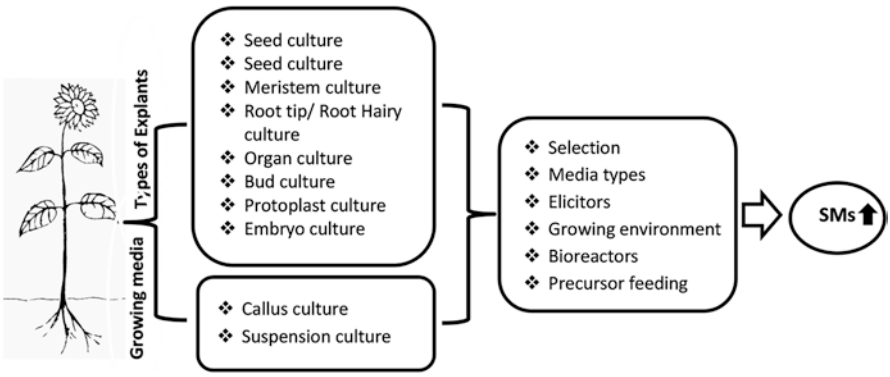

Fig. 2 Systematic representation of SM production under *in vitro* conditions

various chemical factors in the culture (Fischer et al. 1999). *In vitro* methods of SM production relay the best alternative solution and efficient technique for SM production in large scale within the short period of time (Kolewe et al. 2008). *In vitro* production of SM is carried out in two steps such as biomass accumulation and SM synthesis (Yue et al. 2016). Based on the optimization and establishment of highly relevant scientific output in this arena, various types of explant and growing conditions like callus and suspension cultures are hugely used in SM production (Fig. 2). For initiation of the culture, various tissues can be taken such as leaf, shoots, roots, calli and cell suspension culture, etc. Callus cultures are commercially viable for the production of SMs with medicinal relevance (Ogita 2015). This callus can be used to produce multiple clones through micropropagation or for cell suspension culture for the production of SMs through batch or continuous fermentation bioreactors.

Callus cultures are significantly contributed in SM production successfully at commercial level (Ogita 2015). Multiples clones of plant can be produced from this culture and can also be exploited for developing single-cell suspension cultures with the aim of producing the target SMs (Xu et al. 2011). Both types of cultures also provide the possibility to modify the SM biosynthesis pathways; malonate/acetate pathway and the shikimic acid pathways are the key SM biosynthesis pathways in plants (Hussain et al. 2012).

Tropane alkaloid group is mostly synthesized in *solanaceous* genera comprising *Atropa*, *Hyoscyamus*, *Scopolia*, *Mandragora*, and *Duboisia*. It has been reported that tropane alkaloid production could be higher through in vitro techniques. These groups of chemical compounds work as parasympathetic antagonists by blocking the actions of acetylcholine binding to its receptor, consequently having effects on the heart rate, respiration, and central nervous system. Hyoscyamine is a one kind of tropane alkaloid used to treat a variety of stomach/intestinal problems, and it was found to have greater accumulation in callus culture of *Hyoscyamus aureus* (Besher et al. 2014). The maximum level of atropine (236.9μg/g dry weight) and scopolamine (43.1μg/g dry weight) tropane alkaloid was obtained from *Atropa belladonna* leaf callus cultures after the 21 days with the use of elicitor, ornithine, at the rate of 1 mM (Mohamed et al. 2018). *Atropa belladonna* is the most known tropane alkaloid producer, and it was shown that atropine production through callus culture in a significant amount (6.94 mg/g dry weight) as the plant synthesizes atropine normally in leaf (Ogras et al. 2022). α-Tocopherol, in fact, is a type of vitamin E isoform and the most effective fat-soluble antioxidant found in a diverse group of plants. It serves as a scavenger of lipid peroxyl radicals protecting the polyunsaturated fatty acids in membranes and lipoproteins, thereby serving as an antioxidant mainly used for the cure of atherosclerosis. Chemically synthesized alpha-tocopherol is less effective on account of its stereoisomer racemic mixture, so always look for the plant-based extract.

Plenty amount of α-tocopherol is present in normal cultivated plants like oranges and beets (Piironen et al. 1986), cabbage (Lehmann et al. 1986), and sunflower (Velasco et al. 2002). However, by practicing in vitro techniques in sunflower, greater amount of α-tocopherol (19.8μg/g FW) can be collected from hypocotyl-derived callus culture than the normal hypocotyl (11.4μg/g FW) (Sofia et al. 2010).

β-Sitosterol (0.198 mg/g DW) and caffeic acid (4.42 mg/g dw) are the bioactive SMs of *Sericostoma pauciflorum* produced at higher amount from the 6-week-old callus (Jain et al. 2012). Significant amount of SMs ajmaline (0.01 mg/g DW) and ajmalicine (0.006 mg/g DW) was induced though hairy root culture of *Rauvolfia micrantha* through hypocotyl explants (Sudha et al. 2003). Ajmalicine is the best known drug to treat high blood pressure (Wink and Roberts 1998). The treatments of elicitors like salicylic acid, methyl jasmonate, chitosan, and heavy metals in callus cultures enhanced the SM production (DiCosmo and Misawa 1985). Apart from the callus cultures, hairy root culture especially for alkaloids (Sevon and Oksman-Caldentey 2002) and shooty teratoma (tumour-like) cultures for monoterpene production have been well established (Spencer et al. 1993). Therefore, each and every culture is unique and has a potential area for SM production in different MAPs. Some of the important SMs produced by MAPs through in vitro techniques are summarized in Table 1.

Withania is commonly believed to have powerful aphrodisiac, calming, rejuvenative, and life-extension properties in Ayurveda. Moreover, it is employed for geriatric issues and power-boosting tonic called medhya rasayana, which literally translates to "that which enhances wisdom and memory" (Nadkarni 1976; Williamson 2002). The plant was historically used to nourish the growth and development of human body by boosting the production of essential fluids, muscle fat, blood, lymph, semen, and cells in order to support vigour, endurance, and health. Due to the similarities between the above potentials and ginseng roots, ashwagandha roots are popular as Indian ginseng (Singh and Kumar 1998).

Tissue culture of Indian haplotype of *Withania somnifera* was attempted using axillary meristems on MS media accompanied with different hormonal combinations along with coconut milk alone or in combination (Roja et al. 1991). Callus was successfully initiated on media provided with 2,4-D (2 ppm) and 0.2 mg Kin/L. Callus culture was failed to produce withanolides. But the use of shoot tip culture for the development of multiple shoots of *W. somnifera* grown on MS medium containing BA (1 ppm) showed accumulation of 0.04% withaferin A and 0.06% withanolide D (Ray and Jha 2001). Interestingly, the concentration of withanolides was increased substantially on MS liquid medium comprising BA (1 ppm) and coconut milk (10%), which favoured a significant rise in biomass (27 times) and 0.14% of withaferin A from 0.04% (Ray and Jha 2001). Nagella and Murthy (2010) investigated the production of withanolide A in *W. somnifera* cell cultures by optimizing different tissue culture-related parameters. They had observed that the concentration of withanolide A was reached at maximum 2.26 mg/g dry weight in suspension culture added with 2,4-D (2 ppm) in combination with kinetin (0.5 mg/L) followed by 1.82 mg/L with 1 mg/L BA cytokinins.

Table 1 Secondary metabolite production through *in vitro* cultures of various medicinal and aromatic plants

Sr. no.	Bioactive compounds	Crops/plants	In vitro culture	References	Medicinal properties
Alkaloids					
1	Betacyanins, betalains	*B. vulgaris* L.	Hairy root	Shin et al. (2002) and Thimmaraju et al. (2003)	Antioxidant capacities, anti-inflammatory, cancer chemo-preventive activities, protection of low-density lipoproteins (LDLs) from oxidation
2	Berberine	*Tinospora cordifolia*	Callus	Mittal and Sharma (2017)	Used to treat viral infections, cancer, diabetes, inflammation, neurological disorders, psychiatric problems, microbial infection, hypertension, and HIV-AIDS
		Coscinium fenestratum	Callus	Khan et al. (2008)	
3	Piperine	*Piper longum*	Callus	Chatterjee et al. (2021)	Antioxidant, antidiabetes, used in breast and oral cancer, obesity, multiple myeloma, hypertension, Parkinson's disease anti-inflammatory properties
4	N-methylconiine	*A. globuligemma*	Callus	Hotti et al. (2017)	Antagonist to nicotinic acetylcholine receptor blocking of the nervous system, eventually causing death by suffocation in mammals, poisonous to humans and animals

(continued)

Table 1 (continued)

Sr. no.	Bioactive compounds	Crops/plants	In vitro culture	References	Medicinal properties
5	Thebaine, sanguinarine	*Papaver bracteatum* Lindl.	Cell suspension	Dastmalchi et al. (2019)	Antimicrobial, antioxidant, anti-inflammatory, anti-tumour
6	Morphine, codeine, thebaine	Opium poppy (*Papaver somniferum album*)	Embryo	Kassem and Jacquin (2001)	Anaesthesia in severe injuries, cough suppressant
7	Trigonelline	*Trigonella foenum-graecum*	Cell suspension	Radwan and Kokate (1980)	Hypoglycaemic, hypolipidaemic, neuroprotective, antimigraine, memory improvement, antibacterial, antiviral
8	Galantamine	*Narcissus pseudonarcissus*	Callus cultures	Aleya et al. (2021)	Used to treat Alzheimer's disease
Terpenoids					
1	Artemisinin	*Artemisia annua L.*	Shoot culture	Woerdenbag et al. (1993)	Antimalarial, antibacterial, antifungal, antileishmanial, antioxidant, anti-inflammatory
			Callus culture	Baldi and Dixit (2008)	
2	Withanolides	*Ashwagandha* (*Withania somnifera*)	Shoot culture	Mir et al. (2014)	Anticancer, antioxidative, immuno-modulatory, anti-stress, cardio-protective, anti-inflammatory, aphrodisiac, anti-stress, cardio-protective, and neuroprotective
			Root cultures	Sivanandhan et al. (2012)	
	Withaferin A		Cell suspension	Ciddi (2006)	
3	Ginsenosides	Ginseng (*Panax ginseng*)	Adventitious root cultures	Paek et al. (2009)	Antioxidant, anti-inflammatory, vasodilation, anti-allergenic, antidiabetes, anticancer
			Cell suspension	Hibino and Ushiyama (1999) and Thanh and Murthy(2014)	

(continued)

Table 1 (continued)

Sr. no.	Bioactive compounds	Crops/plants	In vitro culture	References	Medicinal properties
4	Monoterpenes-α-terpineol and nerol	*Camellia sinensis*	Cell suspension cultures	Grover et al. (2012)	Antioxidant, anticancer, anticonvulsant, antiulcer, antihypertensive, anti-nociceptive, anti-inflammatory
		Mentha citrate, *Mentha piperita*	Shoot cultures	Hilton et al. (1995)	
5	Monoterpenes-menthol	*Mentha piperita*	Cell suspension culture	Chakraborty and Chattopadhyay (2008)	Anticancer, very effective in alleviating flatulence, menstrual pain, nausea, depression-related anxiety
6	Monoterpenes-β-myrcene	*Ochtodes secundiramea*	Suspension cultures	Jason and Gregory (2018)	Analgesic, sedative, antidiabetes, antioxidant, anti-inflammatory, antibacterial, anticancer
7	Monoterpenes-α-pinene, pulegone, menthol, menthone, and limonene	*Mentha pulegium*	Cell suspension culture	Darvishi et al. (2016)	Antibiotic, anticoagulant, antitumor, antimalarial, antileishmanial, analgesic, antihyperalgesic, anti-pyretic, anti-histaminic
Steroids					
1	Digoxin, digitoxin	*Digitalis lanata*	Shoot tip and single node cultures	Bekhit (2009) and Lee et al. (1999)	Used to treat heart failure and arrhythmias
2	Ecdysteroids	*Achyranthes bidentata* Blume	Cell suspension	Wang et al. (2013)	Lower cholesterol and blood glucose level, effects on the central nervous system, neuromodulatory effects on the GABAA receptor

(continued)

Table 1 (continued)

Sr. no.	Bioactive compounds	Crops/plants	In vitro culture	References	Medicinal properties
3	Steroidal glycosides-saponins, sapogenins	Yucca plant	Root-cell cultures	John and Maccarthy (1985)	Anti-inflammatory, antidiabetes, antioxidant, hepatoprotective activity, lower cancer risks, affect blood glucose response
4	Steroidal lactones withaferin A and withanolide A	*Withania somnifera* (L.)	Hairy root cultures	Doma et al. (2012)	Antioxidant, anti-inflammatory, hormone balancing, immune boosting, benefits for insomnia and joint pain
Quinones					
1	Aloe-emodin	*A. barbadensis* Mill.	Basal and fresh leaf calli	Acurero (2009) and Lee et al. (2013)	Anticancer, antivirus, anti-inflammatory, neuroprotective, hepatoprotective activities
2	Anthraquinones	*Polygonum multiflorum*	Cell suspension cultures	Thiruvengadam et al. (2016)	Laxatives, antimicrobial, anti-inflammatory agents, used to treat arthritis, multiple sclerosis, and cancer
		Rubia tinctorum	Cell and hairy roots cultures	Perassolo et al. (2022)	
3	Sennosides A and B	*Senna alata*	Hairy roots cultures	Putalun et al. (2014)	Treatment for constipation
4	Plumbagin	*Plumbago indica*	Root cultures	Jaisi and Panichayupakaranant (2020)	Used to treat prostate cancer
5	Shikonin	*Lithospermum erythrorhizon*	Callus cultures	Mizukami et al. (1978)	Antioxidant, anti-inflammatory, antithrombotic, antimicrobial, and wound-healing effects

(continued)

Table 1 (continued)

Sr. no.	Bioactive compounds	Crops/plants	In vitro culture	References	Medicinal properties
Phenylpropanoids					
1	Anthocyanins	*Cleome rosea*	Callus cultures	Simoes et al. (2009)	Antioxidant potential, cancer chemo-preventive agents, anti-inflammation, diabetes and obesity prevention, improving memory capacity
2	Coumarins-Psoralen	*Coronilla scorpioides*	Callus cultures	Piovan et al. (2014)	Used in the treatment of vitiligo and psoriasis
3	Eugenol	*Ocimum sanctum L.*	In vitro-generated plantlets	Sharma et al. (2016)	Antibacterial, antifungal, antioxidant, antineoplastic activity
4	Flavonoids	*Sericostoma pauciflorum*	Callus cultures	Jain et al. (2012)	Anticancer, antioxidant, anti-inflammatory, antiviral properties
5	Isoflavonoids	*Genista tinctoria* L.	Suspension culture	Tumova et al. (2014) and Skalicky et al. (2018)	Used in the treatment of osteoporosis, hormone-related cancer, loss of cognitive function
6	Lignans	*Linum* species	Root cultures	Alfieri et al. (2021)	Lowered risk of heart disease, menopausal symptoms, osteoporosis, and breast cancer

3.2 Immobilized Culture

Immobilized culture has received much attention for their efficiency in producing plant secondary metabolites. In this technique, high-density suspension culture cells are confined in an inert matrix such as gel beads of calcium alginate, stainless steel, etc. Then these cultures are shaken in cultured flask with aeration in bioreactor. However, the production of SMs under large scale is quite expensive (Hall et al. 1998).

3.3 Organ Culture

Despite intense efforts, the production of SMs from various useful plants, like morphinan from *Papaver somniferum*, tropanes from numerous *solanaceous* plants, and dimeric indoles from *Catharanthus roseus*, via callus and cell suspension cultures, is not successful. Since the majority of these kinds of chemicals accumulates only when appropriate organs are regenerated from cultured cells. The production of this substance in cultivated cells needs the separation of phytochemical from morphological maturation, which has so far proved ineffective. In this circumstance, organ cultivation is the preferred option. One main drawback of organ culture is that it reduces bioreactor production because the physical form of the shoot or root causes different obstacles, such as handling problems during inoculation and shearing of organ during culture. When the production of tissue-specific monoterpenoid essential oil is concerned, its production with callus or suspension culture could not be the choice because that essential oil is only synthesized in oil secretory aerial part of the plant. At this condition, only shoot tip cultures are considered for the production of target compounds (Severin et al. 1993).

3.4 Hairy Root Culture

Agrobacterium rhizogenes is a soil bacterium that causes a variety of dicotyledonous plants to develop hairy root disease. This phenotype is brought about via genetic modification, much like how *A. tumefaciens* developed crown gall disease. This is similar to *A. tumefaciens*, which causes crown gall disease. The roots, produced after co-cultivation of explants with *A. rhizogenes*, are clearly identified by rapid and highly branched growth of roots on tissue culture medium devoid of hormones (Christey 2001). Plants regenerated from hairy roots frequently display a different phenotype marked by wrinkly leaves, condensed internodes, declined apical dominance, decreased fertility, different flowering, and plagiotropic roots. These alterations come from the transmission and activation of T-DNA loci (rol A, B, C, D) (Christey 2001). Genetic engineering provides a new option to increase the content

of SMs in producing plant species or even producing the metabolite in a heterologous, readily cultivatable plant host system. Hairy root production can be performed in two ways: in vitro and in vivo. In vitro hairy root production is the same as in vitro explant co-cultivation process that is used for *A. tumefaciens*-mediated transformation. The main distinction is that explant is developed on hormone-devoid medium, which allows the identification of hairy root cultures. The medium may be changed with the change of the plant species; however, in maximum cases, Murashige and Skoog medium is reported. In vivo approach includes wounding the stem or petiole of plants using a needle/toothpick immersed in bacterial solution. As moisture is required for the growth of hairy roots, wounding location is frequently covered with gauze to avoid the moisture loss as high humidity is a prerequisite in the development of hairy root. Plant hairy root culture is a promising alternate way to develop chemicals generated in plant roots. *A. rhizogenes*-mediated transformation was exploited for induction hairy roots in plants, allowing in vitro production of SMs of plant roots (Chen et al. 2018).

A successful attempt was made in the production of scopoletin in the cell suspension culture *Spilanthes acmella* Murr. In this investigation, various concentrations of casein hydrolysate and L-phenylalanine were integrated in MS supplemented with BA 15 (μM) and 2,4-D (5μM). It was reported that the scopoletin production was substantially improved in the presence of casein hydrolysate in the nutritional medium with increase in cell biomass. The inclusion of casein hydrolysate up to 75 mg/L promoted scopoletin accumulation, whereas increasing the casein hydrolysate level above 75 mg/L inhibited scopoletin production. Moreover, adding phenylalanine in medium was observed to be more effective in *S. acmella* SM synthesis. The largest concentration of scopoletin was reported in cell suspension with L-phenylalanine (100μM/L), which was 4.51 times more compared to control (Mohammad et al. 2016). *Senna alata* (L.) Roxb. (family Leguminosae) contains anthraquinone glycosides that function as laxatives, such as sennosides A and B. Hairy root culture-based overproduction of sennosides A and B was carried out using *Agrobacterium rhizogenes*. 21-day-old seedlings were co-cultivated *Agrobacterium rhizogenes* strain ATCC 15834 by piercing the plant's stem and leaves with a needle that had been dipped in the bacterial suspension. After 2 weeks of inoculation, hairy roots were stimulated on wounding site on the plant. The roots were grown at 25 °C under a 16-h photoperiod with fluorescent light on hormone-free half-strength MS medium (3% w/v sucrose) supplemented with cefotaxime (500 g/ml).

The microbe-free hairy roots were transplanted into half-strength MS liquid media without hormone after three 14-day passages on medium supplemented with antibiotic. The speedy growth of hairy roots displayed a growth curve from day 5 to day 20, with the maximum root weight reported on day 5. Sennoside A and B levels in hairy roots reduced during day 10 due to hairy root growth. Following lag phase, sennoside A and B production amplified from day 15 and touched its peak in the stationary phase of hairy roots by day 35 (178 15) and (23 2) g g1 dry wt, respectively (Putalun et al. 2014).

Menthol production was significantly increased with cell suspension culture in *Mentha piperita*. In this case, the culture was initiated with leaf segments on simple MS media. Precursor feeding in combination with γ-cyclodextrin and menthone at 35μM showed significant increase in menthol production up to 92 and 110 mg/l compared to 77 mg/l in control (Chakraborty and Chattopadhyay 2008). The production of geraniol was tried in *N. benthamiana*. A gene named *geraniol synthase* of *Valeriana officinalis* was transformed into tobacco plant. The transgenic plants generated through in vitro had the highest geraniol content (48 g/g fresh weight, fw), followed by the transient expression system (27 g/g fw). The transgenics grown hydroponically in a greenhouse, cell suspension cultures, and hairy root cultures showed 16 g/g fw and 9 g/g fw with hairy root cultures (Vasilev et al. 2014).

Plant genetic engineering is favoured over chemical synthesis, which aids in the production of excessive levels of some alkaloids. Isoquinoline alkaloids are among the most significant metabolites produced by plant cell culture (Hay et al. 1988). An alkaloid called berberine known to have antibacterial properties was extracted from *Coptis* (Ranunculaceae). Berberine synthesis in plant cells has been well studied at the enzyme level by Kutchan (1998) and Sato et al. (2001). Geu-Flores discovered that an enzyme called gamma-glutamyl peptidase is responsible for the incorporation of reduced sulphur into glucosinolates via glutathione conjugation. The co-expression of this peptidase increased the return of benzylglucosinolate by 5.7 times, demonstrating the role of primary metabolite resources on natural product output (Geu-Flores et al. 2009).

Similarly, Modlrup et al. (2011) examined the formation of benzyl desulfoglucosinolate, the final metabolite in the benzylglucosinolate pathway, by mobilizing sulphur from primary to secondary metabolism in *N. benthamiana* expression system by co-expressing *adenosine 5′-phosphosulfate kinase*. The 3′-phosphoadenosine-5′-phosphosulfate (PAPS) was provided as co-substrate required for the final step of benzylglucosinolate biosynthesis. They observed a subsequent increase in benzylglucosinolate yield by 16-fold (Moldrup et al. 2011). Mikkelsen et al. (2012) created a flexible platform for *Saccharomyces cerevisiae* to express many gene pathways in a steady manner. This was the first successful generation of glucosinolates in a microbial host achieved by introducing the seven-step indolylglucosinolate pathway from *Arabidopsis thaliana* to yeast. By replacing supporting endogenous yeast activities with enzymes from plants, the synthesis of indolylglucosinolate was significantly improved.

Hughes et al. (2004) studied the efficacy of the hairy root cultures on alkaloid accumulation by better tryptophan accessibility. For testing this association, transgenic hairy root cultures of periwinkle were developed under the control of glucocorticoid-inducible promoter governing the expression of an *Arabidopsis* feedback-resistant alpha subunit of *anthranilate synthase*. Tryptophan and tryptamine yields grew significantly after 6 days of induction, from non-detectable levels to 2.5 mg/g dry weight and from 25 to 267μg/g dry weight, respectively. This suggested that in increasing the alkaloid accumulation, tryptophan and tryptamine concentrations are playing an important role in significant increment in the levels of

most terpenoid indole alkaloids such as lochnericine, which increased to 81% after a 3-day induction.

Rutin is a citrus flavonoid glycoside found in buckwheat (*Fagopyrum esculentum* Moench.). It is also called as rutoside, quercetin-3-rutinoside, and sophorin (Kreft et al. 1997). *Ruta graveolens* is a source of rutin. It is a glycoside composed of the flavonol glycoside quercetin and the disaccharide rutinose. It has a variety of pharmacological properties, like cytoprotective, antioxidant, vasoprotective, cardioprotective, anticarcinogenic, and neuroprotective properties (Javed et al. 2012; Richetti et al. 2011). Rutin has shown a neuroprotective effect in ischaemia of the brain. Rutin administration reduced "ischemic neuronal apoptosis" due to the suppression of p53 transcription and lipid peroxidation, as well as an increase in "endogenous antioxidant defence enzymes" (Khan et al. 2009). It has also been shown to have significant effect in sedative activity (Fernández et al. 2006), neural crest cell survival (Nones et al. 2012), anticonvulsant activity (Nieoczym et al. 2014), and anti-Alzheimer's activity (Wang et al. 2012).

To determine in vitro production of rutin, Lee et al. (2007) developed a hairy root culture by employing infection of *Agrobacterium rhizogenes* strain R1000 on leaf explants of buckwheat. Ten hairy root clones were created, with growth and rutin production rates ranging from 233 to 312 mg dry wt per 30 mL flask and 0.8–1.2 mg/g dry wt, respectively. Clone H8 was superior for rutin production (312 mg dry wt per 30 mL flask and 1.2 mg/g dry wt) and was chosen for further testing. H8 reached its maximum growth and rutin concentration after 30 days in MS medium culture. Among other tested media, half-strength MS medium was shown to induce the maximum growth levels and ultimately for rutin production (1.4 mg/g dry wt) by clone H8 (Lee et al. 2007).

An effort was made to generate hairy root from the seedlings of buckwheat through *A. rhizogenes*. Hormone-free half-strength MS medium was found quite satisfactory to obtained active elongation and high root branching. Insertion of the *RolB* and *Aux1* genes from *A. rhizogenes* (strain 15834) into buckwheat was also confirmed through PCR. Interestingly, in this study, it was identified that the absence of *VirD* gene showed hairy root without bacterial contamination. They had tested the transformed hairy root generated line TB7 on six different media combinations for evaluating the efficacy of its biomass production. The media finalized with half-strength MS liquid medium accompanied with 3% sucrose extended for 20 days resulted in maximal biomass of 13.5 g/l fresh weight, and the accumulation of rutin was achieved to 0.85 mg/g (Huang et al. 2016). Further, hairy root-based suspension culture led to a 45-fold and 4.11-fold accumulation of biomass and rutin content compared to suspension culture of non-transformed roots. They had also observed that the exposure of UV-B stress on hairy roots resulted in an outstanding increase of rutin and quercetin accumulation. The reason for maximal accumulation of these SMs under UV light was due to the dramatic changed in the expression of *FtpAL*, *FtCHI*, *FtCHS*, *FtF3H*, and *FtFLS-1* genes in buckwheat hairy roots (Huang et al. 2016).

In *Ocimum* spp., the increased amounts of ursolic acid and eugenol in *O. tenuiflorum* hairy root cultures matched well with elicitor concentrations, time of

exposure, and culture age (Sharan et al. 2019). Biswas (2020) demonstrated increased rosmarinic acid concentration in non-transformed *O. basilicum* root culture employing methyl jasmonate as an elicitor. Further, Kwon et al. (2021) observed that rosmarinic acid accumulation was higher in hairy root cultures of green basil compared to the purple basil. Elite hairy root lines of *O. basilicum* have previously been created with rosmarinic acid levels that are noticeably greater than non-transformed roots (Srivastava et al. 2016). Somatic hybridization is also employed to create hybrids from distant genera or related species (Grosser 2003). It may be beneficial to use somaclonal modifications to improve the essential oil profile of *Ocimum* species. Plant breeding techniques can be used to include these changes if they have remained genetically stable for several generations (Krishna et al. 2016).

Terpenoids are among the volatile substances that plants release from their aerial parts and play a significant role in interaction with their surroundings. Overexpression of *TPSs* was carried out under constitutively expressing promoters in heterologous system such as *Arabidopsis* (Aharoni et al. 2003, 2006). Transgenic *Arabidopsis* was generated by the expression of two distinct *terpene synthases*. Transformed lines showed the production of linalool and its glycosylated and hydroxylated derivatives in the leaves. In several of the transgenic lines, the sum of the glycosylated components was up to 40–60 times more than the sum of the comparable free alcohols. Recently, a study was undertaken for the accumulation of terpenoid with increased yield of essential oil by overexpression of hydroxymethylglutaryl (*HMGR*) of *O. kilimandscharicum* in several phenylpropanoid-rich *Ocimum* species (*O. basilicum*, *O. gratissimum*, and *O. tenuiflorum*) (Bansal et al. 2018).

Another study on elicitation of withanolide production in ashwagandha hairy root cultures was performed using 150μM jasmonate (MeJ) and salicylic acid (SA) as an elicitors at varied concentrations. Hairy root samples were collected after 4 h of exposure from 40-day-old plants and showed an increase in the production of 32.68 g/FW biomass and 58-fold higher withanolide A (132.44 mg/g DW), 46-fold withanone (4.35 mg/g DW), and 42-fold withaferin A (70.72 mg/g DW) in leaves of ashwagandha. It was also noticed that with an increase in age of plants, the accumulation of withaferin A was observed, but there was a decrease in corresponding withanolide A (Sivanandhan et al. 2013).

Doma et al. (2012) addressed the interesting finding on the accumulation of the withaferin A in hairy root culture induced by *Agrobacterium rhizogenes* at different concentrations of sucrose. From this study, it was confirmed that sucrose in the medium also plays an important role in withanolide accumulation. They had tested different concentrations of sucrose from 2%, 3%, 4%, to 6%, but the accumulation of withanolides was identified only at 6% sucrose with an amount of 1733μ/g dry weight. In fact, the use of triadimefon, a fungicide, in the medium enhanced withaferin A 1626% in hairy roots and 3061% in intact roots, which is not reported earlier (Doma et al. 2012).

4 Factors Affecting SM Production in Tissue Culture

4.1 Media Formulation

Culture media formulation heavily supports on the growth and morphological development of plant tissues. For the effective proliferation and development of cells in tissue culture medium, it should have an optimum concentration of all components in the media formulation comprising macro- and micronutrients, nitrogen supplement (amino acids), vitamins, carbon source (sucrose/glucose), and phytohormones, and in some cases, elicitors are also added. Media formulations such as Murashige and Skoog (MS) media, Gamborg (B5) media, Linsmaier and Skoog (LS) media, Schenk and Hildebrandt (SH) media, White's media, Nitsch and Nitsch (NN) media, Chu (N6) media, and woody plant media (WPM) are commonly used in cell culture. Each medium has its different compositions and used in various in vitro cultures. In 1962, a modified MS medium was designed in *Nicotiana tabacum*, which comprises high amount of ammonium ions along with nitrate and potassium. However, in 1968, for cell and suspension culture of *Glycine max*, a new medium named Gamborg B5 medium was formulated with comparatively lower amount of ammonium ions than MS media. Linsmaier and Skoog medium was developed in 1965 with the aim to optimize organic supplements of the tobacco culture. For the callus and suspension cultures of monocotyledonous and dicotyledonous plants, Schenk and Hildebrandt medium was originally formulated in the year 1972, and in this medium, potassium nitrate was supplemented as the main nitrate source with high amount of copper and myo-inositol. In 1962, R. White formulated White media for root culture, and it was the first media for root culture. This medium is categorized by containing high concentration of magnesium sulphate with low salt, and nitrate content is 19% lower than from MS medium. Another medium that contains greater amount of thiamine, biotin, and folic acid that was specially designed for in vitro another culture of *Nicotiana* called as Nitsch and Nitsch media (1969). Chu media were formulated for another culture in rice with optimized micronutrients and macronutrients in the media. Lioyd and McCrown developed a medium for in vitro culture of woody plant species (*Kalmia latifolia*) in the year 1981 (Rini Vijayan and Raghu 2020).

Therefore, several media formulations are developed for successful in vitro cultures of several species, and its formulation has also influenced the harvest of high-value bioactive compounds. The highest alkaloid content (6.203 mg/g dry weight) was revealed in B5 media suspension culture containing 3% sucrose compared to MS media suspension media (6.021 mg/g dry weight) in *Catharanthus roseus* L. (Mishra et al. 2018a, b). However, it was in contrast with the finding of Zenk et al. (1977) that displayed that MS media formulation was the best medium for the production of alkaloid (serpentine and indole alkaloids) by *Catharanthus roseus* suspension cultures than B5 and white media composition. Full strength of MS media showed the promising response for callus induction and podophyllotoxins production in *Podophyllum peltatum* tissue cultures (Kadkade 1982). Similarly,

callus culture of *Eurycoma longifolia* in MS media showed higher production of 9-methoxycanthin-6-one (Rosli et al. 2009). MS basal medium supplemented with 2, 4-D (0.5 mg/l) and BA (1.0 mg/l) and 6% sucrose was best for leaf callus culture of *C. roseus* for biomass and alkaloid production (Verma et al. 2012). Total alkaloid content was found significantly maximum in MS medium (4.25 g/l dry weight) as compared to B5 medium (7.9 g/l dry weight) in *Hyoscyamus muticus* cell suspension culture. Moreover, among the different strengths of MS media, full strength was revealed the best for nourishing the growth as well as total alkaloid production in *Hyoscyamus* cell culture (Aly et al. 2010). Many researchers described the importance of types of medium and its composition on growth and SM build-up in callus and suspension cultures; among the media, MS and B5 are the two mostly used standard media for cell culture of various plant species.

4.2 Carbon Source and Its Concentration

In vitro culture requires a carbon source in order to fulfil energy loads due to the lack of photosynthesis and that strongly affects the induction and growth of callus as well as cell differentiation. Meanwhile, carbohydrates also have a significant role in the maintenance of osmotic pressure in the medium (Lipavska and Konradova 2004). One of the most commonly used carbohydrate energy source in in vitro culture is sucrose, since it is the form of carbohydrate present in phloem sap of many plant species (Fuents et al. 2000). Apart from this, other carbon sources used in in vitro cultures are mannitol and sorbitol (George 1993), polyethylene glycol (Ramarosandratana et al. 2001), and glucose, fructose, maltose, and lactose. For example, MS media supplemented with glucose as carbon source resulted in higher biomass production (8.3 g/l dry cell weight basis) and podophyllotoxin production (4.9 mg/l) by cell cultures of *Podophyllum hexandrum* than sucrose used as carbohydrate source (Chattopadhyay et al. 2002). The accumulation of SM production in various plants is being influenced by altering the source of carbohydrates and the concentration used in the media, which has long been recognized in plant cell cultures. In *Catharanthus roseus*, higher accumulation of ajmalicine was induced by the media incorporated with glucose as a carbon source (Schlatmann et al. 1995). However, sucrose was shown as best carbon source in shikonin production by *Lithospermum erythrorhizon* (Mizukami et al. 1977). Moreover, in *Cynara cardunculus* cell suspension culture, the highest polyphenol content was recorded in media containing glucose (1307.6μg/g) followed by corn starch (1131.6μg/g), and in sucrose, it was only 911μg/g after 7 days. However, highest polyphenol content was reported maximum in fructose (573.3μg/g) after 14 days (Oliviero et al. 2022). Cell suspension culture of *L. macranthoids* grown in B5 medium supplemented with sucrose (3%) was established as top media for biomass accumulation and SM production (Li et al. 2016). The growth and hyoscyamine accumulation of *Hyoscyamus muticus* developed on media having glucose were significantly reduced than sucrose (Oksman-Caldentey and Arroo 2000).

In the same line, Gertlowski and Petersen (1993) studied the impact of carbon sources on growth and rosmarinic acid accumulation in suspension cultures of *Coleus blumei* and revealed that 5% sucrose used in the medium showed maximum rosmarinic acid. The authors further highlighted that rosmarinic acid accumulation is associated with carbon left in the medium when growth ceases. Therefore, a good carbon source is required not only for cellular growth, but it is necessary for the production of high-value bioactive compounds.

4.3 Nitrogen Source and Its Concentration

In some of the media like MS, LS, and B5, nitrogen is one of the essential components along with the phosphate; these two are the main essential macronutrients required for the plant growth and development. The most commonly used as organic nitrogen in culture media are amino acid mixtures, L-glutamate, L-aspartate, and adenine. Amino acids provide an immediate source of nitrogen in plant cells. Apart from this, nitrogen is supplied in the form of ammonium and nitrate in the medium. Media containing amino acids and proteins exhibited better SM production. Moreover, the amount of nitrogen also impacts the production of the metabolites. A study was done in periwinkle cell suspension culture for the enhancement of alkaloid production through using various levels of nitrogen with phosphate concentration. It was shown that maximum biomass (19.17 and 2.10 g/l fresh and dry weight, respectively) production and total alkaloid content (5.84 mg/g dry weight) were observed in elevated phosphate levels with 3710.10 mg/l of total nitrogen concentration in B5 medium compared to 2850 mg/l of total nitrogen of MS medium (Mishra et al. 2019). The maximum fresh biomass accumulation (294.8 g/l) and total phenol content (76.61 GAE/g dry weight) were registered in *Salvia nemorosa* cell suspension culture in MS media having nitrogen 90 mM. However, media containing 30 and 60 mM of nitrogen showed the maximum rosmarinic acid (16.41 and 16.16 mg/g dry weight, respectively). In this experiment, the researcher used NH_4NO_3 and KNO_3 as the nitrogen sources in constant proportions. Further, they revealed that ammonium and nitrate ratio (NH_4^+/NO_3^-) also affected the growth and accumulation of SMs and found maximum fresh biomass accumulation (296.52 g/l), total phenol (87.30 mg GAE/g dry weight), and total rosmarinic acid (18.43 mg/dry weight) in 10:50 ratio of NH_4^+/NO_3^- (Heydari et al. 2020). In the MS medium containing NH_4^+/NO_3^- ratio of 30:30 mM, elevated quantity of kaempferol epicatechin, quercetin-3-O-glucoside, kaempferol-3-O-rutinoside, and total flavonoid content in callus cultures of *Orostachys cartilaginea* was found (Zhang et al. 2017). However, quercetin production was found maximum in NH_4^+/NO_3^- ratio of 20:40 mM. In the same line, maximum withanolide contents in regenerated multiple shoots of ashwagandha were found in L-glutamine (20 ppm) added in medium along with an appropriate concentration of other media components (Sivanandhan et al. 2012).

Cell suspension culture of *Gymnema sylvestre* in MS media with greater amount of NO3- than NH4+ concentration influenced in better cell growth and gymnemic

acid yield. The NH_4^+/NO_3^- ratio of 7.19/18.80 showed maximum gymnemic acid (11.35 mg/g dry weight) and biomass growth (159.72 and 14.95 g/l fresh and dry weight, respectively) (Praveen et al. 2011). Likewise, SM production was enhanced by modifying NH_4^+/NO_3^- ratio in some other medicinal plants such as *Calendula officinalis* (Legha et al. 2012), *Pueraria tuberosa* (Karwasara and Dixit 2012), and *Bacopa monnieri* (Naik et al. 2011).

4.4 Plant Growth Regulators (PGRs)

PGRs play an important role in tissue culture in a variety of actions including cell division, cell enlargement, callus induction, and organogenesis. Auxins and cytokinins are two mostly used phytohormones, and the ratio of these two hormones generally associated with caulogenesis (low auxin: cytokinin) and rhizogenesis (high auxin: cytokinin) (Djande et al. 2019; Schaller et al. 2015). Direct or indirect organogenesis from the explants or callus cells is stimulated by the use of PGRs (Malik et al. 2007; Yu et al. 2017). The use of hormones in cell culture also provokes yielding of high-value metabolites. The way of PGR crosstalk varies with plant to plant and organs under study (Moubayidin et al. 2009). To obtain high total phenolic content in stem-derived callus of *Bidens pilosa* required moderate to high cytokinin to low auxin ratio in MS media, while total phenolic content was reduced at very high cytokinin concentration with BAP at 8 mg/l (Ramabulana et al. 2021). Further, it was noticed that combined effects of auxins and cytokinins exhibited positive effect on the production of particular metabolites (chlorogenic acid derivatives of hydroxycinnamic acids) in *B. pilosa* cell culture. Li et al. (2016) observed higher biomass and chlorogenic acid production through suspension culture of *Lonicera macrantha* in B5 medium containing 6-BA (2 ppm) and 2,4-D (0.5 ppm). MS medium provided with 2×10^{-6} M 2,4-D in cell suspension culture of *Catharanthus roseus* exposed low accumulation of indole alkaloids ajmalicine and serpentine. This alkaloid content mainly ajmalicine was increased by omitting 2,4-D from the medium (Knobloch and Berlin 1980).

An investigation was made on an exogenous application of plant hormones influenced on growth and coumarin content in hairy root cultures of *Cichorium intybus* L. (Bais et al. 2002). With increase in dedifferentiation under exogenous application of hormone (NAA and kinetin), there has been lost the ability to synthesize the coumarins. Authors further highlighted that media containing 2 ppm of 2,4-D and 0.5 ppm of kinetin showed less amount of esculin (79.8μg per fresh weight culture) and esculetin (51.6μg per fresh weight culture) as compared to the control (316.5 esculin and 226.5 esculetin μg per fresh weight culture) on 28 days. However, in exogenously supplied gibberellic acid (0.5 ppm), enhanced growth and coumarin content were found.

4.5 Culture Conditions and Other Related Factors

Solidified or liquid suspension culture media have substantial influence on growth parameters and accumulation of high-value bioactive compound accumulation in various medicinal plants. Suspension culture of *Garcinia mangostana* L. grown in MS media treated with methyl jasmonate (MeJA) stimulated the thalsimine (alkaloid) and phosphatidyl ethanolamine (fatty acid) production, while callus culture was found significantly increased in the production of thiacremonone (alkaloid) and 7-methylthioheptanaldoxime (glucosinolate) (Jamil et al. 2018). MeJA is used widely in cell culture as elicitor for stimulating SM production. In sweet basil (*Ocimum basilicum*), terpenoid accumulation was enhanced by treating the media with MeJA (Misra et al. 2014); similarly, in *Hypericum perforatum* (Wang et al. 2015a, b) and *Centella asiatica* (Rao and Usha 2015), alkaloid accumulation was enhanced. Besides this, salicylic acid (SA) concentration ranges from 25 to 150μm used in culture media showed enhancement of bioactive compound accumulation. The maximum phenolic (35.4 mg/g dry weight) and total flavonoid (35.4 mg/g dry weight) content occurred at 100μm SA. Therefore, it was clearly shown that the use of elicitors like SA, MeJA, and jasmonate triggers the SM production under in vitro culture (Zhao et al. 2005). Casein hydrolysate was found to be added in culture medium as organic supplement to enhance the desired SMs such as anthocyanin in grapevine (Cetin and Baydar 2014) and sennosides in senna (Chetri et al. 2016).

5 Transgenic-Based Molecular Farming

Many medicinal and aromatic plants have the SMs with anticancer activity like paclitaxel, vinblastine, vincristine, and camptothecin and play a very significant role in prophylaxis and therapy (George et al. 2017). These phytochemicals are not dangerous and less hazardous than synthetic counterparts (Seca and Pinto 2018).

5.1 Sterols

Talking about the sterol, sitosterol and stigmasterol are the promising molecules in the process of drug development for cancer therapy by activating intracellular signalling pathways in several cancers. These molecules reported to act on the Akt/mTOR and JAK/STAT signalling pathways in ovarian and gastric cancers (Bakrim et al. 2022). In addition, stigmasterol has anti-diabetic properties because it lowers fasting glucose, serum insulin levels, and oral glucose tolerance. Additional in vivo research found that this chemical has antiparasitic properties against parasites such as *Trypanosoma congolense*.

Similarly, other anti-carcinogenic compounds biosynthesized in *Withania somnifera* from precursor squalene called withanolides (WTDs) and withaferin A were successfully enhanced by 1.5-fold (WFA; 330 ± 0.87μg dry weight) by expressing *Arabidopsis thaliana Squalene synthase* gene (*AtSQS1*) in *Withania somnifera* (Yousefian et al. 2018).

5.2 Flavonoids and Phenols

A study was undertaken to improve the accumulation of SMs in transgenic *Arabidopsis* through overexpression of *GlPS1* gene. In ancient Chinese medicine, dried root of *Glehnia littoralis* was commonly used to treat lung conditions and currently was also used to fight against the coronavirus disease that caused pneumonia in 2019. *G. littoralis* GlPS1 gene is the candidate gene for the synthesis of furanocoumarin. Expression of this gene leads to the accumulation of 30 differential metabolites in *Arabidopsis*. Of these, twelve coumarin compounds were found significantly up-regulated and six were newly synthesized, which was absent in control or non-transformed plant. Among these furanocoumarins, three compounds, namely, psoralen, imperatorin, and isoimperatorin, were accumulated when transgenic plant was given a salt stress. From this finding, it is also suggested that the adequate stress has significantly increased the economic benefits for enhancing *G. littoralis* quality (Ren et al. 2023).

Besides phenol, flavonoids are the most common types of plant polyphenols, which has a big impact on nutrition and human health. The sulphated forms of flavonoids are more advantageous than their parent compounds in that they are more soluble, stable, and bioavailable. Among the flavonoids, naringenin showed a broad range of biological effects on human health including a reduction in the biomarkers of lipid peroxidation and protein carbonylation, stimulation of carbohydrate metabolism, augmentation of antioxidant defences, scavenging of reactive oxygen species, modulation of immune system activity, and also exerting anti-atherogenic and anti-inflammatory effects (Wang et al. 2015a, b). Additionally, it has been shown to have a strong capacity to control signalling pathways involved in fatty acid metabolism, favouring fatty acid oxidation while hindering lipid build-up in the liver and preventing fatty liver (Zobeiri et al. 2018). The richest source of naringenin is *Citrus* species, tomatoes and figs. An attempt was made to synthesis the sulphated naringenin in *E. coli* by expressing a *Sulfotransferase* (ST) gene from *Arabidopsis* (At2g03770). The mutant strain of *E. coli* was developed using clustered regularly interspaced short palindromic repeats (CRISPR) technique. The synthetic sgRNA produced to repress the *cysH*, a gene encoding 3′-phosphoadenosine-5′-phosphosulfate (PAPS) ST that is mandatory for sulphur metabolism without affecting cell growth, resulted in a rise in intracellular PAPS accumulation of over 3.28-fold. The repressed function of *cysH* gene leads to the increased naringenin 7-sulfate production by 2.83 times than the wild-type control *E. coli* (Chu et al. 2018).

De novo production of naringenin was attempted in *Saccharomyces cerevisiae* because of meager production efficacy from plants and *Escherichia coli; Saccharomyces cerevisiae* found a suitable heterologous system for the production of this metabolite simply from glucose. Five genes, namely, *phenylalanine ammonia lyase* (*PAL*), *trans-cinnamate* 4-*monooxygenase* (*C4H*), 4-*coumaric acid*-CoA *ligase* (*4CL3*), *chalcone synthase* (*CHS3*), and *chalcone isomerase* (*CHI1*), were utilized to produce naringenin in the transformed yeast cell. Increasing the copy number of the *chalcone synthase* gene resulted in a 40 times rise in extracellular naringenin (circa 200μM) in glucose-grown shake-flask cultures. The transformed cell was grown in a 2 L batch bioreactor at pH 5.0 with 20 g/l of glucose (Koopman et al. 2012).

For increasing the production of SMs through gene modulation, overexpression of transcription factor (TF) has also been practised. The TF of MYB family proteins was reported to play an important role in the phenylpropanoid pathway that regulates synthesis of anthocyanin. The expression of the TF *MYB12* in growing seedlings of *A.s thaliana* resulted in an upsurge in total flavonoid (Yang et al. 2012). Similarly, three TFs, viz., *ORCA1*, *ORCA2*, and *ORCA3*, involved in the expression of terpenoid indole alkaloid (TIA) biosynthesis have been reported in *periwinkle*. The overexpression of *ORCA2* or *ORCA3* in *C. roseus* cell suspension/hairy roots increased metabolite synthesis of ajmalicine, serpentine, tryptamine, and catharanthine (Sun and Peebles 2017).

5.3 Lupeol: A Pentacyclic Triterpenoid

Other interesting molecules like lupeol, quercetin, epigallocatechin-3-gallate (EGCG), bergenin, and thymoquinone reported to shrink the serum uric acid levels by stimulating diuresis and altering the stone formation metabolism (Lima et al. 2007). They have numerous potential medicinal properties like anticancer and anti-inflammatory activity (Qiao et al. 2019). An attempt has been made to synthesize the lupeol in yeast by deploying genes from different organisms. Squalene is the precursor of synthesis of lupeol, which has also been employed as an antioxidant and as a possible biofuel. Lupeol naturally occurs at relatively low quantities in plant tissues in many circumstances, severely limiting its industrial applicability.

For these reasons, creating lupeol production in microorganisms is a more appealing option than extracting it from plants. Qiao et al. (2019) made an effort to produce lupeol in *E. coli* and *Saccharomyces cerevisiae* cells by employing the codon-optimized 3 lupeol pathway genes from different organisms specifically *Squalene synthase* from *Thermosynechococcus elongatus* (*tSQS*), *Squalene epoxidase* from *Rattus norvegicus* (*rSE*) and *Lupeol synthase* from *Olea europaea* (*OeLUP*) in *E. coli.* They also evaluated the lupeol pathway in two different yeast strains, namely, WAT11 and EPY300, and found that the engineered strains displayed the best lupeol-producing ability with the maximum lupeol titre of 200.1 mg/l at 30 °C.

5.4 Rutin

A study was conducted to optimize the callus cultures in transgenic tobacco line through expression of a flavonol-specific TF, viz., *AtMYB12*. Transgenic callus showed increased expression of genes involved in the biosynthetic process, resulting in higher build-up of flavonols, especially rutin. At every developmental stage of callus, the rutin content of transgenic callus was many orders of magnitude higher compared to the wild one (Pandey et al. 2012).

5.5 Curcumin

It was used to cure rheumatism, body aches, skin diseases, intestinal worms, diarrhoea, intermittent fevers, hepatic disorders, biliousness, urinary discharges, dyspepsia, inflammations, constipation, leukoderma, amenorrhea, and colic in ancient times on the Indian subcontinent. Curcumin has the extraordinary potential to treat a numerous varieties of inflammatory diseases including cardiovascular diseases, cancer, diabetes, Alzheimer's disease, arthritis, psoriasis, etc., through intonation of many molecular targets (Pari et al. 2008).

The first successful attempt was made through metabolic engineering by rerouting phenylpropanoid pathway for the synthesis of curcumin and its glucoside synthesis in *Atropa belladonna*. Genes, namely, *Diketide-CoA synthase-DCS*, *Curcumin synthase-CURS3*, and *Glucosyltransferase* (*CaUGT2*) genes, resulted in the overproduction of curcumin and its glucoside in transformed hairy root culture. Co-expression of DCS/CURS3 and CaUGT2 gene resulted in higher production of 32.63 ± 2.27μg/g DW curcumin monoglucoside and 67.89 ± 2.56μg/g DW curcumin, whereas co-expression of only DCS/CURS3 gene leads to the production of maximum 180.62 ± 4.7μg/g DW curcumin yield alone (Singh et al. 2021).

5.6 Alkaloids

Putrescine N-methyltransferase (*PMT*) was expressed in transgenic plants of *Atropa belladonna* and *Nicotiana sylvestris* and (*S*)*-scoulerine 9-O-methyltransferase* (*SMT*) in cultured cells of *Coptis japonica* and *Eschscholzia californica*. The overexpression of *PMT* enhanced nicotine content in *N. sylvestris*, but inhibition of endogenous *PMT* activity reduced nicotine content and caused aberrant morphologies. The buildup of benzylisoquinoline alkaloids in *E. californica* was generated by ectopic expression of *SMT* (Sato et al. 2001).

5.7 Paclitaxel

The compound name paclitaxel from *Taxus* bark was reported as an anticancer drug discovered in 1971. It is reported to have an effect on microtubules. This drug stimulates microtubule assembly from tubulin dimers and stabilizes microtubules by preventing depolymerization. Hence, it prevents metaphase-anaphase transitions, inhibits mitosis, and induces apoptosis in a variety of cancer cells (Stage et al. 2018). Currently it is approved for the treatment of various cancers including lung, breast, etc. Harvesting of the paclitaxel from the bark of *Taxus* spp. is not a viable option because the compound levels are extremely low, yew is also a slow-growing species, and extraction is a very destructive process. One treatment requires 2.5–3 g of paclitaxel, which necessitates the use of eight mature yew trees. Chemical synthesis is also not commercially viable. Therefore, various unconventional biotechnological techniques were employed for its production, such as heterologous expression systems and plant cell culture. Biosynthesis of Taxol in yew plants involves 19 steps beginning with the synthesis of geranylgeranyl diphosphate (GGPP) via the condensation of isoprenyl diphosphate and dimethylallyl diphosphate. For Taxol synthesis, many different strategies have been employed to increase the paclitaxel production either by overexpression of *10-deacetylbaccatin III-10-O-acetyltransferase* (*DBAT*) and *Taxadiene synthase* (*TXS*) genes in transgenic *Taxus mairei* (Ho et al. 2005) or in cell culture in *T. umbraculifera var. hicksii (Rehder) Spjut* (Sykłowska et al. 2015). Other studies have found that enhancement of paclitaxel biosynthesis can be obtained by overexpression of another gene named *9-cis-epoxycarotenoid dioxygenase* in transgenic cell lines of *T. chinensis*. In addition, genetic transformation of *N. benthamiana* with a *Taxadiene synthase* (*TS*) gene driven by 35S promoter was found to assist de novo production of taxadiene in *N. benthamiana* and produced 11–27μg taxadiene/g of dry weight; in addition, subsequent elicitor treatment of methyl jasmonate increased the taxadiene accumulation by 1.4 times (Hasan et al. 2014). Similarly, in vitro transformation of T.x media hairy roots and subsequent elicitation permitted the production of paclitaxel; the vector was *A. tumefaciens* carrying the RiA4 plasmid and the binary vector pCAMBIA-TXS-His harbouring the *TXS* gene of *Taxus baccata* L. driven by 35S promoter.

5.8 Vinblastine

Catharanthus roseus (L.) G. Don. is a medicinal plant of excellent pharmaceutical interest due to its ability to biosynthesize more than 130 bioactive molecules known as terpenoid indole alkaloids (TIAs), which include the anti-proliferative drug molecules vinblastine and vincristine, together with the pharmacologic molecules ajmalicine and serpentine (Verma et al. 2017). An experiment was performed to direct the metabolic flux of TIA pathway towards the production of dimeric alkaloids vinblastine and vincristine by overexpression of *Tryptophan decarboxylase* and

Strictosidine synthase in callus and leaf tissues. They did a comparison between the stable and transient methods of transformation for the determination of vinblastine and vincristine content in *Catharanthus roseus*. Callus transformation showed maximum of 0.027% and 0.053% dry wt vindoline and catharanthine production, respectively, whereas the transiently transformed leaves showed 0.30% dry wt vindoline, 0.10% dry wt catharanthine, and 0.0027% dry wt vinblastine contents (Sharma et al. 2018).

5.9 Camptothecin

Camptothecin (CPT) is a monoterpene alkaloid and was first isolated from stem wood of *Camptotheca acuminata* that inhibits topoisomerase I (Top 1), a nuclear enzyme that is involved in DNA repair, recombination, transcription, and replication (Martino et al. 2017). CPT was also isolated from *Nothapodytes foetida* (Wight) Sleumer's bark. The lack of sufficient natural sources for acquiring CPT is a significant barrier. As a result of overharvesting, habitat loss, excessive trading, and unfavourable environmental variables, the natural supply of CPT has become extinct or highly limited (Swamy et al. 2021). Hao et al. (2021) did the time-course expression studies of metabolite analysis to find new transcriptional regulators of camptothecin production in *Ophiorrhiza pumila*. Here, it is demonstrated that camptothecin production increased over the course of cultivation and that there is a strong correlation between camptothecin accumulation and the expression pattern of the gene *OpWRKY2*, which codes for the *WRKY* transcription factor. Overexpression of *OpWRKY2* transcription factor leads to the increase in camptothecin production by more than threefold. Likewise, lower camptothecin levels in the plant were associated with *OpWRKY2* silencing. Additional in-depth molecular characterization using yeast one-hybrid, dual-luciferase, and electrophoretic mobility shift assays revealed that *OpWRKY2* directly binds and activates the *OpTDC* gene, which is involved in the main camptothecin pathway. From the findings of this study, it has been concluded that the *OpWRKY2* function is a direct positive regulator of camptothecin production. Ni et al. (2011) investigated the physiological role of *ORCA3* gene in transformed *Camptotheca acuminate* using *Agrobacterium*-mediated gene transfer technology. HPLC analysis revealed that overexpression of ORCA3 in transgenic hairy root lines can significantly increase camptothecin production by 1.5-fold compared to the control (1.12 mg/g dw).

5.10 Reticuline

A study was undertaken to produce reticuline at the cost of morphine, oripavine, codeine, and thebaine in transgenic *Papaver somniferum* (opium poppy). To increase reticuline alkaloid production, hairpin-based RNAi silencing of all members of

multigene *Codeinone reductase* (COR) family was carried out (Allen et al. 2004). Gene silencing of COR genes showed the accumulation of methylated derivatives of reticuline at a great level. The astonishing increase of (S)-reticuline advocates a presence of feedback mechanism to prevent the intermediate synthesis from general benzylisoquinoline, which is participated in the morphine-specific branch. This the first report of gene silencing where metabolic engineering causes the high yield of the nonnarcotic alkaloid reticuline (Allen et al. 2004).

5.11 Artemisinin

Another important secondary metabolite, namely, artemisinin, identified in *Artemisia annua* has proven its role in the treatment of malaria. The production of this compound in *Artemisia annua* is significantly increased by overexpression of two jasmonic acid-responsive transcription factor *AP2/ERF* proteins (*AaERF1* and *AaERF2*) (Yu et al. 2012). It was well illustrated that jasmonic acid rapidly induces the expression of *AaMYC2* transcription factor, which then binds to the G-box-like motifs of *CYP71AV1* and *DBR2* gene promoter region, which are the key regulator genes of the artemisinin biosynthetic pathway (Qian et al. 2016).

5.12 Stevioside

Novel attempt was made for the production of sweet-tasting steviol glycosides (SGs) in *Stevia rebaudiana* leaves, which is consumed as natural sweeteners. SGs have been widely studied for their exceptional sweetness over the last few decades. SGs may become a basic, low-calorie, and strong sweetener in the burgeoning natural food industry, as well as a natural anti-diabetic therapy, a highly competitive alternative to commercially accessible synthetic medications, in the near future. Many countries have already begun commercial *Stevia* plant farming, as well as SGl extraction and purifying methods from plant material. As a result, the nutritional and pharmacological benefits of these secondary metabolites have become more evident. Metabolic engineering was employed to enhance the production of SGs in *Stevia rebaudiana*. Two enzymes, namely, *Stevia 1-deoxy-D-xylulose-5-phosphate synthase 1* (*SrDXS1*) and *Kaurenoic acid hydroxylase* (*SrKAH*), are required for the SG biosynthesis. Two independent events were generated by overexpressing *SrDXS1* and *SrKAH* genes. The total SG content in *SrDXS1* and *SrKAH* overexpressing transgenic lines was increased by up to 42–54% and 67–88%, respectively, as compared to control plants, indicating a favourable correlation with *SrDXS1* and *SrKAH* expression levels. Furthermore, their overexpression had little effect on the transgenic *Stevia* plants' growth and development (Zheng et al. 2019).

5.13 Shikonin

An effort in the 1970s and 1980s were sparked by the industrial synthesis of shikonin by cell cultures of *Lithospermum erythrorhizon* by Mitsui Chemicals. This was the first large-scale production of a secondary metabolite by dedifferentiated plant cells. The phytohormone ethylene (ET) was identified as an important signalling molecule in the manufacture of shikonin and its derivatives. Shikonin and its derivatives have also been utilized as medicines for antibacterial, anti-inflammatory, and anti-tumour effects in addition to their use as colours. Moreover, they have demonstrated the capacity to treat burns, haemorrhoids, and wounds through the growth of granulation tissue (Kamei et al. 2002; Ordoudi et al. 2011). Structure- and activity-related relationship of shikonin and alkannin was studied in depth from *A. tinctoria* root extract. It was shown that alkannin and shikonin, both oligomeric and monomeric, have strong radical scavenging capacity (Assimopoulou and Papageorgiou 2005).

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Omics Approaches to Study the Biosynthesis of Bioactive Compounds in Medicinal and Aromatic Plants

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Abstract Human beings treat several diseases with plants as medication. The majority of secondary metabolites produced in plants, which function as chemical factories, are either directly used as medicines or indirectly used in the development of pharmaceuticals. Plant breeders still struggle to improve them better through conventional breeding methods. Hence, rapidly developing plant research on omics has increased our grasp on the intricate structure of secondary metabolites, synthesized in medicinal and aromatic plants. Additionally, sequencing techniques and completion of various medicinal and aromatic plant genome sequences have unfurled a lot of options for fine mapping as well as characterization of the genes. The accessibility of various biotechnological techniques along with the studies on quantitative trait loci as well as candidate genes related to secondary metabolites has paved the way for the discovery of innovative approaches for improvement in medicinal and aromatic plants. Over the past three decades, several studies have been conducted to investigate the knowledge of secondary metabolites in medicinal and aromatic plants. This chapter provides detailed insights on the current advancements and integration of omics approaches for improvement in the production of secondary metabolites from medicinal and aromatic plant species.

Keywords Secondary metabolites · Medicinal and aromatic plants · Genomics · Metabolomics

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N. Kumar, R. S. Singh (eds.), *Biosynthesis of Bioactive Compounds in Medicinal and Aromatic Plants*, Food Bioactive Ingredients,
https://doi.org/10.1007/978-3-031-35221-8_2

1 Introduction

People have been exploring nature, mostly plants, in pursuit of novel medicines since ancient times. This has influenced the usage of several medicinal and aromatic plants (MAPs) with therapeutic capabilities to treat a wide range of ailments. Nevertheless, plants or substances derived from plants are still the primary source of health care for more than 80% of the world's population (Barut et al. 2022). In addition to being used as food, they also have medicinal potential and are a source of fiber and other industrial raw materials (Salvi and Katewa 2016). Medicinal plants are harnessed for several bioactive compounds that have biological properties that can enhance human health through the food and pharmaceutical industries. Numerous secondary metabolites including terpenoids, phenylpropanoids, tannins, and alkaloids are being studied for therapeutic development (Sanchita and Sharma 2018). Researchers are using modern biotechnology-based techniques to enhance metabolite production from medicinal plants. Availability of genomic and transcriptome data of medicinal plants using next-generation sequencing (NGS) methods is generating data for several yet-to-be-identified natural compounds from medicinal plants that may be more significant for health (Zhao et al. 2019). The genome sequences, which contain pertinent information about plant origin, evolution, development, inheritable traits, and epigenomic regulation, serve as the foundation and premise for understanding the genome diversity and chemodiversity (especially various secondary metabolites with potential bioactivities) at the molecular level. In addition to focusing attention on the biosynthetic pathways of medicinal compounds, particularly for secondary metabolites and their regulatory mechanisms, high-throughput sequencing of medicinal plants has the potential to significantly advance molecular breeding mechanisms for high-yielding medicinal cultivars (Boutanaev et al. 2015). The next-generation sequencing (NGS) technique has led to an increase in the synthesis of metabolites, communication between co-responses to gene expression, and deep transcriptome analyses of the medicinal plants. The "guilt-by-association" theory states that when two genes are co-expressed, metabolites are produced at a rate that reflects the co-expression of the two genes (Pandita et al. 2021). Modern RNA sequencing techniques enable the global analysis of the expression profiles of transcription factors and enzymes. It is a successful method for obtaining genomic information/transcripts from several medicinal non-model plants that lack a reference genome. The transcriptome investigations aid in characterizing crucial factors involved in the synthesis of secondary metabolites and in examining essential molecular processes involved in pharmaceutically significant mechanisms (Hao et al. 2012). Additionally, proteomics provides a strong platform for exploring proteins that are controlled by medicines in depth and exploring signaling cascades of cell disturbances. The study of proteins' structures, changes, and roles in both in vitro and in vivo protein-protein interactions is involved under proteomics study (Lao et al. 2014). Proteomics research has

revealed several terpenoids, flavonoids, glycosides, and other secondary metabolites found in medicinal plant herbs that have anticancer potential in a variety of tumors. Additionally, proteins important in oxidative stress management, cell signaling, energy metabolic pathways, development, and biosynthesis have been found using the liquid chromatography-tandem mass spectrometry (LC-MS/MS) technique. These methods have created new opportunities for ethnobotanical and phytomedicine research with the goal of proving the use of medicinal plants for the treatment of certain chronic illnesses through the identification of species and biomarkers (Pedrete et al. 2019). Thus, compared to the conventional natural product research approach, metabolomics investigations provide a superior, quicker route to drug development. Metabolomics experiments may be conducted without the requirement for genome data, which is an advantage. The basic goal of metabolomics is to provide a comprehensive qualitative and quantitative analysis of each metabolite present in a biological system. Metabolomics research is being used more often in a bioactivity-driven manner from natural extracts and offers an enhanced accelerated way for plant natural product drug development. It is an interesting concept that may be used to classify natural goods as medications by examining the relationship between the complete metabolome of natural remedies and their biological effects (Salem et al. 2020). As part of the metabolomics experiment procedure, these endogenous metabolites must be effectively isolated before being subjected to qualitative and quantitative analysis. However, unlike other omics technologies, no one analytical platform can analyze all metabolites at once due to their high complexity and wide chemical variability (Alseekh and Fernie 2018). Hence the genes engaged in complex processes that produce bioactive components by integrating transcriptomics, proteomics and metabolomics can aid in predicting the various gene functions (Fig. 1).

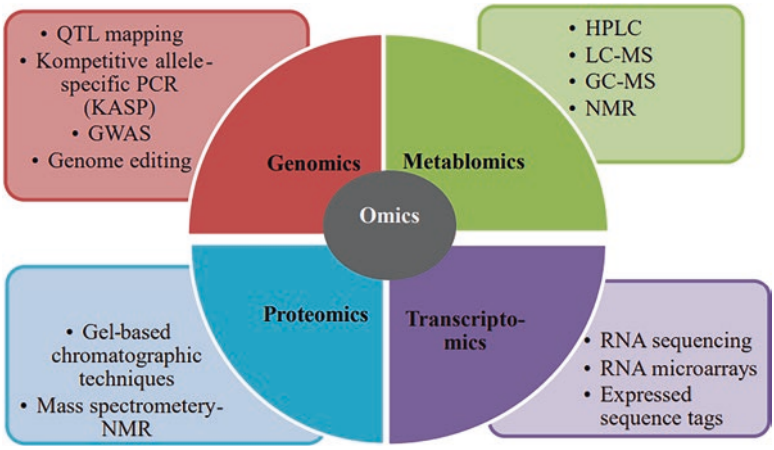

Fig. 1 Omics approaches to study secondary metabolites in medicinal and aromatic plants

2 Omics Approaches in Medicinal and Aromatic Plants

2.1 Genomic Approaches

Omics investigate various kinds of molecules on a big scale by combining interdisciplinary biological sciences using high-throughput methods. These automated techniques provide rapid, precise, and in-depth examination of a large number of samples in a short time period (Porter and Hajibabaei 2018). The genome sequencing is attainable with little time and money expenditure by utilizing bioinformatics techniques. Additionally, they contribute to the discovery of metabolites that are controlled by genes and analyze their effects within cellular lines (Barut et al. 2022) (Table 1). Plant breeding efforts are aided by the genetic profile of plant species. Molecular markers that were previously used for breeding purposes now include new advancements like SCAR (sequence characterized amplified region), LAMP (loop-mediated isothermal amplification), and DNA barcoding, which have made possible the certification of the genetic variety of medicinal and aromatic plant species. Besides this, future reference libraries are being created by using DNA sequences and DNA fingerprints (Ganie et al. 2015). As compared to prior sequencing techniques (like Maxam-Gilbert sequencing method and chain-termination method), NGS as well as third-generation sequencing (TGS) are simpler, less expensive, and capable of being completed much more quickly.

2.1.1 Genetic Engineering and Genome Editing

Identification as well as characterization of genes responsible for synthesis and modification of metabolites is a precondition for enhancing the metabolite production or altering genes present in homologous and heterologous system. Developments in the sequencing techniques have made it feasible to characterize thousands of the genes instantly (Sun et al. 2022). Generally, the aim of such studies is to enhance the

Table 1 Genomic approaches in different plant species

S. no.	Plant species	Approach	References
1	*Salvia miltiorrhiza*	Bioinformatics approach	Sharma and Sarkar (2013)
2	*Sophora flavescens*	Genetic engineering	Yang et al. (2016)
3	*Trichopus zeylanicus*	NGS Illumina	Chellappan et al. (2019)
4	*Santalum album*	Transcriptomics approach	Zhang et al. (2019)
5	*Lithospermum erythrorhizon*	Transcriptomics approach	Auber et al. (2020)
6	*Ocimum basilicum*	Genome sequencing	Bornowski et al. (2020)
7	*Cynara scolymus*	Transcriptomics approach	Hassani et al. (2020)
8	*Panax notoginseng*	NGS Illumina	Cheng et al. (2021a)
9	*Coptis chinensis*	Genome sequencing	Chen et al. (2021)
10	*Taxus wallichiana*	NGS Illumina	Cheng et al. (2021b)
11	*Andrographis paniculata*	Genetic engineering	Adeleye et al. (2022)

amount of specific compounds produced by MAPs or to introduce a mechanism to certain other organisms to produce significant metabolites (Satish et al. 2019). For increasing the production of one or more compounds, two general approaches are being used. Either overcoming particular rate-limiting phase in metabolic pathways, disable competing metabolic pathways and lessen the catabolism of target molecule, so as to change the expression of the gene or changes have been made in regulatory genes that are involved in the regulation of a number of biosynthetic genes. Ogita et al. (2003) observed the transgenic coffee plants with low caffeine concentration by reducing the expression of theobromine synthase gene using RNA interference technology. A metabolite may occasionally be dangerous to humans in one form, or it may occasionally be less prevalent in its natural product. These metabolites can be modified by employing genetic engineering techniques to either a less dangerous or more useful chemical derivative. Rubio et al. (2008) identified four crocin, crocetin and picrocrocin (CCD) genes from *Crocus sativus* and observed a pattern of expression in which CsCCD1a displayed a consistent expression whereas CsCCD1b was particularly expressed in stigma tissue. The largest quantities of carotene and ionone were produced solely by CsCCD4a and CsCCD4b expressing cooperatively throughout the stigma development. Similarly, Ahrazem et al. (2010) isolated and examined the CCD4 genomics DNA areas in *Crocus sativus.* It was discovered that the CCD4a promoter sequence is suitable for initiating β-glucuronidase (GUS) expression in saffron flower, especially in the pollen.

Genetic engineering techniques have gained a lot of attention, but the conventional approaches to genetic engineering have several drawbacks, including the complexity that involves in manipulating the large genomes of the higher plant species and social acceptance, which is a major obstacle to the commercialization of genetically engineered crops. Currently, a number of tools are available to assist with the challenges of accurate plant genome editing (Aftab and Hakeem 2021).

Recent advances in genome editing have offered biologists a new technique for precisely altering plants on a massive scale that is not attainable with conventional genetic engineering techniques. Only few studies have so far demonstrated that secondary metabolic engineering may be accomplished through genome editing in medicinal and aromatic plants (Jansing et al. 2019). With this aim in mind, CRISPR/Cas9 (clustered regularly interspaced short palindromic repeats), ZFN (zinc finger nucleases), and TALENS (transcription activator-like effector nucleases) are currently artificially engineered. CRISPR/Cas9 system is most innovative and intriguing gene modification technique that enables metabolic engineering of the plant by modifying various genes in the same region of chromosome and creating smart plant varieties (Wilson and Roberts 2014). The number of studies on the application of the CRISPR/Cas9 system in medicinal and aromatic plants has progressively increased in recent years. The technique was employed by Ma et al. (2015) and Li et al. (2016) in *Salvia miltiorrhiza* to block the rosmarinic-acid synthase gene (SmRAS) and mute the main gene of tanshinone metabolic pathway. Alagoz et al. (2016) employed the type II CRISPR/Cas9 system to delete the 4′OMT2 gene, which controls the production of benzylisoquinoline alkaloids (BIA). They demonstrated that the transgenic plants had considerably less BIA production like thebaine

and morphine than normal. Additionally, they also found a unique, unidentified alkaloid that was present solely in CRISPR/Cas9 modified plants. Consequently, the CRISPR/Cas9 system was used in demonstrating for the first time how medicinal and aromatic plants can control BIA metabolism and production. Feng et al. (2018) recorded that a directed mutation in the gene Dzfps inhibited the activity of farnesyl pyrophosphate synthase (FPS) enzyme in *Dioscorea zingiberensis*, resulting in 1.6 times decrease in the squalene concentration than in the plants of the wild type.

The medicinal plant *Symphytum officinale* has anti-inflammatory as well as analgesic effects and also possesses pyrrolizidine alkaloids that are thought to be hazardous to humans. Zakaria et al. (2021) used the CRISPR/Cas9 method to introduce alterations into the homospermidine synthase gene in the hairy roots of *Symphytum officinale*. However, homospermidine synthase gene synthesized homospermidine, which have a particular antecedent for pyrrolizidine alkaloids. The homospermidine synthase encoding gene was successfully knocked out; as a result, pyrrolizidine alkaloids were completely eradicated. These results offer new insights into the genome editing of medicinal and aromatic plants and provide a new and powerful tool for precise plant breeding.

2.1.2 Quantitative Trait Locus (QTL) Mapping

QTL mapping analysis ought to be enhanced as it is among the most practical methods for revealing the heritability of secondary metabolites and scanning the entire genome to identify the QTL regulating traits associated with the production of secondary metabolites in medicinal and aromatic plants. Graham et al. (2010) revealed a QTL map for *Artemisia annua* that accounts for a significant proportion of variation in the essential attributes that regulate artemisinin yield. The requirement for artemisinin is anticipated to rise in near future, due to its treatment in malaria. Similarly, Celik et al. (2016) examined 15 cultivars and 103 Turkish landraces of *Papaver somniferum* for exploration of the QTLs that regulate the morphine content. They discovered several loci that were significantly associated with morphine content; also their entire set of detected markers offered preliminary data for the marker-assisted trait evaluation.

Choi et al. (2020) constructed an single nucleotide polymorphism (SNP)-based genetic linkage map of *Allium cepa* to locate QTLs for the total anthocyanin content by employing the genotyping-by-sequencing (GBS) method that relies on reference gene set. Their analysis unveiled one significant QTL (qAS7.1) for the synthesis of anthocyanin and two major QTLs for the anthocyanin content. Further, the genetic linkage map created and also QTL information will bestow the development of markers in the future for the higher anthocyanin content in the bulbs of *Allium cepa*. Vallejo and Warner (2021) reported the identification of two QTLs for the production of steviol glycoside in *Stevia rebaudiana* by utilizing transcriptome-derived 97 simple sequence repeats (SSRs). However, their study delineates the initial report of QTLs in *Stevia* but will assist future efforts for the development of molecular markers and discovery of gene for economically significant characteristics in *Stevia*.

2.1.3 Genome-Wide Association Studies (GWAS)

Biotechnologists and agronomists are forced to develop innovative cultivars with enhanced qualitative as well as quantitative characteristics due to the rising market demand for medicinal and aromatic plants. With respect to yields and secondary metabolite compositions, there is significant genetic variability observed among the plant species in nature. Genetic inheritance studies in several medicinal and aromatic plants unveiled the qualitative as well as quantitative characteristics of genes responsible for the synthesis of secondary metabolites. In a variety of plant species, success stories from GWAS have provoked a contentious debate. However, the development of NGS and other genotyping techniques has greatly expanded the potential for extremely quick marker production, thus enabling the genome-wide testing of the markers in unsequenced as well as uncharacterized medicinal and aromatic plants. Otto et al. (2017) utilized the GBS method to analyze the genetic composition of cultivated chamomile types and carried GWAS to find associated markers that were related to alpha-bisabolol concentration. In a study of four *Matricaria discoidea* and 91 *Matricaria recutita* plants, GBS investigation discovered 6495 SNP markers of high quality. Multiple cluster and principal coordinate analysis revealed that *Matricaria recutita* evolved from the *Matricaria discoidea* plants and certain tetraploids such as alpha-bisabolol were present in higher proportion with 71 SNPs involved in alpha-bisabolol production. From their findings, they concluded that GWAS data paved the way for future research on the genomics of chamomile attributes, the determination of marker-trait relationships, and the generation of credible markers for plant breeding that aided in marker-assisted breeding, QTL identification, gene localization for metabolites, and GWAS. Fan et al. (2020) identified the SNP markers linked to ginsenoside biosynthesis pathway in *Panax notoginseng*.

2.1.4 Kompetitive Allele-Specific PCR (KASP)

KASP, a uniplex SNP genotyping method, is now recognized as a benchmark technology on a global scale and produces more than a million data sets every year. In *Panax ginseng*, Jang et al. (2020) analyzed the whole ginseng mitogenome on the basis of long-read data set from the nanopore sequencing method. Out of 278 variations discovered using ten SNPs, they created ten KASP markers to identify 59 Korean ginseng genotypes followed by the elucidation of mitogenome diversity. These ten KASP markers along with the complete mitogenome sequencing will be beneficial for ginseng breeding in the near future. Similarly, the KASP method was used by Ruzicka et al. (2021) to assess the *Symphytum* plant, which was created by using the NGS strategy, resulting in the formation of six different genetic clusters from the plants. Though rosmarinic acid was not related to any cluster, some compounds like globoidan A and allantoin had significantly distinct clusters. Similarly, they observed low pyrrolizidine alkaloid levels, which were associated with a

specific genetic cluster and could serve as useful gene pools for pyrrolizidine alkaloid reducing levels via breeding.

2.2 Transcriptomics

Several important secondary metabolites produced by medicinal and aromatic plants are valuable for modern pharmacy. Out of a wide variety of phytochemicals, metabolites like carotenoids, flavonoids, lignans, and phenolic acids have a variety of biological functions (Chen 2020). Their study has been substantially aided by recent advancements in molecular biology, genomics, and functional genomics as well as high-throughput technologies (Table 2). Over the past century, various active ingredients are isolated from medicinal and aromatic plants such as artemisinin for malaria, huperzine A for Alzheimer's disease, ephedrine for colds, camptothecin for cancer, and tetrandrine against Ebola virus (Sakurai et al. 2015; Yang et al. 2016). For a better understanding of gene activity, regulatory mechanisms, and molecular techniques, several breeding traits are selected for higher productivity of beneficial secondary metabolites using transcriptomics study. Medicinal plant transcriptomes might have a comparable upstream pathway to produce a small number of comparable precursors that provide insightful and helpful data on the activation or expression of the genes (Wang et al. 2015). Xu et al. (2014) studied high-throughput transcriptome Illumina GA-II sequencing technique in the Amur grape (*Vitis amurensis* L.) and observed a total of 6850 transcripts that participated in the controlled

Table 2 Transcriptomics approaches in different plant species

S. no.	Plant species	Approach	References
1	*Ophiorrhiza pumila*	Transcriptome sequencing using the NGS technology	Yamazaki et al. (2013)
	Cassia obtusifolia	Transcriptomics analysis	Deng et al. (2018)
2	*Dendrobium officinale*	mRNA-Seq (Illumina)	Chen et al. (2019)
3	*Saussurea lappa*	De novo transcriptome assembly	Bains et al. (2019)
4	*Salvia miltiorrhiza*	Transcriptome sequencing using the NGS technology	Chang et al. (2019)
5	*Rheum officinale*	mRNA-Seq (Illumina)	Hei et al. (2019)
6	*Dendrobium nobile*	Transcriptomics analysis	Wang et al. (2020)
7	*Rheum emodi*	High-throughput transcriptome sequencing	Liu et al. (2020)
8	*Dendrobium huoshanense*	De novo assembly	Zhou et al. (2020)
9	*Fritillaria roylei*	De novo assembly and functional annotation	Sharma et al. (2021)
10	*Polygonum cuspidatum*	mRNA-Seq (Illumina)	Zheng et al. (2021)
11	*Rheum tanguticum* Maxim	De novo assembly	Hu et al. (2022)

mechanism and sighting the path for cold stress tolerance by *Vitis* species. Li et al. (2017) reported comprehensive transcriptome of 18 libraries from different organs of *Dracocephalum tanguticum* and assembled 1, 87,447 transcripts including unigenes de novo that were assigned to metabolic pathway for the production of rosmarinic acid, which is a multifunctional phenolic bioactive compound. Similarly, Singh et al. (2017b) sequenced the transcriptome of *Trillium govanianum* using paired end sequencing technique and discovered 69,174 transcripts in total as well as several genes. Their study identified routes for the biosynthesis of secondary metabolites and steroid saponins. The identification of potentially biologically dynamic metabolites was made possible by this discovery, which also contributed to the development of useful molecular marker resources in this plant. Hou et al. (2018) designed an investigation into the therapeutic plant *Cornus officinalis* that produces a multifunctional phenolic active molecule, rosmarinic acid with antiviral and antibacterial properties. 4585 significant differentially expressed genes (DEGs) and 56,392 uni-genes were observed from the the transcriptome of leaf and fruit tissues along with 1392 genes which were upstream regulated and 3193 genes down-regulated. Chen et al. (2018) performed RNA sequencing to profile the leaf transcriptomes of the two chemotypes of *Cinnamomum camphora* and identified a total of 2863 unigenes that were differentially expressed, where 1714 unigenes were upregulated and 1149 downregulated. In addition, three monoterpene synthase genes were upregulated with borneol type compared to linalool type that increased the expression of genes. In order to build the link between miRNAs and their targets using techniques like high-throughput technology or microarray, researchers developed numerous web-based databases connected to miRNA interactions that have connection to the emergence of diseases. There has been a growing interest in the study of miRNAs and the interactions between them and their targets among several researchers (Chou et al. 2018). Choudhri et al. (2018) sequenced the transcriptome in leaf and root samples in *Aloe vera* using the Illumina paired-end sequencing technique and observed 16 genes related to the synthesis of carotenoids, lignins, anthraquinones, and saponins. Fu et al. (2019) selected 2579 unigenes for enrichment analysis because of their distinct expression patterns in flowers and leaves after sequencing the transcriptome of *Dysphania schraderiana*. Their study opened the door to more investigations in the physiological functions and production of secondary metabolites in *Dysphania schraderiana.*

Vu et al. (2020) investigated the transcripts from the stems, leaves, and roots of the medicinal plant *Populus alba* and optimized 11,343 expressed sequence tag-simple sequence repeat (EST-SSR) primers for polymorphism confirmation. These findings have a significant role in *Populus alba* restoration, conservation, and better management practices. Lade et al. (2020) investigated the genetic variation of 96 *Tinospora cordifolia* medicinal plants collected from various locations in India using 7611 EST-SSRs, where four primer pairs were found to have a high potential for genetic variation. Hina et al. (2020) used the Illumina technology and de novo assembly to sequence the transcriptomes of two *Menispermum* species. A total of 78,921 unigenes were obtained, and 521 polymorphic EST-SSRs with high transferability against *Menispermum* species were discovered. Their study concluded that

the newly developed marker will be useful for future *Menispermum* genetics research. He et al. (2020) used microsatellite software to analyze SSR sites in *Paeonia lactiflora* and discovered 86,195 unigenes, with 21,998 SSR sites spread across 17,567 unigenes. Out of 100 randomly selected primers, 45 showed high polymorphism. These highly polymorphic primers were used to group 16 *Paeonia lactiflora* cultivars. These newly discovered markers will be useful for future *Paeonia lactiflora* genetic research. Yan et al. (2020) investigated the metabolome and transcriptome of green and purple *Tetrastigma hemsleyanum* leaves. A total of 4211 transcripts and 209 metabolites were discovered to be differentially expressed in the leaves, with 16 compounds revealed to be significantly related and 14 transcripts implicated in the anthocyanin biosynthesis pathway. This has a wide range of therapeutic applications due to the sesquiterpene lactones produced by it. Tripathi et al. (2020) studied in-depth transcriptomic analysis in *Withania somnifera* where 75,280 transcripts were completely annotated covering nearly all genes related with withanolide biosynthetic pathway. In fact, they also performed the tissue-wide gene expression analysis in the leaf, root, and berry to reflect the transcripts that were linked with terpenoid pathway. The metabolome map generated was used to study transcripts related to 143 metabolic pathways interconnected with each other. As the primary transcriptional regulators of secondary metabolism, a significant distribution of transcription factor genes including MYB; early light-inducible protein (ELIP); minichromosome maintenance 1, Agamous, Deficiens, and serum response factor (MADS); and WRKY was noted. The comprehensive and comparative analyses of *Withania somnifera* transcriptome data across the three tissues as well as across other Solanaceae plants (*Nicotiana*, *Solanum*, and *Capsicum*) with regard to main pathways were associated for metabolome regulation. Xu et al. (2021) performed RNA sequencing in *Ardisiapusilla* and successfully annotated 49,623 unigenes out of total of 88,444 where 4101 showed differential expression. The metabolic pathways of the toluene response genes were mapped, and it was discovered that the differentially expressed genes were primarily involved in carotenoid biosynthesis, phenylpropanoid biosynthesis, flavonoid biosynthesis, starch and sucrose metabolism, glycolysis/gluconeogenesis, and glycerophospholipid metabolism. Through this experiment, 53 transcription factors from 13 different transcription factor families were discovered that have a good correlation between RNA sequencing transcripts and differentially expressed genes that are associated with major metabolic pathways. Shukla et al. (2021) identified long non-coding RNA transcript sequence data in enormous quantities by using high-throughput RNA sequencing analyses. They developed bioinformatic pipelines to study long non-coding sequences from genomes of many medicinal and aromatic plant species. These long non-coding RNAs are important as effective gene expression regulators. Kapoor et al. (2021) observed the unprecedented biodiversity and valuable secondary metabolites from the Northwestern Himalayan region that are unique to the dynamic geo-climatic region. The experimental and bioinformatic workflow with sequencing-based transcriptomic studies enabled differentially expressed genes, transcription factors, cytochrome P450 enzymes, and KEGG (Kyoto Encyclopedia of Genes and Genomes) for various medicinal plants such as *Aconitum heterophyllum*,

Dactylorhiza hatagirea, *Fritillaria roylei*, *Picrorhiza kurrooa*, *Nardostachys jatamansi*, and *Trillium govanianum*. *Mangifera indica* (Dashehari) de novo transcriptome assembly and analysis were carried out by Illumina sequencing in order to comprehend the molecular mechanism for mango scent. The identification of key genes for terpenoids, carotenoids, flavonoids, lactones, lipoxygenases, aromatic amino acids, alkaloids, and phenylpropanoid pathways are possibly involved in aroma biosynthesis. Five different mango varieties (Dashehari, Banganpalli, Ratna, Mallika, and Alphonso) underwent comparative mRNA expression study to identify changes associated with their ripening and variety. The changes in the expression patterns of some genes in the multigene family were observed, suggesting that the related enzymes may have variable substrate specificities (Pathak et al. 2022). Zhang et al. (2022) studied the important characteristics of aromatic leaves and bulbs in fennel (*Anethum foeniculum*) for their color and aroma. They used integrated transcriptomics and metabolomics approach to distinctively examine the anthocyanin color and scent production in purple fennel. The crucial genes involved in the manufacture and regulation of anthocyanins and volatile phenylpropanoids were identified and thoroughly examined. Moreover, ten primary bioactive compounds were identified and measured using ultra-high-performance liquid chromatography combined with quadrupole Orbitrap high-resolution mass spectrometry (UHPLC-Q-Orbitrap-HRMS). Studies on gas chromatography-mass spectrometry (GC-MS) revealed that the decreased volatile phenylpropanoids, such as isoeugenol, trans-isoeugenol, and apiol, are primarily responsible for the distinctive scent alterations and anthocyanin production observed in purple fennel.

2.3 Metabolomics

Several thousands bioactive compounds were observed in the minute plants samples for various metabolical activities. Metabolomics is a large scale study that involves the foundational understanding of numerous domains of science such as biochemistry, analytical chemistry and statistics (Aderemi et al. 2021). It provides a comprehensive knowledge of the qualitative and quantitative alterations in the metabolic profile of the plant species (Deidda et al. 2015) (Table 3). It also provides a crucial information about the endogenous metabolites of plant species in response to numerous intrinsic and extrinsic perturbations such as environmental stresses, diseases, spatial-temporal changes, and comparative analysis between plant species (Clish 2015). Several metabolomics tools such as Metscape, VANTEB, Metaboanalyst, PathWhiz and Met PA are used to investigate the metabolic pathways of the target metabolites as well as their effect and roles in the underlying biological systems (Beale et al. 2018).

Table 3 Metabolomics approaches in different plant species

S. no.	Plant species	Approach	References
1	*Cannabis sativa*	NMR approach	Andre et al. (2016)
2	*Artemisia annua*	HPLC approach	Xie et al. (2016)
3	*Bidens pilosa*	LC-MS technique	Cortes-Rojas et al. (2016)
4	*Papaver somniferum*	LC-MS technique	De Filippis et al. (2016)
5	*Withania somnifera*	Reverse-phase HPLC	Agarwal et al. (2018)
6	*Vaccinium vitis-idaea*	UHPLC-MS/MS technique	Bujor et al. (2018)
7	*Carissa carandas*	HPLC-MS technique	Le et al. (2019)
8	*Severinia buxifolia*	HPLC approach	Truong (2019)
9	*Arthrocaulon macrostachyum*	UHPLC-ESI-MS/MS technique	ElNaker et al. (2020)
10	*Moringa oleifera*	HPLC-PDA-ESI/MS technique	Bennour et al. (2020)
11	*Vaccinium myrtillus*	HPLC-MS, LC-MS technique	Ziemlewska et al. (2021)

2.3.1 High-Performance Liquid Chromatography/High-Pressure Liquid Chromatography (HPLC)

HPLC is an analytical method for qualitative and quantitative analysis of the bioactive compounds present in the sample mixture (Ghanjaoui 2020). Molecules are being analyzed on the basis of retention time depending upon their interactions between the stationary phase and the solvent(s) used (Petrova and Sauer 2017). Singh et al. (2017a) studied the bioactive compounds of *Bergenia ciliata* and recorded the presence of bioactive compounds, such as gallic acid, catechin, and bergenin, used to cure kidney and gallbladder stones, ophthalmia, fever, and lung infections. Bergenin was recorded in all the plant extracts, whereas gallic acid and catechin were present only in methanolic extracts. Thus, methanolic extracts of this plant serve as a potent source of novel natural drugs. Similarly, Hodaei et al. (2021) studied the bioactive compound profiling and quantification of potent polyphenolic compounds in the Iranian *Chrysanthemum morifolium* flowers using the HPLC method. This medicinal plant has well-established antibacterial and antioxidant activities owing to its bioactive compounds, especially polyphenols. The HPLC analysis showed the presence of the following compounds: chlorogenic acid, gallic acid, ferulic acid, p-coumaric acid, quercetin, luteolin, rutin, and apigenin.

2.3.2 Liquid Chromatography-Mass Spectrometry (LC-MS)

LC-MS is an analytical technique that combines the separation capability of HPLC and mass analysis of mass spectrometry for identification and quantification of various components present in the sample (Pratima and Gadikar 2018).

Das et al. (2022) used the LC-MS method to identify the major bioactive compounds present in the traditional medicinal herb *Houttuynia cordata* with the aim of finding the potent inhibitor for SARS-CoV-2 replication proteins. LC-MS revealed the presence of 97 bioactive phytocompounds, among which the major were 6-hydroxyondansetron, quercitrin, canthaxanthi Nα-Acetyl-L-glutaminen 8-hydroxydesmethylondansetron and porphobilinogen. Dinore and Farooqui (2022) analyzed the bioactive phytocompounds of the methanolic leaf extract of *Cajanus cajan* using the LC-MS technique. The leaves of this plant has well-established pharmacological activities such as antimicrobial, anti-malarial, anti-ulcer, anticancer, and anti-hyperglycemic activities, owing to its bioactive compounds. The LC-MS investigation showed the presence of compounds in the leaves of this plant, namely, essential oils, flavonoids, fatty acids, alkaloids, selidin (coumarin), and tamarixetin (monomethoxyflavones).

2.3.3 Gas Chromatography-Mass Spectrometry (GC-MS)

GC-MS studies are used to determine the bioactive compounds, namely, glycosides, terpenoids, alkaloids, polyphenols, and flavonoids, from the entire medicinal and aromatic plants (Olivia et al. 2021). GC-MS is an analytical method that separates the chemical mixtures and detect the masses of diverse bioactive compounds present within a sample (Chauhan et al. 2014). In GC-MS analysis, gas chromatography differentiates and segregates the semi-volatile, volatile, and heat-resistant bioactive compounds in a sample, and mass spectroscopy fragments them to be identified on the basis of their mass/electrostatic charge (m/z) ratios (Chauhan 2014). El-Said et al. (2021) studied some wild-growing aromatic plant species, namely, *Juniperus procera*, *Lavandula pubescens*, and *Pulicaria incise* from the Arabian flora, with the aim of finding the natural sources of potent antimicrobial agents. They hydrodistilled the essential oils from the aerial parts of these aromatic plants, and they were subsequently analyzed by the GC-MS method. The GC-MS analysis showed the presence of potent bioactive compounds in *Lavandula pubescens*, namely, β-bisabolene, carvacrol, and methyl carvacrol. The major constituents in the essential oil of *Pulicaria incisa* were found to be chrysanthenone, linalool, cis-chrysanthenol, and eugenol, and in *Juniperus procera*, they were alpha-pinene and beta-3-carene. The authors concluded that further research in the field of mode of action of these bioactive compounds of aromatic plants would provide a potent natural source of antimicrobial agents. Vijaya et al. (2022) carried out the GC-MS analysis of the methanolic leaf extracts of *Costus igneus* in order to find the natural plant-based antihyperglycemic agent. *Costus igneus*, commonly known as insulin plant, has been in use as folklore medicine traditionally for the treatment of diabetes. GC-MS screening indicated the presence of 21 compounds.

2.3.4 Nuclear Magnetic Resonance (NMR) Spectroscopy

The metabolic profiling and structural elucidation of natural bioactive compounds can be obtained by using NMR spectroscopy. While this spectroscopy offers numerous advantages over other spectroscopic techniques such as easy sample recovery, non-destructiveness, and quantification without utilizing the internal standards, it is less sensitive than other spectroscopic techniques like LC-MS and GC-MS (Kumar 2016). NMR spectroscopy focuses more on the physical characteristics of the bioactive compounds, such as the number and arrangement of carbon atoms and the presence of protons, carbon isotopes, and hydrogen atoms. Additionally, it detailed the arrangement of atoms in a molecule (Abubakar and Haque 2020). Mohotti et al. (2020) used NMR spectroscopy to elucidate the structure of isolated antimicrobial flavonoids from *Derris scandens*. Miran et al. (2020) isolated and characterized the phthalides from the roots of the herb *Levisticum officinale*. They used NMR spectroscopy to elucidate the structures of the phthalides and also discovered the new phthalide from the root of this plant, namely, 7-methoxy-3-propylidenephthalide. Medicinal and aromatic plants are gold mines of bioactive compounds for future potent herbal medicine. Mohammed et al. (2021) studied the metabolic profiling and structure elucidation of the aromatic herb *Pulicaria jaubertii* using NMR and MS spectroscopy and discovered around six bioactive compounds on the basis of NMR and MS analysis, namely, pseudo-taraxasterol, pseudo-taraxasterol acetate, 3β-acetoxytaraxaster-20-en-30-aldehyde, calenduladiol-3-*O*-palmitate, stigmasterol, and α-tocospiro B.

2.4 Proteomics

Medicinal plants have extensively served as the global foundation of herbalism and played a significant role in biomedical innovation. In spite of various efforts to screen plant-derived natural products, there is still great potential of plant elements that could be useful sources as therapeutic agents. Therefore, for the future identification of active molecules, an understanding of the metabolic networks in medicinal plants and their systemic regulation is essential. A proteomic strategy describes medicinal plant research to clarify plant metabolic pathways that produce bioactive chemicals (Hashiguchi et al. 2017). Proteomic methods have been utilized to study the synthesis of the bioactive chemicals that give medicinal plants their health-promoting characteristics and allow the evaluation of systemic changes occurring during cellular metabolism (Table 4). Isobaric tags for relative and absolute protein quantification (iTRAQ) in conjunction with RNA sequencing allowed the identification of four proteins from the CYP718A subfamily of enzymes that are crucial for triterpenoid saponin production in *Anemone flaccida* (Zhan et al. 2016). Proteomics has made possible the precise and improved protein identification for a variety of medicinal plants (Ma et al. 2017). The purification of proteins with low profusion using combinatorial peptide ligand libraries (CPLL) or polyethylene glycol

Table 4 Proteomics approaches in different plant species

S. no.	Plant species	Approach	References
1	*Pithecellobium dumosum*	SDS-PAGE and MALDI TOF/TOF MS analysis	Oliveira et al. (2007)
2	*Mesua ferrea*	HPTLC proteomics approach	Gupta and Chaphalkar (2015)
3	*Zingiber zerumbet*	UniProtKB analysis and bioinformatics approach	Mahadevan et al. (2015)
4	*Nigella sativa*	Bioinformatics databases	Alanazi et al. (2016)
5	*Pseudostellaria heterophylla*	(iTRAQ) MS/MS and bioinformatics approach	Hua et al. (2016)
6	*Bauhinia forficata*	Spectrophotometrically, SDS-PAGE analysis	Andreia et al. (2016)
7	*Chamaecostus cuspidatus*	MALDI TOF/TOF MS analysis	Hardikar et al. (2016)
8	*Taxus brevifolia*	MS/MS data sets approach	Hashiguchi et al. (2017)
9	*Pseudostellaria heterophylla*	Mass spectrometry and multidimensional liquid chromatography	Zaynab et al. (2018)
10	*Chrysobalanus icaco*	Liquid chromatography tandem mass spectrometry	Pedrete et al. (2019)
11	*Picrorhiza kurroa*	MALDI-TOF technique	Tyagi et al. (2021)

fractionation treatment has been proven to be successful for the proteomic investigation of secondary metabolism in medicinal plants. The bioactive substances including oridonin, a diterpenoid from *Rabdosia rubescens*; curdione, a sesquiterpenoid from *Curcuma aromatica*; and geniposide, an iridoid glycoside from *Gardenia jasminoides* have all been studied using proteomics. In a two-dimensional electrophoresis analysis, stathmin was observed to be a target of *Rabdosia rubescens*-derived oridonin in human hepatocarcinoma cells and also has implications in cancer therapy. Using gel-free proteomic techniques on curdione-treated human platelets, proteins involved in focal adhesion, such as talin 1 and beta-1 tubulin, were identified with regard to the antithrombotic actions of *Curcuma aromatica*, thus elucidating the molecular mechanism of action by *Gardenia jasminoides* (Zhang et al. 2017). Studies on protein-protein interactions in green cardamom have revealed that the PP1-HDAC2-p53 and ERK1/2-p53 pathways were involved in the bisabolene-induced mitochondrial apoptosis. This phosphoproteomic-based research is helpful for the development of new anticancer medications from γ-bisabolene (Al-Obaidi 2021). On comparing the metabolite variations in ginger collected from Ghana and China and correlating them with the proteins, it was observed that the metabolism-related enzymes are responsible for the variations present within the ginger species (Yin et al. 2018). Due to its numerous biological properties, fennel flower seed oil (also known as black seed) has potential anticancer and antidiabetic effects (Jan et al. 2019). Gel-free proteomics identified proteins connected to vanillin production, including ACC synthase, chalcone-flavanone

isomerase, and vanillin synthase, suggesting that another species from this *Vanilla* species may be a potential alternative source for the production of vanilla (Lopes et al. 2019). Contreras et al. (2019) observed more than 800 proteins with five novel cytochromes P450 (CYPs) and five novel alkaloids through proteomic analysis of hairy root materials that were used to study the changes in proteins during the cultivation of red sage hairy roots. These proteins are thought to be responsible for the synthesis of tanshinones, which have antioxidant and anti-inflammatory properties. Another proteomic study performed by Mahadevan et al. (2015) on *Zingiber zerumbet* plant revealed proteins from the barley stripe mosaic virus together with the plant proteins, which confirms the infection of the plant and observed various proteins that are involved in changes related to defense, developmental processes, and secondary metabolite enzymes. Moreover, it demonstrates high degree of tolerance to diseases affecting cultivated ginger. Datta et al. (2018) observed 21 differentially expressed proteins from the leaf membrane, including membrane trafficking proteins like ADP ribosylation factor and channels and receptor proteins like potassium channels and receptors with an AKT1-like structure in plant-disease interaction. One-third of those proteins were found to be connected to defense against fungal infection. Proteomics helps to discover the enzymes involved in drug metabolism in addition to its elaboration on the physiological alterations that are systematic in medicinal plant research. The widely used herb *Centella asiatica*, which possesses saponins as its active ingredients, has gained notoriety in Asian nations (Gray et al. 2017). Gonulalan et al. (2020) observed two cytochrome P450 family proteins, P450 71B11 and P450 94B3, that are positively correlated with brain-derived neurotrophic factor (BDNF) activity. Proteomics investigations enabled highly sensitive detection of several components even at extremely low quantities. The whole metabolite and proteome pool of the four distinct plants was identified, and the metabolite's and protein's positive and negative correlations with BDNF expression were established. Georgiadou et al. (2018) reported the impact of three heavy metals such as nickel (Ni), copper (Cu), and zinc (Zn) on basil plant. The allergenic protein profiling was more concentrated after treatment with Cu, although the concentration of total proteins (presumably as a result of proteolysis) and antioxidant capacity decreased with increasing Cu and Zn concentrations. It was also observed that under extreme Cu stress, particular proteins involved in transpiration and photosynthetic activities accumulated. Based on these results, Ni stress in basil plants was observed to be harmful with lower potential for allergens than Cu and Zn stress, while Cu-stressed basil plants showed the worst consequences and produce the most allergens.

3 Conclusion

Medicinal and aromatic plants are repositories of numerous potent nutraceutical and pharmaceutical bioactive compounds. They have unexplored potential for use in the discovery of novel natural plant-based drugs. Moreover, bioreactor and genetic engineering methods are the coherent ways for the mass production of these

compounds for industrial use. However, for the discovery of novel plant-based drugs and their mass production, complete insight knowledge of the biosynthetic pathways of these bioactive compounds and their underlying regulatory mechanism is required. The emerging omics technologies including genomics, transcriptomics, proteomics, and metabolomics and their integration provide a relevant knowledge to elucidate the biosynthesis pathways of these secondary metabolites. The integration of multiomics data and their analysis requires the development of advanced bioinformatics tools and software. Nowadays, the large biological data extracted from the multiomics technologies helps to discover the metabolites, metabolic pathways and their enzymes, gene and gene networks, and proteins and their interactions.

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Advances in Medicinal Plant Improvement in Perspective to the Changing Environment

Mohammad Javad Ahmadi-Lahijani, Faegheh Jangjoo, and Saeed Moori

Abstract Global climate change has altered the natural ecosystems, and it is expected to worsen the conditions for plant growth and productivity. Elevated CO_2 and temperature impose diverse direct and indirect effects on plant species. Different biotic and abiotic stresses might adversely affect the plants. Medicinal plant species may also be threatened by changing temperature and precipitation regimes, increases in pest and pathogen populations, and natural habitat alteration. The changing environment, poverty and the loss of traditions, easy access to medicinal plant habitats, lack of sufficient knowledge about the amount and methods of sustainable harvesting of medicinal plants, the existence of a profitable trade market, and the lack of legal policies are among the effective factors in the excessive exploitation of medicinal plants and reduction of their genetic diversity. Genetics and crop improvement are technologies that can contribute to plant adaptation to the changing environment. The goals of crop improvement are to reduce adverse environmental effects, preserve heritage resources, and increase the quality and quantity of medicinal plants. It is necessary to identify the needs of each species and the degree of compatibility against negative environmental factors. Recent advances in the field of plant genetics and breeding have helped to strengthen research in crop improvement studies. However, how to breed medicinal plants to adapt to the changing climate needs more investigation. In this chapter, recent advances in medicinal plant improvement in perspective to the changing environment and environmental challenges are discussed.

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N. Kumar, R. S. Singh (eds.), *Biosynthesis of Bioactive Compounds in Medicinal and Aromatic Plants*, Food Bioactive Ingredients,
https://doi.org/10.1007/978-3-031-35221-8_3

Keywords Abiotic stresses · Crop improvement · Medicinal plant breeding · Secondary metabolites · Selective breeding method

1 Introduction

Environmental changes have negatively affected agricultural production and natural ecosystems (Arunanondchai et al. 2018). Two major problems in the twenty-first century are global climate change and food insecurity. Climate change is characterized as the change in climate (long term) resulting from human activities such as ozone layer destruction and greenhouse gas emissions and natural processes (Kotir 2011). Climate changes include atmospheric CO_2 elevation, temperature increase, and precipitation pattern variations, leading to floods, drought, and various biotic and abiotic stresses (Ziska 2016). The intensity of global warming is rising gently and has disrupted the ecosystem. The greatest disturbance in the global ecosystem is still related to human activities. In 2022, the global average temperature, obtained from key monitoring stations data, was estimated to be ~1.15 °C above the average of the second half of the nineteenth century, and 2015 to 2022 were probably the eight warmest years on record (Pörtner et al. 2022).

Global warming has an important role in the challenges facing the world and threatens future development planning, global food security, prosperity, economics, water resources, and human health worldwide (Tripathi et al. 2016). Agricultural ecosystems are affected by global climate changes that can affect all aspects of agriculture such as biodiversity, productivity, and food security (Allmendinger 2018). These effects are directly related to global warming, which means that extreme changes in weather affect all regions of the world. Climate change affects agricultural systems directly (morphology, physiology, phenology, crop production, and adaptability), indirectly (soil fertility, biotic and abiotic stresses, sea level rise), and socioeconomically (food value, food security, and food trade) (Raza et al. 2019). Climate changes alter several levels of biology such as physiology, morphology, and genetic diversity, populations, species (size and location, habitat quality and quantity), communities (productivity, biomass, species relationships), and ecosystems (functions and processes) (Scheffers et al. 2016).

Medicinal plants are valuable to human life and a main portion of health care for many populations worldwide. In developing countries, the primary medicine materials for ~80% of people are supplied by medicinal plants, and they are increasingly utilized by many people in developed countries (Robinson and Zhang 2011). Worldwide climate changes have negatively affected medicinal plants and may impact the accessibility, productivity, and phytochemical content of medicinal plant populations. Environmental stresses reduce biomass production of some medicinal species and may change chemical content and affect the quality of medicinal products (Fig. 1). Therefore, conservation and local cultivation of valued medicinal plants, sustainable harvesting, preservation of traditional knowledge, programs to

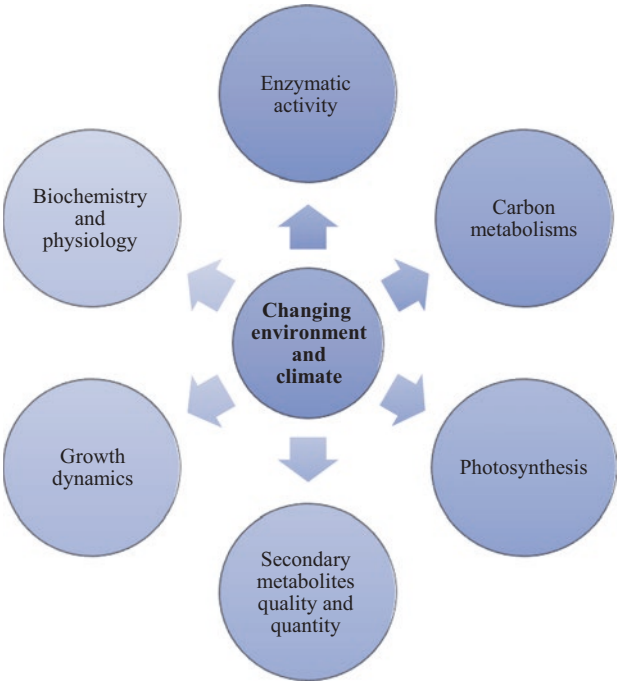

Fig. 1 Effects of changing environment on growth, physiology, and biochemistry of medicinal and aromatic plants

monitor raw material quality, and crop improvement and breeding to mitigate environmental change effects are recommended.

Plant breeding is the function of developing various plant varieties that can effectively help cropping and production systems. Population growth increases the need for food supply, and to feed the population, breeding is an ever-serious issue. Creating germplasm to solve obstacles in agriculture and enhancing value for producers, consumers, and society are the main goals of plant breeding programs (Kholová et al. 2021). Therefore, plant breeders endeavor to increase plant productivity and quality and diminish negative environmental impacts on plants. Plant breeding is important to cope with the effects of climate change and supplement crop management and policy interventions to ensure global food security (Xiong et al. 2022). Climate change has altered the breeding objectives' priority; for example, resilience breeding was put at the top priority (Langridge et al. 2021). With progressed knowledge of the gene and the development of technologies such as gene editing, plant breeders still face challenges due to limited genetic grades and indistinct climates across the globe (Zabel et al. 2021).

It is predicted that plant species will become more sensitive to pathogens and pests under climate changes, leading to a decrease in their quantity and quality and food security (Van der Fels-Klerx et al. 2016). The ecosystems will eventually be disrupted due to extreme weather changes and the increased frequency of global

warming. Therefore, improving plant species, here medicinal plants, to environmental changes and global climate changes can lead to mitigating the potential adverse effects on the productivity of plants. In this chapter, recent advances in medicinal plant breeding, with a glance at the changing climatic conditions, are discussed.

2 Effects of Climate Change on Medicinal Plants

Many ecological aspects such as temperature, nutrition, light, water availability, and CO_2 concentrations increase the secondary metabolites (SMs) such as total flavonoids and phenolic content in medicinal plants (Clark and Menary 1980). SMs are bioactive compounds and might be altered by environmental stimuli. Unfavorable environmental factors affect the growth and primary metabolites of medicinal plants, which in turn affects the biosynthesis of SMs.

The SMs of medicinal plants show a wide range of adaptations to changing environments (Mishra 2016). Elevated CO_2 is predicted to alter the plant carbon/nitrogen ratio in carbon-based secondary metabolites (Heyworth et al. 1998). For example, phenolic compounds, hypericin, pseudohypericin, and hyperforin of *Hypericum perforatum* were increased at elevated CO_2 (Zobayed and Saxena 2004). The alkaloid concentrations of *Papaver setigerum* were also enhanced by raising the CO_2 concentration (Ziska et al. 2008). However, a decrease in SMs was also observed at elevated CO_2. Snow et al. (2003) observed that elevated CO_2 decreased *Pseudotsuga menziesii* terpenes, specifically monoterpenes. Time of exposure to elevated CO_2 is also a determinant factor affecting SMs. Working on *Hymenocallis littoralis*, a bulbous medicinal plant, Idso et al. (2000) observed that exposure of plants to the elevated CO_2 enhanced the alkaloids only in the first year but they decreased in the year after.

Drought is the most limiting factor of plant productivity worldwide and an undeniable climate change consequence. Drought and water scarcity adversely affect crop yield. Plant growth is practically influenced by drought; nevertheless, it depends on the growth stage and the duration, intensity, and severity of the stress (Zarghami Moghadam et al. 2021). Drought stress causes stomatal closure, limits gas exchanges, and inhibits metabolism and photosynthetic rate in plants, which subsequently leads to plant death (Vazquez and Dunford 2005). Drought stress induces different plant structural changes including morphological (root and shoot shape and growth), physiological (gas exchange variables), and biochemical (metabolic) alterations to cope with the stressful conditions. However, the duration and intensity of drought, plant species, and growth stage affect the plant's durability and its survival ability in stressful environments (Vazquez and Dunford 2005). The growth, essential oil, and proline content of *Calendula officinalis* L. plants were increased under drought conditions resulting from the increased plant height, leaf area, flower diameter, and spike stem diameter (Metwally et al. 2013). Fresh and dry

weights of *Ocimum* sp. (Khalid 2006) and *Satureja hortensis* L. (Baher et al. 2002) plants were diminished by water deficit.

Seed germination behaviors are affected by genetic factors, climate change scenarios, and ecological parameters. One of the most crucial limiting factors for germination and plant establishment is the temperature (de Souza and Válio 2001). However, based on the climate change scenarios, the world temperature is rising gradually and is expected to increase by ~2 °C by 2050. This temperature increase would negatively affect all plant species in various ways. Working on *Catharanthus roseus* and *Mentha piperita* medicinal plants, Alhaithloul et al. (2019) found that a combination of drought and heat decreased total phenol, flavonoid, and saponin contents, plant height, fresh weight, and dry matter, while tannins, alkaloids, and terpenoids were increased. They concluded that the antimicrobial and anticancer activities of plants were significantly reduced when exposed to drought and heat stress.

3 Medicinal Plant Breeding: Importance and Challenges

The effects of climate change and its critical implications for food security are forcing plant breeders to act quickly, necessitating the improvement of plant species in a shorter time, which is a challenge for current "slow but successful" breeding efforts (Challinor et al. 2016). In addition to adapting to new abiotic and biotic stresses, improved crops must meet other urgent needs arising from climate change such as agricultural migration to new areas and crop management to achieve climate mitigation such as reduced fertilizer utilization, tillage, and other changes in planting practices (Henry 2020; Heredia et al. 2021).

Medicinal plants with a large number of plant species and diverse biological characteristics have a smaller cultivated area compared to other crop plants, and due to the high cost, a limited capacity for breeding investigation is considered (Pank 2009). Breeding of medicinal plants is more complex than other crop species breeding because the production, growth cycle, medicinal parts, and SMs of the plants must all be taken into consideration. Quality determines the value of medicinal plant production, which makes their breeding different in particularity. The term "top-geoherbs" is determined as medicinal substances with demonstrated higher quality produced in particular geographical zones, which has been suggested based on the relationship between the habitat of medicinal plants and their yield quality (Huang et al. 2011).

A major prerequisite for medicinal plant cultivation is the availability of germplasms with high germination rates, uniform germination, high yield quality, and economic value. While crops such as wheat, maize, rice, potato, tomato, and soybean are mainly bred for high yield, medicinal plants should be bred for stable yield and high quality (Wang et al. 2020). Environmental stresses such as extreme temperatures, drought, and salinity inhibit the growth and development of plant species

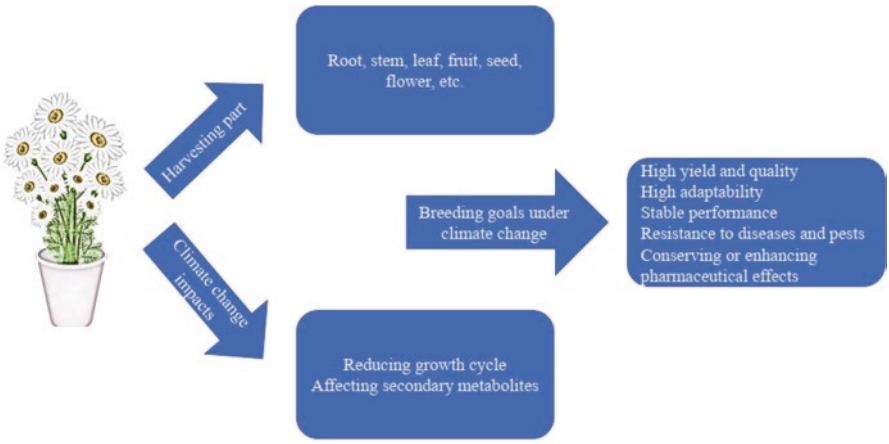

Fig. 2 Medicinal plant breeding goals under a changing climate

and might stimulate the accumulation of SMs such as alkaloids and flavonoids in medicinal plants (Huang and Guo 2007), indicating the differences between medicinal and crop plant breeding purposes (Fig. 2).

4 Recent Advances in Medicinal Plant Improvement

Medicinal and aromatic plants play vital roles in human life. Plants' biochemical and metabolic processes are genetically controlled. Controlled genetic diversity to improve performance and create environmental stability can play a role in fulfilling human goals. The creation of new diversity can occur by methods such as selective breeding, hybrid breeding, mutation breeding, tissue culture, polyploidy breeding, DNA marker-assisted breeding, and transgenic breeding (Table 1). Among the criteria that are followed in medicinal plant breeding, high adaptability and stable performance, resistance to diseases and insects, high functional value, and safety, and saving cost and sustainable production can be mentioned. In addition, drought and salt stress resistance, low input cultivation, high SM amount for economic extraction, low content of harmful metabolites to avoid heavy costs to remove them, and high dry matter content can also be considered (Ahmadi and Shabani 2020).

In general, the improvement of medicinal plants is carried out in two stages:

1. Research stage: At this stage, there is no knowledge of the ecological requirements of the medicinal plant; therefore, the plant is transferred to experimental plots in various sexual and asexual ways, and all factors such as appropriate soil type, planting depth, irrigation cycle, within- and in-row distance, and soil fertility are available to the plant as much as possible.

Table 1 Recent advances in medicinal plant species improvement

Medicinal plant species	Common name	Breeding method		Results	Climatic conditions	Reference
Pseudostellaria heterophylla (Miq.) Pax.	False starwort	Selective breeding		Higher polysaccharide content, root yield, and disease and lodging resistance		Xiao et al. (2016)
Trigonella foenum-graecum L.	Fenugreek			High ecological temperature range for germination, adaptation, and eco-evolutionary, rapid seedling growth, lower risks of low productivity and extinction	Temperature	Solouki et al. (2022)
Panax ginseng	Ginseng		Pure-line selection	Disease resistance, root shape, and root yield		Yang et al. (2006)
Coriandrum sativum L.	Coriander			High capacity of essential oil and fruit yield	Drought	Gholizadeh et al. (2019)
Bupleurum chinense DC	Bupleurum			Saikosaponin content, agronomic and morphological characteristics		Zheng et al. (2010)
Schizonepeta tenuifolia	Hairy sage			High quality and production, high active constituent levels, disease resistance		Cao et al. (2009)
Trigonella foenum-graecum L.	Fenugreek			Higher branch number/plant and 1000 seeds Weight, survival rate, and yield	Freezing stress	Mirmiran et al. (2021)
Calendula officinalis	Pot marigold			Higher flower numbers and proline content	Drought stress	Zarghami Moghadam et al. (2021)

(continued)

Table 1 (continued)

Medicinal plant species	Common name	Breeding method		Results	Climatic conditions	Reference
Gastrodia elata Bl.	Tianma	Hybrid breeding	Reciprocal crossbreeding	New hybrid lines with stable and high yields		Wang and Guo (2001)
Gastrodia uralensis	Tianma		Crossbreeding	Vigorous growth and high glycyrrhizin and total flavonoid contents		Ozaki and Shibano (2014)
Fritillaria lichuanensis P.	Checkered lily			Higher seed-setting rate, higher germination rate, and enhanced disease resistance		Ruan et al. (2004)
Platycodon grandiflorus				A low number of lateral roots and processing suitability, high saponin content and extraction suitability, and low crude fiber content, edibility, and medicinal suitability		Wei et al. (2011)
Rehmannia glutinosa (Gaertn.)	Rehmannia	Mutation breeding	Interspecific hybridization	Higher seed-setting rate of the progeny		Li et al. (2012)
Isatis indigotica fortune	Woad		Microwave radiation	Enhanced germination rate, shortened germination time, and accelerated plant growth		Chen (2005)
Chamaecrista rotundifolia (Pers.) Greene	Round-leaf cassia		Gamma rays	Squaring period, flowering period, pod stage, and mature stage		Weng et al. (2004)
Catharanthus roseus (L.) G. Don	Madagascar periwinkle		Ethyl methane sulfonate (EMS)	Rapid growth and high indole alkaline content		Xiusheng et al. (2004)
Celosia cristata L.	Cock's comb	Space mutation	High-altitude balloon	Enhanced total flavonol content of inflorescences		Debao et al. (2002)
Dendrobium nobile	Noble dendrobium		Satellite launched	Higher alkaloid and polysaccharide contents		Peng and Ye (2017)
Glycyrrhiza uralensis	Chinese liquorice		Spaceflight	Higher liquiritin and glycyrrhizic acid contents in the seeds		Zhang et al. (2011)

Echinacea purpurea	Coneflower	Tissue culture	Plant growth hormone	Enhanced transplanted seedling survival		Wang et al. (2005)
Pogostemon cablin (Blanco) Benth.	Patchouli	Polyploid breeding		Increased stem thickness and the size of leaves and stomata, higher patchouli alcohol content		Yan et al. (2016)
Stevia rebaudiana (Bertoni) Hemsl.	Candyleaf			Higher stevioside content		Xu et al. (2014)
Citrus limonia Osb.	Canton lemon			Higher resistance to hydric stress, improved membrane stability	Water deficit	Vieira et al. (2016)
Dioscorea zingiberensis	Yam			Lower electrolyte leakage, malondialdehyde contents, superoxide anions, and hydrogen peroxide, stronger antioxidant defense system, and increased heat tolerance	Heat	Zhang et al. (2010)
Citrus reticulata	Mandarin			Higher antioxidant enzymes and heat shock proteins	Salt stress	Podda et al. (2013)
Panax notoginseng	Notoginseng	DNA marker-assisted breeding		Reduced root rot and rust rot in seedlings		Dong et al. (2017)
Perilla frutescens (L.) Britt.	Beefsteak plant	Transgenic breeding		High yield and high resistance		Shen et al. (2017)
Papaver somniferum L.	Opium poppy			High morphinan alkaloid content		Frick et al. (2005)
Atractylodes macrocephala Koidz.	Bai Zhu		Gene gun-mediated method	Disease resistant		Mao et al. (1994)
Pogostemon cablin (Blanco) Benth.	Patchouli		*Agrobacterium*-mediated transformation	High disease resistance		Zhang et al. (1997)

2. Planting stage: After conducting research, the best method of planting with the highest yield (quantitative and qualitative) is obtained. When reaching this basic information, the plant has been domesticated (Afkar and Karimzade 2009).

Although crops have been bred for high yield with good quality and adaptability under certain cultivation conditions, medicinal plants are mainly cultivated using wild types or local varieties without any selection or screening (Wang et al. 2020). For instance, *Rosmarinus officinalis* Linn is widely used for ornamental and culinary purposes and has many antibacterial and anti-inflammatory effects, while no cultivars of which have been specifically selected or bred for medicinal purposes (Begum et al. 2013).

Typically, it is expected that cultivated and domesticated medicinal plants be different from their wild-type ancestors owing to the wild-type species usually growing in relatively harsh environments. For example, cumin (*Cuminum cyminum*), which originated in Western Asia including Iran, grows naturally in mountain areas with harsh climates, and it may affect the pharmaceutical effects of the plants. The rhizomes of wild *Pinellia ternata* (Thunb.) Breit. with antitussive and expectorant effects are superior to those of cultivated varieties (Gao et al. 2010). However, cultivation does not always alter the genetic factors of these plants compared with the wild types (Wang et al. 2020). Recent studies showed that some cultivated medicinal species had higher levels of active ingredients such as volatile oil in *Atractylodes lancea* (Thunb.) DC (Huang et al. 1990) and matrine in *Euchresta japonica* Hook. f. ex Regel (Yang et al. 2006) than those found in the wild counterparts.

The most important breeding method in the early stages of medicinal plant breeding has been the screening of the varieties with better performance from populations. For successful breeding using the selection method, the traits for which changes are desired should be defined clearly. Mass selection from resources of *Pseudostellaria heterophylla* (Miq.) Pax. from different areas in China helped to breed a new variety (Shitai No. 1) with high lodging and disease resistance, root yield, and polysaccharide content (Xiao et al. 2016). Solouki et al. (2022) found that the Mashhad ecotype at 39.7 °C showed the highest ecological temperature range (TR) for germination among eight ecotypes. Furthermore, 11 cultivars of Korean ginseng have been selected using the pure-line-selection method with improved root yield, root shape, and disease resistance (Yang et al. 2017).

Zarghami Moghadam et al. (2021) evaluated 13 calendula cultivars (*Calendula officinalis*) under drought stress. They found that drought stress reduced the traits attributed to flowers such as diameter and flower number while proline content was increased; however, the cultivars differed in their response to the stressful conditions. Although most of the flower numbers of the cultivars (11 out of 13) were decreased by drought stress, Citrus Cocktail and Oopsy Daisy cultivars showed higher flower numbers at 50% of field capacity (FC) than their respective under 100% FC. Only four cultivars of calendula showed a significant increase in proline content exposed to drought stress. They ultimately selected premium cultivars Neon and Candyman as the most resistant to water stress.

Seed germination and plant establishment are the most important stages in a plant life cycle; however, poor germination and plantlet establishment limit crop yield (Windauer et al. 2007). The final yield of crops can be determined by faster seed germination and desirable plant establishment (Bybordi and Tabatabaei 2009). Solouki et al. (2022) quantified cardinal temperatures of eight fenugreek (*Trigonella foenum-graecum* L.) ecotypes at nine constant temperature levels (0 °C, 5 °C, 10 °C, 15 °C, 20 °C, 25 °C, 30 °C, 35 °C, and 40 °C) by nonlinear regression models. They identified Intersected-lines and Dent-like models as the best explainable models for different fenugreek ecotypes. By increasing temperature to optimum, the germination rate and reciprocal time to median germination (R_{50}) increased. The Mashhad ecotype at 39.7 °C showed the highest ecological temperature range (TR) for germination. The adaptation and eco-evolutionary of the Mashhad ecotype for higher germination result in rapid seedling growth and avoid the risks of low productivity and extinction in the presence of abiotic stresses caused by climate change.

In hybrid breeding, the good traits of two or more varieties are combined through hybridization, where one of the most important steps in the breeding program is parent selection. The progeny shows superior performance compared with that of either parent (Fridman 2015). Despite promising results, considerable work is necessary to produce new varieties via hybrid breeding. Using reciprocal crossbreeding, four new hybrid lines of *Gastrodia elata* Bl. with stable and high yields were selected (Wang et al. 2020). The crossbreeding of a strain with vigorous growth as the father and a strain with high glycyrrhizin content as the mother resulted in releasing a new strain of *Gastrodia uralensis* (C-2) with vigorous growth and high glycyrrhizin and total flavonoid contents (Ozaki and Shibano 2014). Distant hybridization is sometimes preferred to common hybridization since it might result in varieties with more obvious heterosis. In a study on *Rehmannia glutinosa*, it was observed that interspecific hybridization of cultivated and wild types led to a higher seed-setting rate of the progeny (Li et al. 2012). Therefore, interspecific hybridization is sometimes more advantageous than intraspecific hybridization. However, the opposite results were also obtained (Wang et al. 2012; Wang et al. 2020).

Mutations induced in organisms via physical, chemical, or space processes are called mutation breeding; among those, physical mutagenesis has become one of the most effective means of obtaining new germplasm resources (Wang et al. 2020). For instance, using a CO_2 laser, a new variety (Si Jiyi 78–1) of *Coix lacryma-jobi* L. was obtained with larger seeds, greater tiller number, and dwarf form (Qiao and Cui 1981). However, some species may show significant sensitivities to radiation, or unpredictable outcome may be obtained. It was observed that increasing the intensity of gamma radiation had inhibitory effects on plant growth, especially reductions in germination rate, flowering, and fertility rate in *Melilotus officinalis* (L.) Pall., *Melilotus dentatus*, *Melilotus albus*, and *I. indigotica* (Wang et al. 2006). In *Chamaecrista rotundifolia* (Pers.) Greene under gamma rays, five varieties with various squaring periods, flowering periods, pod stages, and maturation stages were obtained (Weng et al. 2004). Chemical mutagenesis has rarely been applied in medicinal plant improvement and has usually been applied in combination with tissue culture. For example, exposure of *Catharanthus roseus* (L.) G. Don and

Lavandula angustifolia Mill. to ethyl methane sulfonate (EMS) resulted in mutant cell lines with rapid growth, higher indole alkaloid content, and enriched 1,8-cineole and borneol (Desautels et al. 2009).

Induced mutation in germplasm materials exposed to near-space physical and chemical factors for breeding of new varieties is referred to as space mutation, which is carried by return satellites and high-altitude balloons, or in high-altitude simulation tests (Yan and Lei 2002). Space breeding of two *Celosia cristata* L. varieties with a high-altitude balloon enhanced the total flavonol content of inflorescences (Debao et al. 2002). In another study, the satellite-launched *Dendrobium nobile* obtained significantly higher alkaloid and polysaccharide contents than the earth-grown plants (Peng and Ye 2017).

Polyploidy is another plant breeding method used in medicinal plant improvement and is reported to increase plant environmental stress tolerance (Zhang et al. 2002; Zhang et al. 2010). In polyploid breeding, it is tried to obtain materials through chromosome doubling to improve varieties to meet customer demands in a certain environment. It leads to obtaining polyploid plants with larger vegetative organs and higher contents of active ingredients than those of typical plants (Niazian and Nalousi 2020). Zhang et al. (2010) observed that tetraploid plants of *Dioscorea zingiberensis* showed lower electrolyte leakage and contents of malondialdehyde, superoxide anions, and hydrogen peroxide and stimulated antioxidant enzyme activities. They concluded that tetraploid plants had a stronger antioxidant defense system and increased heat tolerance. New polyploid varieties of *Citrus limonia* Osb. with higher resistance to water deficit stress have also been bred (Vieira et al. 2016).

DNA marker-assisted breeding has also been considered as a method to improve crops as well as medicinal species. This method of breeding can contribute to the breeding of new varieties with high yield, high quality, and high resistance (Dong et al. 2017). Since traditional breeding methods are time-consuming and a long-term endeavor, molecular marker-assisted breeding can accelerate and facilitate the breeding of medicinal plants; however, a few reports on the marker-assisted breeding of medicinal species have been released. In a study on *Panax notoginseng* (Burkill), DNA marker-assisted selection and systematic breeding led to introduce a new variety with reduced seedling root rot and rust rot (Dong et al. 2017). A new variety with high yield and resistance was also bred in *Perilla frutescens* (L.) Britt. using marker-assisted methods (Shen et al. 2017).

5 Conclusions

Medicinal plants have many benefits for humans, and their cash values are mainly higher than other crops. Nevertheless, less research, development, and progress of breeding programs have been conducted on medicinal plants due to limited knowledge about their genetic background, growth cycle, and heterozygosity. Besides, the diversity and various ecological habitats of medicinal species and the growth of species in a changing environment have made their breeding more complex.

Although many crops have been selected and bred for high yield, adaptability, improved stress resistance, efficiency, and productivity, to date fewer medicinal species have been bred successfully. Crop improvement programs for the fluctuating environment and climatic conditions should carry out toward introducing more resistant varieties to environmental stresses with higher productivity, yield quality, and quantity.

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Applications of CRISPR/Cas9 in the Synthesis of Bioactive Compounds from Medicinal Plants

Mridul Jamwal, Bhawna Ghora, Saajan Kumar, and Ajai Prakash Gupta

Abstract Due to the high advantages and ease of operation, the clustered regularly interspaced short palindromic repeats (CRISPR)/Cas gene editing technology has opened a new era of genome engineering. With the help of this technology, several plant species were used to select site-directed editing of genes. But in the case of medicinal plants, the application of CRISPR/Cas technology is still in the early stages of development. In this chapter, we assess the research history of the CRISPR/Cas system and its working mechanism and discuss their application in medicinal plants. Further, we assess the CRISPR/Cas technology for editing genes in medicinal plants as a gene editing method for the synthesis of bioactive compounds in medicinal plants. The main focus is to allow a direction for the implementation of this technology in the study of genome function, genetic improvement, synthetic biology, and innovation of germplasm in medicinal plants. The CRISPR/Cas technology helps to improve the medicinal properties and agricultural values of plants.

Keywords CRISPR/Cas9 system · Gene editing technology · Bioactive compounds · Medicinal plants

1 Introduction

In earlier times, when gene editing technology emerged, there were many problems in which one of the problems is target gene random integration into the receptor of the genome; this leads to poor identification, and also there are other problems like gene silencing and surprising variations. So the technique in which targeted genes are edited and this technology promptly modifies the details of the locus in the genome helps to attain selected deletion of the gene, insertion of a gene, or replacement of a gene and decreases the effect on the receptor genome. With site-specific

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N. Kumar, R. S. Singh (eds.), *Biosynthesis of Bioactive Compounds in Medicinal and Aromatic Plants*, Food Bioactive Ingredients,
https://doi.org/10.1007/978-3-031-35221-8_4

nucleases genome editing, several modifications are to be done in the genome like the reverse genetics, genetic engineering, and targeted transgene integration; this research is to be carried out in a very efficient and precise manner. Gene editing technology includes the addition of DNA double-strand breaks (DSBs) that act as a target to which an engineered nuclease is used; this stimulates the cellular DNA repair mechanisms in the cells. Basically, the CRISPR/Cas9 genome editing system is discovered to study the defense mechanisms of some bacterial species. CRISPR/Cas9 stands for clustered regularly interspaced short palindromic repeats/CRISPR-associated protein 9, which is the third-generation technique usually using an RNA-guided endonuclease technology.

In this technique, the system uses short RNA sequences to proceed with the degradation of foreign nucleic acids. In the case of bacteria and archaea, this system uses genetically encoded RNA to direct the cleavage of foreign DNA by a CRISPR-associated (Cas) nuclease, which is an endogenous sequence-specific nuclease system; that is, it has the property of cleavage. CRISPR loci are short spacers that are variable in nature separated by small repeats further transcribed into the type of RNA, i.e., non-coding RNAs. In this system, the Cas9 enzyme has RNA-guided endonuclease activity; it guides the single-guide RNA, i.e., sgRNA, to identify a targeted site and produces a DSB, i.e., double-strand break. When the double-strand breaks are produced, there is the production of modified DNA basically in two ways: one is non-homologous end joining (NHEJ), resulting in the insertions of nucleotides, deletions of nucleotides, and substitutions of nucleotides in the genome, and the other way is homologous recombination (HR); this is performed if homologous donor templates are present in the genome; it facilitates the insertion of DNA fragments.

CRISPR/Cas9 is an effective genome editing technique that results in complete and persistent loss of gene function. This technique helps in the healing studies of many organisms, especially plants. In the light of the CRISPR/Cas9 genome editing system, it made a great contribution to plant breeding, and this system has pioneered the production of efficient products. Studies in plant species are generally focused on improving desirable properties, increasing yield, and developing resistance to various stress factors. The CRISPR/Cas9 technology helps in the synthesis of bioactive compounds.

The bioactive compounds are developed by plants as secondary metabolites that have some pharmacological and toxicological activities in humans and animals. In plants, these bioactive compounds are produced to help plants survive, which is mainly aimed at plant growth and development. These compounds are not needed for plants in daily use but are functional during stress conditions of plants.

In plants, bioactive compounds include alkaloids, i.e., compounds containing nitrogen, terpenoids natural products that are basically available in plant essential oils, coumarins, i.e., they are mainly antiviral in nature, flavonoids, i.e., they are polyphenolic in nature, compounds that contain nitrogen, organosulfur compounds that contain sulfur bonded to carbon atoms, phenolics, etc. These bioactive compounds exhibit activities such as anti-inflammatory, i.e., pain relieving and reduced swelling properties, and immunostimulatory, i.e., immunity stimulating, antimicrobial, anticancer, antioxidant, etc.

1.1 Biologically Active Compounds

The word "bioactive" consists of two words: "bio," which means life, and "active," which means dynamic or involved in an activity (Bernard and Dromard 2011). It is also called "biologically active" (Cammack et al. 2006). Consequently, a bioactive compound simply possesses a substance that consists of some biological activity in it (Guaadaoui et al. 2014).

Bioactive compounds can be categorized into essential and non-essential in which essential compounds include compounds that are produced regularly and are needed for the growth and development of plants, called primary metabolites, and non-essential compounds include compounds that are not usually active under normal circumstances but are produced during some instances, usually called secondary metabolites (e.g., polyphenols, alkaloids, flavonoids, etc.). These bioactive compounds exist in nature; they are the contributors to the food chain, i.e., exhibit a part of it, and can also affect the health of humans (Biesalski et al. 2009).

These bioactive compounds may be derived from different natural resources; they may be plants, animals, microorganisms (e.g., fungi, etc.), or marine organisms (e.g., lichens, etc.) (Swamy and Akhtar 2019). The quantity of these biologically active compounds is naturally low in its natural product (Patel et al. 2019). These bioactive compounds are found in plant matrix. These biologically active compounds are produced in very small amounts, and the concentrations are different in different plant organs or parts such as leaves, roots, stems, fruits, and the whole plant. For the isolation of compounds, there may be further processes required after extraction to purify desired compounds (Fotsing Yannick Stéphane et al. 2022).

1.2 History of Gene Engineering

The technologies that help in the editing of genome consist of three primary site-specific nucleases (SSN) systems. These technologies are as follows:

- The zinc-finger nucleases (ZFNs) technology.
- The transcription activator-like effector nucleases (TALENs) technology.
- The other most recent one which is the clustered regularly interspaced short palindromic repeats/CRISPR-associated protein 9 (CRISPR/Cas9) system technology.

To edit plant genomes, ZFNs were the first-generation SSN system that was in progress (Lloyd et al. 2005). ZFNs are enzymes that usually have the function of binding and cutting the distinct sequences of double-stranded DNA (dsDNA) (Hansen K, Coussens MJ, doi: 10.3791/3304. PMID: 22732945; PMCID: PMC3476380). ZFN sequences are tough to design and construct to use in distinct organisms, which also include plants. The ZFNs are affected by indeterminate DNA sequence identification, and also it costs high while using this technology (Ramirez

et al. 2008). Subsequently, a more effective tool, which is TALENs, is obtained from *Xanthomonas* bacteria; it was basically evolved for gene editing in plants; hence, it was used as a gene editing technique in plants (Christian et al. 2010). But it has one drawback, i.e., its intricated tandem repeat property in the DNA binding domains of TALEN; here, the TALEN protein forms the establishment of TALEN genes, which is slow and difficult in nature.

Soon after the emergence of the TALEN system, there was another technology that was under development, i.e., the CRISPR/Cas9 system genome editing tool. This CRISPR/Cas9 technology evolved after TALEN in the year 2012. Although after its emergence and its effectiveness, CRISPR turns almost into a promising technology for editing genes in the genome, this technique is not a new or fresh idea and is also not the first technology to be developed for editing DNA sequences. However, it was much more not possible to perform a targeted gene insertion into the genome of a cell by this technique. This disadvantage has led researchers worldwide to make the effort to form distinct gene-targeting technologies. In 2005, zinc-finger nucleases (ZFNs), which are the first system to target genes, evolved. With the introduction of this technology, specific sequences of DNA can be identified, and also targeted insertion could be executed into the genome (Lozano-Juste and Cutler 2014). In the year 2010, after a few years later, another technology arrived, i.e., TALENs (Liu and Fan 2014). *Streptococcus pyogenes*, a bacterium strain discovered with the use of the CRISPR/Cas9 technology, shows that it can be adapted for genetic engineering technology. This system comprises the "clustered regularly interspaced short palindromic repeat"; in this, a DSB is formed with the help of an RNA, which performs the function of guide, and the Cas9 protein, which works as an endonuclease finally allows the double-strand breaks (DSBs) (Mahfouz et al. 2014).

The difference in CRISPR in comparison to that of earlier perspectives is to make use of a small RNA sequence, which serves as a unique specific-site determination element for DSBs. In the case of prokaryotes, the CRISPR/Cas9 system originally provides protection against movable genetic components, specifically bacteriophages (bacteria-eating viruses) and plasmids. In recent years, various types of CRISPR have been engineered to develop the technique's programmable nature with minimal requirements. The application of the CRISPR technology concurrently leads to various practical and technological obstacles mainly linked with off-target and on-target actions, delivery strategies, the control of repair pathways, and controversial ethical problems (Xu et al. 2016).

1.3 General Applications of CRISPR/Cas9 in Plants

By using this technology, there are various improvements in crop characteristics, which include produce, crop quality, resistance to disease, and resistance to herbicides.

- *As Non-homologous End Joining (NHEJ) Gene Knockouts*

CRISPR/Cas9 helps in gene knockouts, which means inactivating the gene in an organism to interpret how the single or multiple target genes perform their function (e.g., enzyme genes or microRNAs). This knockout of genes is done by performing mutation of genes in DNA sequence (Liu et al. 2016)

- *As Homology-Directed Repair (HDR) Gene Replacement and Gene Knock-In*

HDR is a very beneficial repairing pathway for double-strand breaks that gives rise to error-free gene knock-in or gene replacement. Still, there is a limitation that only a few cases have successfully been done using target genes editing CRISPR/Cas9 with HDR. The challenge in HDR-related CRISPR/Cas9 genome editing is mainly related to the transfer of the donor DNA template and the synthetic endonuclease simultaneously into the tissue of the plant. Thus, on the successful transfer of this donor DNA template and the synthetic endonuclease, the gene knock-in or the gene replacement in an organism or plant boosts. By using this technology in medicinal plants to carry out the expected traits, such as resistance to pests, high productivity, and high quality can be acquired (Liu et al. 2016)

- *Increasing the Yield of Plants*

By manipulating cytokinin homeostasis, we can enhance the yield of cereals. By modifying the C-terminal of the plant gene *Oryza sativa LOGL5*, a gene that encodes for the cytokinin-activation enzyme in the case of rice, it increases grain production in different environmental conditions (Wang et al. 2020). Also, in the case of wheat, cytokinin oxidase/dehydrogenase (CKX) is the gene that encodes to show a knock-out effect, which results in the enzyme that catalyzes cytokinin degradation and generates high-yield phenotypes in wheat (Zhang et al. 2019b). Using this technology also helps to increase the production of fruit crops produced by editing genes, namely, CLV (Rodriguez-Leal et al. 2017) and ENO (Yuste-Lisbona et al. 2020); these genes control the size of meristematic tissue and rice lines that have low amylose contents; it can be done by altering the amino acid sequence of GBSS1 with a CBE (Xu et al. 2020).

In the case of wheat grains, the gluten proteins are responsible for coeliac disease in susceptible individuals which these proteins are encoded by almost 100 similar loci in the wheat genome; earlier, there was the use of traditional methods of breeding which does not help in decreasing gluten content, but by using CRISPR/Cas, we can target the particular conserved region of genes that encodes gluten, which results in such a wheat line having low-gluten content, which is up to 85% loss of immunoreactivity (Sánchez-León et al. 2018). CRISPR/Cas also helps in increasing or decreasing the quality content of the crop, which helps in the production of high-quality crops with an abundance of carotenoids (Li et al. 2018a; Dong et al. 2020), γ-aminobutyric acid (Li et al. 2018a, b, c), reduced phytic acid (Khan et al. 2019), and high oleic acid contents (Do et al. 2019) in plants.

- *Disease Resistance in Plants*

CRISPR/Cas helps in protecting plants against biotic stress. Rice production is strongly under the threat of bacterial blight, which is caused by *Xanthomonas oryzae pv. oryzae*. During the introduction of infection in the plant, a group of bacterial factors activate the transcription of the SWEET genes; their products are needed for disease susceptibility. The mutation is done in the promoter region of *O. sativa SWEET11*, *O. sativa SWEET13*, and *O. sativa SWEET14* using CRISPR/Cas; researchers procured rice lines with a wide range of resistance to *X. oryzae* pv. *oryzae* (Oliva et al. 2019; Xu et al. 2019). By using this strategy, researchers have confirmed plant immune systems against viruses like geminivirus (Ji et al. 2018) and caulimovirus (Liu et al. 2018).

2 Classification of the CRISPR System

CRISPR systems are of six types; moreover, they are categorized into two classes, which are established on the basis of sequence and the structure of Cas proteins. Of these six, type I and type III are identified earlier, and the type II CRISPR system is thoroughly studied, so it is most widely used in the CRISPR technique; types IV–VI are the types of the CRISPR system that have only recently been identified.

The type II systems are the simplest; type I and type III are tough to use, so these are not used in the modification of the genes due to their complications. In the case of the type I system, which consists of a protein named Cas3, this protein uses the DNase domain and helicase to disintegrate the sequence, i.e., targeted sequences. The subtypes of type II systems have the Cas9 genes, and the type II-B subtype has Cas1, Cas2, and Cas4 genes used in gene therapy. Several mutations, i.e., point mutations in the Cas9 gene, have been incorporated to issue even more specificity (Niu et al. 2021). The main aim in using this technology is to decrease the size of working nucleases, which makes it simple to wrap up their genes for distribution in the sequence.

3 Components of the CRISPR/Cas9 System

The CRISPR/Cas9 method consists of three elements, i.e., an endonuclease (Cas9) gene and two small RNAs – CRISPR RNAs (crRNAs); these crRNAs are sequence-specific targeting components, and trans-activating crRNA (tracrRNA) helps to create a link between an endonuclease Cas9 gene and the crRNA, which is a sequence-specific targeting component (Pourcel et al. 2005). The endonuclease Cas9 gene is a great multifunctional and also a multidomain DNA endonuclease. Its main function is to create a cut at the specific desired position in the genome. The endonuclease Cas9 consists of all the important elements for the following:

- Joining to the gRNA, in this binding allows Cas9 endonuclease to create a cut at specific genomic locus out of different attainable loci.
- Joining to the targeted DNA in the existence of a gRNA, it provides that the target present is upstream (i.e., 5′) of a protospacer adjacent motif.
- Cutting of the target DNA, which out-turn in the emergence of double-strand break (Barrangou et al. 2007).

The Cas9 protein consists of two different lobes, in which one is the recognition lobe, i.e., REC, and the other one is the nuclease lobe, i.e., NUC. It further consists of two endonuclease domains, i.e., the RuvC-like nuclease domain and the HNH-like nuclease domain; these are very important for the performance of the Cas9 protein in the final step. Through the cutting of the targeted sequence of the DNA, the RuvC domain and HNH-like nuclease domain make a cut in both of the DNA strands, which leads to the formation of DSB three base pairs upstream of the motif, i.e., PAM motif. The other HNH-like nuclease domain did the cutting of the complementary strand, while the other RuvC-like domain cleaved the other non-complementary strand of the sequence (Mahfouz et al. 2014). The Rec I domain is larger, and it helps in the joining of sgRNA; the bridge helix has an important function for the start of cutting deliberately, and the PAM-interacting domain is important for specificity in pairs upstream of the motif (PAM) (Cong et al. 2013).

- *the gRNA, i.e., guide RNA*, which is also known as single-guide RNA (sgRNA) in Cas9 responsible for the target location with the help of two RNAs: one is the crRNA, which helps in the identification and joining with the help of sequence, which has almost 20 nucleotides present within the genome, i.e., targeted genome, and the other RNA element, which is tracrRNA, helps in the joining of the crRNA to Cas9, and this helps in the maturation of crRNAs from pre-crRNAs.
- *PAM, i.e., protospacer adjacent motif*, which is used for the joining of Cas9 endonuclease to the genomic locus that is targeted and further consists of 20-nucleotide-long complement sequence of the sgRNA; it pairs with the sequence with three base pairs called PAM. It is a small sequence present on the DNA strand, mainly the targeted DNA strand, which is important for the Cas9 endonuclease activity. If this sequence is absent or the short sequence is absent or the whole complementary sequence cannot be identifiable, this results in the necessity of PAM in the genome is considered one of the main limitations of this approach (Cong et al. 2013).

4 CRISPR/Cas Structure

Basically, the CRISPR/Cas system consists of a protein family, i.e., Cas gene protein family, and the other one is the CRISPR array that further consists of three sequences. These sequences are as follows: repeat sequences, spacer sequences, and leader sequences. The initiation of CRISPR transcription is carried out by the leader sequences as the name suggests, i.e., to lead the transcription; this sequence is

present upstream of the CRISPR array and initiates the transcription. Then the other one repeats as the name suggests, which has the property of repetition. These repeats are small sequences of 21–48 nucleotides in length. These sequences form a structure that resembles the hair loop-like structure. These sequences are not fixed; they may change from species to species. These sequences may be ranged from a few to hundreds. Then the last one is spacer sequences; as the name suggests, they act as space between the two. These spacer sequences act as space between the two repeat sequences. These spacer sequences are almost 26–72-nucleotide-long sequences (Grissa et al. 2007). Then the Cas protein consists of different properties as it helps in different activities in which one of the important activities is to cleave the DNA sequences (Bland et al. 2007). This Cas protein is encoded by the Cas gene, and its coding sequences are present on the upstream region of the CRISPR array. These coding regions encode specific conserved nucleic acid-related Cas protein (Richter et al. 2013).

5 The Working Mechanism of CRISPR/Cas System

The CRISPR/Cas system mechanism exhibits three steps: acquisition by enzyme, expression of protein, and interference of sequence. The initial step is mainly done by the protein complex, i.e., Cas1 complex and Cas2 protein complex, which are same in all till known CRISPR/Cas systems, and sometimes, it involves other Cas proteins. This complex of protein leads to recognition of the spacer, i.e., protospacer and PAM in non-native, i.e., an alien nucleic acid that is directionally collected and merged as fresh CRISPR spacers into a CRISPR array that is made apart by repeat sequences; it leads to generating an "immune memory" of intrupting genetic components (Pourcel et al. 2005). Again when the similar ectogenous gene is re-invaded, the CRISPR locus is transcribed into a precursor CRISPR RNA transcript (pre-crRNA); now this pre-crRNA is under processed into a small mature crRNA, with the help of ribonuclease III (RNase III) enzyme. The processed crRNA consists of partial CRISPR spacer sequences that are joined together to partial CRISPR repeat (Barrangou 2015).

There is another transcript that is trans-activating crRNA (tracrRNA); this transcript is encoded by the CRISPR locus, and it has interrelated to the repeat regions of crRNA (Deltcheva et al. 2011). Single or multiple Cas nucleases are encoded by the CRISPR locus. For example, in the case of type II CRISPR/Cas9 system, the Cas9 protein is very necessary because it actively plays a role in the crRNA maturation and destroys the intrupting ectogenous nucleic acids. The fusion of the crRNA with the tracrRNA leads to the formation of the single-guide RNA (sgRNA) that forms the complexes with Cas9 (Jinek et al. 2012). Afterward, an effector ribonucleoprotein complex is formed by binding sgRNA to Cas9, which is a reason for the demolition of incorporating nucleic acids that are duly spaced from a required 5′-NGG-3′ PAM sequence (Garneau et al. 2010). The PAM sequence is necessary to identify, distinguish, and cleavage between self-DNAA and foreign DNA (Marrafni and Sontheimer 2010).

The Cas9 protein is indicated by a pair of nuclease domains that consist of two domains, which are RuvC domain and HNH domain; they perform cutting tasks between these two domains; the HNH domain cleavage is done on the complementary strand of the targeted DNA; this targeted DNA is present at a position of three nucleotides upstream of the PAM sequence (Gasiunas et al. 2012), whereas on the other hand the another RuvC domain done the cleavage on the other non-complementary strand at the similar site; eventually, it leads to ectogenous DNA double-strand breaks (DSBs) (Gasiunas et al. 2012).

In the case of eukaryotic cells, they start the DNA damage repair mechanisms, the very eminent being non-homologous end joining (NHEJ) and homology-directed repair (HDR); these two factors rectify broken double-stranded spaces to reach gene-targeted editing. NHEJ consists of errors in its mechanism that helps in the rejoining of the two free ends of a DSB with the help of arbitrarily frequent small nucleotide deletions or insertions, which results in the phenomenon of frame-shift mutations and also deletions; this results in the formation of targeted gene knockout.

So HDR can attain the error-free editing of target genes, so in the presence of exogenous homologous donor templates, there is a formation of insertion or replacement of a specific nucleotide sequence in HDR. However, the HDR-mediated gene targeting is very demanding due to the low unprompted effectiveness of HDR and the restrictions in delivery of donor template in cell (Jacobs et al. 2015).

The mechanism of CRISPR/Cas9 in medicinal plants has three steps (Fig. 1):

- The recognition of the specific site on DNA for the action of sgRNA.
- Then after the recognition, the cleavage is done on the specific site.
- At last, there is repairing of the particular site, which undergoes cleavage during the process.

6 Application of the CRISPR/Cas9 Technology in Medicinal Plants

The CRISPR/Cas9 system is a type II system of the CRISPR/Cas technology; it has many applications in different fields of biological sciences, but in the case of medicinal plants and its applications are mainly emphasize on the model plants that have whole genetic details and very effective systems of genetic transformation.

6.1 Application in the Case of Salvia miltiorrhiza *Plant*

Salvia miltiorrhiza is used in the treatment of cardiovascular diseases and cerebrovascular diseases for a very long time (Ren et al. 2019). It is a member of the family Labiatae, which is known to be a traditional Chinese medicinal herb. It consists of

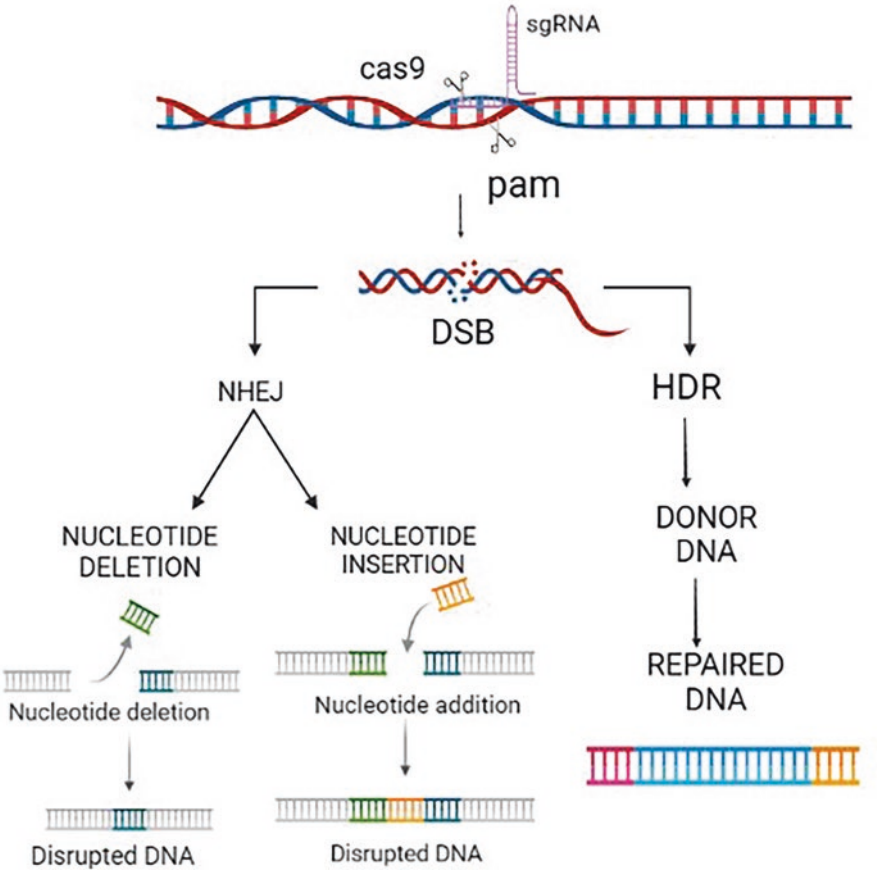

Fig. 1 Mechanism of CRISPR/Cas9

lipid-soluble compounds and also water-soluble compounds that are as follows: tanshinones and phenolic acids, including rosmarinic acid, salvianolic acid, and lithospermic acid; due to the presence of these compounds, they exhibit pharmacological activities (Luo et al. 2014). This plant after the genome dissection is widely used in medicinal plant studies (Xu et al. 2016). The CRISPR/Cas9 technology known to perform the knockout mechanism of the SmCPS1 gene, which is known for the formation of diterpene and the gene is called diterpene synthase gene mainly intricated in the potent elements of tanshinone biosynthesis (Li et al. 2017). *Agrobacterium*-mediated transformation is done to obtain the mutants in the medicinal plant; there are three homozygous and eight chimeric transgenic hairy root mutants in the case of *S. miltiorrhiza*; this mutation is done to make transformations in the content and varieties of secondary metabolites between the mutant ones and the wild ones, which are further collated using a technique called the liquid chromatography mass spectrometry (LC-MS) technique.

The main bioactive compounds of tanshinones include tanshinone I, tanshinone IIA, and cryptotanshinone; these compounds are totally absent in homozygous mutants. This research shows that gene SmCPS1 is the main gene for the synthesis of the tanshinone in its biosynthesis pathway. The targeted knockout results in the formation of pure *S. miltiorrhiza* hairy root mutant with the help of the transformed rosmarinic acid (RA) synthase gene, i.e., SmRAS; this gene comes in function by the water-soluble phenolic acid biosynthetic pathway by using the CRISPR/Cas9 system (In 2018, Zhou et al.). From the 16 independent transgenic hairy root lines, there is the formation of mutants that are as follows: five biallelic, two heterozygous, and one homozygous mutant. In this, there is a decrease in levels of phenolic acids, including RA and salvianolic acid B (SAB), whereas there is an increase in the levels of the RA precursor 3,4-dihydroxy phenyl lactic acid in the mutants. So these results proved the function of the SmRAS gene.

There are more than 20 genes that are knocked out of the laccase family of *S. miltiorrhiza* by using the technology, i.e., the CRISPR/Cas9 dual-locus editing technology (2021, Zhou et al.). In the editing lines, the accumulated RA, SAB, and lignin are lowered due to the gene expression of the target laccase gene and phenolic acid biosynthesis; both of them are the main gene expression levels for their accumulation. Also, in the CRISPR lines, it was observed that the levels of phenolic acids, including RA and salvianolic acid B (SAB), were decreased. These observations play important roles in the development and also the lignin production in the root of *S. miltiorrhiza* and are important for biosynthesis of phenolic acid. Other experiment is done by using overexpression (OE) of genes and the CRISPR/Cas9 technology to aim the SmbZIP2, a novel basic leucine zipper transcription factor separated from *S. miltiorrhiza* (Shi et al. 2021). Its analysis shows that the transgenic lines were observed with the reduction of phenolic acid in the overexpression lines and elevated content of phenolic acid in the case of CRISPR/Cas9 lines. It was found that SmbZIP2 plays a negative regulatory part in the biosynthesis of phenolic acid, which leads to the production of a novel biosynthesis scheme for phenolic acid.

6.2 *Application in* Dendrobium officinale *Plant*

Dendrobium officinale is a member of the genus *Dendrobium* and the family Orchidaceae. It has been used for more than 2000 years, and this medicinal plant is one of the most important medicinal herbs that have been used in traditional medical herbal treatment. This plant possesses various pharmacological activities, such as hepatoprotective (i.e., protection of the liver from damage) (Liang et al. 2018), anti-tumor (Liang et al. 2019), hypoglycemic (i.e., lower blood glucose level) (Chen et al. 2020), gastro-protective (Zhang et al. 2019a), and anti-inflammatory (Yang et al. 2020) functions. In the year 2020, the National Health Commission of China stated that the plant *D. officinale* is a binary-used plant with food applications and botanical medicine. Due to the overuse of *D. officinale*, the price of the plant increased, but there were also the reasons that the plant growth rate is very slow and

the germination rate is also slow, which leads to the expensing of this plant and demand of this plant also increases (Hou et al. 2012). So these reasons were enough to seek new strategies for the good production of *D. officinale*; for this, a new gene editing technology was used in the production of stable and free inheritable characteristic varieties of *D. officinale*.

Here, the CRISPR/Cas9 system is successfully used, which gives five targeted genes for editing in different pathways like the lignocellulose biosynthesis pathway, in which a number of enzymes play an important role in the biosynthesis of lignocellulose biosynthesis pathway and the enzymes are mostly coumarate 3-hydroxylase (C3H), cinnamate 4-hydroxylase (C4H), 4-coumarate: coenzyme A ligase (4CL), cinnamoyl-coenzyme A reductase (CCR), and irregular xylem (IRX); also there is another finding that it helps in studying the mutation rates of various target sites between 10% and 100%; it is possible by using a technology called polymerase chain reaction (PCR) technique, which is an amplification technique and a sequencing technique (In 2016, Kui et al.). These studies result in the successful application of a CRISPR/Cas9 mediated genome editing system in *D. officinale* genome editing, resulting that the technology has a huge development potency as a tool for the molecular breeding and genetic investigation of *D. officinale*.

6.3 Application in Cannabis sativa *Plant*

Cannabis sativa has delta-9-tetrahydrocannabinol, also called THC, and cannabidiol, also called CBD; these are the compounds present in cannabis that show pharmacological effects and play an important role in medicinal property that can help in treating numerous diseases of humans, like neurological diseases and cancer (Schultz et al. 2020; Devsi et al. 2020). The CRISPR/Cas9 system provides prominent target programmability and precision in the genome of cannabis, so it is very effective in the case of the discovery of cannabinoid synthesis genes and also in the case of genetic improvement in cannabis.

With the help of CRISPR/Cas9 technology, a gene is obtained, which edits the phytoene desaturase gene (CsPDS1); it is known to be a marker gene, i.e., a gene common in nature and used in testing genetically manipulated tools and finally formed for transgenic cannabis seedlings which are four in number that have a different phenotype, i.e., albino phenotype (In 2021, Zhang et al.). For the production of transgenic cannabis plants, there is a formation of a stable transformation and regeneration method development. There is the development of a genetic system called *Agrobacterium*-mediated transformation system, which was specially constructed to have stable incorporation of T-DNA in the cannabis genome, and also this system was stable and justified.

6.4 Application in Comfrey Plant

Symphytum officinale belongs to the family Boraginaceae and is a medicinally active plant with some properties like anti-inflammatory, analgesic, and proliferative effects (Staiger 2012). However, the pharmaceutical applications of this plant show that it contains high content of some toxin, i.e., pyrrolizidine alkaloid (PA); this toxin is present in the whole plant and causes toxic effects, which leads to hepatic toxicity in humans even at a very low dose (Stickel and Seitz 2000). The important profitable characteristics of comfrey's useful metabolites are medicinally limited (Allgaier and Franz 2015; Knutsen and Alexander 2017). The CRISPR/Cas9 system was used to incorporate destructive mutations in the homospermidine synthase (HSS) gene sequence; this gene results in the first specific enzyme of a very important pathway, which is the pyrrolizidine alkaloid (PA) biosynthesis pathway (In 2021, Zakaria et al.). Using this technology, HSS-deficient hairy roots (HRs) were conveniently achieved, and the analysis determined that the extent of homospermidine synthase enzyme and pyrrolizidine alkaloid (PA) was lowered in the hairy roots. This experimentation shows the implementation potency of CRISPR/Cas genome editing techniques helps in targeted gene editing and breeding of low-toxic comfrey transgenic varieties.

7 Summary of the CRISPR/Cas9 Technology in Editing Genes in Medicinal Plants

- *Salvia miltiorrhiza* has a number of genes that are targeted, named SmCPS1, SmRAS, SmLACs, SmbZIP2, and their promoters, specifically called Cas9/sgRNA promoters, are CaMV35S/AtU6–26; CaMV35S/AtU6–26, OsU3; AtUBQ/AtU6; CaMV35S/AtU6–26, respectively; the plant undergoes a number of mutations by these genes from 11.5% to 90.6%. The results show the number of characters such as heterozygous and homozygous hairy root mutants.
- *Dendrobium officinale* target genes are C3H, C4H, 4CL, CCR, and IRX, and their Cas9/sgRNA promoters are MttHP, CVMV, MMV, OCISV, and CaMV35S/OsU3. This results in DoLACs-deficient in hairy roots. *Dendrobium* Chao Praya Smile target genes are DOTFL1, and its Cas9/sgRNA promoter is Ubi/OsU3, OsU6a, which results in 13 homozygous mutant plants with a mutation frequency of 10.1%.
- A *Cannabis sativa* target gene is CsPDS1 with the Cas9/sgRNA promoter CaMV 35S/AtU6, resulting in CsPDS1-deficient seedlings with a mutation rate of 2.5% to 51.6% for the homozygous and chimeric mutants.
- *Comfrey* with the target gene of HSS with Cas9/sgRNA promoter AtU6–26 with HSS-deficient mutant plants.
- The CRISPR/Cas9 technology performs gene editing in the medicinal plants to produce bioactive compounds in a way that first the sgRNA are designed by the

recombinant DNA technology and loaded in the vector commonly Agrobacterium by the use of biolistic bombardment on the cell wall of *Agrobacterium*; also there is the addition of several important elements. Then the treated colonies are observed for regeneration and screened in next-generation sequencing machines. This results in the production of CRISPR/Cas9 gene editing medicinal plants.

8 Strategies of Medicinal Plants with New Omics Technologies

The new technology, i.e., the omics technologies in which the genome, transcriptome, proteome, metabolome, and other omics help to study the CRISPR/Cas gene editing with the help of bioinformatics. The use of bioinformatic studies helps in the prediction of the functional active sites in the CRISPR/Cas genome, i.e., the key genes. It helps to identify the functional genes in CRISPR and also helps in studying the regulation of metabolic pathways. The omics technology reveals the reverse genetics of gene function, genetic improvement, and germplasm innovation and also the synthetic biology of effective components.

9 Discussion and Future Prospects

For basic research and also applied plant research, a technology called CRISPR/Cas9 technology has evolved as a revolutionary tool for the betterment of medicinal plant research. With the properties of mutations in the genes, it also induces particular manipulations in the genome with its massive capability of gene editing; these particular tools play a great role in creating a number of crop varieties that have properties like improvement in agronomic performance and that create a huge revolution in breeding technologies that are very important in this era of the greater population.

CRISPR/Cas9 technology helps to synthesize the crucial bioactive compound of plants. There are a lot more concepts of this technology to be identified. Great work would be done in the future regarding the synthesis of bioactive compounds by this technology. By using this technology we can enhance the yield of bioactive compounds from medicinally active plants which are precisely low in content in the case of plants.

Acknowledgments CSIR-Indian İnstitute of Integrative Medicine, Jammu, India is gratefully acknowledged. The authors would also like to kindly acknowledge Dr. Ajai Prakash Gupta for giving support during the earliest phases of writing this article. Miss Diksha Manhas SRF from CSIR-IIIM, Jammu, India, is acknowledged for her fruitful suggestions and discussions.

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Exploring Endophytes for In Vitro Synthesis of Bioactive Compounds in Medicinal and Aromatic Plants

Delin Xu and Zhaogao Li

Abstract Symbiosis with microorganisms is an influential pathway in plant evolution, and many related components have yet to be discovered. Medicinal and aromatic plants are favoured by a wide range of researchers as natural sources for the development of pharmaceutical and aromatic products for humans. Endophytes are the "second genome" of plants and play an essential role in plant growth and development. Therefore, the analysis of molecular mechanisms of symbiosis can help shed light on the interplay between plants and endosymbionts and thus provide a basis for the application of plant microbial resources. In this chapter, we review the interactions between plants and microorganisms and the effects of endophytes on plant growth and the synthesis and accumulation of secondary metabolites. In addition, the research hotspots and application progress of plant endophytes are presented as a reference for understanding the regulatory role of plant microorganisms and to provide future research directions.

Keywords Endophytic bacteria · Secondary metabolites · Medicinal plants · Interactions · Regulation of growth

1 Introduction

Plants are one of the most important producers in nature, and the stability of plant resources is of great significance to the Earth's ecosphere. However, the general degradation of plant resources is a serious global challenge at present, and the chain

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N. Kumar, R. S. Singh (eds.), *Biosynthesis of Bioactive Compounds in Medicinal and Aromatic Plants*, Food Bioactive Ingredients,
https://doi.org/10.1007/978-3-031-35221-8_5

effect of this phenomenon has led to dramatic dynamic changes in the Earth's ecology, such as global warming and atmospheric deterioration. The plants in the Earth's biosphere are mainly divided into aquatic plants and terrestrial plants according to their habitat, among which terrestrial plants are the most widely studied. To date, almost all of the more than 300,000 extant species of land plants are derived from the two branches of bryophytes and vascular plants, with extensive ecological specificity (Xue et al. 2022). As an extremely important part of natural ecosystem functioning, land plants play a vital role in protecting the stability of surface and underground environments, such as by maintaining the surface energy balance, increasing the albedo, improving climate, and regulating water cycling, weathering, nutrient element cycling, and soil formation and maintenance (Chapin et al. 2011; Beerling and Butterfield 2012).

As an essential natural biological resource, plants have attracted attention because they produce abundant and diverse active substances (Xiang et al. 2021). Plants produce a variety of active compounds that are functionally divided into primary metabolites, which are necessary for growth and development processes such as photosynthesis and respiration, and secondary metabolites. The latter accumulate as unique substances during the growth of the plant and are not consumed as intermediates. Although the functions of these diversified active metabolites in plant metabolism, physiology, and biochemistry are still unclear, an increasing number of studies have shown that such metabolites play crucial roles in plant–environment interactions; for example, some metabolites can act as signalling molecules to activate plant immune regulation and stimulate plant growth (Liu et al. 2022a, b). The action of these metabolites can enable plants to resist pathogen invasion (Schiering et al. 2017), induce insect attachment to promote pollination (Bao et al. 2019), and improve plant resistance to abiotic stress (Liu et al. 2018).

Humans have an innate affinity for plants, which may come from our long-term dependence on them. Before the advent of modern advanced drugs, human treatment of diseases invariably relied on the discovery of therapeutic substances derived from plants, and even now, a considerable part of the population still relies on natural plant-derived substances for disease treatment. Many plants are being developed for commercial use. Of the hundreds of thousands of known plants, only approximately 12.5% have been documented to have medicinal properties, and only a few hundred have been cultivated artificially (Schippmann et al. 2002). With the decline in natural resources and the increase in global research interest, plant resources are becoming increasingly scarce. Under natural conditions, plants are vulnerable to biotic and abiotic stresses such as pathogen invasion (Liu et al. 2021), temperature shifts (Jung et al. 2020), salinity (Liu et al. 2022a, b), heavy metals (Lv et al. 2022), water deficits (Takahashi et al. 2018), and flood disasters (Lou et al. 2017). Humans have achieved a series of technical developments in response to the shortage of plant resources in medical treatment, agriculture, and food production, including but not limited to transgenic plant production (Maher et al. 2020; Lou et al. 2017), cell culture (Krasteva et al. 2020), and variety improvement (Yang and Hwa 2008), but the results are unsatisfactory. It is difficult to develop crop varieties with stable production, and this difficulty is reflected in elevated costs and greater time

consumption. Therefore, it is essential to explore and apply simple, efficient, environmentally friendly, low-cost, and cost-effective methods to improve plant production and human health.

All organisms encounter various biotic and abiotic environmental factors that stimulate and threaten them over their lifetime. Unlike other living things such as animals, plants are sessile. Therefore, plants must evolve more efficient and deeply targeted strategies to adapt to the challenges brought by dynamic changes in the environment. Mutualism with microorganisms, namely, endophytes, is a crucial strategy for plant selection. However, due to restrictions at the developmental level, research on medicinal and aromatic plants mainly focuses on macroscale factors such as pharmacological action, substance composition, and growth traits and ignores the microsystem of the plant internal environment (Qi et al. 2022; Cui et al. 2018). Since the discovery of endophytes in plants, people have started to re-examine the factors affecting the quality of medicinal plants from microscale perspectives such as microorganisms and the plant internal environment (Li et al. 2023). Therefore, combining botany, microbiology, and pharmacology to study the microecosystems of endophytes and host plants, their species structures and the relationships between them can enable a more profound and comprehensive understanding of medicinal and aromatic plants.

In this study, based on the interaction mechanism between endophytes and host plants, we explored the correlation between endophytes and host plants and increased the understanding of the role of endophytes in regulating the synthesis of plant secondary metabolites from a new perspective. The findings are conducive to more effective plant quality control and provide a reference for wide applications in the medical, agricultural, and food industries.

2 Plant Recruitment: From Microorganisms to Endophytes

The potential value of plant-related microorganisms has been widely experimentally confirmed, and many relevant studies have been conducted on the influences of plants on microorganisms, including successful endophytes (Bergelson et al. 2021; Xiong et al. 2021). In a suitable environment, plants actively regulate their microbial communities according to their growth and development needs by recruiting microorganisms, screening beneficial microorganisms, resisting pathogen infestation, and regulating interactions between microbial communities (Timilsina et al. 2020). Different species or even the same species under different cultivation conditions have different phenotypes and genotypes, which results in significant differences in the recruited functional microbial community structure (De Vries et al. 2020). Studies have found that plants can stimulate immune system response by regulating the synthesis and accumulation of metabolites, thus affecting the activity and structure formation of the microbial community (Venturi and Bez 2021). When plants are affected by adverse factors, defence genes and symbiotic genes rapidly reorganize microbial communities by integrating stress signals and plant response

signal transmission pathways and by using root secretions to gather and recruit beneficial microorganisms, thus resisting biotic or abiotic stress threats (Gao et al. 2021).

During the growth process, plants actively recruit microorganisms from the surrounding environment to build the necessary microbial community (Fig. 1). In soil, plant roots not only anchor plants and serve as organs for absorbing water and nutrients but also provide a unique ecological niche for the soil microbial community and attract various microbial communities (Compant et al. 2019). The area approximately 5 cm above and below the plant base is called the rhizosphere. It is the most active area of microbial life and one of the most complex ecosystems in the world. The microorganisms gathered in the rhizosphere play an essential role in the growth and development of plants, so they are also called the "second genome" of plants. As the most significant component of the plant microbial community, the rhizosphere microbial community is involved in various biological and biochemical processes of rhizosphere soil and has an essential influence on plant growth, development, and environmental adaptation.

Plant roots recruit microorganisms through secretions. Under biotic and abiotic stress, plant roots can secrete abundant and diverse compounds to affect the composition of the surrounding microbial structure; such compounds include plant growth

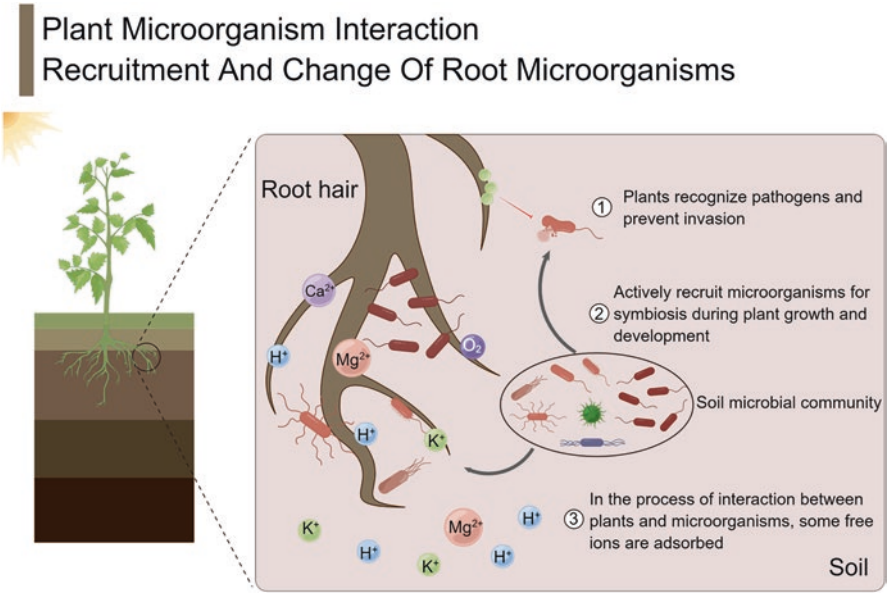

Fig. 1 Interaction forming between plants and microorganisms. Plants produce special substances for their own growth needs and release them into the soil, attracting soil microorganisms to gather around their roots. Different microorganisms use different invasion methods (such as contact invasion, chemical attraction, etc.) to act on the roots of plants. Plants differentiate between pathogenic and beneficial microorganisms through their immune systems. In the process, pathogenic bacteria are killed or inhibited, and beneficial microorganisms take up most of the niches under the plant's active action, thus stimulating root reactions and nutrient uptake. (By Figdraw)

regulators, organic acids, nucleotides, sugars, pressors, sterols, vitamins, amino acids, fatty acids, and phenols (Sasse et al. 2018). Different plant species affect the composition of their rhizosphere microbial communities by secreting varying types and amounts of substances. In this process, some microorganisms continuously form closer interactions with the plant; accordingly, these microorganisms are called endophytes (Chen et al. 2022). In contrast to the rhizosphere microbial community, the stems, leaves, fruits, and other parts of plants provide different, unique ecological niches for the survival of microorganisms. However, the living environment of successfully colonized endophytes is significantly different from that of microorganisms on the outer surface of tissues. Typically, endophytes are derived from soil, seeds, and air, with soil being the main source. Microorganisms in the process of adapting to the conditions of the stems, leaves, fruits, and other parts of plants are also affected by uncontrollable factors such as soil, cultivation measures, and the environment (Compant et al. 2019). In long-term interactions, a large number of compounds and even genetic materials are transferred between plants and microorganisms, especially endophytes. Therefore, there is a strong functional correlation between plants and microbial communities, especially endophytes, which is of great significance for plant growth and development.

3 Plant Interactions: An Important Mode of Microbial Survival

A plant is not an individual, but a large functional body composed of numerous microorganisms. In nature, virtually all growing and developing plants are internally and externally enriched with a large number and variety of microorganisms; these microorganisms together constitute the microbial community that interacts with plants and further become endophytic bacteria with mutualistic and symbiotic relationships (Cheng et al. 2019). Plants provide a stable living environment for the survival, growth, and reproduction of microorganisms. Among plant–microbe interactions, bacteria and fungi occupy many ecological niches in the plant microecosystem due to their strong adaptability (Trivedi et al. 2020). Plant health and high diversity are related to the dynamic microbial community, which plays an essential role in plant growth and development (Müller et al. 2016; Beilsmith et al. 2019) (Fig. 2). Microorganisms are crucial in regulating plant growth, promoting the absorption of inorganic elements, improving disease resistance, resisting abiotic stress, regulating the accumulation of medicinal ingredients, improving the quality of medicinal materials, and resisting insect predation (Cordovez et al. 2019; Bai et al. 2022). At present, plant–microorganism interactions and the underlying molecular mechanisms are the focus of life science research and the key bottleneck in expanding the applications of functional microorganisms.

Microbial regulation is a fundamental means of improving plant quality. To date, a large number of microorganisms with different functions have been isolated from plants with abundant and diverse local sources. Plant microorganisms can be divided

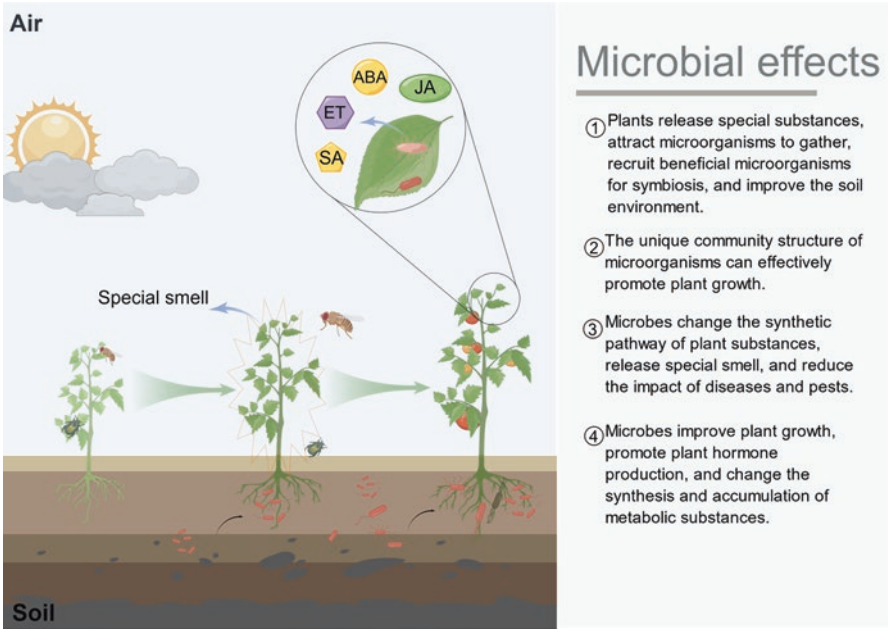

Fig. 2 The role of endophyte in plant growth and development. Endophytes form dynamic microbial communities in plants. Through the overall regulation of host plants, it can improve plant growth status and help plants avoid the impact of biological/abiotic stresses that may be encountered in the growth environment. At the same time, it can promote the synthesis and accumulation of plant metabolic substances and form new substances under certain conditions. (By Figdraw)

into root microorganisms (Berendsen et al. 2012), interfoliar microorganisms (Trivedi et al. 2020; Schlaeppi and Bulgarelli 2015), and intraspecies microorganisms according to the location from which they are obtained (Walsh et al. 2021); these microorganisms have direct or indirect regulatory effects on plant growth and development (Compant et al. 2019; Turner et al. 2013). Because of their abundant and diverse functions, microorganisms are considered critical resources for realizing sustainable ecology (Toju et al. 2018; Lemanceau et al. 2017).

Medicinal plants are the main source of traditional and even modern medicines and are important in the treatment of human diseases. The regulatory effect of microorganisms on such plants is reflected not only in improving the ability of the plants to promote growth and resist stress but also in promoting the synthesis and accumulation of secondary metabolites and improving their quality. In addition, the generation of new metabolites with active medicinal ingredients can effectively alleviate the shortage of medicinal plant resources and even replace the production of medicinal plants to a certain extent (Wu et al. 2021a, b; Huang et al. 2018). Therefore, the microbial community of medicinal plants is a valuable resource for the high-quality development of medicinal plants at present and in the future.

4 Regulatory Mechanism of Endophytic Bacteria on Plant Growth

4.1 Regulation of Plant Growth and Development

There are extensive interactions between endophytes and plants, among which endophytes can significantly promote the growth and development of medicinal plants. During the growth and development of medicinal plants, endophytes regulate plant hormone levels or help the host plants absorb more nutrients through their ability to fix nitrogen, dissolve phosphorus, dissolve potassium, and produce iron carriers and plant hormones (Abdelshafy Mohamad et al. 2020). A large number of studies have found that the growth rate of plants living in symbiosis with microorganisms is significantly faster than that of plants not living in symbiosis with microorganisms. *Bacillus velezensis* SQR9 is a typical symbiotic endophyte. Cucumber inoculated with *B. velezensis* showed significantly increased growth and improved salinity tolerance (Sun et al. 2022). Nine strains of endophytes isolated from turmeric rhizomes could not only fix nitrogen and solubilize phosphorus but also produce IAA to stimulate plant growth. In addition, some strains could produce ferric carriers, which promoted the leaf number, stem height, and fresh weight of turmeric stems and rhizomes (Kumar et al. 2016).

4.2 Regulating the Accumulation of Plant Secondary Metabolites

The accumulation of plant secondary metabolites is affected not only by genetic and abiotic factors but also by endophytes to a large extent (He et al. 2020). First, endophytes themselves act as inducers to promote the synthesis and accumulation of plant secondary metabolites. In a suspension culture of periwinkle, it was found that the synthesis and accumulation rate of vinblastine significantly increased under the action of endogenous fungi, and the yield was 2–5 times higher than that of the control group (Namdeo et al. 2002). Thomloudi isolated halotolerant *Bacillus* Hil4 from the leaves of the medicinal plant *Hypericum* and found that the microorganism significantly increased the content of secondary metabolites in the host in vitro and exhibited many growth-promoting traits (Thomloudi et al. 2021). Tan et al. explored the induction mechanism of endophytes and believed that these inducers could significantly promote the accumulation of active ingredients in medicinal plants (Tan et al. 2013). Such a relationship indicates that in a specific physiological environment, the receptors on plant cells can specifically bind to endophytes infecting the host, and a series of complex signal transductions occur in the plant cells; these signals then stimulate the expression of relevant gene fragments involved in the secretion of secondary metabolites, changing the activity of key enzymes and thus promoting the accumulation of plant active ingredients.

Endophytes affect the synthesis of secondary metabolites in the host by producing proteins with special functions that change the speed or direction of a reaction in the plant or by participating in the synthesis of secondary metabolites that the host plant cannot produce independently. The endophytic fungi *Penicillium simplicissimum* CN7, *Talaromyces flavus* BC1, and *Trichoderma konilangbra* DL3 isolated from stevia significantly increased the IAA content and promoted the growth of stevia. Thus, the accumulation of active medicinal ingredients can be regulated (Huong et al. 2022). *Fusarium oxysporum*, *Penicillium steckii*, *Enterobacter cloacae*, and *Serratia marcescens* were inoculated singly or in combination into Tripterygium wilfordii, and the results showed that the synthesis and accumulation of triptolide and celastrol significantly increased (Song et al. 2020). Endophytes from the rhizosphere of *Salvia miltiorrhiza* significantly promoted the accumulation of the phenolic acids, rosmarinic acid and salvianolic acid B (You et al. 2017). In addition, the rhizosphere of *Salvia miltiorrhiza* is associated with the dominant fungi *Cladosporium tenuissimum*, *Aspergillus terreus*, *P. steckii*, *Mucor circinelloides*, and *Acremonium* sp. This rhizosphere community significantly promotes the synthesis and accumulation of cryptotanshinone and tanshinone IIA in *S. miltiorrhiza* (Chen 2020).

4.3 In Vitro Synthesis of Active Medicinal Ingredients and Their Analogues

Since the discovery of endophytes, continuous and in-depth research has revealed that in addition to promoting the synthesis of plant active ingredients, endophytic bacteria can produce the same or similar compounds as plant secondary metabolites, which is the basis of in vitro synthesis by endophytic bacteria. The first antibiotic from endophytic fungi used in humans was penicillin, which was first discovered by Alexander Fleming in 1928 from *Penicillium* and can effectively inhibit the growth of *Staphylococcus*. The discovery of this substance opened the door to the production of drugs using microorganisms (Wainwright and Lederberg 1992). In 1993, when the endophytic fungus *Taxomyces andreanae* was isolated from *Taxus chinensis* and found to produce the same anticancer substance (paclitaxel) as the host, research on functional endophytic strains in medicinal plants was initiated (Stierle et al. 1993). Endophytes produce the same or similar secondary metabolites as medicinal plants, which provides a new method for the development of new microbial drugs and the protection of medicinal plants. Anjum and Chandra isolated endophytes, *Microbacterium* sp., from Catharanthus roseus that can produce Vindoline, which is used in the treatment of Hodgkin's disease and acute leukaemia (Anjum and Chandra 2019). Liu isolated the endophytes *Microbacterium* and *Burkholderia*, which can convert berberine, from the stems and leaves of Chinese pistache; their findings enriched the diversity of varieties of Chinese pistache (Liu et al. 2020). *Bacillus subtilis* produces abundant products through solid fermentation, and such products have been widely used in the treatment of ischaemic vascular-related diseases (Yin et al. 2019).

Engineered strains obtained by scientists through genetic engineering and other molecular biological means can also produce rare and precious drugs (Song et al. 2009). In addition, in 2003, Martin transferred the amorpha-4,11-diene synthase gene and isopentadiene pathway-related gene of yeast into *E. coli*. This technique enabled heterologous synthesis of artemisinin intermediates. Gene transfer research also strongly supports the in vitro synthesis of secondary metabolites of medicinal plants produced by endophytes (Martin et al. 2003).

4.4 Regulation of Barriers to Continuous Cropping in Plants

There are serious obstacles to continuous cropping of plants, and medicinal plants are no exception. Modern studies have shown that there are three main factors limiting continuous cropping: deterioration of soil physical and chemical properties, allelopathy and autotoxicity of medicinal plants, and aggravation of soil-transmitted diseases (Huang et al. 2021; Zheng et al. 2020). At present, obstacles to the continuous cropping of medicinal plants are alleviated mainly by the cultivation of different plant varieties resistant to these obstacles, soil sterilization, soil improvement, the establishment of a reasonable tillage system, the application of organic fertilizer, the application of endophytic fertilizer agents, and other methods (Tan et al. 2017; Xie et al. 2017, 2019). Among the above methods, endophytes have attracted the attention of numerous researchers due to their advantageous low cost and environmental friendliness. Endophytes form bacterial fertilizer through self-degradation, which can improve soil fertility and soil structure, regulate soil microbial community structure, inhibit diseases and insect pests, and improve the yield and quality of medicinal plants in practical cultivation (Li et al. 2020). The use of microbial fertilizer containing beneficial microorganisms in the continuous cropping of *Radix pseudostellariae* could not only increase the yield and total polysaccharide content of *R. pseudostellariae* but also regulate the abundance of the beneficial microbial community in rhizosphere soil and reduce the invasion degree of pathogenic bacteria; thus, the obstacles to continuous cropping of this species were effectively alleviated (Wu et al. 2021a, b).

4.5 Enhancing the Stress Resistance of Medicinal Plants

During the growth and development process, plants constantly suffer from adverse factors and form a certain degree of resistance through long-term confrontations. Plant stress can be classified into biotic stress and abiotic stress. Biotic stress mainly includes pest infestation, diseases, herbivore feeding, and insect attachment, while abiotic stress includes changes in natural environmental parameters, such as salinity, temperature, drought, oxidation, and heavy metal toxicity. Dynamic changes in the endophyte community can significantly affect the ability of plants to adapt to the

environment (Li et al. 2022). On the one hand, endophytes enhance plant stress resistance by affecting the growth of insect larvae; on the other hand, they produce unique substances to compete with other organisms for ecological niches by changing metabolic pathways in plants. Endophytes can also induce immunity to produce certain resistance in plants. Induced systemic resistance (ISR) and systemic acquired resistance (SAR) are two hotspots of the current microbiology research; research has shown that the immune effect produced by endophytes plays an important role in enhancing plant immunity especially broad-spectrum resistance to pathogenic bacteria (Dubey et al. 2020).

Endophytes are also important in resistance to abiotic stress. By producing phenazine compounds, *Pseudomonas* promoted the expression of drought-resistance genes and the recovery of water deficit-related pathways in wheat seedlings; these effects led to a longer adaptation period for the seedlings, thus improving their drought and stress resistance (Mahmoudi et al. 2019). In a natural saline–alkali environment, an isolated endophytic strain could effectively reduce salt stress and multiple heavy metal stress, protect plants from fungal infection, and promote the growth of tomato seedlings (Masmoudi et al. 2019). Interestingly, Ulrich studied the effects of the rhizosphere endophyte community on the physiological response of plants to drought and found that endophytes can exert a positive influence on the drought tolerance of plants in the early stage of drought, but in severe drought, they have a certain negative influence (Ulrich et al. 2019). These results indicate that endophytes have positive effects on the drought tolerance of plants, but the range of the effects is limited, perhaps because endophyte tolerance to adverse environments varies. At a certain threshold stress level, endophytes compete with the host plants for ecological niches based on their survival needs, resulting in a significant reduction in plant stress resistance.

5 Research Hotspots and Application Progress of Endophytes in Plants

5.1 Systematic Study of the Evolutionary Process of Endophyte–Plant Interactions

In recent years, with the rapid development of high-throughput sequencing technology and computer science, the focus of microbiology research has shifted from environmental microorganisms to plant microbiomes (De-Medeiros-Azevedo et al. 2021; Wang et al. 2021; Gao and Chu 2020). This shift has also led to opportunities for developing research on medicinal plant microbiomes. Given the pharmacological properties of medicinal plants, the study of their microbiome must include secondary metabolites. Secondary metabolites are also related to the evolutionary level

and kinship of species. Studies have found that the closer the kinship is, the more likely species are to produce the same or similar chemical components (Park et al. 2021). For example, ranunculin is the most characteristic chemical component in members of Ranunculaceae, and aristolochic acid and its derivatives are the most characteristic in species of *Aristolochia*. In addition, major compounds such as phenolic compounds, triterpenoids, saponins, cardiosides, and alkaloids are commonly found in Moraceae plants (Park et al. 2021; De-Pádua-Lúcio et al. 2018). Therefore, systematic classification has been carried out according to compound type or botanical family, genus, and species, and multispecies and multiomics methods have been used for joint analysis of the microbiome of medicinal plants to characterize the distribution law. In the following sections, we will explore specific endophytes that can produce substances closely related to the secondary metabolites of medicinal plants and provide fresh insights into the coevolutionary process of the interaction between medicinal plants and endophytes.

5.2 *Exploring the Regulatory Effects of Endophytes on Plants by Spatiotemporal Analysis*

Plant metabolites vary according to geographical distribution, developmental period, years of growth, seasonal changes, tissue sites, and other factors (Park et al. 2021). This dynamic variation was not only found in plant secondary metabolites but also in the microbiome of medicinal plants, especially in the community structure and species composition of endophytes (He et al. 2020). An interesting example is the dynamic changes in endophytic fungal community structure and composition in *Agastache rugosa* in different seasons, resulting in significant differences in secondary metabolites (Yeo et al. 2021). However, in *Atractylodes lanceolata*, there are differences in the dominant endophyte genera in different tissue parts. Studies have shown that *Fusarium*, *Penicillium*, *Rhizoctonia*, and *Myriangium* compete for a large number of ecological niches in the rhizome, and *Penicillium*, as the only dominant fungus in leaves, symbioses with *Atractylodes* (Cao et al. 2010). In addition, due to differences in the geographical environment, the community structure and composition of endophytes of medicinal plants from different sources differ greatly (He et al. 2020). To date, a large number of studies have shown that endophytes can significantly regulate the content and composition of plant secondary metabolites, which indicates that the synthesis and accumulation of medicinal ingredients in medicinal plants and the interactions of endophytes are also related to some extent. Therefore, it is necessary to carry out dynamic studies on plant microbiomes at both temporal and spatial scales and to elucidate the role of endophytes in the synthesis and accumulation of plant secondary metabolites.

5.3 Production of Core Plant Endophytes for Application Purposes

The core microbiome was originally developed to represent the common microbial groups in humans within a certain range, which are shown as overlapping areas in the Venn diagram. Subsequently, the concept was expanded to plants for research purposes (Segata et al. 2016; Thomas et al. 2016; He and Chung 2020). Scientists are interested in the interactions between hosts and microorganisms, between microorganisms and the environment, and between microorganisms and microorganisms, and research emphasizes the function of the core microbial group in a geographical or environmental context and in host ecosystems and further extends the function and behaviour of the microbial community (Shade and Handelsman 2012). As a hotspot in current microbiology research, the core microbiome is widely used in sustainable agriculture, and it is expected to be an invaluable biological repository for large-scale applications in the fields of medicine and food. With changes in the environment and the continuous progress of science and technology, the development of modern crop growth agents, pesticides, and plant growth conditioners has become crucial, and this development phenomenon has gradually spread to include the cultivation of medicinal plants (Ma et al. 2021). Although many countries have issued policies that slow the use of pesticide fertilizers, the effect is unsatisfactory. Microorganisms are considered to have certain advantages, such as high efficiency and safety, and they are important in the cultivation of medicinal plants. At present, the core endogenous bacterial community of medicinal plants has become the target for the development of medicinal plant ecosystems. Although the role of the microbiome is well established, there is no consensus on the definition of the core microbiome. Therefore, the study of the core microbial groups of medicinal plants also requires joint multiomics analysis, which reveals the composition of the core microbial community at different levels and explores the intercorrelation between medicinal plants and endophytes. In the future, the artificial construction of an appropriate microbial community and the symbiotic evolution of medicinal plants with this community may be able to reduce the use of fertilizers and pesticides in medicinal plant cultivation. Further, use endophytes to promote the efficient synthesis and accumulation of medicinally active ingredients of medicinal plants to improve the quality of medicinal materials and high-quality production.

5.4 Gene Co-modification in Plants and Endophytes

A plant is a symbiotic functional organism that coexists with endophytic bacteria, and its genes are composed of a plant genome and an endophytic microbial genome (Gopal and Gupta 2016). For a long time, humans have been deliberately selecting original varieties of plants with certain desirable traits and domesticating them to meet different needs. In this process, some natural genotypes have been removed

without notice. In recent years, although some studies have focused on genotypic changes, they have only focused on the plant genome rather than the whole genome including endophytic microbial communities (Gopal and Gupta 2016). In addition, studies have shown that domestication not only leads to the loss of plant genetic diversity but also reduces the diversity of plant-related microorganisms, thus causing plants to lose the ability to interact with beneficial microorganisms (Pérez-Jaramillo et al. 2016). Different genotypes of plants have different microbial community compositions, and microorganisms can modify the phenotype of plants. Therefore, the influence of the microbial community should be further considered when selecting different plant varieties to generate new traits or promote plant growth and nutrient absorption without changing plant genomic information related to resistance to biotic and abiotic stresses (Wei and Jousset 2017). Wild plants have evolved specific microbial communities, but human domestication has disrupted this particular symbiotic relationship (Gopal and Gupta 2016). The development of symbiotic functional theory provides a modern basis for genetic variation in plant breeding, which emphasizes the role of the plant microbiome, especially endophytic bacteria (Nogales et al. 2016). The breeding of current plant varieties is still in its initial stage, so variety confusion and uneven quality are still common. Due to the essential roles of microorganisms in plant growth and development, quality improvement, and so on, it is particularly vital to consider microorganisms in plant cultivation. How to obtain excellent traits by co-modifying the genes of plants and interacting microorganisms, especially endophytes, or to promote the synthesis and accumulation of active metabolites is a problem that needs to be solved at present.

5.5 Application of Endophytes in the Development of Natural Medicinal Products

Endophytes play an important role in drug development. Most of the raw materials of clinical drugs on the global market are derived from the natural active ingredients of medicinal plants. Paclitaxel, a unique substance produced in *Taxus chinensis*, is used in the clinical treatment of gastric cancer, lung cancer, digestive tract cancer, breast cancer, and other cancers. It was found that the endophytic bacteria of medicinal plants with paclitaxel synthesis ability are *Taxomyces andreanae*, *Fusarium*, *Aspergillus niger*, *Alternaria*, *Rhizoctonia*, *Cephalosporium*, etc. (Kang et al. 2011). Kumar isolated the endophytic fungus *Fusarium oxysporum* from Indian Vinca, which produces the anticancer drugs vinblastine and vincristine and is widely used in the treatment of Hodgkin's disease, acute and chronic lymphocytic leukaemia, malignant lymphatic tumours, various cell tumours, and breast cancer (Kumar et al. 2013). It is also one of the most widely used natural plant antitumour drugs (Palem et al. 2015). *Paecilomyces variotii* Bain., isolated by Shukla from the root of *Ocimum sanctum* Linn., showed a high level of antioxidant activity in free radical scavenging tests and was used in antioxidant drug development (Shukla et al. 2012).

In addition, the excellent ketone derivative (2′)-2-(2-acetoxypropyl)-7-hydroxy-5-methylchroone was extracted from the endophytic strain *Alternaria brassicae* JS959, which can effectively inhibit high-density lipoprotein and copper-induced low-density lipoprotein oxidation in human plasma. It can be used as an effective factor in treating heart disease (Rimsha et al. 2019).

5.6 Limitations of Endophyte Applications

Endophytic microorganisms are tissue specific, and their symbiosis and function in the host are affected by tissue type, host genotype, and the environmental conditions in the microecosystem. In addition, studies on the distribution of endosymbiosis in endophyte hosts are relatively scarce, resulting in an incomplete understanding of the widespread endophyte community in plant tissues, which seriously hinders research on the function of endophytes in various fields (Harrison and Griffin 2020). It is important to note that comprehending the function of endophytes and implementing the widespread use of related products depend on an understanding of how endophytes colonize plants. To ensure that endophytes and host resources can be used repeatedly and consistently, reliable delivery methods of endophytic inoculants should be developed to improve productivity in practical applications. Harnessing the relationship between plants and endophytes is crucial to advancing sustainable development. Therefore, further research is needed to understand the biogenic bacteria in hosts to improve the viability of endophytes assisting the host plant, especially in the important area of facilitating the synthesis and accumulation of secondary metabolites in medicinal plants.

6 Perspectives

Medicinal plant resources are highly abundant and urgently need to be developed. Under the influence of species and regional differences, diverse endophytic bacterial resources have been cultivated in plants. With the rise of emerging technologies such as microbiomics, genomics, and synthetic biology, endophytic bacteria research has attracted increasing attention, and a large number of species resources have been accumulated. At present, there are still some problems in the study of endophytic bacteria. Theoretical research is extensive, but the level of industrialization is low; therefore, great efforts should be made to apply and modify endophyte theory in accordance with production and human needs. Research on endophytes mainly focuses on the screening and identification of secondary metabolites, but research on their functions is still relatively lacking. Knowledge of endophyte functions could promote the exploration of functional genes in plant breeding and the development of functional enzymes in medical and food fields. In addition, there is a close relationship between endophytes and plants, but the study of their

interaction is still relatively weak. Endophytes play an essential role in plant growth, and the microecological effects and functions of endophytes need to be explored further. With the arrival of the omics era, single-strain studies can no longer meet current needs. In the next step, omics technology can be applied to study individual plant microbiomes to further reveal the close relationship between endophytic bacteria and host plants.

Since paclitaxel, a secondary metabolite with anticancer activity, was discovered in endophytes, a large number of studies have focused on the in vitro synthesis of medicinally active components from endophytes. It is worth noting that a variety of valuable compounds, including secondary metabolites, have been discovered and isolated from endophytes. Endophytes are regarded essential sources of a wide range of biological metabolites. The secondary metabolites produced by endophytes have extremely promising application prospects; however, there are still numerous problems to be solved at the current stage of development and application, and the tiny output scale is far from meeting the huge scale of human needs. Although some modified endophytic strains have performed adequately at the laboratory scale, there is still a certain gap regarding application at a large industrial scale. In practical applications, it may also be necessary to focus on potential risks, such as whether the artificial addition of microorganisms will have a negative impact on the structure and composition of the native microbial community of plants. In general, research on the interaction between endophytes and plants, especially medicinal and aromatic plants, is a promising area for resource development, functional research, and product application. The combination of different technologies and methods, such as high-throughput sequencing technology, metagenomic technology, and genome mining, may lead to unexpected research results.

The influence of the plant microbiome on secondary metabolites during plant growth and development and the interaction mechanism of these microorganisms with plants represent the current frontier and focus of research worldwide. Microbial genes, as the "second set of genomes" of plants, are a future direction of focus. With the development of macroomics, further combined analysis of the metagenomes and metaproteomics of medicinal and aromatic plant microbiomes and the host plants can effectively help elucidate the interaction mechanisms between the two. The results of such combined analysis can be applied to plant cultivation to account for the original interactions between plants and microorganisms. In addition, the application of synthetic microbial communities (SynComs) to medicinal and aromatic plants can broaden the genetic diversity of plants and expand the possibilities. In the future, culturing and screening core microorganisms will enable further exploration of the function and role of the microbiome and strengthen the utilization of beneficial microorganisms, which will actively promote the sustainable protection of medicinal and aromatic plant resources.

Acknowledgements This work was supported by the National Natural Science Foundation of China (32260089, 31960074), Innovation and Entrepreneurship Education of Guizhou Ordinary Undergraduate Colleges (2022SCJZW10), Future Outstanding Teachers Training Program of Zunyi Medical University, Postgraduate Teaching Reform Project of Zunyi Medical University

(ZYK105), Undergraduate Education and Teaching Reform Project of Zunyi Medical University (XJJG2022-22), Joint Bidding Project of Zunyi Science & Technology Department and Zunyi Medical University (No. ZSKHHZ[2020]91), Science and Technology Department Foundation of Guizhou Province of China (No. QKPTRC [2019]-027), and Science and Technology Plan Project of Honghuagang District, Zunyi City (No. ZHKHNZT(2020)04).

Contribution Statement *Zhaogao Li*: Conceptualization, Writing, visualization, and investigation

Delin Xu: Writing, reviewing, and editing; supervision, project administration, and funding acquisition

Declaration of Competing Interests The authors declare no potential conflicts of interest concerning the research, authorship, and/or publication of this article.

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RNA Interference for Improvement of Bioactive Compound Production in Plants

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Abstract Plants that consist of bioactive compounds such as alkaloids, carotenoids, flavonoids, etc. have high commercial value due to their numerous uses in medicine, agriculture, cosmetics, pharmaceutical, food industry, etc. In response to various biotic as well as abiotic stresses, these bioactive compounds are produced. There is a great rise in demand for bioactive compounds because of their significant importance; therefore, there is a drastic need to increase the formulation of these compounds. RNA interference (RNAi) is a natural gene-silencing phenomenon that has lately been widely employed in agriculture to enhance crop improvement, disease control, plant growth, and so on. RNA interference (RNAi) has attracted various researchers all over the world for studies in relation to plant improvement. So far, various researchers have investigated the miRNA and siRNA interference for enhancing the bioactive compounds in various plants, responsible for controlling the various traits. This book chapter is, to sum up, all that scattered information related to RNA interference (RNAi) for improving bioactive compound production in plants; it will also be helpful in facilitating future research.

Keywords RNA interference · Bioactive compounds · siRNAs · miRNAs · Secondary metabolite

1 Introduction

Plants have always performed significant functions in the world. The vital well-being of around 80% of the worldwide people is reliant on plant-based materials (Winter and Tang 2012; Yuan et al. 2016). Growing conscientiousness of public lifestyle choices has boosted the market for natural and organic goods. Due to its health advantages over synthetic medications in terms of cost and safety, interest in

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N. Kumar, R. S. Singh (eds.), *Biosynthesis of Bioactive Compounds in Medicinal and Aromatic Plants*, Food Bioactive Ingredients,
https://doi.org/10.1007/978-3-031-35221-8_6

medicinal plants has grown in the last two decades (Ekor 2013; Thomford et al. 2018; Anand et al. 2019). Phytochemicals are present in abundance among plants, which have the remarkable potential to medicate disorders and are thus used in a wide range of trades, including nutraceuticals, cosmetics, and medicines (Nasri et al. 2014). The therapeutic qualities of plants are due to the presence of bioactive molecules, also referred to as secondary metabolites that perform biological functions, and these vary in structure and metabolic processes (John Wiley & Sons 2009). In fruits, vegetables, and grains, well over 5000 distinct phytochemicals have been retrieved and recognized (Liu 2013). Bioactive compounds are well suited for use in pharmacogenetics, agrochemicals, cosmetics, the food sector, and nanotechnology. These bioactive chemicals are employed in agro-alimentary, fragrances, flavors, colors, and pharmaceutical preparations because of how versatile their effects are (Naik and Al-Khayri 2016). Major roles about these bioactive compounds are to safeguard plants against various ailments, insects, etc. as well as to help them for surviving against biotic or abiotic stresses (Shitan 2016). Targeting various signal transduction pathways in plants is one technique to boost the synthesis of these chemicals. Moreover, a limited amount of natural pathways for bioactive compound production and their synthesis is dependent on diverse factors, consisting of physiological stages of plants (Dixon 2001). Plant elicitation is another method for gradually boosting the synthesis of these bioactive compounds (Namdeo 2007; Parola-Contreras et al. 2020). Plants primarily produce active secondary metabolites in response to stress, which can be imitated using a variety of elicitors, which can be abiotic stresses and biostimulants (Vargas-Hernandez et al. 2020). Despite not being necessary for plant growth as well as development (Rosenthal 1991), these metabolites have a significant function in signaling and defense mechanisms as well (Ncube and Van Staden 2015). Due to their use in flavors, drugs, fragrances, dyes, insecticides, and other products, they are regarded as economically significant products (Guerriero et al. 2018). These secondary metabolites are generated by different energy-generating routes such as glycolysis and photosynthesis like metabolic pathways. Depending on how they are biosynthesized, how they are in structure, and how they function, they are divided into many groups, i.e., terpenoids, enzyme cofactors, terpenes, steroids, alkaloids, saponins, and lipids are the several categories they fall under, and these bioactive compounds/secondary metabolites in plants are also referred to as phytochemicals (Hussein and El-Anssary 2018; McMurry 2015).

As there is a swift upgrade in world population, in turn, demand for plants increases (Brown and Funk 2008; Lobell et al. 2008; Godfray et al. 2010). Traditional plant genetic techniques are well renowned and are still used, for the quality and quantity of plants, but they are tedious, burdensome, and subject to a number of other ecological, physiological, and biological drawbacks. A large range of new genes and characteristics that may be successfully introduced into crops for increasing productivity and nutritious value and impart retardation to abiotic and biotic challenges have been made available by precision biotechnologies, particularly genetic engineering (Sharma et al. 2002). However, the use of this technology in modern agriculture, biosafety regulations, and the environmental impact of

genetically engineered crops have raised public concerns and doubts (Wolfenbarger and Phifer 2000; Herdt 2006). Concerns have been expressed regarding the potential risks to humans and the environment that have been raised by the ability to transfuse and expression of the same genes from diverse sources mainly other than plants into economically important edible crops. Significant concerns include eroding genetic diversity, ecological disturbances, and transgene transfer to other diverse strains and wild relatives resulting in monstrous yield. As a result, intricate tests are performed on transgenic plants before they are made available for general use to try to assess the hazards and ensure safety. Thus, more effort, money, and knowledge are needed for the creation of transgenic crops. Therefore, it is necessary to formulate new ecologically adequate and sustainable methods for enhancing plant attributes without altering their genomes. In this context, several findings indicate that RNA interference induction may be used for the downregulation of certain gene expression for monitoring disease resistance, growth processes, stress tolerance, and also other plant features (Kamthan et al. 2015). RNAi has attracted various researchers all over the world for studies in relation to plant improvement. Small interfering RNAs (siRNAs) and microRNAs (miRNAs) can silence genes by cleaving mRNAs and blocking protein production, and the process is referred to as RNA interference (RNAi). RNA interference, a phenomenon that naturally silences genes, has become frequently used in agriculture to improve features linked to crop enhancement, growth of plants, and disease control. In order to silence particular genes that control target traits, double-stranded RNAs (dsRNAs) are expressed by transgenic plants in applications of RNAi technology in agriculture (Qi et al. 2019). RNAi discovery has contributed to our understanding of the regulation, function, and analysis of genes and introduced new directions for the development of an enticing approach that has an enormous ability to use in genetic analysis, plant conservation, an increase in the synthesis of bioactive compounds, and more areas connected to crop improvement. RNAi's potential to affect plant growth, morphogenesis, generation of bioactive compounds, polarity, development, and other physiological processes has been extensively demonstrated. Based on these discoveries, the idea of developing artificial RNAi was developed. This technology allows for the inhibition of targeted genes or silences, particular factors required for crop improvement. However, this chapter intends to summarize current scattered information regarding RNAi for the improvement of bioactive compound production related to crop improvement for describing and being able to comprehend the function of RNAi.

2 RNA Interference (RNAi) and Its Discovery

RNA interference in the nematode has been discovered a decade ago; firstly in *Caenorhabditis elegans*, this mechanism was discovered (Fire et al. 1998). Co-suppression, or silencing of genes through a sense of transgene, is one of these phenomena. Co-suppression is the process of suppressing both the transgene's own expression and the expression of endogenous homologous genes. Later research

revealed that co-suppression can occur through either PTGS or TGS, i.e., posttranscriptional gene silencing or transcriptional gene silencing, respectively (Kusaba 2004). There is another phenomenon related to RNAi, i.e., coat protein-mediated protection (CPMP). A coat protein transgene confers virus resistance. Primitively, it was believed that the coat protein was what caused protection, but it was later discovered that transgenes for untranslated coat proteins could also confer virus resistance. It was believed that coat protein-mediated protection (CPMP) and posttranscriptional gene silencing (PTGS) shared similar mechanisms because it was observed that CPMP works posttranscriptionally (Waterhouse et al. 2001; Kusaba 2004). Mahmoud and Croteau proposed gene silencing in 2001 to reduce the impact of the undesired menthofuran in *Mentha x piperita*, a medicinal plant by antisense suppression in the mfs gene, allowing for the cytochrome P450(+) menthofuran synthase. In the RNAi process of dsRNA-mediated gene silence, only the mRNA corresponding to dsRNA is selectively destroyed.

RNA interference is an evolutionarily sustained defensive process that occurs in nature. Small RNA disrupts the target mRNA transcript's translation in this biological process, ultimately repressing the gene expression. The dsRNA cleavage products known as microRNA (miRNA) and small interfering RNA (siRNAs) produce small noncoding RNAs. A Dicer-like enzyme (DICER) is a ribonuclease that performs cleavage (Pare and Hobman 2007). RNA-induced silencing complex (RISC), Argonaute (AGO), and other effector proteins work together to perform RNAi- or RNA-induced silencing (Ender and Meister 2010; Riley et al. 2012; Wilson and Doudna 2013; Redfern et al. 2013). This phenomenon has become an efficacious tool for functional genomics as well as genetic engineering. It requires a significant amount of time and works to enhance agricultural plants by changing characteristics using conventional plant breeding methods. Researchers have switched biotechnology strategies for crop enhancement over the previous two decades. RNAi can now be used to quickly manipulate the expression of the genes for quality traits in crops.

There is a self-complementary sequence in hairpin RNA (hpRNA), which is homologous with respect to the target gene and is intended to be expressed by the RNAi construct. Efficiency is increased by inserting a spacer sequence, typically an intron, that expresses ihpRNA (intron hairpin RNA), between two complementary sequences (Wesley et al. 2001). For improved RNAi efficiency in certain plants, the implementation of appropriate vector, marker, promoter, and transformation procedures is required. The role and function of small noncoding RNAs (sncRNAs) in posttranscriptional gene silencing (PTGS) as well as in transcriptional gene silencing (TGS) regulatory processes have been studied since the dawn of the twenty-first century. The results of the experiments demonstrated the effects of gene silencing for a better understanding of processes. Diverse model organisms have so far discovered the numerous small noncoding regulatory RNA classes, which include small vault RNA (svRNA), QDE-2-interacting RNA (qiRNA), PIWI-interacting RNA (piRNA), miRNA, and siRNA. Each of which had a unique biochemical process for its biogenesis (Aalto and Pasquinelli 2012). When creating their respective dsRNA precursors, miRNA and siRNA biogenesis begins differently.

Later, DICER (Dicer-like enzyme), dsRNA-specific endonuclease, a member of RNase III family, cleaves dsRNA precursors to produce siRNAs and miRNAs (Hutvagner et al. 2001; Bernstein et al. 2001). RISC (RNA-induced silencing complex), AGO (Argonaute), and other effector proteins collaborate by small noncoding RNAs to silence genes. Small RNAs in plants are also known as siRNAs and miRNAs (Ruiz-Ferrer and Voinnet 2007). Small noncoding RNAs (ncRNAs) play a role in both transcriptional gene silencing and posttranscriptional gene silencing. While beginning with dsRNAs, the transcriptional gene silencing (TGS) and post-transcriptional gene silencing (PTGS) pathways use several ways of machinery and mechanisms to complete their processes. Posttranscriptional gene silencing (PTGS) is typically used for HIGS, i.e., host-induced gene silencing, which involves host plants that have been genetically modified to develop small RNAs, i.e., siRNAs or miRNAs, averse to the target gene. Two modern applications of RNA interference for crop improvement, i.e., spray-induced gene silencing (SIGS) and transgene-mediated HIGS, share a common fundamental idea of processing. SIGS is a potential "non-transgenic" crop-protection technique that is still relatively fresh and evolving. Spraying dsRNA onto plants has effectively silenced genes from a variety of insects, pests, and diseases while also suppressing transgenes and endogenous genes in the target crop (Wang and Dean 2020). Both small RNAs and microRNAs are the effector molecules for PTGS (Bartel 2004; Zamore and Haley 2005; Vazquez 2006). Long dsRNA is processed to produce siRNAs and miRNAs, which are both 20–24 nt long. Their origins, primary precursor structures, biogenesis pathways, and modes of action are all different (Axtell 2013).

2.1 Short-Interfering RNAs (siRNAs)

The dsRNAs from either exogenous sources or endogenous sources initiate the formation of siRNAs (Fire et al. 1998; Tuschl 2001). Using DICER (DCL) of the RNase III endonuclease family, these anomalous dsRNAs are recognized by the plant cell as foreign objects, and they are then degraded into 21–25 nt tiny siRNA duplexes (Hamilton and Baulcombe 1999; Hammond et al. 2000; Bernstein et al. 2001). The siRNAs are short, 5′-phosphorylated dsRNAs that are produced by DICER from larger dsRNAs and have two nucleotide overhangs at the 3′ ends (Bernstein et al. 2001; Elbashir et al. 2001). To distinguish between two siRNA strands as sense or antisense, siRISC (siRNA-induced silencing complex) is called upon, which causes the sense strand degradation. siRNA's antisense strand and RISC are then sequentially combined with the target mRNA. RISC with AGO (Argonaute) and other effector proteins block translation by cleaving target messenger RNA. Activated RISC is responsible for causing the posttranscriptional gene silencing (PTGS) process, which can frequently take part in the degradation of mRNA, in turn inhibiting the synthesis of protein. As cotranscriptional silencers of gene expression through chromatin regulation, siRNAs are also involved in this (Burkhart et al. 2011; Fagegaltier et al. 2009). It has been demonstrated that

transcriptional gene silencing (TGS) occurs when siRNA binds to a number of DNA- and histone-modifying proteins, including the cytosine methyltransferase CMT3. These proteins work together to create a silent chromatin state with low transcriptional activity (Ossowski et al. 2008; Wang and Dean 2020).

2.2 MicroRNA (miRNAs)

In *Caenorhabditis elegans*, initially, miRNA was identified as a regulator of the juvenile to adult alteration (Lee et al. 1993; Reinhart et al. 2000). It has been discovered that this class of short regulatory RNAs also regulates a variety of developmental transitions in plants (Wu and Poethig 2006; Wu et al. 2009). The mRNAs of protein-coding genes are paired with this class of short regulatory RNAs to control their suppression, which mediates important gene-regulatory activities. Plant miRNA (microRNA) biogenesis begins with the endogenous pri-miRNA (primary miRNA) precursor, transcribed by RNA polymerase II in the nucleus, and has a partially double-stranded stem-loop structure (Jones-Rhoades et al. 2006; Zhu 2008). DCL1 (Dicer-like 1), an RNase III enzyme, and additional proteins, i.e., SE, HYL1, and HEN1, further process the pri-miRNA to create the 70–110-nucleotide-long pre-miRNA (precursor miRNA). Pre-miRNA is cut into a 22–24-nt-long miRNA duplex by DCL1, which is then transported toward the cytoplasm by HASTY protein. Sense miRNA is destroyed by the SDN protein, which then recruits matured miRNA duplex into RISC complex and activates it. In order to mediate their targets' cleavage or translational obstruction, mature miRNAs typically attach to target mRNAs in the 3′ UTR (untranslated region) (Bao et al. 2004; Khraiwesh et al. 2010). The silencing mechanisms can be influenced by a number of variables, including cellular state, developmental stage, cell type, target site, etc. There are two ways to suppress gene expression. Firstly miRNA complex is formed, which prevents the joining of ribosome subunit or translational initiation and causes nascent polypeptide chain to degrade prematurely, increasing ribosome drop-off, and the second is the induction of deadenylation, plant growth and development, the manufacture of secondary metabolites, abiotic and biotic stress responses, and other processes, which are all impacted by mRNA instability of the target microRNA expression (Huntzinger and Izaurralde 2011). For the development of plants with desirable traits, modifications to their expression and biosynthesis may be advantageous (Pareek et al. 2015).

3 The Basic Mechanism of RNA Interference

RNA interference mechanism was first studied and explained in a nematode, i.e., *Caenorhabditis elegans*; after the study, this term (RNA interference) was introduced and the term interference was proposed. In plants, this mechanism that leads to

RNA silencing or RNA interference in plants drives at minimum three different stages these are: mRNA cleavage done as a result of dsRNA cytoplasmic silencing or interference; microRNAs endogenously silencing the mRNAs that regulate negatively gene expression, leading to cleavage of RNA or blocking of protein translation known as PTGS (posttranscriptional gene silencing); and the methylation of DNA in sequence specific manner responsible for this silencing, in turn suppressing the transcription referred to as transcriptional gene silencing (TGS) (Mansoor et al. 2006). Generally RNA interference approach involves cloning as well as inserting the targeted gene in adequate plasmid to create a recombinant plasmid. *Agrobacterium*, a good vector for plant transformation, is used to transfect the recombinant plasmid. Generally, small interfering RNAs (siRNAs), which are 21–24 nucleotides in number, initiate the RNA interference, cleaved from DICER, an enzyme from ribonuclease III type family (Hamilton and Baulcombe 1999; Zamore et al. 2000), and then RISC (RNA-induced silencing complex) is incorporated in these 21–24-nt-long siRNAs, which contains numerous Argonaute (AGO) proteins as well (Baumberger and Baulcombe 2005; Vaucheret 2008). Double-stranded siRNA is unwound by the ATP-activated RISC. Loss of sense strand in siRNA duplex has been taken place due to the activity of RNA helicase, and then antisense strand of the siRNA is integrated in the RISC complex containing nuclease (Kusaba 2004). Base-pairing interaction of RISC incorporated in antisense strand of siRNA sequence along with homologous transcript degrades targeted mRNA and inhibits the synthesis of protein (Bartel 2004). In this way, RNAi technology may have several benefits, especially more precise, sequence-based gene silencing.

4 RNAi for the Improvement of Bioactive Compound Production

The primary sources of pigments, aromas, medicines, food additives, pesticides, etc. come from plant secondary metabolites like flavonoids, terpenoids, alkaloids, etc. The primary care needs of 70–80% of population in the world are reportedly met by herbal remedies, which are in turn made from plant secondary metabolites (Canter et al. 2005). Because of its immense biological significance, understanding the biosynthesis regulation pathway of bioactive compounds is crucial. These secondary metabolites are produced by an intricate network of genes. For almost two decades, model systems and crops were thoroughly researched by researchers, and a vast quantity of data was gathered. Noncoding RNAs, particularly their regulatory functions in the production of bioactive compounds, are just starting to gain attention as a study topic for medicinal plants. Over the period, advancement in molecular techniques allowed better understanding of the genes, enzymes, proteins, etc. that are implicated for secondary metabolite production pathway. Although there is flourishing knowledge about the regulation of many processes by

miRNAs, miRNA's role in controlling biosynthesis of secondary plant products is still not fully understood (Gupta et al. 2017). So far, hundreds of miRNA candidates have been discovered in over 50 medicinal plant species, including *Panax ginseng*, *Digitalis purpurea*, and *Salvia miltiorrhiza*. Among these, the production of more than 30 miRNAs has been expected to be regulated. The miR397-LAC module, the miR12112-PPO module, the miR156-SPL module, the miR828-MYB module, the miR858-MYB module, and other siRNA and lncRNA regulatory pathways are only a few examples of the various regulatory routes and modules that may be employed to achieve control. The quality as well as quantity of medicinal plants can be improved with the help of further functional analysis of herbal ncRNAs (Li et al. 2021). RNAi is a known method for controlling secondary metabolites (Borgio 2009). Numerous physiological, ecological, and biological problems are connected to the current breeding and enhancement efforts. Recently, genetic engineering using RNA interference (RNAi) has demonstrated its potential for enhancing bioactive compound synthesis in plants that are valuable for several agronomic traits.

4.1 Production of Non-narcotic Alkaloids

The finest illustration of metabolic engineering by RNAi was the substitution of the nonaddictive alkaloid reticuline in the opium poppy (*Papaver somniferum*) for morphine. Numerous genes involved in several phases of a convoluted metabolic process have been shown to be silenced by RNAi (Allen et al. 2004). They created an hpRNA construct at the same time that triggered all COR (codeine reductase) gene family members' downregulation. The buildup of a non-narcotic alkaloid precursor (S)-reticuline, at the cost of opium, codeine, morphine, was brought in transgenic plants by silencing the COR (codeine reductase) gene family.

4.2 Role of RNAi on Caffeine

Caffeine stimulates the circulatory, central nervous, and respiratory systems. Additionally, it provides protection from type II diabetes, liver diseases, and Parkinson's disease. Nevertheless, excessive caffeine use results in sleeplessness, agitation, and palpitations. Only 10% of the global coffee market is made up of decaffeinated coffee (DECAF). The caffeine level of the transgenic plant that was silenced by CaMXMT1, i.e., 7-N-methylxanthine methyltransferase, also known as theobromine synthase, was reduced up to 70% (Ogita et al. 2003). Similar to this, less production of caffeine in tea was created by downregulating the gene of caffeine synthase (CS) while maintaining its arousing properties (Mohanpuria et al. 2011).

4.3 *In* Picrorhiza kurroa*: Role of miRNAs in the Terpenoid Biosynthesis*

In six transcriptomes from the shoot, root, and stolon organs of *Picrorhiza kurroa* that varied in growth, development, and culture conditions, miRNAs were identified. miRBase entries for every plant miRNA that is currently in existence were utilized as backend datasets. In *Picrorhiza kurroa*, 18 conserved miRNAs were discovered, and their potential impact in the regulation of varied biological processes was discussed through target prediction and functional annotation. When compared to samples from viticulture and field-grown samples, miR-5532 as well as miR-5368 showed much lower expression, indicating that they are involved in regulating *P. kurroa* growth in culture environments (Vashisht et al. 2015). According to miRNA validation and expression study performed by qRT-PCR and 50 RACE, miRNA-4995 may have a function in the control of terpenoid biosynthesis, ultimately influencing the creation of picroside.

4.4 *Role of Smi-miR397 and Smi-miR408 in the Posttranscriptional Regulation of* S. miltiorrhiza LAC *Genes*

By the use of the 5′ RACE method, experimental validation and high-throughput small RNA sequences are analyzed, which indicates that Smi-miR397 targets the 23 *SmLACs* in *Salvia miltiorrhiza*. It was also analyzed that Smi-miR408 also targeted three of them. This advocate the advantage of miR397 as well as miR408 in the posttranscriptional regulation of *SmLAC* genes, responsible for the production of phenolic compounds (Li et al. 2019a, b).

4.5 *In* Punica granatum *L.: Role of miRNAs on Bioactive Compound Production*

The pomegranate fruit includes a range of natural substances including terpenoids, phenolics, alkaloids, and fatty acids that play a role in several health-promoting processes (Heber 2008). To extract natural substances like punicalagin, product of glucose as well as gallic acid, and anthocyanins, a family of water-soluble phenolic chemicals imparts pink to red fruit (Ismail et al. 2012). From pooled RNA samples of pomegranate plant's young seedling to mature fruits, a small RNA library from 29,948,480 high-quality reads was identified. About 50% of the pool of small RNAs between 15 and 30 nt were 24 nt. With variations within each family, the miR157 was the most prevalent family, accompanied by the miR156, miR166, and miR168

families. The expression of dominant and new miRNAs in various fruit development phases, male and female flowers, leaves, and stem-loop RT-qPCR was employed. The expression of miR156, miR156a, miR159a, miR159b, and miR319b increased along with developing fruit. It was observed in the later development of fruit that elevated expression of miR156 positively regulates the biosynthesis of anthocyanin by reducing the SPL transcription factor (Saminathan et al. 2016).

4.6 *In* Vitis vinifera *L.: Role of miR828 and miR858 in the Accumulation of Anthocyanin and Flavonol*

Studies on various plants have discovered some microRNAs and other siRNAs that target the MYB transcription factors, which control the phenylpropanoid metabolic pathway. In grape lines, conserved miRNAs like miR159, miR166, miR168, and miR319 were found. By using miRProf analysis of miRNA, abundance has been taken, which indicates that there were several miRNAs (Moxon et al. 2008; Stocks et al. 2012). Few miRNAs significantly varied in their accumulation between lines, linked to secondary metabolic pathways. The majority of miRNAs that accumulated in greater quantities in anthocyanin were miR3632, miR403, miR828, and miR858. miR858 has the most conserved sequence among all chosen miRNAs that can target MYBs. miR828 and 22-nt miRNA that is responsible for initiating TAS4 cascade silencing pathways are highly expressable but not conserved well. By the use of sRNA sequencing, mRNA sequencing, proteome analysis, and degradome analysis, it is demonstrated that anthocyanin-rich content in grape lines represents two MYB targeting miRNAs extensively, which results in variable MYB protein expression in some cases. The coding domains of specific helix motifs in the mRNA sequences of MYB proteins are specifically targeted by miR828 and miR858. Secondary siRNAs in a cascade that relay on RNA-dependent RNA polymerase were produced as a result of miR828 targeting, which also caused MYB RNA to decay. In comparison with a flavonol-rich grape line, grape lines with high anthocyanin content had more strong cascade silencing and MYB suppression. It was determined that microRNA-mediated silencing enhanced anthocyanin biosynthesis, resulting in high anthocyanins by targeting the repressor class of MYBs (Tirumalai, V et al. 2019).

4.7 *Role of RNAi in Fruit Coloring and Anthocyanin Biosynthesis of* Actinidia arguta

As significant regulators, miRNAs could be essential in regulating the fruit color. By using high-throughput sequencing of small RNAs, three developmental phases of the *A. arguta* fruit were examined. Forty-six miRNA families were created from 482 conserved microRNAs in total, which correspond to 526 pre-miRNAs, and 581

novel microRNAs, which correspond to 619 pre-miRNAs. Analysis and target gene prediction resulting that miR858 were found to be a part of the biosynthesis of anthocyanins, which is what gives the fruit its color. Utilizing UPLC-MS/MS, the number of anthocyanins present in *A. arguta* was determined. The data on the role of miRNA in *A. arguta* was first time reported, and it will be very helpful for future research into how miRNAs are regulated in the production of anthocyanins and the color of fruits (Li, Y et al. 2019).

4.8 In Curcuma longa*: Role of RNA Interference in the Development and Bioactive Compound Synthesis*

Turmeric has long been a plant used for medicine because it contains a variety of bioactive substances. microRNAs are known to control gene expression posttranscriptionally through translation repression or transcriptional cleavage. In turmeric, it has been observed that miRNAs actively regulate bioactive compounds. In turmeric, 18 miRNA families were found and also observed that 16 miRNA families regulate the 238 target transcripts. To demonstrate the biological function of the targets regulated by the putative miRNAs, gene annotation and pathway analysis were used. The miRNA-mediated gene regulation network also revealed co-regulated targets, which were controlled by two or more miRNA families. Rhizome development was found to be influenced by miR156 and miR5015. miR5021 regulates isoquinoline alkaloid and terpenoid biosynthesis pathways. The mechanism for the manufacture of flavonoids was shown to be under the control of miR2919. Three miRNAs, i.e., miR1858, miR1168.2, and miR156b, were discovered to be involved in curcumin production (Singh and Sharma 2017).

4.9 In Ferula gummosa*: Role of RNAi in Terpene Biosynthesis*

Ferula gummosa is a medicinal plant and is very well known for its resin named galbanum. The primary bioactive compound of galbanum is terpenes. RNA-seq data gathered from the plant's flowers and roots were used to assess miRNAs and their targets using computational techniques. The direct or indirect regulatory effects of miRNAs on the targets involved in terpene biosynthesis were also examined using biological network analyses. In *F. gummosa,* 220 miRNAs from 94 families have been discovered for the first time. Five highly abundant miRNAs are miR5658, miR1533, miR5021, miR414, and miR1436. Six miRNAs from five different miRNA families, namely, miR838, miR5251, miR2919, miR5021, and miR5658, were discovered to be related to the terpene biosynthesis pathway as per these results of KEGG and PlantCyc and were found to be united with biosynthesis

pathway of terpenes. Furthermore, network analysis revealed that three miRNAs consisting of miR5021, miR1533, and miR5658 were presumed to be regulating the three TF regulating terpenes, i.e., SPL7, SPL11, and ATHB13, respectively (Sobhani et al. 2018).

4.10 Role of RNA Interference in the Withanolide Production

Withania somnifera is a medicinal plant also known as Indian ginseng. Because of its medicinal properties and genes involved in biosynthetic processes, this plant has been extensively investigated. The goal was to pinpoint the miRNA transcriptome, specifically in the root and leaf tissues separately, that controls withanolide production. For the purpose of identifying miRNAs, the transcriptome data from plant root and leaf tissues grown in vitro was taken into consideration. In the leaf tissues and root tissues, 39 and 24 miRNA families were identified in total respectively, among them 15 miRNA families in root tissues, and 27 miRNA families in leaf tissues have demonstrated their participation in many biological processes. The function of target genes in the metabolism of withanolide has also been investigated. Endogenous leaf-miR477, leaf-miR530, root-miR5140, and root-miR159 control withanolide production (Srivastava et al. 2018).

4.11 Role of RNA Interference in Achieving Biotic Stress Resistance

Multiple miRNAs are expressed either more or less when there is biotic stress (Li et al. 2012; Khraiwesh et al. 2012; Singh and Sharma 2017). Against soybean cyst nematodes (SCN), miRNA expression varied across susceptible and resistant soybean cultivars (Li et al. 2012). Overexpression of osa-mi7696 gave protection to rice *(Oryza sativa*) against blast fungus, i.e., *Magnaporthe oryzae* (Campo et al. 2013). This miRNA in rice inhibits the natural resistance-associated macrophage protein-6 (OsNramp6). As a result, RNA interference is emerging as a viable alternative strategy for building resistance in plants against biotic stresses.

4.12 RNA Interference Effects on Bioactive Compound Production for Increasing Fruits' Shelf Life

Numerous vitamins as well as minerals are abundantly present in fruits and vegetables. For use as food, they are collected, transported, and stored. Losses from diseases, pest infestation during storage, improper handling, spoilage, and transportation

are included during postharvest crop losses. One method for minimizing postharvest losses is a delayed ripening process. Ethylene work as a ripening hormone that starts, controls, and coordinates the expression of numerous genes related to ripening. Climacteric fruits react to the ripening process in accordance with the concentration of ethylene. The ripening process can be delayed, and fruit and vegetable shelf life can be increased by blocking ethylene-mediated signaling, ethylene biosynthesis, and ethylene response elements using RNA interference, a characteristic that is desired in the postharvest or transportation industries (Xiong et al. 2005). ACC synthase, a crucial enzyme in the ethylene biosynthesis pathway, catalyzes the production of the ethylene precursor ACC. It was discovered that silencing all three ACC synthase homologs simultaneously improved the regulation of ethylene production (Gupta et al. 2013). They found a delay in ripening and an improvement in shelf life for roughly 45 days in transgenic tomatoes due to decreased ethylene production after expressing the chimeric dsRNA derived from off-target-free sequences of three tomato ACS homologs under the control of fruit-specific promoter 2A11. They also noted that the reduced expression of ethylene in RNAi plants affects the expression of genes that are sensitive to ethylene. Fruits' carotenoids build up throughout the ripening phase as well. STAY-GREEN (SlSGR1) protein inhibits the main carotenoid biosynthesis gene (SlPSY1). Through the control of ethylene signaling and gene expression, SlSGR1 also works in unison with the ripening process. The generation of H_2O_2 was reduced, and the ethylene-mediated signal transduction in tomatoes was altered by the low expression of the SlSGR1 protein, which improved the ripening process by increasing the production of ethylene and carotenoids (Luo et al. 2013).

4.13 Role of RNAi *in Raphanus sativus*

Under high salt conditions, 22 novel miRNAs that control salt-responsive genes such as squamosa promoter-binding-like proteins, auxin response factors, and nuclear transcription factor Y, i.e., SPLs, ARFs, and NF-Y, respectively, were discovered in *Raphanus sativus* (Sun et al. 2015). The transcription factor encoded by the NAC gene is needed for plant development and resistance to various environmental stresses. In order to sustain resistance to various abiotic stresses, miRNAs upregulate and downregulate the transcripts of their targeted genes.

4.14 Role of RNA Interference in Achieving Biotic Stress Resistance

The plant doesn't wilt in a drought because the increase in endogenous sterol level caused by SQS silencing reduces the density of stomata and stops water loss through transpiration. The CCCH type zinc finger protein OsTZF1 is expressed in response

to drought, salinity, and ROS. OsTZF1's activity for abiotic stress tolerance is indicated by the fact that silencing of the OsTZF1 gene leads to improvement in rice plants' resistance to extreme salinity and water-deficient conditions (Jan et al. 2013). Under conditions of high salinity, low expression of the OsTZF1 gene retains the internal constancy of plants by altering hormonal expression at the molecular as well as cellular levels. Similarly to this, RNA interference reduces plants' ability to tolerate cold temperatures by suppressing the proteins that are proline-rich in *Poncirus trifoliata*. PtrPRP protein has been seen to accumulate in extreme cold conditions. A number of miRNAs are upregulated when under heat-stressed conditions. In conditions of heat stress, high levels of mir398 suppress the genes for Cu/Zn superoxide dismutase (CSD). It has been found that overexpressing miRNA398 decreases the resilience of *Arabidopsis thaliana* and common bean (*Phaseolus vulgaris*) plants during heat stress because it causes miRNA-mediated degradation of CSD mRNAs (Guan et al. 2013; Naya et al. 2014).

5 Limitations of RNAi

The foremost drawback of RNAi is the time-taking and precious process. Significant controls as well as concerns with public knowledge were also necessary for these genetically engineered plants. In spite of its many benefits, VIGS types of RNAi technology have several drawbacks. The most common effect of RNAi is the random silencing of genes across the infected plant, which may lead to incorrect expositions when the silencing is unrelated to targeted phenotype. Perhaps the issue was resolved with including a positive control in the RNAi vectors, which makes silenced genes' vision easier (Burch-Smith et al. 2004). Additionally, VIGS is in charge of mutating difficult-to-understand nontarget genes, particularly at the time when there is an unavailability of genome sequence of the understudied species. Plant genomic content has been increased through HIGS technology to increase resistance to several plant diseases. However, because partial silence of mRNA do not guarantee the action of the protein, silencing a single gene from the plant may not be sufficient to control the abovementioned plant trait. The solution to this issue is to first examine the silencing structures using the transient system (Qi et al. 2019). When one or more sections in the 3′ untranslated regions of a nontargeted mRNA (or mRNAs) and seed region, i.e., positions 2–7 or 2–8 of siRNA complement one other, off-targeting occurs. The RISC complex can encourage off-target mRNA cleavage if it is unable to discriminate between the guide strand and passenger strand, i.e., antisense strand and sense strand, respectively. Erroneous effects might bring cellular malfunction as well as unfavorable genetic changes (adverse mutations). Other restrictions are the means of distribution and how to reach target tissues with little adverse effects (Srivastava et al. 2018). The off-target effects of HIGS are another drawback that might be remedied by employing bioinformatic tools to find off-targets. The specific process of RNA molecule transportation is yet to be researched; however, SIGS is another cutting-edge and groundbreaking

approach for improving bioactive compound production in plants (Koch et al. 2016 and Akbar et al. 2022).

6 Conclusion and Future Prospect

This chapter summarizes and concludes the most recent findings on RNA interference in plants and their regulatory functions in bioactive compound production. RNAi is a reliable and efficient method for developing plants with improved production of bioactive compounds or secondary metabolites. A high-yield variety can be developed by using RNAi technology. A variety of regulatory pathways and modules, including to achieve regulation, one may employ miR156-SPL module, miR397-LAC module, miR858-MYB module, miR12112-PPO module, miR828-MYB module, and various siRNA and lncRNA regulatory pathways. The quality and quantity of medicinal plants could be improved by further functional analyzing the noncoding RNAs of the plants. Future advancements in science and research may be able to overcome all current constraints.

Acknowledgments CSIR-Indian Institute of Integrative Medicine, Jammu, India is gratefully acknowledged. The authors would also like to generously acknowledge Dr. Ajai Prakash Gupta for providing assistance during the initial phases of writing this article. Miss Diksha Manhas from CSIR-IIIM, SRF, Jammu, is acknowledged for fruitful suggestions and discussions.

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Hairy Root Cultures: A Versatile Tool for Bioactive Compound Production

Shweta Singh and Manish Kumar

Abstract "Hairy root" finds its route through a century-long path, from being a disease to a tool for enhanced production of bioactive compounds, the essence of which lies on the "natural genetic engineer" *Rhizobium rhizogenes*. To date, hairy root cultures have been established in hundreds of plant species including several threatened species, offering opportunities to produce a large amount of bioactive compounds in an eco-friendly manner. Diverse strategies are being supplemented to enhance the production of desired metabolites from hairy root culture. A combination of strategies, along with upscaling of the hairy root culture, is a way forward for the commercial production of bioactive compounds.

Keywords Hairy root · *Rhizobium rhizogenes* · Bioactive compounds · Secondary metabolites · Elicitors

1 Introduction

Plants have served mankind with their nutrients since time immemorial. Besides nutrients, other useful bioactive compounds which range from drugs, colors, flavors, pesticides, cosmetic additives, etc. are in daily use of modern society. Secondary metabolites, which elicit certain pharmacological or toxicological impact on life of human or animals, may be designated as bioactive compounds of the plant. Secondary metabolites in plants are produced as a defense against different biotic and abiotic stress, as attractants, and for signaling. Along with primary

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N. Kumar, R. S. Singh (eds.), *Biosynthesis of Bioactive Compounds in Medicinal and Aromatic Plants*, Food Bioactive Ingredients,
https://doi.org/10.1007/978-3-031-35221-8_7

metabolites, essential for plant growth and development, secondary metabolites are produced in addition and are therefore regarded as products of biochemical "side tracks" in plant cells, not needed for the primary metabolic functions of the plant. Despite massive progress in synthetic chemistry, plants are the raw material for more than 25% of all prescribed medicines. To obtain these bioactive compounds at a commercial level, harvesting plant resources from nature has led to overexploitation, bringing them under threat of becoming vulnerable to extinction. To fulfill the demand-driven industries dependent on bioactive compounds from plants, various alternative technologies for their production have surfaced.

In vitro techniques, wherein plant cells, tissues, and plants are cultivated in a sterile condition, free of natural environmental conditions and geographical barriers, offer alternatives for obtaining bioactive compounds. Manipulation in the technique of natural bacterial transformation of roots of higher plants referred to as "hairy root culture" is emerging as a tool to fulfill the need of growing industries for obtaining bioactive compounds from the plants.

2 Hairy Root Culture: Historical Background and Establishment

During the 1930s, several investigations were carried over to understand the mechanism underlying crown gall and hairy root diseases. By the end of the 1970s, natural genetic engineering of phytopathogenic bacteria, *Agrobacterium tumefaciens* and *Agrobacterium rhizogenes* (syn. *Rhizobium rhizogenes*) belonging to family *Rhizobiaceae* causing these diseases, was understood and the molecular mechanism involved was elucidated (Chilton et al. 1977, 1980).

Recent advances in tissue culture coupled with genetic engineering particularly genetic transformation approaches have been pursued for enhanced productivity of plant secondary metabolites (Verpoorte et al. 2002; Georgiev et al. 2007). Generally, in vitro multiplied medicinal plants act as a starting material for the biotransformation and metabolomic research, aimed at the enrichment of bioactive secondary metabolites (Singh et al. 2017). Though hairy root transformation experimentation started during the early 1930s, the exploitation of this disease for enhanced production of secondary metabolites took wing during the mid-1980s.

Initially, during the 1930s, studies were conducted on hairy root infection of apples from the gram-negative, nonacid-fast, rod-shaped bacterium with further positive results upon inoculation on rose, sugar beet, bean, etc. Out of the collected 96 overgrowths of hairy root types from apples, 78 yielded the hairy root type. Natural and induced hairy root infections and their seasonal influences were studied (Riker et al. 1930; Riker and Hildebrand 1934). During the mid-1980s, several experimentations were carried out for enhanced production of plants' secondary metabolites, particularly alkaloids from hairy root systems. In *Datura stramonium*, hairy root culture on leaf tissue yielded a 55-fold increase in cell mass with 0.3% hyoscyamine, a quantity comparable to that obtained from pot plants. In *Atropa*

belladonna, the amount of atropine and scopolamine obtained from hairy root culture were comparable or even higher than those obtained from field-grown plants (Kamada et al. 1986; Spano et al. 1987; Payne et al. 1987).

The intensity of hairy root transformation in different plants studied was found to be dependent on several factors, including types of explants selected and coculture period along with the strains of *R. rhizogenes*. In *Bacopa monnieri*, stable hairy root line was obtained with different strains of *R. rhizogenes* where maximum transformation frequency was obtained upon infection with strain SA_{79} using leaf explants followed by internode explants. The maximum frequency of transformation was observed at 2 days of cocultivation period and 10 min of infection (Bansal et al. 2014). In *Withania somnifera*, effect of different explants, coculture periods, and strains of *R. rhizogenes* on hairy root culture were studied (Sivanandhan et al. 2014). In *Rauwolfia serpentina*, virulence of five strains of *R. rhizogenes* on comparison showed the A4 strain to be of greatest prominence (Mehrotra et al. 2015). Cotyledons and hypocotyl of 3-week-old aseptically grown plantlets were used for hairy root culture in *Eurycoma longifolia* (Ngoc et al. 2016).

The hairy root system for secondary metabolite production has also been reported in plant species belonging to the family Acanthaceae. In *Andrographis paniculata*, different strains of *R. rhizogenes* were tested for their transformation efficiency using different explants and cocultivation periods (Marwani et al. 2015). The effect of signal compounds for the enhancement of andrographolide production in hairy roots of *A. paniculatus* was reported (Sharmila and Subburathinam 2013).

The attractive features of the hairy root system, including high genetic stability (compared to undifferentiated cell and tissue culture) and a relatively fast growth rate (compared to normal roots), make it a potential tool for producing valuable metabolites. By 2015, more than 155 plant species of 41 families, transformed by *R. rhizogenes* strains of diverse host range and virulence, have been reported to produce secondary metabolites (Tian 2015). Hairy root transformation of *Atropa belladonna*, *Duboisia myoporoides–D. leichhardtii* hybrid, *Cephaelis ipecacuanha*, *Digitalis lanata*, and *Papaver somniferum* through *R. rhizogenes* infection and production of medicinally important secondary metabolites have been well demonstrated. In this effort, the production of low morphine *Papaver somniferum* plant which mainly contained codeine, was achieved for the first time through *R. rhizogenes*–mediated transformation (Yoshimatsu 2008).

In secondary metabolite production, hairy root culture has been found biochemically much more stable and more significant in production capacity than cell suspension culture (Deus-Neumann and Zenk 1984; Toivonen 1993; Guillon et al. 2006; Verma et al. 2007; Yue et al. 2016). The growth of hairy root has been reported to be much higher than the of non-transformed roots (Huang et al. 2014). The transformed roots are reported to produce secondary metabolites with higher efficiency (Kamada et al. 1986; Flores et al. 1999; Giri and Narasu 2000). In different species of *Valerian*, growth rate and valepotriate content in hairy roots were observed much higher than in the in vitro or in vivo grown roots (Banerjee et al. 1998; Granischer et al. 1992). *R. rhizogenes* strain A4 was observed to contain a higher amount of valepotriate when compared with strain LBA 9402 (Banerjee et al. 1998). Cerebral

malaria, an infectious disease caused by parasitic protozoa *Plasmodium falciparum*, can be treated with artemisinin, a sesquiterpene endoperoxide lactone found in herb *Artemisia annua* L. Large-scale production of artemisinin using hairy root cultures has been reported by several scientists (Wang et al. 2001; Patra and Srivastava 2014, 2016). *R. rhizogenes*-mediated transformation and hairy roots offer metabolic engineering interventions which may further increase the productivity of desired secondary metabolites (Hughes et al. 2004; Kai et al. 2011; Sun and Peebles 2016).

3 *Rhizobium rhizogenes*: A Natural Genetic Engineer

The hairy root is a plant disease resulting in neoplastic growth in plants caused by virulent strains of *Rhizobium rhizogenes*. These hairy root tumors result in massive proliferation of roots, originating from the site of infection. The genes responsible for the hairy root phenomenon harbor a large-sized (>200 kbp) conjugative and replicative Ri (root-inducing) plasmid possessing all the major genes for pathogenicity (Fig. 1). Ri plasmid from different strains of *R. rhizogenes* generally have several features in common. The plasmid contains an origin of replication, one or more T-DNA (transfer-DNA) regions, a vir region, genes for the catabolism of opines (a class of amino acid/sugar conjugates).

T-DNA region of Ri plasmid is defined by the presence of a small (24 bp) right and left border sequence. The DNA between the borders gets transferred into the genome of the host plant. The genes that are responsible for the transfer of the T-DNA region into the host plant are situated on the Ti plasmid, around 40 kb region outside the T-DNA, known as vir (virulence) region. T-DNA region of Ri plasmid gets transferred to the plant tissues when induced with the polyphenolic exudate from the plant wounds (Fig. 2). These polyphenolic exudates from wounded plant cells signal and induce the Vir A gene (Vir A, a membrane-linked sensor kinase), which autophosphorylates, subsequently phosphorylates, and activates Vir G. Vir G then induces the expression of all other vir genes. The products of these vir genes are responsible for the transfer of T-DNA to the host plant and integration of the segment in the plant genome. Vir D1 and Vir D2 are involved in single-stranded T-DNA production, protection, and release to the host cell. Products from Vir B gene form transfer channels. Vir D2-attached single-stranded T-DNA gets exported through this transfer channel along with the gene product of Vir E2. Within the host plant cell, Vir E2 coats the T-DNA protecting it from the undesirable effects of plant products. The diverse arrays of vir genes are involved in the successful transfer and integration of secondary metabolites, along with the abovementioned genes. Horizontal gene transfer and tumorigenesis initiation with A4 strain of *R. rhizogenes* in the host plant *Nicotiana glauca* were first shown by White et al. (1982).

Once transferred, the T-DNA integrates into the plant's nuclear DNA. The T-DNA contains a set of genes that are functionally divided into two groups: oncogenes, which cause malignant transformation of the host cells resulting into hairy roots, and opine biosynthesis genes, which direct in the production and secretion of

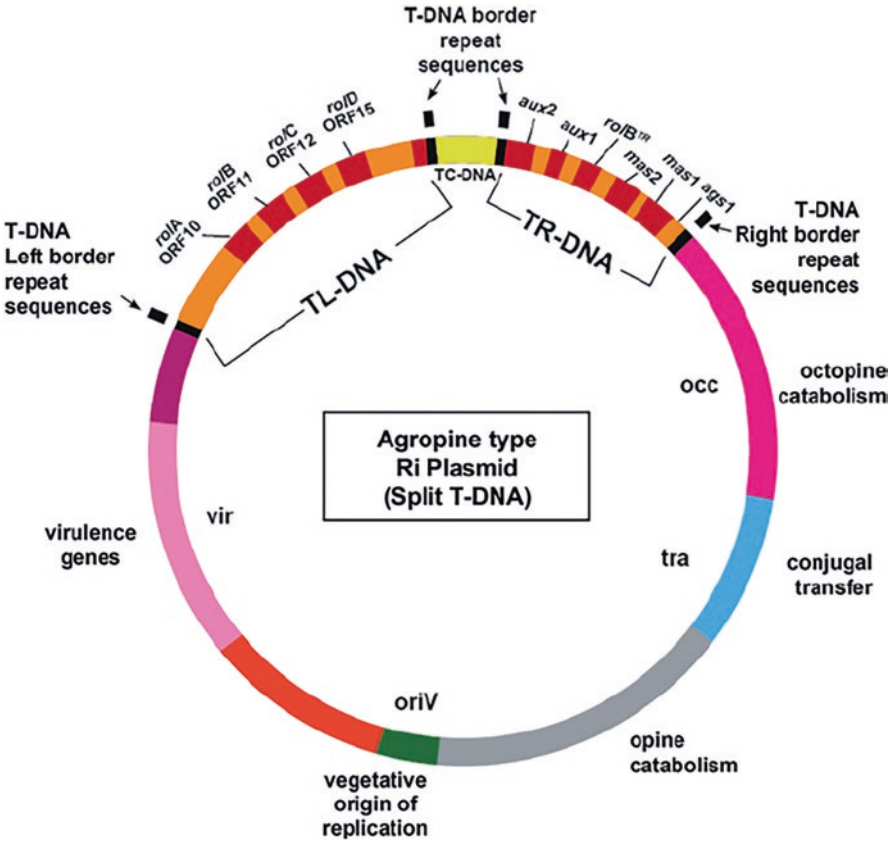

Fig. 1 Schematic diagram of Ri plasmid. (Ozyigit et al. 2013)

opines for catabolism in *R. rhizogenes* (Schell et al. 1979). These opines are utilized by *R. rhizogenes* as the sole source of carbon and, in some instances, nitrogen. In a few strains (e.g., agropine and octopine strains) of *R. rhizogenes*, T-DNA is fragmented into two arms, T_L-DNA and T_R-DNA that can be independently transferred to the plant (Vladimirov et al. 2015). T_L-DNA is essential for the induction of hairy root syndrome. It carries 18 open reading frames (ORF), four of which have genes *rolA*, *rolB*, *rolC*, and *rolD*, essential for hairy root formation corresponding to ORF 10, ORF 11, ORF 12, and ORF 15, respectively. T_R-DNA contains two genes responsible for the biosynthesis of auxins (*iaaM* and *iaaH*) along with the genes responsible for synthesis of the opines, mannopine (*mas1′* and *mas 2′*), and agropine (*ags*).

This natural phenomenon has been exploited in plant biotechnology to generate transformed root culture. During the mid-1980s, alkaloid production in a few plant hairy roots provided insight for the utility of this biological transformation in secondary metabolites. Research in the 1990s highlighted several advantages of hairy

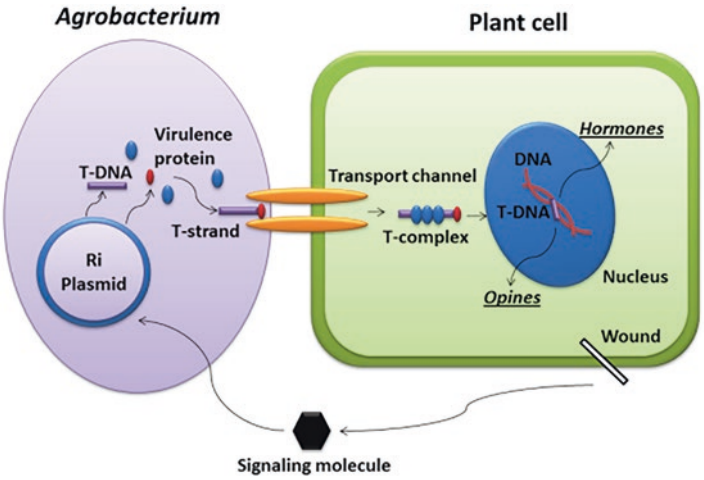

Fig. 2 Schematic diagram of infection of plant cell with *R. rhizogenes*. (Adopted from Altpeter et al. 2016)

roots including their relatively large growth in hormone-free media, genetic and metabolic stability, and lack of geotropism. Several commercial companies are currently interested in large-scale industrial production of secondary metabolites from hairy plant roots. Apart from the production of standard secondary metabolites of the parent plant, hairy roots are the potential source of new compounds with pharmaceutical value.

4 Secondary Metabolites

Secondary metabolites are produced in plants for their survival in their environment. Defense against diseases and pests, attractants to pollinators, etc. are some of the roles of secondary metabolites. These secondary metabolites have found commercial applications such as drugs, insecticides, flavoring and fragrance, dye, etc. Plant secondary compounds are classified according to the biosynthetic pathways followed by them. Three broad families of secondary metabolites are phenolics, terpenes, and steroids and alkaloids. Various other bases for grouping of secondary metabolites include their chemical composition (e.g., N containing), structural differences (e.g., compounds possessing rings, glucosyl group, hydroxyl group), their solubility, and the pathway of their synthesis (e.g., umbelliferone, derived from phenylalanine). In a simplified way, secondary metabolites are classified into three main groups: the terpenes (largest group, synthesised through mevalonic acid, composed mostly of carbon and hydrogen only), phenolics (comprising simple sugar, hydrogen, and oxygen, also containing benzene rings) and nitrogen containing compounds including alkaloids (extremely diverse group).

5 Procedure for *Rhizobium rhizogenes*-Mediated Transformation

The procedure used to induce hairy roots includes the cultivation of wounded plant parts (explants) with suspensions of *R. rhizogenes* under aseptic conditions. Explants are usually excised from in vitro grown shootlets, though sterilized plant parts from the field are also being used with limited success. Explants (leaves, shoot tip, or nodal explants) can be infected with *R. rhizogenes* either through direct inoculation with bacterial suspension or through cocultivation of explants in liquid media for a certain time period. In either of the cases, explants are transferred on solid media with antibiotics (Claforan or penicillin derivatives) for incubation, usually about 72 h later, to eliminate bacteria (Rahman et al. 2004). Alternatively, post-coculture, the explants may be washed with cefotaxime (antibiotics) followed by inoculation in harmone-free basal media (Murashige and Skoog's or Gamborg's B5) (Singh et al. 2020). The neoplastic, transformed roots with abundant branching appear (usually within 1–4 weeks) in non-geotropic manner under aseptic, controlled culture conditions.

Successful genetic transformations can be confirmed/validated indirectly or directly by detecting opines or T-DNA, respectively. Opine production, in some case, is not stable or may even cease; therefore, direct method is a preferred one. Detection of T-DNA (*rol* genes) in transformed plants can be done through either polymerase chain reaction (Singh et al. 2020) or through southern hybridization (Xie et al. 2001). After a short period of adaptation, hairy root culture can be used for the production of secondary metabolites either in solid or liquid media.

6 Elicitors: Route for Enhancement in Bioactive Compound Production by Hairy Root Culture System

The term elicitors refer to chemicals obtained from various biotic or abiotic sources, as well as physical factors, that can trigger defense response in plants resulting in the production and accumulation of secondary metabolites. The studies on induction by *Phytophthora megasperma* resulting in phytoalexins accumulation in soybean proved that small molecules of pathogen origin may act as an elicitor and trigger the same response in the plant as pathogen itself (Keen 1975). Elicitors are, therefore, useful tools for enhancing the production of desired bioactive compounds (secondary metabolites).

Classification of Elicitors

Elicitors have been classified, in general, on the basis of their origin and molecular structure (Table 1). According to the characteristics of elicitors and plants, each type of elicitor can induce specific response that depends on the interaction between elicitor and plant hairy root culture. Elicitors may be of biotic or abiotic origin.

Table 1 Classification of elicitors

Biotic elicitors			Abiotic elicitors	
Exogenous		Endogenous		
Defined composition	Complex composition		Physical	Chemical
Chitosan	Fungi homogenate	Cell wall	Thermal stress	Sodium orthovanadate
Alginate	Yeast extract	Plant signaling compounds (jasmonic acid, methyl jasmonate, salicylic acid, etc.)	Osmotic stress	Vanadyl sulfate
Chitin	Fungal spores		UV irradiation	Heavy metal (Ag, Cd, Cu)
Pectin	Bacterial lysate		Wounding	Mannitol, sorbitol
Naphthaleneacetic acid			Salinity	Ozone
Elicitin				pH

Biotic elicitors usually originate from pathogens and sometimes from the plant itself (endogenous elicitor). Biotic elicitors may be of defined composition where their molecular structures are known or of complex composition, where the chemical identity of the elicitor becomes impossible to ascertain. Abiotic elicitors are further classified as physical factors and chemical compounds of inorganic origin. Apart from abovementioned classification, elicitors may also be classified as "general elicitors," which may trigger defense response both in host and nonhost plants and "race-specific elicitors," which trigger defense response only in specific host cultivars.

6.1 Factors Influencing Elicitation

The effectiveness of elicitation strategy for enhanced production of secondary metabolites in plant hairy root system depends upon optimization of different parameters, such as elicitor specificity, concentration and treatment duration, treatment intervals, culture conditions, etc.

Elicitor Specificity: A few elicitors have been reported to stimulate secondary metabolism in different plant hairy root cultures, whereas certain hairy roots are responsive to diverse types of elicitors. Also, an elicitor may not stimulate a different group of bioactive compounds (secondary metabolites) in the same species as well as similar secondary metabolites in all species. Though the class of

metabolites produced depends on the plant species, the kinetics of induction and accumulation level of the metabolites vary with different elicitors.

Concentration, Treatment Duration, and Treatment Intervals: The concentration of elicitors greatly influences the intensity of the production of bioactive compounds from hairy roots. Concentration, treatment duration, and treatment intervals are the factors that need optimization for enhanced production of secondary metabolites from any specific plant hairy root system. In general, two types of dose response have been observed: first in which higher doses do not affect the hairy root system, and second, where optimization for enhanced production becomes essential (Dixon et al. 1981).

Culture Conditions: The growth stage of hairy roots, media composition, light, etc. influence the time of addition of elicitors. Literature suggests that maximum production takes place during the exponential growth phase of hairy roots (Hussain et al. 2022) as the enzymatic machinery is at its maximum functional state, and the response to the elicitors, as a consequence, becomes highest. The chemical composition of media, the presence of growth hormones such as auxins, and precursor feeding, for example, cholestrol, L-arginine, etc., to the basal media along with elicitors have resulted in enhanced secondary metabolite production in the hairy root system (Ooi et al. 2016). In some instances, dark periods have been found to stimulate secondary metabolite production upon the addition of elicitors (Walker et al. 2002), while in some cases, the light stimulus becomes essential (Vazquez-Flota and De Luca 1998).

Elicitation of hairy root culture offers a feasible alternative route for the commercial production of important bioactive compounds from plants. Detailed information on the effect of different types of elicitors on the scaling up of secondary metabolite production in hairy root culture can be found in the review paper by Halder et al. (2019).

7 Future Prospects

Recent advancements in the use of *Rhizobium rhizogenes*-mediated transformation techniques for the hairy root system and consequently enhanced production of bioactive compounds have been found successful in a number of plant species, including rare and threatened plants of therapeutic values, offering an attempt to preserve biodiversity and maintain ecological balance in nature. Through enhancement in the knowledge of metabolic pathways in plants and the mechanism of their regulation, furthermore, enrichment in bioactive compounds' productivity by hairy root culture can be achieved. Overall, strategies available and under rigorous research suggest that hairy root technologies hold a threshold for the new era of bioactive compound synthesis at the commercial level.

Acknowledgments The authors are grateful to Birla Institute of Technology, Mesra, Ranchi and Birsa Agricultural University, Ranchi for providing infrastructure support throughout the research work.

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Role of Induced Mutation and Stresses in the Production of Bioactive Compounds in Plants

Binit Baraik, Tanupa Kumari, and Shalini Lal

Abstract Humans have used the plants as medicine for a variety of ailments for a long time. Because of their broad pharmacological activities, excellent safety margins, and lower prices, medicinal herbs are widely used as a primary healthcare option in both developed and developing countries. Secondary metabolites from plants are an outstanding source for medicines, food additives, flavorings, and other industrial applications. Higher plants produce a variety of secondary metabolites, which are often involved in ecological processes but only slightly affect the basic functions of the plant. Induced mutagenesis is one of the most efficient strategies that have been extensively applied to create genetic variation for economically valuable traits. Induced mutations have been efficiently used to create improved varieties of some medicinal plants. In addition, it is believed that in response to environmental stress, the plant stimulates the production of bioactive molecules. Plant cells exhibit both morphological and physiological reactions in response to environmental factors. This chapter describes the role of induced mutation and stresses in the production of bioactive compounds in plants. This chapter discusses the various biotic and abiotic elicitors used to induce the biosynthesis of secondary metabolites in several medicinal plants as well as the factors that affect the elicitation process. This chapter also briefly discusses the elicitation mechanism and how it affects the gene that codes for an enzyme for secondary metabolite biosynthesis.

Keywords Secondary metabolites · Induced mutation · Elicitors · Medicinal plants

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N. Kumar, R. S. Singh (eds.), *Biosynthesis of Bioactive Compounds in Medicinal and Aromatic Plants*, Food Bioactive Ingredients,
https://doi.org/10.1007/978-3-031-35221-8_8

1 Introduction

Throughout human history, medicinal plants have been identified and used. Different nations use plants as medicine, and they are the source of a number of strong and potent drugs (Petrovska 2012). The World Health Organization reports that traditional medicines continue to be the primary method of primary healthcare for the majority of populations. These medications are typically less expensive and safer than synthetic or contemporary drugs. The formation of these biologically active compounds is strongly influenced by the different growth stages of the plant. Secondary metabolites that are used commercially as biologically active chemicals are obtained from medicinal plants and are typically high value, low volume products, as opposed to primary metabolites. Because their chemical synthesis is either extremely challenging or impractical from an economic standpoint, the majority of these bioactive molecules are currently isolated from wild or domesticated plants. A great number of herbs and trees, which are the primary source of highly valuable drugs, are losing their natural habitat as a result of anthropogenic activities. Many researchers across the world are developing various strategies to address this worrying situation with the goal of qualitatively and quantitatively enhancing biologically active compounds. All of these efforts are intended to meet the demand for extremely expensive drugs and their precursors.

2 Role of Induced Mutation in the Production of Bioactive Compounds

Crop improvement requires the presence of genetic variability, which breeders can obtain through natural sources or artificially induced mutations. Mutagenesis is the process by which physical, chemical, or biological agents cause sudden, heritable changes in an organism's genetic makeup (Fig. 1). Physical mutagens include electromagnetic radiation and particle radiation, whereas chemical mutagens primarily include alkylating agents, base analogues, deaminating agents, acridine dyes, and metal ions (Kodym and Afza 2003).

2.1 Induced Mutagenesis

Induced mutagenesis is one of the most efficient strategies that have been extensively applied to create genetic variation for economically valuable traits that may be challenging to incorporate via traditional breeding. Plant cultivars with improved traits, such as greater production, quick and efficient cultivation periods, disease resistance, stress resistance, greater metabolite output, etc., can be developed using induced mutation. Although there are a number of methods for inducing mutations,

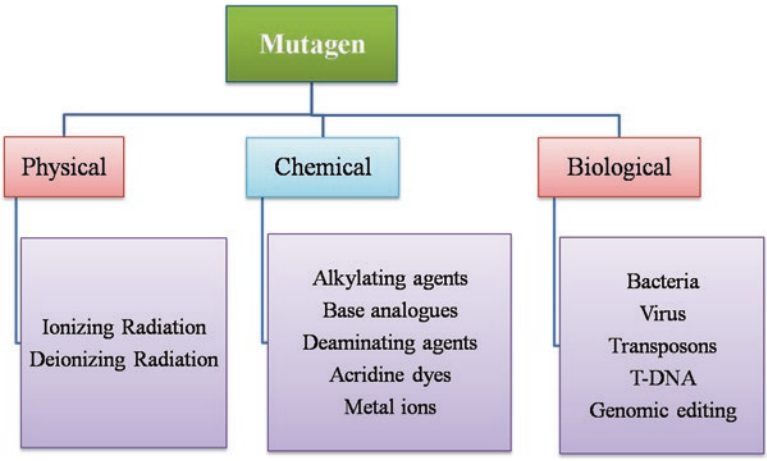

Fig. 1 Types of mutagen used in induced mutagenesis

including physical, chemical, and insertional mutagen treatments, these approaches are not preferred due to their high cost and time- consuming nature. However, millions of mutations can now be studied in a very short amount of time owing to advances in biotechnology, including next-generation sequencing (NGS) techniques and whole-genome sequencing (Chaudhary et al. 2019). When treating crops with mutagenic agents, seeds are the easiest and most common option. As they are easy to manage, preserve, and transport, large quantities of seeds can be used for the mutagenic treatment. Furthermore, the conditions for the mutagenic treatment can be easily replicated in order to obtain reproducible results. In addition to treating seeds, whole plants and their parts, or in vitro regenerated tissue, can also be used (Kodym and Afza 2003; Oladosu et al. 2016). Identifying appropriate genotypes with desired traits among present varieties, or creating them if they are not already present in nature, is the initial step in plant breeding. In order to create mutations in seeds as well as other planting materials, chemical and physical mutagenesis are both used. The majority of mutant lines may be eliminated during the first generation of selection for agronomic traits. Through phenotypic stability, the agronomic characters are ascertained in the second and third generations, and other assessments are made in the later generations. Then, only desirable mutated lines are chosen as a parent line for crossbreeding (Oladosu et al. 2016).

2.2 Induced Mutagenesis in Medicinal Plant Improvement

Induced mutations have been successfully used over the past decades to create improved varieties of crop plants (Suprasanna et al. 2015). However, there hasn't been much work done to improve medicinal and aromatic plants (Oladosu et al. 2016). Most medicinal and aromatic plants have very limited genetic diversity.

Table 1 Application of induced mutation in some medicinal plant improvement

Plant name	Mutagen used	Improved effect	References
Hyoscyamus niger	Ethyl methane sulfonate	Increased accumulation of scopolamine and hyoscyamine	Shah et al. (2020)
Papaver somniferum	Gamma rays, ethyl methane sulfonate	High seed yield, high seed oil	Sharma et al. (1999)
Catharanthus roseus	Spermine, jasmonic acid, methyl jasmonate, putrescine, and cold plasma treatments	Significant increase in antioxidant enzyme activity	Marzban et al. (2022)
Catharanthus roseus	Ethyl methane sulfonate and X-rays	Delayed seed pod development	Mistry et al. (2022)
Catharanthus roseus	Sodium azide, X-ray	Higher catharanthine, vindoline, and vinblastine content	Mistry et al. (2022)
Catharanthus roseus	Colchicine	Larger stoma and more branches and leaves Increase the contents of terpenoid indole alkaloids	Xing et al. (2011)
Gloriosa superba	Ethyl methane sulfonate	Significant improvement in seed production	Padmapriya and Rajamani (2017)
Nigella sativa	Colchicine	Total flavonoid and phenolic and total antioxidant significantly elevated	Gupta et al. (2021)
Chamomilla recutita	γ-rays	High flower and essential oil yield	Lal et al. (2019)

Additionally, the gene pool is being depleted at an exponential rate due to overexploitation. In this situation, inducing genetic variability through deliberate mutations may help produce plants with higher levels of active compounds. Despite the lack of research, many scientists are working to improve medicinal plants through induced mutations (Table 1).

3 Elicitation and Production of Secondary Metabolite

Secondary metabolites are typically isolated from wild or cultivated plants and are a major source for the pharma, agro, and aroma industries. For the huge in vitro production of plant secondary metabolites, plant tissue culture is considered an effective technique (Rao and Ravishankar 2002; Vanisree et al. 2004). As a result of various stresses, including biotic and abiotic, these substances accumulate in plants. Elicitation is the process by which plants stimulate or improve the synthesis of active biomolecules. Elicitors, the substances that cause the synthesis of secondary metabolites, could either be abiotic or biotic (Fig. 2). It can also be divided into exogenous and endogenous categories depending on their "origin" (Namdeo 2007). They have particular binding sites that serve as receptors and are known to change

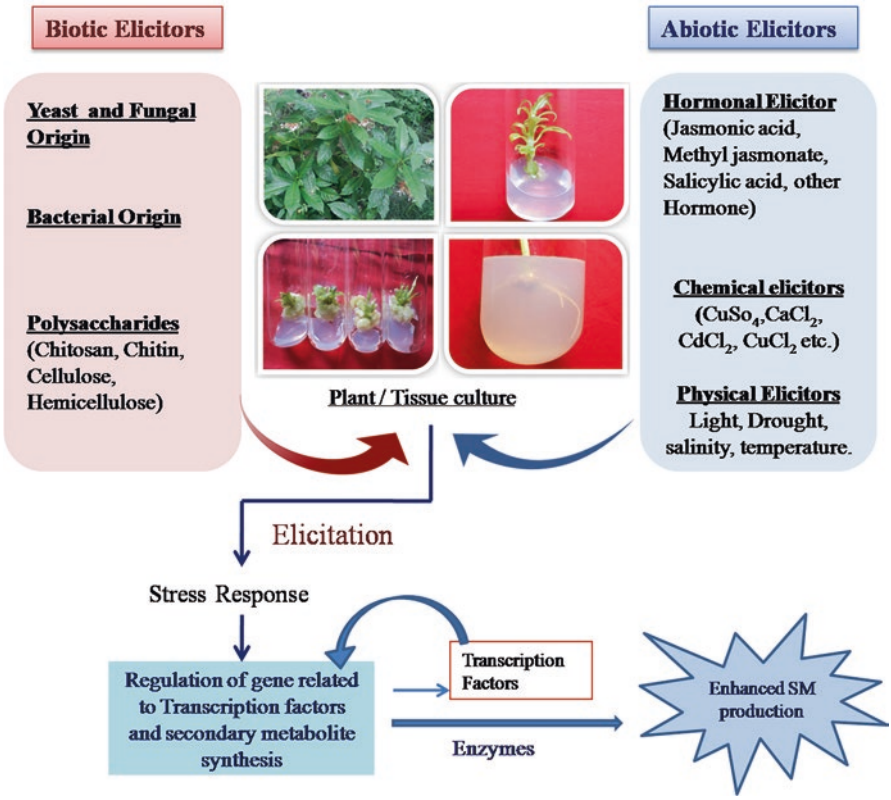

Fig. 2 Diagrammatic representation of different types of elicitors used for secondary metabolite production in medicinal plants and the mechanism of elicitation

the permeability of plasma membranes. The production of active biomolecules is triggered by this interaction of elicitors and binding sites. Such interactions of elicitors with binding sites trigger secondary metabolite production inside the cell (Sudha and Ravishankar 2003).

3.1 Molecular Mechanism of Elicitation in Medicinal Plants

Plants react to stressful situations by triggering tolerance mechanisms at various organizational levels. Plants typically start responding to elicitors at the plasma membrane of the cell. Different elicitors are perceived by distinct membrane receptors, despite the fact that they may activate the same signaling pathways. Second messengers act during the transduction of the elicitor signal, which amplifies the signal for additional downstream reactions. The depolarization of the cell membrane caused by elicitor perception activates plasma membrane channels such as the

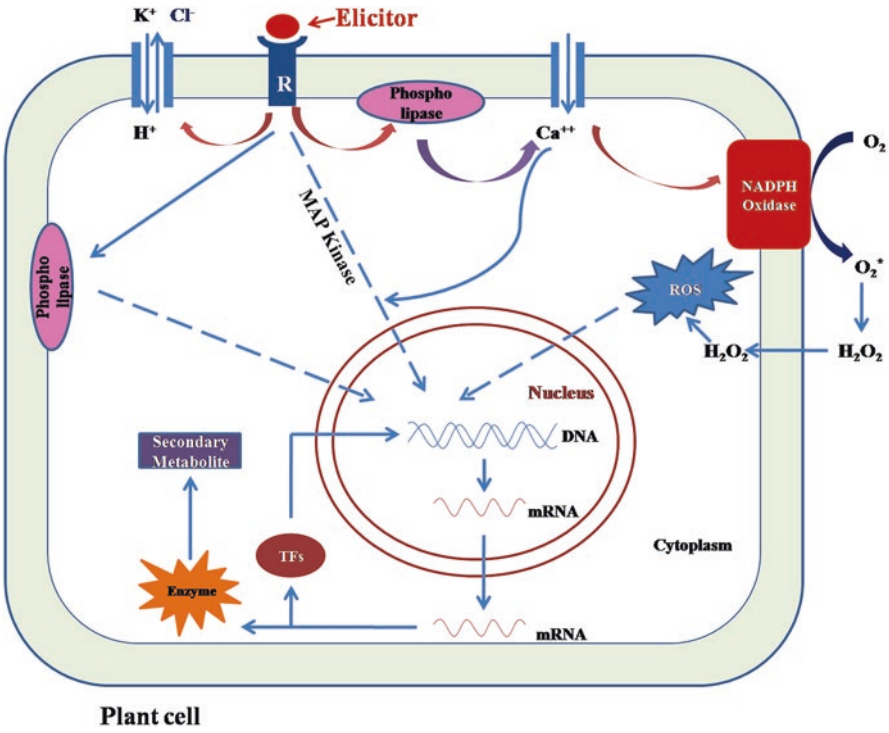

Fig. 3 Diagrammatic representation of molecular mechanism of elicitation in plant cell (Abbreviation: *R* receptor, *ROS* reactive oxygen species, *MAP kinase* mitogen-activated protein kinase, *TFs* transcription factors) (adopted and modified from Ramirez-Estrada et al. 2016)

Cl- and K+/H+ antiport channels. The extracellular alkalinization and cytoplasmic acidification caused by ion fluxes can serve as indicators for the synthesis of secondary metabolites. Elicitors also activate mitogen-activated protein kinase (MAPK) cascades, which in turn phosphorylate transcription factors (TF) regulating the expression of genes that encode enzymes involved in the biosynthesis of specific bioactive compounds or secondary signals, such as ethylene, salicylic acid, and jasmonic acid (Fig. 3) (Zhao et al. 2005; Ferrari 2010; Ramirez-Estrada et al. 2016).

3.2 Effect of Elicitors on Gene Related to Metabolic Pathway

The metabolic pathway leading to secondary metabolite formation is a very complex metabolic process that is still not completely understood. In numerous plants, the impact of elicitation on the expression of a few genes connected to a metabolic pathway has now been investigated. A number of researchers have found a link between the amount of secondary metabolite formation in response to elicitation and the transcript richness of the genes required for their synthesis.

On a variety of plants, many authors have studied the influence of methyl jasmonate (MeJA) on the regulation of the genes involved in metabolic pathways. Two crucial genes of the mevalonate pathway, the metabolic pathway leading to the production of diosgenin, were upregulated by methyl jasmonate: Sterol-3-glucosyl transferase (STRL) and 3-hydroxy-3- methylglutaryl-CoA reductase (HMG) in *Trigonella foenum-graecum*. Notably, MeJA was shown to have a dose-dependent effect on both target gene expression and diosgenin levels, with a decreasing effect with increasing MeJA concentration (Chaudhary et al. 2015). Kim et al. (2013) studied the impact of methyl jasmonate on the rosmarinic acid biosynthesis of *Agastache rugosa* cell cultures. With the addition of 50 M MeJA, the transcript levels of the phenylpropanoid biosynthetic genes ArPAL (phenylalanine ammonia lyase), Ar4CL (4-coumarate:CoA) ligase), and ArC4H (cinnamate 4-hydroxylase) increased in comparison to untreated controls (Kim et al. (2013). Additionally, after being exposed to methyl jasmonate (MJ), cultured cells of *Lithospermum erythrorhizon* showed a marked increase in rosmarinic acid (RA) content by increasing phenylalanine ammonia lyase (PAL) and 4-hydroxyphenyl pyruvate reductase (HPR) activities (Mizukami et al. 1993). Tyrosine aminotransferase (TAT) and phenylalanine ammonia lyase (PAL) expression in *Cannabis sativa* cell suspensions is enhanced by methyl jasmonate in combination with precursor feeding (tyrosine) (Gabotti et al. 2019). MeJA stimulates the growth of the culture and accelerates metabolite synthesis by controlling the expression of the genes responsible for the metabolite biosynthesis in *Codonopsis pilosula*. Following MeJA treatment, expression of the gene CpPMK increased, an enzyme in the mevalonic acid (MVA) pathway involved in terpenoid synthesis that may influence atractylenolide III synthesis in *C. pilosula* (Ji et al. 2019). In a two-stage system, Sabater-Jara et al. (2014) investigated the synergistic effect of cyclodextrins and methyl jasmonate on taxane production in Taxus x media cell cultures and its connection to the transcription of the genes associated with taxol metabolism. Transcript abundance and taxol biosynthesis clearly benefited from the combined effect of methyl jasmonate and cyclodextrins, with levels of production 55 times greater than in unelicited cultures (Sabater-Jara et al. 2014). In cultured cells of *Glycyrrhiza glabra*, methyl jasmonate (MeJA) increases soyasaponin biosynthesis by increasing the mRNA level and enzyme activity of beta-amyrin synthase (AS), an oxidosqualene cyclase, and squalene synthase (SQS), an enzyme involved in triterpene and sterol biosynthesis (Hayashi et al. 2003).

In *Bupleurum falcatum*, the squalene synthase (BfSS1) gene was studied to determine its regulatory function in the biosynthesis of phytosterol and triterpenes. Methyl jasmonate (MeJA), ABA, and ethephon exposure cause BfSS1 mRNA to accumulate widely in plant organs (Kim et al. 2011). Salicylic acid as well as yeast extract increased the levels of the biosynthetic genes farnesyl diphosphate synthase and isopentenyl pyrophosphate isomerase, which led to the formation of mono- and sesquiterpenoids in root cultures of *Panax ginseng* (Rahimi et al. 2014). In *Adhatoda vasica* cell cultures, salicylic acid and sorbitol stimulated anthranilate synthase activity, which serves as an essential step in the biosynthesis of pyrroloquinazoline alkaloids (Singh et al. 2017).

Studies on the effect of biotic elicitors on the abundance of gene transcripts and the formation of bioactive molecules also reveal a positive correlation between them. The endophytic fungal elicitor has the ability to activate a variety of specific secondary metabolic pathways, which can result in marked increases in the expression of specific genes that increase the biosynthesis of bioactive compounds (Zhai et al. 2017). In *Catharanthus roseus*, the pathway for terpenoid indole alkaloid (TIA) synthesis is differentially controlled in response to various stresses. *Aspergillus flavus* increased the transcript profile of the genes involved in TIA biosynthesis in the suspension culture, which in turn encouraged the accumulation of terpenoid indole alkaloids (Liang et al. 2018). By modifying the gene expression involved in the TIA biosynthetic pathway, endophytes can improve the level of ajmalicine and serpentine in *C. roseus*. Strictosidine synthase, tryptophan decarboxylase, and geraniol 10-hydroxylase are three key genes whose expression has been upregulated by endophytic strains. In plants inoculated with endophytes, upregulation of transcription activators and downregulation of transcriptional repressors improved the expression of genes responsible for active biomolecule production (Singh et al. 2020). By altering gene expression, the polysaccharide fraction from the endophytic fungus *T. atroviride* D16, isolated from the root of *Salvia miltiorrhiza*, altered the chemical composition of the host plant (Ming et al. 2013). In the six different genetic resources, a positive correlation was found between the transcript abundance of one early and two late TIA biosynthetic pathway genes, which are involved in the accumulation of alkaloids (Dutta et al. 2005). In contrast, low-temperature stress negatively affects the TIA biosynthetic pathway (Dutta et al. 2007). *Escherichia coli* (1.5%) upregulates the genes cycloartenol synthase and 3-hydroxy-3-methylglutaryl-CoA reductase to boost diosgenin biosynthesis in suspension cultures of *Helicteres isora* (Shaikh et al. 2020). In some plants, the impact of other elicitors on the modulation of genes involved in metabolic pathways has also been investigated. There is a positive relationship between the amount of active component formation in response to elicitation and the abundance of transcripts associated with its syntheses. Oligogalacturonic acid (OGA) induced the production of saponin by expressing the gene encoding the saponin biosynthesis enzymes squalene synthase and squalene epoxidase in suspension cultures of *Panax ginseng* (Hu et al. 2003). In the hairy root cultures of *Isatis tinctoria*, elicitation with chitosan significantly increased the transcription of related genes involved in the flavonoid biosynthesis pathway (Jiao et al. 2018). In *W. somnifera,* supplemental UV-B increases the accumulation of secondary metabolites and their biosynthetic enzymes (Takshak and Agarwal 2014).

4 Secondary Metabolite Production in Medicinal Plant Cultures Using Biotic Elicitors

Biotic elicitors are substances derived from microorganisms and plant cell walls, such as chitin, pectin, and cellulose. One of the best methods to increase the manufacturing of active compounds in plant cell cultures is the use of biotic elicitors.

In order to enhance the production of secondary metabolites in plant cultures, yeast, fungus, and bacteria are frequently used. Prior to identifying the active ingredient, pathogen-derived biological mixtures were created for elicitation. Researchers from all over the world have noted appreciable increases in the biosynthesis of different active compounds in various plant cultures in response to biotic elicitors.

4.1 Yeast Origin

Yeast extract is frequently employed for studies on the production of secondary metabolites as biotic elicitors. In cell suspension culture, it promote the synthesis of sanguinarine in *Argemone Mexicana* (Trujillo-Villanueva et al. 2012), andrographolide in *Andrographis paniculata* (Gandi et al. 2012), asiaticoside in *Centella asiatica* (Loc and Giang 2012), benzophenanthridine in *Escherichia californica* (Byun and Pedersen 1994), rosmarinic acid in *Ocimum sanctum* (Hakkim et al. 2011), acrid-one epoxide in *Ruta graveolens* (Eilert et al. 1984), and solasodine in *Solanum hainanense* (Loc et al. 2014). After treating a suspension culture of *Mitragyna speciosa* with 250 mg of L^{-1} yeast extract, the highest mitragynine content was found (Zuldin et al. 2013). It promotes the synthesis of hyoscyamine in *Datura metel* (Ajungla et al. 2009), noradrenaline in *Portulaca oleracea* (Pirian and Piri 2013), diterpenoid tanshinones, and rosmarinic acid in *Salvia miltiorrhiza* (Qiong et al. 2005; Yan et al. 2006) in root culture. In the callus culture of *Boswellia serrata,* yeast extracts enhanced accumulation of the alkaloids KBBA, AKBBA, and BBA by 27-fold, 23-fold, and 42-fold, respectively (Ghorpade et al. 2011). When yeast extract was added to the hairy root cultures of *Brugmansia candida*, the intracellular concentration of the alkaloids scopolamine and hyoscyamine increased by seven times (Pitta-Alvarez et al. 2000). Bacchotricuneatin C, guaiazulene, isochiapin B, and p-benzoquinone sesquiterpenoid production were induced by yeast extract in *Panax ginseng* adventitious root cultures (Rahimi et al. 2014). Yeast extract at 50 mg L^{-1} increased ginsenoside level to 1.57 times higher in *Panax quinquefolium* hairy roots than that attained in control medium (Kochan et al. 2017). In agitated microshoot cultures of *Schisandra chinensis*, the production of lignan was also markedly increased by elicitation with yeast extract (about 1.8-fold) (Szopa et al. 2018).

4.2 Fungal Origin

In various plant cell cultures, it has been demonstrated that biotic elicitors obtained from fungi promote the manufacturing of active compounds. Elicitor preparations based primarily on pathogenic fungi have so far been widely employed for this purpose. Some common elicitors used to trigger the production of secondary metabolites in plants include *Aspergillus niger*, *Fusarium moniliforme*, *Trichoderma viride*,

Fusarium oxysporum, *Alternaria sp.*, *Curvularia lunata*, *Pythium aphanidermatum*, and their preparations. An increase in the production of ajmalicine, an antihypertensive drug used to treat high blood pressure, has been noticed in *Catharanthus roseus* cell culture elicited by *Aspergillus niger*, *Fusarium moniliforme*, and *Trichoderma viride* (Namdeo et al. 2002). *Aspergillus niger* stimulates the production of flavonoids in *Andrographis paniculata* (Mendhulkar and Vakil 2013a), psoralen in *Psoralea corylifolia* (Ahmed and Baig 2014), and taxol in *Taxus chinensis* (Wang et al. 2001). *Penicillium expansum* enhanced the production of flavonoid and andrographolide in *Andrographis paniculata* (Mendhulkar and Vakil 2013; Vakil and Mendhulkar 2013). The isoeuphpekinensin as well as euphol contents of the *Euphorbia pekinensis* suspension cultures are increased by the endophytic fungi E5 (*Fusarium* sp.) to 5.81- and 3.56-fold higher than those of the control, respectively (Gao et al. 2011). On the growth and production of solasodine by free and alginate-entrapped cells of *Solanum elaeagnifolium*, a fungal elicitor isolated from *Alternaria* sp. has been studied. Fourteen-day-old cultures elicited with autoclaved homogenates increased solasodine production by up to 65% in suspension cultures and about 95% in entrapped cells (Quadri and Giulietti 1993). *Aspergillus niger* cell extract stimulated the gymnemic acid production by ninefold in elicited cell culture of *Gymnema sylvestre* (Devi and Srinivasan 2011).

Endophytic fungi, an essential component of medicinal plants' surroundings, are known to coexist with them in stable, long-lasting, and mutually advantageous symbioses. In medicinal plants, the endophytic fungal elicitor stimulates the expression of particular genes that may activate a number of particular secondary metabolic pathways, leading to the significant accumulation of active compounds (Zhai et al. 2017). Endophytic fungi *Fusarium oxysporum* increases active compound production in *Catharanthus roseus* and *Dioscorea zingiberensis* cell cultures (Tang et al. 2011; Li et al. 2011). Endophytic fungi, *Phoma* spp., and *Nigrospora sphaerica* dried cell powder in the suspension cultures of *Calophyllum inophyllum* leaf- and stem-derived callus stimulate inophyllum A and inophyllum B production, respectively. Similarly, *enhanced biosynthesis of* inophyllum C and inophyllum P in the suspension culture of leaf-derived callus supplied with a culture filter of *N. sphaerica* has been reported (Pawar et al. 2011). The best polysaccharide for increasing diosgenin synthesis in *Dioscorea zingiberensis* cell culture was discovered to be the mycelial polysaccharide of the endophytic fungus *Fusarium oxysporum* Dzf17. In comparison to controls, it increased cell dry weight, diosgenin content, and diosgenin yield by 1.34-fold, 2.85-fold, and 3.83-fold, respectively (Li et al. 2011). The mycelial extract and polysaccharide fraction *of Trichoderma atroviride D16*, an endophytic fungus isolated from the root of *Salvia miltiorrhiza*, enhance tanshinone biosynthesis in *S. miltiorrhiza* (Ming et al. 2013). Gymnemic acid formation in *Gymnema sylvestre* cell suspension cultures was consistently increased by dried mycelium and the culture filtrate of the endophytes *Polyancora globosa* and *Xylaria sp*. separated from the leaves of *G. sylvestre* (Netala et al. 2016). Researchers investigated how the elicitors from the endophytic fungus *Coniothyrium palmarum*, which was isolated from *Taxus baccata*, affected the paclitaxel biosynthesis in

Corylus avellana cell suspension culture. The highest stimulation of paclitaxel biosynthesis was achieved by the combination of the *C. palmarum* cell wall (2.5% v/v) and methylcyclodextrin (Farhadi et al. 2020).

In the callus culture of *Boswellia serrata*, *Fusarium oxysporum* increased the production of alkaloids (Ghorpade et al. 2011). It also stimulates the production of caffeic acid, rosmarinic acid, carnosic acid, and carnosol in *Rosmarinus officinalis* (Khaleel et al. 2011). *Verticillium dahliae* increased the bioproduction of withaferin A up to tenfold in the callus culture of *Withania somnifera* (Chitturi et al. 2010).

In root cultures of different plants, such as *Azadirachta indica*, *Datura metel*, and *Arnebia euchroma*, increased active metabolite synthesis on elicitation with fungal elicitors has been reported. *Curvularia lunata* increases azadirachtin production in *Azadirachta indica* (Srivastava and Srivastava 2014). Alkaloid production in *Datura metel* has been increased by *Aspergillus niger*, *Alternaria sp.*, and *F. moniliforme* (Ajungla et al. 2009). It has been reported that cell wall fragments of *Phytophthora megasperma* and *Pythium aphanidermatum* stimulated the synthesis of alkaloids in *Datura stramonium* and rosmarinic acid in *Coleus blumei*, respectively, in cell culture (Ballica et al. 1993; Szabo et al. 1999).

4.3 Bacterial Origin

Different physiological and developmental responses in plants are influenced by bacteria. Researchers have noted significant increases in the synthesis of active compounds in some plant cultures in response to elicitors of bacterial origin. *Pseudomonas aeruginosa* accelerated flavonoid production in the cell cultures of *Drypetes roxburghii*, *Codiaeum variegatum*, and *Baliospermum montanum* (Bijekar and Gayatri 2014). Production of 5′- phosphodiesterase was enhanced up to 10.68-fold and 20-fold in *Catharanthus roseus* cell culture elicited with the marine bacterium *Alteromonas macleodii and a* mixture of *A. macleodii* and alginate oligomers, respectively (Aoyagi et al. 2006). The hypersensitive response (HR) elicitor from *Xanthomonas* spp. increased gymnemic acid biosynthesis in *Gymnema sylvestre* cell culture (Devi et al. 2012). Elicitations with *Bacillus sp.* cell lysate cause a higher formation of coumarin in *Ruta graveolens* shoot culture (Orlita et al. 2008). In the cell, callus, and hairy root cultures, autoclaved lysates of bacteria (*Enterobacter sakazaki*) enhance the production of bioactive molecules in *Ammi majus* (Staniszewska et al. 2003). In recent studies, it has been found that biotic elicitation of *Dionaea muscipula* cultures using *Cronobacter sakazakii* bacteria lysate combined with rotary shaking increased total phenolic compounds and phenylpropanoids in comparison to conventional tissue cultures. Phenylpropanoids and phenolic compounds were increased in the plant culture treated with 5% of bacterial lysate to levels that were 2.43 and 1.74 times higher than in the control plants, respectively (Makowski et al. 2020).

4.4 Polysaccharides

4.4.1 Chitin and Chitosan

Chitosan and chitin are substances found naturally that may be applied in agriculture to protect and cure plant diseases (Hadrami et al. 2010). The exoskeletons of arthropods and the cell walls of fungi contain chitin, which is the second-most prevalent polymer in the world (Rinaudo 2006). In contrast to chitin, chitosan is uncommon in nature and is only present in fungi that produce deacetylase enzymes. Chitosan improves the ability of plants to grow and mitigates the negative effects of unfavorable conditions. It influences a number of physiological responses, including plant immune function and defense mechanisms against harmful conditions, which involve various enzymes like phenylalanine ammonia lyase, polyphenol oxidase, and tyrosine ammonia lyase (Katiyar et al. 2015; Yin et al. 2016). The production of bioactive molecules is influenced by the use of chitin and chitosan as elicitors in a variety of medicinal plants. L-dopa and beta-thujaplicin formations in *Mucuna pruriens* and *Cupressus lusitanica* cell culture, respectively, have been found to be stimulated by chitin (Raghavendra et al. 2011; Zhao et al. 2001). Chitosan stimulated the production of flavonoids in *Andrographis paniculata*, 20-hydroxyecdysone in *Vitex glabrata*, and *Stemona alkaloids* in *Stemona sp.*, which have also been observed (Mendhulkar and Vakil 2013a; Chamnipa et al. 2012; Chaichana et al. 2012). In the cell culture of *Morinda citrifolia*, chitosan also promotes the biosynthesis of anthraquinones, phenolics, and flavonoids (Baque et al. 2012). Numerous secondary matabolites, including withanolide A and B, withaferin A, withanone, 12-deoxy withastramonolide, withanoside V and IV, and withanolides, have all been produced in greater quantities in *Withania somnifera* cell culture upon chitosan elicitation (Sivanandhan et al. 2014). *Isatis tinctoria* hairy root cultures that were 24 days old and exposed to 150 mg L^{-1} chitosan for 36 h had a 7.08-fold rise in total flavonoids compared to the control (Jiao et al. 2018). Chitosan increases the accumulation of dibenzocyclooctadiene lignans by up to 1.35-fold in *Schisandra chinensis* (Szopa et al. 2018).

4.4.2 Pectin

Pectin, an important polysaccharide, is used in a variety of industries, including food and medicine (Thakur et al. 1997). Pectin increased L-dopa production in *Mucuna pruriens* cell suspension culture by 18.45-fold (Raghavendra et al. 2011). Additionally, it has been observed that elicitation with pectin increased the production of the metabolites hypericin and pseudohypericin in *Hypericum adenotrichum* seedling culture (Yamaner et al. 2013).

4.4.3 Cellulose and Hemicellulose

Cellulose- and hemicellulose-stimulated enhanced production of active compounds has also been observed in a few plants. Increased capsaicin production was observed after the addition of cellulose to the suspension and immobilized cell culture of *Capsicum annuum* (Islek et al. 2014). Hemicellulose-elicited root cultures of *Brugmansia candida* enhanced alkaloid production by 100–200% (Pitta-Alvarez et al. 1999). *Glycyrrhiza glabra* hairy root cultures were treated for 7 days with 200 g mL^{-1} cellulase, which increased the yield of glycyrrhizin by 8.6 times (Srivastava et al. 2019).

5 Secondary Metabolite Production in Medicinal Plant Cultures Using Abiotic Elicitors

Abiotic elicitors are substances that come from nonliving sources and fall into three categories: hormonal, chemical, and physical.

5.1 Plant Growth Regulators as Elicitors

Elicitation studies have made extensive use of a variety of plant hormones. The most investigated are salicylic acid, methyl jasmonate, and jasmonic acid (JA), which play important roles in the plant defense system. Jasmonic acid (JA) and its methyl ester (methyl jasmonate, MeJA) are cyclopentanone-based compounds derived from linolenic acid (LA) and are widely found in plants (Creelman and Mullet 1997).

5.1.1 Methyl Jasmonate and Jasmonic Acid

Methyl jasmonate (MeJA) is a plant growth regulator that acts as a signaling molecule, allowing for inter- and intracellular communication and is known to mediate plant defense responses to biotic and abiotic stresses (Dar et al. 2015). Treatments with jasmonates (JAs) and its derivatives before and after harvesting plants can boost the production of bioactive molecules (Reyes-Díaz et al. 2016). In cell suspension culture, the use of methyl jasmonate as an elicitor significantly increased the production of a variety of bioactive compounds. It increased the biosynthesis of rosmarinic in *Ocimum sanctum*, solasodine in *Solanum hainanense*, rosmarinic acid in *Coleus blumei*, L-dopa in *Mucuna pruriens*, beta-thujaplicin in *Cupressus lusitanica*, 20-hydroxyecdysone in *Vitex glabrata*, andrographolide in *Andrographis paniculata*, reserpine in *Rauvolfia serpentina*, baccatin III in *Taxus baccata*, and flavonoids in *Hypericum perforatum* (Hakkim et al. 2011; Loc et al. 2014; Bijekar

and Gayatri 2014; Raghavendra et al. 2011; Zhao et al. 2001; Chamnipa et al. 2012; Sharma et al. 2015b; Harisaranraj et al. 2009; Bonfill et al. 2007; Wang et al. 2015). The increased biosynthesis of saponin in the plant culture of *Calendula officinalis* on elicitation with methyl jasmonate and silver nanoparticles has been observed (Ghanati and Bakhtiarian 2014).

On elicitation with methyl jasmonate, a noticeable increase in the production of diosgenin was seen in the 12-day-old seedlings of six fenugreek (*Trigonella foenum-graecum*) varieties. (Chaudhary et al. 2015). It has also been reported that methyl jasmonate stimulated peruvoside production in *Thevetia peruviana* cell cultures grown in Schenk–Hildebrandt (SH) medium (Zabala et al. 2010). MeJa increased the concentration of total phenols, flavonoids, and the flavonoid compound acacetin in *Scrophularia kakudensis* cell culture (Manivannan et al. 2016). When *T. peruviana* cell suspension cultures were exposed to MeJA at a concentration of 3 M, it was found that the contents of phenolic compounds and flavonoids increased 1.49 and 2.55 times, respectively, in comparison to the control culture (Mendoza et al. 2018). In agitated shoot cultures of *Centella asiatica*, 50 M MeJa was the most effective elicitor for the accumulation of centellosides and flavonoids (Skrzypczak-Pietraszek et al. 2019).

Moreover, methyl jasmonate also induces secondary metabolite production in root culture. Methyl jasmonate stimulated increased production of artemisinin in *Artemisia annua*, pyrrolizidine alkaloids in *Echium rauwolfii*, and gossypol in *Gossypium barbadense* (Patra et al. 2013; Abd El-Mawla 2010; Frankfater et al. 2009). In *Gloriosa superba*, colchicine production increased up to 50-fold on elicitation with methyl jasmonate (Ghosh et al. 2006).

Jasmonic acid (JA) increased azadirachtin production in *Azadirachta indica* hairy root culture by six times (Satdive et al. 2007). When *Catharanthus roseus* was stimulated with jasmonic acid, the production of ajmalicine and serpentine increased (Rijhwani and Shanks 1998). Transformed root cultures of *Brugmansia candida* elicited with JA also increased scopolamine production (Spollansky et al. 2000). JA and MeJA significantly increase phenolic accumulation in *Cannabis sativa* cell cultures by 42% and 52%, respectively (Gabotti et al. 2019).

5.1.2 Salicylic Acid

It has been observed that plants respond in a variety of metabolic and physiological ways to salicylic acid (SA), an endogenous plant regulator. Exogenous application of low concentrations of salicylic acid provides considerable protection against various biotic stresses and promotes plant growth and development (Hayata et al. 2010). Salicylic acid stimulated asiaticoside production in *Centella asiatica*, flavonoids in *Andrographis paniculata*, and taxuyunnanine C in *Taxus chinensis* cell cultures (Loc and Giang 2012; Mendhulkar and Vakil 2013; Qian et al. 2006). In root culture, it stimulates the synthesis of tropane alkaloids in *Datura metel* and *Brugmansia candida* and azadirachtin in *Azadirachta indica* (Ajungla et al. 2009; Pitta-Alvarez et al. 2000; Satdive et al. 2007). *Stemona* sp. plantlets exposed to

100 μM salicylic acid for one week produced 1.69 times more 1′,2′-didehydrostemofoline and 1.61 times more stemofoline than the control (Chaichana and Dheeranupattana 2012). Salicylic acid (0.5 and 1 mM) foliar application increased phenolic compound concentration in *Mentha piperita* (Figueroa-Pérez et al. 2014). Salicylic acid induces accumulation of farnesol, isochiapin B sesquiterpenoids, camphor, and cineole monoterpenoids in root cultures of *Panax ginseng* (Rahimi et al. 2014).

5.1.3 Phytohormones

Some plant hormones also function as elicitors in the synthesis of active biomolecules. α- Naphthyl acetic acid has been shown to stimulate vasicine biosynthesis in the cell suspension cultures of *Justicia adhatoda* (Pa and Mathew 2012). ABA increased salvianolic acid production in *Salvia miltiorrhiza* hairy root culture (Hao et al. 2012). In callus cultures, it has been investigated how plant growth regulators affect the accumulation of indolizidine alkaloids and isoflavone phytoestrogens in *Securinega suffruticosa* and *Genista tinctoria*, respectively. The combination of 0.5 mg L^{-1} kinetin and 5.0 mg L^{-1} 2, 4-D produced the highest isoflavone content and the fastest biomass growth in *Genista tinctoria* (Luczkiewicz et al. 2014). However, *Securinega suffruticosa* calluses grown in the presence of 0.5 mg L^{-1} indole acetic acid and 5.0 mg L^{-1} kinetin had the highest concentrations of securinine and allosecurinine (Raj et al. 2015). Additionally, it was reported that the presence of various plant growth regulators in the culture medium had a sizable impact on the volatile composition of *Agastache rugosa* (Zielinska et al. 2011).

5.2 Chemical Elicitors

Copper sulfate- ($CuSO_4$)-accelerated flavonoid productions in *Codiaeum variegatum*, *Drypetes roxburghii*, and *Baliospermum montanum* cell suspension cultures have been observed (Aoyagi et al. 2006). It also stimulated the bioproduction of withaferin A in *Withania somnifera, an* alkaloid in *Brugmansia candida*, and bacoside in *Bacopa monnieri* in callus, hairy root, and shoot cultures, respectively (Srivastava and Srivastava 2014; Pitta-Alvarez et al. 1999; Sharma et al. 2015a). $CuSO_4$ and DMSO enhanced grindelic acid production in *Grindelia pulchella* cell suspension culture (Hernandez et al. 2005). In root culture, calcium chloride ($CaCl_2$) stimulated the enhanced production of the alkaloids in *Datura metel* and colchicines in *Gloriosa superba* (Ajungla et al. 2009; Ghosh et al. 2006). Following $CaCl_2$ elicitation, the production of rosmarinic acid and rosmanol in *Rosmarinus officinalis* callus culture also rises to 1.16 and 1.09 times, respectively (Khaleel et al. 2011). Sodium sulfate (Na_2SO_4) stimulated the enhanced accumulation of both AKBBA and KBBA in *Boswellia serrata* callus culture (Ghorpade et al. 2011). Silver nitrate

($AgNO_3$) significantly increased scopolamine release in the root culture of *Brugmansia candida* (Pitta-Alvarez et al. 2000). In terms of the formation of gymnemic acid among the different salts examined, $CdCl_2$ showed the greatest response at a concentration of 2 mM after 24 h, whereas $AgNO_3$ showed the least response after 48 h of incubation at 1 mM (Ch et al. 2012). The total lignan content of the agitated *Schisandra chinensis* microshoot cultures increased by up to twofold after elicitation with $CdCl_2$ (Szopa et al. 2018). Aluminum salt ($AlCl_3$) also stimulated the production of the alkaloids scopolamine and hyoscyamine in root cultures of *Datura metel* and *Brugmansia candida* (Ajungla et al. 2009; Spollansky et al. 2000). In *Gloriosa superba*, $AlCl_3$ enhanced the intracellular colchicine content of the root (Ghosh et al. 2006). Ammonium nitrate (NH_4NO_3) stimulated aloin production in *Aloe vera* callus culture (Raei et al. 2014). With the addition of copper chloride ($CuCl_2$), reserpine production in the callus culture of *Rauvolfia serpentina* increased by 1.41-fold (Nurcahyani et al. 2008). Cadmium chloride ($CdCl_2$) addition also enhanced intracellular colchicine content in *Gloriosa superba* root culture (Ghosh et al. 2006). The abiotic elicitor cadmium stimulated the maximum output of inophyllums A and C and calophyllolide in suspension cultures of stem callus, whereas it induced the maximum output of inophyllums B and P in suspension cultures of leaf callus. Chromium increased the production of inophyllum D in suspension cultures of *C. inophyllum* stem callus (Pawar and Thengane 2011). In *Panax ginseng* hairy root culture, the addition of 0.5 mM selenium or 20 microM $NiSO_4$ increased saponin content (Jeong and Park 2006). In addition to their potential for toxicity, some nanoparticles (NPs) may also be useful as novel and effective elicitors for the in vitro synthesis of active compounds that can be used in pharmaceutical applications. In comparison to the control, nickel oxide increased the content of glaucine and quercetin in *Nigella arvensis* (Modarresi et al. 2020). Nanosilver increased the production of aloin in *Aloe vera* and atropine in *Datura metel* callus and root culture, respectively (Raei et al. 2014; Shakeran et al. 2015). In *Cupressus lusitanica* cell culture, the production of beta-thujaplicin had also been increased by sodium alginate, a gum made from the cell walls of brown algae (Zhao et al. 2001).

It was found that sucrose elicited a significant rise in thymol synthesis in *Origanum vulgare* callus culture (Al-Jibouri et al. 2012). The biosynthesis of rosmarinic acid in *Ocimum sanctum* and 20-hydroxyecdysone in *V. glabrata* increased on elicitation with sucrose in cell culture (Hakkim et al. 2011; Thanonkeo et al. 2011). Pseudohypericin and hypericin synthesis increased in seedling cultures of *Hypericum adenotrichum* on elicitation with mannan (Yamaner et al. 2013). 100 mg/L of mannan after 1 week increased ajmaline concentration up to 2.9-fold in a hairy root culture of *Rauvolfia serpentina* (Srivastava et al. 2016). Mannan at a dose of 10 mg L^{-1} in the hairy root cultures of *G. glabra* produced up to 7.8 times as much glycyrrhizin after 10 days of stress (Srivastava et al. 2019). Polyamines stimulated the production of both salvianolic acid A and salvianolic acid B in the hairy root culture of *Salvia miltiorrhiza* (Hao et al. 2012). Upon elicitation with phenylalanine, increased rosmarinic in *Ocimum sanctum* and flavonoids in *Baliospermum montanum, Codiaeum variegatum,* and *Drypetes roxburghii* in cell suspension culture have been observed (Hakkim et al. 2011; Aoyagi et al. 2006).

Exogenous phenylalanine incorporation in *Citrullus colocynthis* liquid culture increased total quercetin yield (Meena et al. 2014). Total alkaloid production in *Datura stramonium* cell culture also increased on elicitation with phenylalanine and ornithine (Szabo et al. 1999). *Justicia adhatoda* cell culture with mannitol increased vasicine production (Pa and Mathew 2012). In the callus culture of *Origanum vulgare*, the production of thymol was stimulated by the addition of proline (Al-Jibouri et al. 2012). After quercetin elicitation, pyrrolizidine alkaloid production increased in the root culture of *Echium rauwolfii* (Abd El-Mawla 2010). Trifluoroethyl salicylate enhanced taxuyunnanine-C production in *Taxus chinensis* cell culture (Qian et al. 2006). By feeding tryptophan and sorbitol at a concentration of 50 mM to the *Adhatoda vasica* cell cultures, an increase in the production of vasicinone (12-fold) and vasicine (8.3-fold) has been achieved (Singh et al. 2017).

5.3 Physical Elicitors

Light, drought, salinity, and thermal stress are physical elicitors that have an impact on the biosynthesis of bioactive compounds in medicinal plants.

5.3.1 Light

Solar radiation energy is among the most significant environmental factors required for plant growth and development. Now, it is also well established that light affects metabolite production in plants. Light signals are perceived by the plant and integrated into its complex signaling network, which influence plant growth and development. UV-B radiation has significant physiological and ecological effects on the synthesis of a variety of bioactive molecules, including flavonoids, tannins, and lignin (Rozema et al. 1997). In *Eurycoma longifolia* callus culture, exposure to UV light enhanced the production of canthin-6-one alkaloid and pyrrolidine (Parikrama and Esyanti 2014). When *Boswellia serrata* callus culture was exposed to UV-C, increased accumulation of AKBBA and BBA was observed (Ghorpade et al. 2011). UV-C irradiation applied to callus cultures of *Cyclopia subternata* also stimulated the accumulation of hesperidin, calycosin, and pseudobaptigenin without negatively influencing callus growth (Kokotkiewicz et al. 2014). In the cultures of *Ruta graveolens* L., both white light and blue light were found to positively influence the total production of phenolic acids, but the total output of furanocoumarins was undoubtedly strengthened by blue light in *R. graveolens* (Szopa et al. 2012). *Schisandra chinensis* was found to produce phenolic acids and dibenzocyclooctadiene lignans best under blue light (Szopa and Ekiert 2016). *Cyclopia subternata* callus cultures were maintained under a variety of lighting conditions to determine their effects on biomass growth and bioflavonoid accumulation. After 28 days of testing, 14-day-old calluses grown under blue light produced the highest amounts of hesperidin and isoflavones (Kokotkiewicz et al. 2014). UV-C irradiation seems to be

an effective factor for increased secondary metabolite production in *Vitis vinifera*. UV-C radiation remarkably promotes accumulation of phenolic, flavanol, and catechin content in the callus culture of *Vitis vinifera* when compared to a control. Among all phenolics, ferulic acid best responded to UV-C irradiation and increased up to sixfold (Cetin 2014).

5.3.2 Drought

The key environmental factor that influences a plant's quality and productivity is drought. While limiting plant growth, drought stress enhances the production of bioactive compounds. The yield of total flavonoids in *Glechoma longituba* has been observed to increase with water treatment with 80–85% field capacity (Zhang et al. 2012). Water stress has been shown to increase the amount of anti-inflammatory saikosaponins in *Bupleurum chinense* and the total amount of flavonoids in *Camellia sinensis* (Zhu et al. 2009; Wang et al. 2016). However, it markedly reduced the amount of tanshinone IIA and markedly enhanced the production of salvianolic acid B in *Salvia miltiorrhiza* (Liu et al. 2011). Polyethylene glycol at 1% stimulated the biosynthesis of glycyrrhizin in root cultures of *G. glabra* by up to 5.4-fold after being exposed for 24 h (Srivastava et al. 2019).

5.3.3 Salinity

Salinity, which also prevents plant growth and development, affects a wide range of physiological and metabolic processes. Increased solasodine production in *Solanum nigrum* shoot culture as a result of elicitation with NaCl has been observed (Sutkovic et al. 2011). Although the addition of NaCl up to 100 mM increased the bacoside A content, higher salt concentrations prevented the biosynthesis of bacoside A in regenerated shoots of *Bacopa monnieri* (Ahire et al. 2013). In hairy root cultures of *Rauvolfia serpentina* and *Solanum khasianum*, respectively, NaCl at a concentration of 100 mM stimulated the production of ajmalicine up to 14.8 times and solasodine up to 4.0 times (Srivastava et al. 2019).

5.3.4 Temperature

Temperature stress is known to have an impact on the synthesis of secondary metabolites in plants, in addition to causing a number of physiological, biochemical, and molecular alterations in plants (Zobayed et al. 2005). *Vitex glabrata* cultures grown at 25 °C stimulated higher cell growth and 20-hydroxyecdysone production than those grown at 30 °C (Thanonkeo et al. 2011). It has been investigated how temperature affects the bioflavonoid accumulation in *Cyclopia subternata* callus cultures. When applied over the course of the entire 28-day growth cycle, low-temperature (13 °C) treatment increased calycosin content by over 1500%

while also resulting in a 95% reduction in culture growth when compared to the reference culture maintained at 24 °C. Contrarily, the content of calycosin and pseudobaptigenin increased by over 300% and 500%, respectively, when a higher temperature (29 °C) was applied in the second half of the growth period (Kokotkiewicz et al. 2014).

6 During Elicitation, There Are Various Factors That Can Affect the Production of Secondary Metabolites

A new area of research has opened up because of the role of elicitation in the biosynthesis of secondary metabolites in plants. The biosynthesis of a plant's active compound is influenced by a variety of factors. These factors include cell lines, age of culture, nutrient content, elicitor concentration, and exposure time.

6.1 Elicitor Concentration

In the elicitation process, elicitor concentration is crucial. In *Datura metel* cultures treated with varying concentrations of elicitor extracts from *Aspergillus niger*, *Alternaria sp.*, *and Fusarium moniliforme*, higher accumulations of hyoscyamine and scopolamine have been reported. Higher concentrations (1.0 mg L^{-1}) of elicitor extracts resulted in higher levels of hyoscyamine and scopolamine accumulation in the cells than lower concentrations (0.1 mg L^{-1}). However, increasing the concentration to 1.5 mg L^{-1} had a negative impact on the synthesis (Ajungla et al. 2009). When cholesterol was fed to *Vitex glabrata* cell culture at a rate of 5 mg/L, the cells produced more 20-hydroxyecdysone than the control cells. The production of 20-hydroxyecdysone was reduced when cholesterol was increased further to 10 mg L^{-1} (Thanonkeo et al. 2011). Although the addition of NaCl up to 100 mM increased the bacoside A content, higher salt concentrations prevented the accumulation of bacoside A in regenerated shoots of *Bacopa monnieri* (Ahire et al. 2013). A study by Abd El-Mawla (2010) observed higher accumulation of pyrrolizidine alkaloids in *Echium rauwolfii* cultures when treated with different concentrations of elicitor methyl jasmonate and quercetin (Abd El-Mawla 2010). Methyl jasmonate significantly increased the contents of seven alkaloids, as determined by GC-MS in the root cultures of *Rhazya stricta*, as compared to the control (Akhgari et al. 2019).

6.2 Age of Culture

Vakil and Mendhulkar (2013) reported higher yield of andrographolide in 10–day- and 8–day-old culture of *Andrographis paniculata* on elicitation with *Aspergillus niger* and *Penicillium expansum*, respectively (Vakil and Mendhulkar 2013). It was

observed that the optimum time to introduce heavy metal elicitors for the production of dipyranocoumarins was on the tenth day after beginning the suspension cultures of *Calophyllum inophyllum* (Pawar 2011). Mendhulkar and Vakil (2013) also observed higher yields of total flavonoids in 24-h-old cultured cells and 4-day-old cultured cells of *Andrographis paniculata* on elicitation with chitosan and *Aspergillus niger*, respectively. But after 24 h and 4 days, respectively, there was a decrease in total flavonoid production (Mendhulkar and Vakil 2013a).

6.3 Duration of Exposure to Elicitor

The length of exposure to the elicitor influences the production of active compounds. As per Ghorpade et al., as UV exposure increases, the biosynthesis of active molecules declines. In the callus culture of *Boswellia serrata*, an optimal UV exposure time of 5 min was effective for both the accumulation of AKBBA and BBA and the total boswellic acid content (Ghorpade et al. 2011). Similar findings for UV-B radiation-induced flavonoid production in *Passiflora quadrangularis* callus cultures were also noted (Antognoni et al. 2007). In addition, it was also found that *Moringa oleifera* had concentrations of the antioxidant compounds crypto-chlorogenic acid, isoquercetin, and astragalin after exposure to UV light for 10 min (Patchang 2014). Therefore, the ideal exposure time for elicitors is crucial for the biosynthesis of active molecules.

6.4 Nutrient Composition

The production of rosmarinic acid by *Coleus forskohlii* hairy root cultures in different liquid media elicited by different elicitors like yeast extract, salicylic acid, and methyl jasmonate was investigated. B5 medium supplemented with 0.01 m yeast extract, 0.1 M salicylic acid, and 1.4 M methyl jasmonic acid was found to be the best culture media for producing the key metabolic acid (Li et al. 2005). It has been observed in *Datura stramonium* that the carbon and nitrogen sources supplied to cell cultures in the culture phase affect the synthesis of tropane alkaloids (Ballica et al. 1993). It was also investigated how *Azadirachta indica* hairy root cultures responded to various culture media and elicitation to promote azadirachtin growth and production. The highest yield of azadirachtin was produced by hairy roots cultured on Ohyama and Nitsch's basal medium, compared to Gamborg's and Murashige and Skoog's (Satdive et al. 2007).

7 Conclusion

Plants have been used for medicinal purposes long before the prehistoric period. A number of plants are currently being studied to determine their medicinal efficacies because many drugs have plant origins. Because of the presence of numerous metabolites such as alkaloids, steroids, flavonoids, terpenoids, saponins, tannins, and others, many plants have pharmacological effects. These plants were very commonly available in abundance. During recent years, due to overexploitation and deforestation, many of these natural sources have been destroyed. Several pharmaceutically important plant species are already on their way to extinction and face a serious threat. However, the demands for these valuable plants are increasing throughout the world. So there is a great need to meet the high demand for these valuable medicines obtained from medicinal plants. It is only possible by increasing both the quality and quantity of the bioactive compound by using some important biological techniques. Induced mutagenesis is one of the most efficient strategies that have been extensively applied to create genetic variation for economically valuable traits that may be challenging to incorporate via traditional breeding. A few aromatic and therapeutic plants were effectively subjected to mutation breeding to improve different attributes like growth, yield, secondary metabolite contents, etc. Some of the many mutagenesis studies on medicinal plants are listed in this chapter. Through mutational breeding, medicinal plants can be improved in terms of physiological function as well as the biosynthesis of active compounds. Therefore, there is huge potential for improving medicinal plants through mutation breeding. Another crucial method for increasing the production of bioactive compounds found in plants is the use of elicitors to induce secondary metabolite production. Elicitors are known to alter the permeability of plasma membranes and have unique binding sites that function as receptors. This interaction of elicitors and binding sites promotes the expression of important genes that lead to the generation of biologically active compounds. A variety of abiotic and biotic elicitors can increase the synthesis of a large number of compounds with pharmaceutical value. For elicitation studies, various elicitors are used, including fungi, bacteria, yeast, jasmonic acid, salicylic acid, phytohormones, and many different types of chemical compounds and their mixtures. Additionally, certain physical factors like light, drought, salinity, and temperature encourage the production of metabolites. The most widely used culture systems for the production of pharmaceutically significant phytochemicals include cell suspensions, callus, and hairy root cultures. Elicitation depends on the type of elicitors, their concentration, their duration, and the age of the culture. Additionally, for the induction of bioactive compound synthesis in cell culture, an ideal level or dosage of the elicitor is needed. The underlying mechanisms of elicitation are still unknown, despite the fact that it increases the in vitro synthesis of bioactive compounds in plants or plant cells. In order to understand the molecular mechanisms underlying plant cells' responses to elicitors and the accumulation of bioactive molecules, extensive research is necessary in the fields of plant metabolism, biochemistry, molecular biology, and biotechnology. It might aid in increasing both the

quantity and purity of the metabolites produced. Understanding the plant biosynthetic pathways that lead to the production of bioactive compounds and ultimately finding out how to manipulate those pathways depend on the integration of omics technologies—genomics, proteomics, transcriptomics, and metabolomics.

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New Insights for the Production of Medicinal Plant Materials: Ex Vitro and in Vitro Propagation

H. I. G. K. Anuruddi, Niluka Nakandalage, and D. L. C. Kumari Fonseka

Abstract Medicinal plants are a vital input of raw materials for the manufacture of pharmaceuticals to treat various ailments of humans and animals. The excessive demand for natural medicinal therapies has encouraged intensive harvesting of medicinal plants from the wild reserves without proper replenishment. This has put tremendous pressure on many valuable medicinal plant resources, pushing them toward extinction. Therefore, it is urgent to identify alternative production techniques for medicinal plants to fulfill the growing demand while safeguarding their natural stands. The production of therapeutic herbs has evolved from traditional methods to modern techniques including ex vitro and in vitro propagation and biotechnology. In vitro methods are presented as alternative and complementary techniques of propagation providing solutions for the drawbacks associated with conventional propagation techniques. In addition, to in vitro plant propagation, Synseed production, cell suspension cultures, cryopreservation, short-to-medium-term storage, in vitro metabolite production, and root cultures in vitro are some other important aspects of biotechnological interventions for medicinal plant material production. In this context, studying and distributing knowledge on novel involvements of their production are important to promote collaboration and sharing of information among researchers, scientists, and industry professionals leading to more rapid advancements in the field. Therefore, this chapter highlights the recent knowledge based on medicinal plants giving special reference to ex vitro and in vitro production methods emphasizing the most recent advances, limitations, and future prospects.

Keywords Ex vitro · In vitro · Medicinal plants · Novel technologies · Propagation

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N. Kumar, R. S. Singh (eds.), *Biosynthesis of Bioactive Compounds in Medicinal and Aromatic Plants*, Food Bioactive Ingredients,
https://doi.org/10.1007/978-3-031-35221-8_9

1 Introduction

Like a symphony of healing, medicinal plants (MPs) harmoniously blend the melodies of nature and science to offer a natural remedy for the ailments of mankind. MPs cover a broad spectrum of species including foods, condiments, aromatics, and cosmetics (Schippmann et al. 2006; Smith-Hall et al. 2012; Slikkerveer 2006). According to Hamayun et al. (2006), for a plant to be classified as a MP, it should contain active chemical compounds in any of its parts that generate a clear physical response when treating for various disorders in both humans and animals.

Salmerón-Manzano et al. (2020) evaluated more than 100,000 reports of scientific literature until 2019 published in the Scopus database to identify some notable plant families used for medicinal purposes. According to them, plants in the family Fabaceae, Asteraceae and Lamiaceae are the most prominent families to be studied by researchers for medicinal uses. Certainly, there are still a majority of plant species in the kingdom of Plantae that have not yet been identified to have active chemical compounds; many plants are continually screened for their pharmacological activities (Evans 2008). On the other hand, there are some species having proven medicinal properties although there may not be enough scientific evidence to support their efficacy. These plants are ought to be recognized as therapeutic herbs (Sofowora et al. 2013).

Many scientific shreds of evidence are available for the nutraceutical properties of different MPs and their primary use in treating infectious and chronic diseases around the globe. Approximately 50,000 plant species are cited in the literature to have therapeutic characteristics (Barboza et al. 2009). Some plants reported to have anticancer properties, *Calotropis gigantea* L. (Mutiah et al. 2017), *Andrographis paniculata* L. (Malik et al. 2021), *Phyllanthus emblica* L. (Kumar et al. 2021a, b), and *Curcuma longa* L. (Zhang et al. 2023); antidiabetic properties, *Momordica charantia* L. (Liu et al. 2021), *Cinnamomum verum* J. (Deyno et al. 2019), and *Ocimum sanctum* L. (Das et al. 2020); anti-inflammatory properties, *Curcuma longa* L. (Abd El-Hack et al. 2021), *Centella asiatica* L. (Sun et al. 2020), and *Emblica officinalis* L. (Almatroodi et al. 2020); antihypertension, *Allium sativum* L. (Chan et al. 2020) and *Convolvulus pluricaulis* L. (Somarathinam et al. 2023); and antidiarrheal properties, *Zingiber officinale* L. (Momoh et al. 2022), *Emblica officinalis* L. (Hussain et al. 2021), etc.

MPs are a beneficial non-timber forest product because they encourage access to low-cost healthcare and livelihood stability (Larsen et al. 2005). Smith-Hall et al. (2012) assert that using medicinal herbs provides benefits for consumers, producers, and society as a whole. The indirect nonmonetary benefits that consumers receive from medicinal herbs are often hard to quantify. Producer advantages are frequently monetary and include direct gains from the cultivation of MPs, trading in plant-based drugs, and the provision of healthcare services. Social benefits of the MP industry include employment opportunities for processors, retailers, and healthcare providers in addition to tax revenues collected by the government (Smith-Hall et al. 2012).

Official figures on the sales and usage of MP products are sparse and not informative since they are frequently a part of the informal economy (Smith-Hall et al. 2012). Nevertheless, in the middle of the twentieth century, the pharmaceutical industry upsurged, and the usage of MPs became less popular as researchers favored using synthetic compounds to treat ailments (Hamayun et al. 2006). But today, this pattern is reversing, and according to estimations, almost 80% of the global population depends on some sort of conventional medicine (Builders 2018). In general, people are becoming more conscious of the negative consequences of artificial products and the advantages of living a more natural lifestyle. The reasons for this improvement are given as that in contrast to synthetic chemicals, medicinal herbs have fewer or no side effects and include natural components which are chemically balanced, effective, and least harmful (Hamayun et al. 2006).

The global trade value of medicinal and aromatic plants (MAPs) was $800 million per year in 2018 and is expected to increase by 15–25% by 2050, reaching a market value of $50 trillion (UN Comtrade database, 2018). China, France, Germany, Italy, Japan, Spain, and the United Kingdom boast the world's largest markets for MAPs, with the United States and Japan possessing the highest per capita global phytomedicine consumption (Heidarzadeh et al. 2016). According to Sofowora et al. (2013), more than 90% of traditional medicine recipes and treatments consist of medicinal herbs. One of the cornerstones of African natural medicine is the utilization of medicinal herbs, which is considered to be the most ancient and varied of all therapeutic modalities (Mahomoodally 2013). Similarly, the Indian subcontinent claims a vast stockpile of MPs that have been used in traditional medical strategies as well as in modern medicine (Pandey et al. 2013). Traditional tribal medicines account for around 40% of all medical consumption in China (Hamayun et al. 2006). In spite of the prevalence of contemporary medicine in Sri Lanka, traditional and indigenous systems of care are still regularly used by people, and their error-free treatment methods without side effects are popular among the general public (Napagoda et al. 2019).

When particular plants, like those used in traditional medicine, suddenly spark public interest, their local wild reserves are soon depleted (Evans 2008) leading to the dwindling of their genetic diversity (Sofowora et al. 2013). The wild reserves of MPs are one major harvesting option for collectors (Astutik et al. 2019). Primarily, wild harvesting is associated with the consumption of medicinal herbs either for subsistence or financial benefits (Astutik et al. 2019). In Bangladesh, for instance, the purpose was for either personal consumption (63%) or commercial purposes (37%) (Mukul 2007). In contrast, in western Nepal, over 67% of the harvest was sold and the remainder was consumed domestically (Kunwar et al. 2015). Studies have shown that overexploitation and unsustainable harvesting are significant problems within wild-gathering systems (Astutik et al. 2019). According to Kankanamalage et al. (2014), the primary concerns in wild gathering systems in Sri Lanka are price fluctuations, insufficiency of expertise in plant recognition, the limitations of wild reserves, and a scarcity of research and innovation. Rahman et al. (2011) report that the issues concerned in Bangladesh include illegal and excessive harvesting and regulations that do not suit the demands of the local population.

Nonetheless, some advantages related to improving the wild harvesting of MPs exist, such as low production costs, elevated income on land and labor, superior product quality, and increased supply potential with a good price (Astutik et al. 2019).

Domestication and cultivation lessen wild harvesting pressure (Canter et al. 2005), and cultivation is considered a way to improve local economic conditions (Phondani et al. 2016). Cultivated species account for 10–70% of family income in China (Shengji et al. 2010). The use of biotechnology to address issues inherent in the production of herbal medicines is also made possible by cultivation (Canter et al. 2005). The exploitation of medicinal herbs from the wild compels the search for substitute production methods in order to satisfy rising demand, pay off habitat deterioration, and maintain natural vegetation. Some studies suggest cultivation has drawbacks despite its benefits. For instance, Schippmann et al. (2002) explain that certain species are hard to grow for their biological features (slow growth rate, lower germination) or ecological requirements (special soil requirements).

In Sri Lanka, in-depth research has not yet been conducted to assemble the medicinal herbs with local therapeutic uses, despite the existence of a vast repository of indigenous knowledge on MPs (Napagoda et al. 2019). The general public has to be aware of the importance of preserving MPs and the traditional knowledge associated with them. This knowledge can be used to develop sustainable harvesting and cultivation methods that minimize the impact on wild populations. Additionally, individuals should be aware of the quality and sustainability of the production processes of commercially available MP products and support the promotion of bio-industries with green concepts.

The emergence and commercialization of bio-industries based on MPs in developing nations depend on the accessibility of resources and knowledge on the bio-processing, extraction, purification, and marketing of MPs. Most of our understanding of MPs has come from trial-and-error approaches, often based on speculation and superstition (Hamayun et al. 2006). However, scientific knowledge is still rather fragmented when it relates to the potential of different MP production systems and their therapeutic usages. There is a prevalence of uncertainty over future studies and legislative changes related to the production and commercialization of MPs and their effects on both household and national economies of Asian nations (Astutik et al. 2019). Therefore, many attempts are required to explore the prospects of medicinal plant cultivation as a way of ensuring their conservation and propagation and as a potential economic venture.

The continuous demand for a regular supply of therapeutic herbs, the rapid exhaustion of forest reserves, and the impact of environmental pollution on natural populations support the argument for expanding the rare and elite MP species through their cultivation. Numerous research findings indicate that the cultivation of MPs has increased in various Asian countries targeting the preservation of valued species, produce revenue for native residents, and promote economic advance (Astutik et al. 2019). Abundant medicinal plants, particularly aromatic plants, are cultivated in home gardens, sometimes as field crops and as plantation crops (De Padua et al. 1999).

Conventional cultivation techniques are typically ex vitro (in vivo production of plants outside the laboratory settings) approaches involving asexual and sexual propagation methods. Sexual methods involve using seeds, while clonal propagation produces new plants from any vegetative parts of the plant (Shah, et al. 2020). Vegetative propagation aids in the preservation of genetic identity and populations of plants (Deepak et al. 2016). There are many scientific shreds of evidence for using ex vitro techniques for the propagation of MPs.

Ex vitro cultivations under controlled growth systems such as greenhouses, controlled environment chambers, hydroponic, aeroponic, and aquaponic systems are economically viable and novel options for cultivating difficult-to-grow MPs. These novel methods provide many benefits: producing consistent and high-quality plant materials by controlling temperature, humidity, light, and other environmental factors, year-round production regardless of seasonal variations and weather, increased plant densities and faster growth rates, prevention of the pest and disease incidences, and influence on phenotypic disparity in the accumulation of the active compounds. Apart from that, high cost associated with energy and technology and lack of genetic diversity are disadvantages of controlled growth systems of MPs.

Apart from ex vitro propagation methods, using in vitro culture techniques is popular in modern MP production. In vitro methods are presented as alternative and complementary methods of propagation which provide solutions for the drawbacks associated with conventional propagation techniques. In addition to in vitro plant propagation, synthetic seed production, cell suspension cultures, cryopreservation and short-to-medium-term storage, and in vitro metabolite production are some other important aspects of biotechnological interventions important in this context. Plant tissue culture is the sterile and optimal in vitro growth of all plant parts (explants), whether a single cell, tissue, or organ (Ahmad et al. 2013). Tissue culture techniques and genetic manipulation are used in in vitro metabolite synthesis to change target metabolite production pathways from MPs (Canter et al. 2005). These innovative biotechnological techniques for cultivating plant cells and tissues enable the preservation and fast multiplication of valuable, uncommon, and endangered MPs (Nalawade and Tsay 2004).

The purpose of this chapter is to summarize the recent knowledge based on MPs giving special reference to ex vitro and in vitro production methods, emphasizing the most recent advances, limitations, and future prospects.

2 Ex Vitro Production of MPs

MPs are the crucial source of raw materials for obtaining biologically active chemicals for the production of pharmaceuticals. The ever-increasing demand for herbal remedies has severely depleted plant resources, leading many MPs to the edge of their extinction. MPs can be conserved using improved biotechnological approaches or by improving their cultivation practices. Biotechnological mediations are costly

and require expertise. In this context, the development of cost-effective propagation techniques for MPs is important for their successful cultivation.

In principle, there are two ways to produce plants: vegetatively (asexually, often known as cloning) and generatively (sexually, by seeds). When sexual propagation is ineffective because seeds are not produced or are not produced in sufficient quantities or when seeds lose their viability, vegetative propagation is commonly employed. Ex vitro vegetative propagation via different propagules, such as cuttings, splitting or division, layering, budding, and grafting, plays a major role in plant production.

2.1 Ex Vitro Clonal Propagation of MPs

Ex vitro clonal propagation is carried out using different types of propagules depending on the species. Semi-hardwood cuttings were successful with *Salacia reticulata* Wight (Nayana et al. 2015) and *Evolvulus alsinoides* L. (Nakandalage and Anuruddi 2022); Fig. 1c. *Salacia oblonga* Wall., a woody climber found in Sri Lanka and India was successfully propagated by using stem cuttings with and without leaves and root cuttings (Deepak et al. 2016). Hardwood cuttings of *Vitex negundo* L. and softwood cuttings of *Flueggea leucopyrus* Willd. treated with IBA

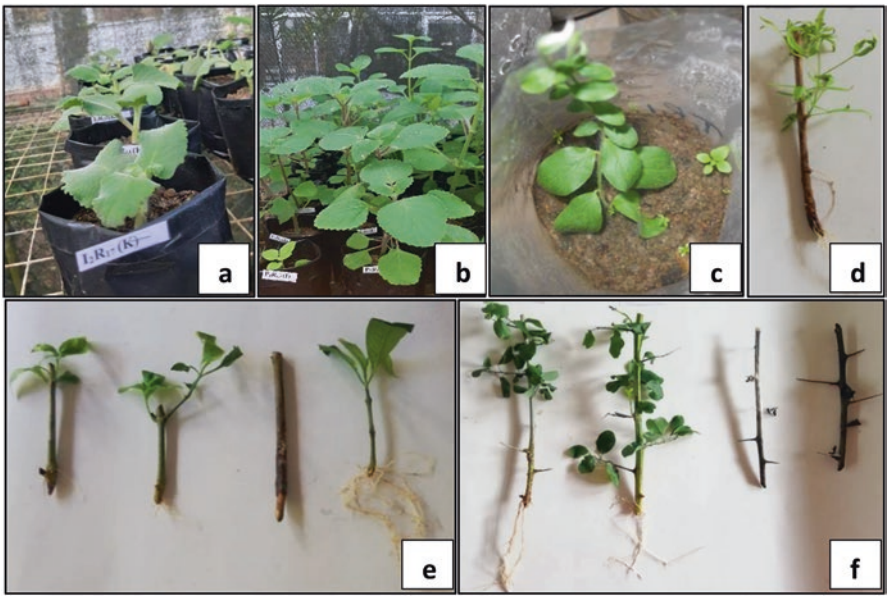

Fig. 1 Ex vitro clonal propagation of some medicinal plants using stem cuttings: (**a**) *Plectranthus amboinicus* Roxb., (**b**) *Plectranthus zeylanicus* L., (**c**) *Evolvulus alsinoides* L., (**d**) *Vitex negundo* L., (**e**) *Adhatoda vasica* L., (**f**) *Flueggea leucopyrus* Willd

and planted in sand–coir dust 1:1 media displayed promising results (Samaranayake et al. 2022); Fig. 1d, f. Clonal propagation of *Plectranthus amboinicus* Roxb., *Plectranthus zeylanicus* L., and *Adhatoda vasica* L. with stem cuttings also showed successful results (Nakandalage 2022 Unpublished data); Fig. 1a, b, e. Ex vitro rooting of *Siraitia grosvenorii* Swingle in a perlite media using in vitro-propagated shoots as propagules produced morphologically robust and well-developed root system capable of supporting plantlets in field conditions upon transplantation (Yan et al. 2010). *Aloe barbadensis* L. is propagated using root suckers or rhizome sections (Das and Chatopadhyay 2004). Wedge grafting of *Terminalia chebula* Retz using *Terminalia catappa* L. root stock and patch budding with *Terminalia bellirica* Roxb. were successful with 78% and 67% survival rates, respectively (Nakandalage et al. 2021). Examples of some other clonal propagation methods are given in Table 1.

Ex vitro plant propagation of MPs offers numerous advantages. The plants raised after ex vitro rooting have better developed root systems compared to the ones raised after in vitro rooting of strawberry (Bozena 2001). Besides, the ex vitro techniques reduce the risk of genetic alterations of plants and enhance the disease resistance. Also ex vitro propagation methods are economical, save time, and require fewer inputs. But offspring from clonal propagation may find it difficult to survive under changing climatic conditions, and competition with the parent plants is observed when colonized and overcrowded in the same area.

2.2 Ex Vitro Sexual Propagation of MPs

Seed propagation is the primary technique by which plants reproduce in nature, as well as one of the most effective and extensively utilized propagation strategies for cultivated crops. Different ex vitro seed propagation protocols have reported to propagate a wide range of MAPs. Mature seeds of *Coscinium fenestratum* L. were successfully germinated by exposing to direct sunlight followed by treating with 2250 mg/L gibberellic acid (GA_3) solution for 24 h (Warakagoda and Subasinghe 2015). Seed propagation of *Andrographis paniculata* L. and *Barleria prionitis* L. were successful with the application of GA_3 and with dry storage (Jayawardhane et al. 2021). Seed germination of *Azadirachta indica* L. was successful when pretreated with 1 M hydrochloric acid (HCl) (Abubakar et al. 2019) (Table 1). Seed propagation is ideal to develop plants with a well-developed root system and is especially vital when clonal propagation is unsuccessful. But sometimes sexual propagation carries disadvantages as well. Their progenies are not true to type and not suitable for commercial cultivation when uniform quality and high-yielding plants are required. Also, seeds may lose their viability within a limited time period.

Table 1 Ex vitro propagation protocols for some selected MPs

No	Scientific name	Family	Propagule	New improvement in ex vitro propagation method	Successful ex vitro media	[a]Medicinal value	References
1	*Echinacea purpurea* L.	Asteraceae	Seedlings with four real leaves	A new growing media with various perlite particle sizes and its mixture with peat moss is tested for hydroponic-based production	The medium containing very fine-grade perlite (less than 0.5 mm) and 50:50 v/v perlite to peat moss ratio	Treat anxiety, depression and inflammations	Ahmadi et al. (2021) and [a]Manayi et al. (2015)
2	*Salacia oblonga* Wall.	Celastraceae	Stems without leaves (S) and stem with leaves (SL) (10–15 cm) and roots (5–10 cm)	Maximum shooting in S and SL is observed with 300 ppm IBA and rooting is maximized with 200 ppm IBA	Fine soil/sand/ vermiculite at 2:1:1 ratio	Treat diabetes,skin diseases, rheumatism	Deepak et al. (2016) and [a]Matsuda et al. (2002)
3	*Zingiber officinal*e L.	Zingiberaceae	Germinated rhizomes	Biomass production, primary and secondary metabolite synthesis, and antioxidant activities are enhanced by enriching the controlled environment atmosphere with CO_2	Soilless mixture includes burnt rice husk and coco peat with ratio at 1:1	Treat cold, colic, diarrhoea, spasm, influenza	Ghasemzadeh and Jaafar, (2011) and [a]Riazur et al. (2011)
4	*Artemisia vulgaris* L.	Asteraceae	Seedlings	Propagate in a deep pool floating raft system (FRT)	Hydroponic nutrient solution	Treat malaria	Papadopoulos et al. (2000) and [a]Bamunuarachchi et al. (2013)
5	*Withania somnifera* L.	Solanaceae	Seedlings	Propagate in hydroponic culture chambers controlling temperature and humidity and CO_2. Plants are settled in containers filled with rockwool	Hydroponic nutrient solution	Treat brain and immune disorders and cancer	Kaul et al. (2016)

6	*Ocimum basilicum* L.	Lamiaceae	Two-week-old seedlings	Hydroponic system (deep water culture), aeroponic system, soilless substrate (peat moss slabs)	Nutrient solution is consisted with ca$(NO_3)_2$, KNO_3, K_2SO_4, KH_2PO_4, $MgSO_4$	Treat headaches, coughs, and diarrhea	Khater et al. (2021) and [a]Joshi (2014)
7	*Siraitia grosvenorii* Swingle	Cucurbitaceae	Cuttings with two nodes	Use perlite as a substrate for root induction	Dip the base of the cuttings in 100 mg/L NAA for 1-min and insert into perlite substrate in the seedling bed	Treat diabetics, tumors, and inflammations	Yan et al. (2010) and [a]He et al. (2022)
8	*Panax pseudo-ginseng* wall.	Araliaceae	Rhizomes and roots	Inclined root cuts enhance the propagation than horizontal cuts	Decayed wood powder, sand, and top black soil at 1:1:3 ratio	Treat diabetics, inflammation, and liver diseases	Jamir et al. (2016) and [a]Liu et al. (2020)
9	*Andrographis paniculata* L.	Acanthaceae	Seeds	Application of dormancy breaking treatments (GA_3) and dry storage for 1 or 3 months	Sand/garden soil at 1:1 ratio	Treat diarrhea, flu, leprosy, and leptospirosis	Jayawardhane et al. (2021) and [a]Kumar et al. (2021a, b)
10	*Helichrysum odoratissimum* L.	Asteraceae	Seedlings	Grow seedlings in a recirculating aquaponic system. Seedlings are transplanted into a net pot containing a mixture of perlite and coco coir as a substrate	Nutrient-rich fish wastewater is utilized and recycled water is returned to the fish tank	Treat heart diseases, relief of chest pains, and use for calming	Zantanta et al. (2022) and [a]Serabele et al. (2023)

[a]References for the medicinal values of the plant

2.3 New Insights of Ex Vitro Propagation of MPs

2.3.1 Production of MPs Under Controlled Environments

MP cultivation is prevalent, and the desire for new methods of cultivation is growing rapidly. The composition of active ingredients included in MPs is subjective to several factors such as cultivation conditions, harvesting time, drying methods, and genetic factors (Yoshimatsu 2012). Under these conditions, a long-term solution for a sufficient and continuous supply of MPs is well identified. Advances in controlled environment cultivation such as hydroponic, aeroponic, and aquaponic systems are very popular due to the results it showed recently in the cultivation of vegetables and other food crops and applicable for the production of MPs.

There are a few reported issues with the plant-based medicines that justify the need for their production under controlled environments. Biotic and abiotic contamination caused by bacteria, fungi, insects, and pest invasion of MPs potentially endanger the quality and amount of secondary metabolites. Adulteration with weeds and misidentification of MPs due to the indistinguishable morphological characteristics with other species, alterations occurred in the active ingredients due to the number of sources of variations such as environment and agronomic practices, the effect of growing conditions such as photoperiod and light intensity, postharvest handling practices including drying and storage, and extinction of wild species arise the need for developing new technologies for efficient mass production of MPs.

Cultivation of MPs under a controlled environment will avoid the difficulties when gathering raw materials from the wild, and it will produce quality and uniform shoot and root materials enriched with a high percentage of bioactive substances. Also, it provides opportunities to improve consistency of production to enhance the biomass in a large-scale production with multiple harvests while extending the growing season.

Hydroponic systems provide nutrients in the liquid form with or without a substrate to anchor the plants. These systems will enhance the purity of the MP materials by avoiding the fortuitous adulterations caused by weeds, soil and toxins, and heavy metals by providing solutions for the conservation, characterization, and commercial production of MPs under a pesticide-free environment (Hayden 2006). Aeroponics is a method of growing plants in an air or mist environment without the use of soil. The plants are grown in a closed or semi-closed environment, and their roots are suspended in the air while they are misted with a nutrient solution. The roots are exposed to the nutrient solution and oxygen, which allows them to absorb the nutrients they need to grow while reducing the risk of disease. This method is known for its high efficiency, versatility, and sustainability. Aeroponic systems are more productive when leaves and flowers are harvested for medicinal purposes (Hayden 2006) (Fig. 2).

The process of production of MPs under controlled environments includes different steps such as screening superior germplasm, in vitro protocol development, setting up control environments, mass-scale in vitro production, ex vitro establishment, optimization of biomass production and postharvest technologies, and quality

Fig. 2 Production of *Vetiver zizanioides* L. in a hydroponic system

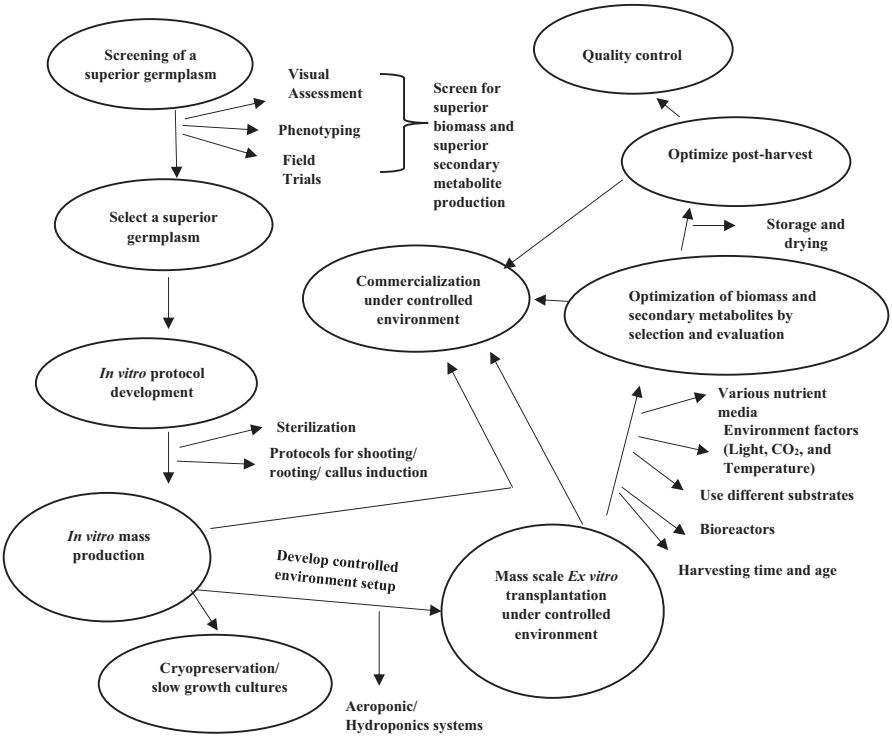

Fig. 3 Procedure for the production of MPs under controlled environment

control. Finally, mass-scale ex vitro production of MPs is practiced under controlled environments (Fig. 3).

According to Afreen, et al. (2005), glycyrrhizin content in the roots of *Glycyrrhiza uralensis* Fisch. was highest when cultivated under hydroponic conditions

providing red light. Leafy medicinal plants such as *Scutellaria lateriflora* L. and *Mentha piperita* L. have produced high yields of leaves and flowers in aeroponic system, and multiple harvests per year were also possible over traditional field practices. But rhizomatous crops are difficult to manage in hydroponic systems since they produce shoots in unpredictable patterns in their multi-branching rhizomes (Hayden 2006). *Arctium lappa* L. harvested for its roots and used as a blood-detoxifying agent (Chan et al. 2011) was successful in aeroponic system. Pagliarulo et al. 2004 conducted research to evaluate the best hydroponic/aeroponic system for *Urtica dioica* L. compared to a control established in another soilless media. Results revealed that the aeroponic system produced high roots but fewer rhizomes compared to the control. But aeroponically grown plants permitted multiple harvests. The roots and vegetative parts of *Urtica dioica* L. are used in herbal dietary supplement production. *Achillea millefolium* L., *Inula helenium* L., *Stellaria media* L., *Taraxacum officinale* L., and *Valeriana officinalis* L. cultivated in Floating Raft System (FRT) successfully increased their root dry weight compared to field-grown plants (Papadopoulos et al. 2000). Khater et al. (2021) report that *Ocimum basilicum* L. recorded the highest values for vegetative parameters and essential oil content when cultivated in aeroponic system compared to deep water culture system and peat moss slab cultivations. Amarasinghe et al. (2008) proposed a successful protocol for the production of *Vetiver zizanioides* L. in a hydroponic system (Fig. 2).

3 In Vitro Propagation of Medicinal Plants

Biotechnological approaches either preserve elite germplasm or produce high-value MPs compared to conventional methods (Singha and Pradhan 2015). The advancement of genetic engineering and tissue culture has created a new path for the massive and effective manufacturing of biologically active compounds. In vitro propagation, also known as micropropagation, is a biotechnological intervention producing a significant number of progeny plants from distinct somatic cells, tissues, or organs of plants under controlled environments within a fairly short period of time (Singha and Pradhan 2015). Plant tissue culture not only promotes the multiplication of MPs but also their conservation and metabolite production in higher levels (Niazian 2019) so that wild harvesting can be prevented. These modern techniques will be used in conjunction with other molecular studies on endangered or extinct species. Over the last three decades, in vitro technologies have been employed to propagate numerous rare and endangered MPs to evaluate their sustainable applications by optimizing the media and addition of PGRs (Sharma et al. 2020; Rajasekharan and Wani 2020). Tissue culture techniques have typically been used when traditional methods of propagation kept failing or demonstrated inadequate. The performance of tissue-cultured plants is governed by several factors, such as the selection of explant, media composition, PGRs, and environment.

3.1 Stages of Micropropagation of MPs

In vitro propagation involves five steps (0–4). Stage 0, which is the basic stage, involves germplasm collection. Before initiation of micropropagation, donor plant selection is crucial. Selection of a disease-free mother plant is important to avoid contamination. Generally, pretreatment of mother plants with suitable growth hormones, foliar sprays, fungicides, and insecticides is practiced to improve the quality and certain growth parameters. Stage 01 is where aseptic cultures are established. Maintaining aseptic conditions for in vitro explant establishment is a necessary requirement for the effective implementation of tissue culture technologies. The physiology of the mother plant, condition of the explant, methods of sterilization, and tissue culture medium are pivotal factors that decide the successful establishment (Sharma and Pandey 2015b). The type (Rajasekharan and Wani 2020), age, size, position of the stem, and polyphenol oxidation of the explant (Sharma et al. 2020) chosen for micropropagation are extremely important in defining the in vitro regeneration capacity of MPs. The primary explant size may range from 0.1 mm to 1 cm (Sharma et al. 2020).

Explant sterilization is one other major concern in successful in vitro establishment. Different agents, such as calcium hypochlorite, sodium hypochlorite, ethanol, and mercuric chloride, are used for explant sterilization (Mihaljevic et al. 2013). The choice of sterilant should be determined by the physical traits, such as firmness and pliability, of the explant (Yadav and Singh 2011). According to Ramandi et al. (2019), the best surface sterilization protocol for seeds of *Catharanthus roseus* L. was 3% sodium hypochlorite for 5 min. *Salacia reticulata* Wight nodal cuttings were sterilized using running tap water, Teepol, sodium hypochlorite, 70% alcohol, and 0.1% mercuric chloride (Dhanasri et al. 2013). Highest number of survived plants were observed in *Aloe barbadensis* L. when explants were sterilized with Tween 20 (5 drops) for 10 min, Bavistin 1% for 10 min, and $Ca(ClO)_2$ (3.25.0%) for 6 min (Abbasi 2017) (Table 2).

Among different growth media, Murashige and Skoog's (MS) medium (Murashige and Skoog 1962) is frequently used. A tissue culture medium is an artificial nutrient supplement consisting of organic and inorganic nutrients. It comprises a carbon source (usually sucrose), macro- and micronutrients, plant growth regulators (PGRs), and vitamins along with other organic substances. Sometimes, the medium is enriched with hormones to augment metabolite synthesis. The type and strength of growth regulators vary based on the species, genetic variation, source of the plant material, and method of reproducing the plant (Sharma et al. 2020).

Stage 02 is the multiplication stage where the number of propagules is increased. Plant regeneration in vitro refers to the process where explants, such as plant tissues or organs, undergo cell division and differentiation to grow into new plants during a specified period (Bidabadi and Jain 2020). The regrowth of an explant can either occur through organogenesis or somatic embryogenesis, depending on the type of plant and the growing conditions (Sharma et al. 2020). In organogenesis, new organs

Table 2 Different in vitro protocols for production of some selected MPs

Number	Scientific name	Family	Explant	Sterilization method	Successful multiplication medium	Successfulrooting medium/ encapsulation medium	New improvement in in vitro propagation method	References
1	*Siraitia grosvenorii* Swingle	Cucurbitaceae	Shoot tips 0.5–1 mm	Wash under running tap water +75% alcohol for 5 s, + 0.1% $HgCl_2$ solution for 10 min + three repeated washes with sterile water.	**Shoot induction:** MS basal medium supplemented with 1.0 mg/L 6-benzylaminopurine (BAP), 0.01 mg/L NAA, 3% (w/v) sucrose, and 4.0 g/L agar **Axillary shoot proliferation:** MS basal medium supplemented with 0.5 mg/L BAP, 0.01 mg/L NAA, 3% (w/v) sucrose, and 4.0 g/L agar	MS basal medium +0.1 mg/L NAA, 3% (w/v) sucrose +4.0 g/L agar	Replacement of agar with perlite produces lateral roots with very less- or nondeveloped callus	Yan et al. (2010)
2	*Celastrus paniculatus* Willd.	Celastraceae	Nodal segments 2–4 mm	Wash under running tap water + wash with detergent "Teepol" for 5 min + wash with 10% Clorox for 10 minutes + wash (3–4 washes) with sterile double-distilled water.	**Shoot proliferation**: MS medium supplemented with 0.1 mg/L TDZ	Slightly modify of MS basal medium by devoiding calcium salt and agar and fortified with 3.0% sucrose and sodium alginate	Develop encapsulated micro-shoots (synthetic seeds)	Fonseka et al. (2019)

3	*Taxus chinensis* L.	Taxaceae		Seeds: Wash under running tap water for 1 h + surface sterilize with 5% sodium hypochlorite (w/v) for 20 min + wash with sterile distilled water for 3 times. Excised embryos: Endosperm cells can be shaken off by rinsing in sterile distilled water for three times.	Woody plant medium +0.5 mg L^{-1} GA_3 + 0.5 mg L^{-1} IAA + 0.5 mg L^{-1} BA +1 g L^{-1} activated charcoal		In vitro embryo culture	Song et al. (2014)
4	*Swertia chirayita* Roxb.	Gentianaceae	In vitro leaf explants	Surface sterilize in vivo grown leaves with 0.2% Bavistin (fungicide) + tween- 20, (5–6 drops/100 ml) solution for 20 min + 0.1% $HgCl_2$ w/v for 8 min + wash out with sterilized distilled water for 4–5 times.	**Embryogenic callus induction:** MS medium supplemented with 0.5 mg/L 2,4-D and 0.5 mg/L kinetin **Synthetic seed germination:** MS medium supplemented with 1.0 mg/L BA +0.5 mg/L NAA	4% w/v sodium alginate gel and dipped into the calcium chloride ($CaCl_2 \cdot 2H_2O$)	Synthetic seed of *S. chirayita* through somatic embryogenesis	Kumar and Chandra (2014)

(continued)

Table 2 (continued)

Number	Scientific name	Family	Explant	Sterilization method	Successful multiplication medium	Successfulrooting medium/ encapsulation medium	New improvement in in vitro propagation method	References
5	*Amomum tsao-ko* L.	Zingiberaceae	Seeds	Pretreat the seeds with 25% HCl for 15 min + wash with liquid soap for 10 min + rinse directly under running tap water + wash with 70% ethanol for 30 s + immerse in 0.1% (w/v) aqueous mercuric chloride for 10 min + rinse in distilled water for 4–5 times.	MS medium enriched with macronutrients diluted to 1/16 strength		Pre treatment for the seeds before in vitro germination Best pre treatment method is the application of mechanical scarification	Khuat, et al. (2022)
6	*Rauvolfia tetraphylla* L.	Apocynaceae	Seeds	Shake in 2–3 drops of tween-20 for 5–10 min + wash with sterilized distilled water + wash with 70% ethanol for 5–10 min + wash thoroughly with sterilized distilled water for 2–3 times + shake gently in 0.1% $HgCl_2$ for 10–15 min + wash with distilled water for 3–5 times.	MS medium with 3% sucrose without supplementing with any PGR		First, give cold treatment to the seeds for 7–10 days at 4 °C. + Cutting one edge of the seed to enhance germination	Hoque et al. (2020)

such as axillary and adventitious shoots and or even whole plants are formed. It begins with distinct shoot and/or root meristem organization within the explant (direct organogenesis) or from the callus (indirect organogenesis). Axillary bud proliferation is the most commonly used and effective method for plant micropropagation, and it ensures the genetic stability of the newly regenerated plants. The unsprouted axillary buds are caused by the dominance of the shoot tip, known as apical dominance, and their growth can be boosted by synthetic cytokinins such as 6-benzylaminopurine (BAP) (Sivaji et al. 2020).

There are multiple methods of indirect organogenesis in tissue culture, such as protoplast culture, cell suspension culture, and somatic embryogenesis which involve the formation of callus tissue, which is then induced to form new plants. Typically, callus induction in cultures is achieved through the use of auxins like 2,4-dichlorophenoxyacetic acid (2,4-D). Varying proportions of auxins and cytokinins play a crucial role in determining the differentiation of shoots into callus. Callus-mediated regeneration has special significance since it provides the scope of manipulation and exploitation of the somaclonal variation. Leaves of *Withania somnifera* L. proved to be the best explant over shoot tips and nodal segments for optimum callus induction with a combination of 2,4-D (0.5 mg l^{-1}) and KN (kinetin) (0.2 mg l^{-1}) (Chakraborty et al. 2013). Stem and leaf tissues of *Trichosanthes kirilowii* maxim showed the best response (100%) for callus initiation on MS medium supplemented with 4.5-μM 2,4-D (Zhao et al. 2018). Stem segments of *Vitex leucoxylon* L. cultivated on MS media fortified with 5.37 M NAA (naphthalene acetic acid) + 2.22 M BAP (6-benzylaminopurine) developed dense and greenish callus from internodal segments (Chordia et al. 2010). Successful callus induction of *Swertia chirayita* Roxb. was attained from in vitro-regenerated roots on MS medium supplemented with 13.32 μM BA and 0.90 μM 2,4-D (Pant et al. 2012).

Somatic embryogenesis is one of the common methods of indirect organogenesis. In somatic embryogenesis, undifferentiated callus mass is treated with specific hormones to induce the formation of a structural cells similar to a zygotic embryo, and finally an entire plant is regenerated (Ikeuchi et al. 2016). Somatic embryogenesis has several advantages over other methods of tissue culture. It is a more controlled process that allows for the selection of specific embryos with desirable traits. Additionally, it is an effective tool for plant breeding, genetic engineering, and conservation of endangered species.

Stage 03 is distinguished by the cessation of fast multiplication and the development of fully formed plantlets. At this stage, elongation of shoots and root formation along with the formation of storage organs are taken place. Rooting is achieved through auxins such as IAA, IBA, and NAA either singly or in different combinations (Sharma et al. 2020). Rooting media comprised with high auxin: cytokinin ratio is preferred. Supplementing rooting media with riboflavin and other adjuvants will be beneficial (Sivaji et al. 2020).

The final step is acclimatization and establishment/transplanting of the plantlets under ex vitro conditions. Complete regenerated plantlets with sufficient roots after removal of adhering media by immersing in water should be transferred to the field/pots containing a suitable growing media. Tissue-cultured plantlets are easily

subjected to light and temperature shock as they lack a protective cuticle layer and hairy roots (Sivaji et al. 2020). Moreover, plants grown through tissue culture are delicate and their vascular systems are underdeveloped. To compound these issues, the plantlets are subjected to a hardening process by gradually changing the light and humidity.

3.2 In Vitro Propagation Methods of MPs

Different vegetative parts of plants can be used as starting materials for in vitro culture establishment: nodal segments, shoot tips, rhizomes, florets, roots (Rajasekharan and Wani 2020), and leaf discs (Singha and Pradhan 2015). Besides meristems, axillary buds, adventitious buds, anthers, and seeds/embryos are also used as propagules in in vitro propagation. Nodal culture involves the use of nodal segments, which are sections of stem that contain both shoot- and root-forming tissues. Nodal segments have been used in *Celastrus paniculatus* Willd. (Silva and Senarath 2009), *Aegle marmelos* L. (Akter et al. 2013), *Nardostachys jatamansi* (Pant et al. 2021), *Salacia reticulata* Wight (Dhanasri et al. 2013), and *Spilanthes paniculata* L. (Fig. 4). Shoot tip culture use small pieces of shoot tip tissues that contain actively dividing cells used to produce new shoots and roots. Shoot tips are used in *Celastrus paniculatus* Willd. (Silva and Senarath 2009) and *Aegle marmelos* L. (Fonseka et al. 2021) (Fig. 6). Leaf culture involves the use of leaf explants to produce new shoots via direct organogenesis or indirectly through callus culture. Leaf discs are used in *Celastrus paniculatus* Willd. (Silva and Senarath 2009) and *Aegle marmelos* L. (Pathirana et al. 2020). Meristem culture involves the use of shoot or root meristems, the regions of the plant where cell division occurs, to produce new shoots or roots. Meristem explants of *Centella asiatica* L. were successfully induced shoots in MS medium supplemented with 0.5 mg/ l BAP + 0.1 mg/ l NAA (Siddiqui and Thomas 2019). Direct regeneration of *Zingiber officinale* L. from immature inflorescence was practiced in the MS medium supplemented with 10 mg/L BA and 0.2 mg/L 2, 4-D, and rooting was enhanced by using MS liquid medium with 1 mg/L NAA (Nirmal Babu et al. 1992). Some other examples

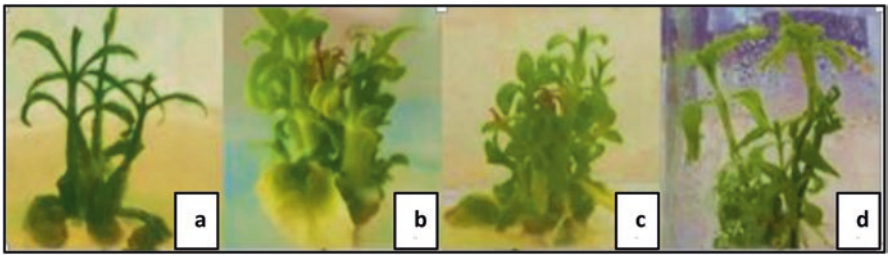

Fig. 4 In vitro clonal propagation of *Spilanthes paniculata* L. using nodal segments grown in MS media fortified with different concentrations of NAA and BAP

are provided in Table 2. Some advantages of in vitro clonal propagation include the genetic consistency of plants, faster propagation, permitting mutation induction, and ability to preserve elite germplasms. Meanwhile, lack of adaptability and hybrid vigor, dependency on certain plant parts that may not always be available, and limited genetic variations are identified as disadvantages of in vitro clonal propagation.

In vitro methods can greatly enhance seed germination and the development of seedlings in certain species where conventional methods typically show limited or no seed germination due to the presence of dormancy or specific conditions needed for germination. In some MPs, germination is prevented because the embryo is encased by a tough outer layer (seed coat), resulting in a dormant state. Therefore, it is possible to release the dormancy by removing the surrounding structures by means of different pretreatment methods under in vitro conditions (Sharma et al. 2020) or by embryo and endosperm culture (Mabundza et al. 2010). Seed pretreatment methods include scarification (allow water and oxygen to penetrate the seed coat and reach the embryo), stratification (cold stratification will stimulate the conditions that the seed would experience in nature), soaking (softening the seed coat and enhancing the water uptake and embryonic cells will be activated, and some seeds are treated with hormones such as GA_3 to promote the germination). The seeds of the endangered antidiabetic plant, *Swertia chirayita* Roxb., were able to sprout successfully after being soaked overnight in a solution of 1.146 M GA3 and then planted in ½ MS medium (Joshi and Dhawan 2007). Seeds of *Aegle marmelos* L. were successfully propagated using splitted seeds (Fonseka et al.2021) (Fig. 5), and in vitro shoots tips were obtained for further multiplication (Fig. 6). Not all plants require pretreatment before in vitro seed germination. The specific pretreatment required depends on the type of plant and the desired outcome. Some seeds may germinate without any pretreatment. *Andrographis paniculata* L. seeds were successfully germinated in MS medium supplemented with 2.0 mgL^{-1} 2, 4 D + 1.0 mgL^{-1} NAA without any pretreatment (Ranaweera et al. 2020).

The process of embryo culture involves growing embryos in a sterile environment in a laboratory using a nutrient-rich medium. This technique is beneficial for

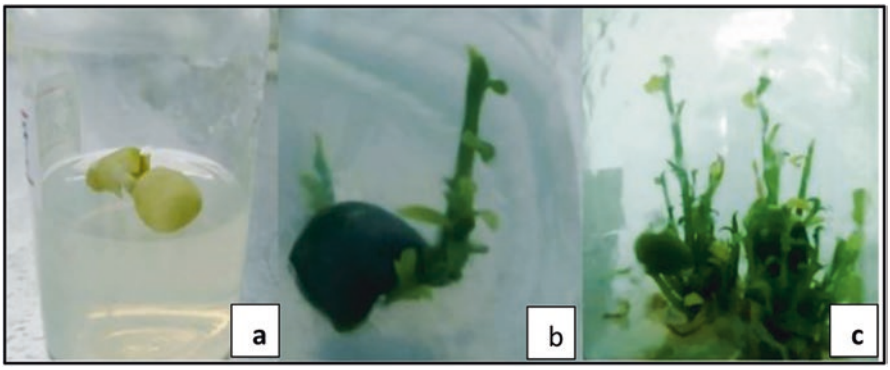

Fig. 5 Different stages of in vitro seed propagation of *Aegle marmelos* L. (Fonseka et al. 2021)

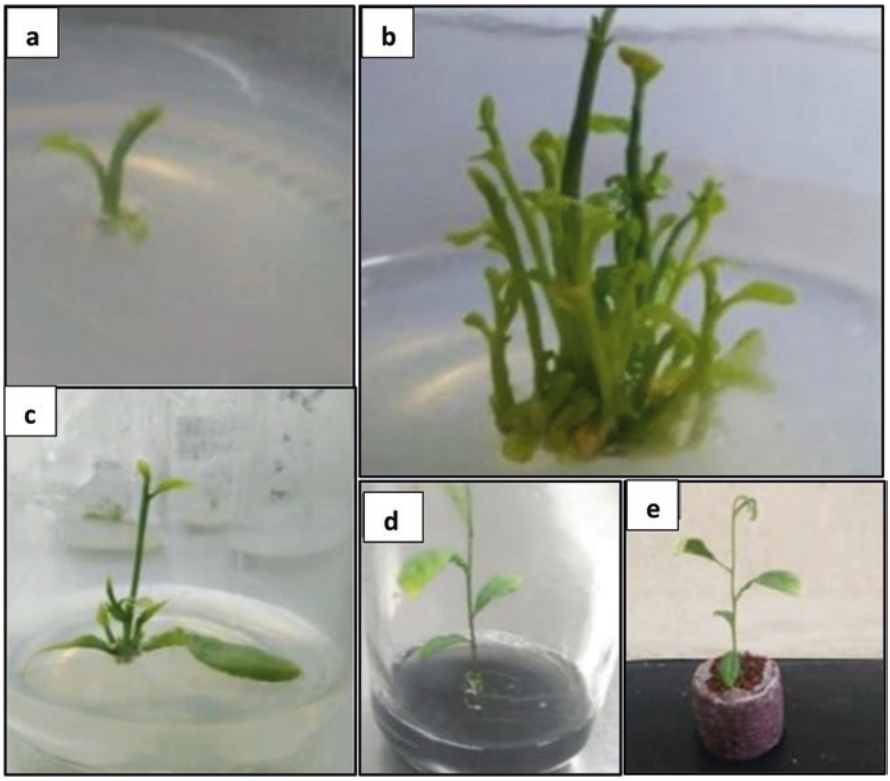

Fig. 6 Different stages of in vitro propagation of *A. marmelos* L. by in vitro shoot tips. (Fonseka et al. 2021)

awakening dormant seeds and reducing the time needed for breeding. According to Song et al. (2014), the addition of GA_3, (IAA), 6-benzylaminopurine (BAP), and organic additives such as casein hydrolysate and yeast extract in media promotes the germination and seedling development of embryos in the darkness of *Taxus chinensis* L. var. mairei. The use of zygotic embryos helped for germination to occur within 7 days of *Strychnos pseudoquina* seeds which normally spend 65 days to germinate (Leite et al. 2021).

3.3 New Insights of In Vitro Propagation

3.3.1 Synthetic Seed Production

When conventional seeds are inadequate for propagation, then synthetic seed technology is a possible solution. Synthetic seeds are produced by enveloping various micropropagules, such as nodal segments, axillary buds, meristem tips,

or somatic embryos, with a calcium alginate gel that is commonly used as a matrix material. In addition to providing physical support, the encapsulating matrix also supplies moisture and nutrients to support the regrowth of axillary buds (Zarei et al. 2022). To slow down the metabolic rate of the encapsulated propagules, synthetic seeds produced are stored at low temperatures (kinci et al. 2019). Compared to the conventional propagation of MPs, synthetic seed technology offers an efficient method for widespread reproduction, preservation of plant genetic information, and the transfer of genetic materials across international boundaries. The Synseed technology serves as the foundation for producing commercially certified plant materials that are free of pests and diseases (Adhikary et al. 2021). Zarei et al. (2022) conducted research on a dependable method for producing synthetic cannabis seeds through encapsulating nodal segments from both ex vitro and in vitro sources. Plantlet regrowth efficacy of ex vitro- and in vitro-derived seeds were 90% and 70%, respectively, when stored under 6 °C under 50 μmol S^{-1} m^{-2} light for 150 days. A protocol for producing synthetic seeds of *Celastrus paniculatus* Willd., an endangered medicinal plant in Sri Lanka, was developed efficiently through in vitro multiple shoot proliferation. The shoot tips remained healthy and green when stored at a temperature of 5 °C for 8 week (Fonseka et al. 2019) (Figs. 7 and 8). Synseed of *Zingiber officinale* L. was developed by using 1–3 mm size in vitro-developed shoots/embryoids with the apical dome. In vitro shoots/somatic embryos were drenched in MS medium supplemented with 4% (w/v) sodium alginate (NA),

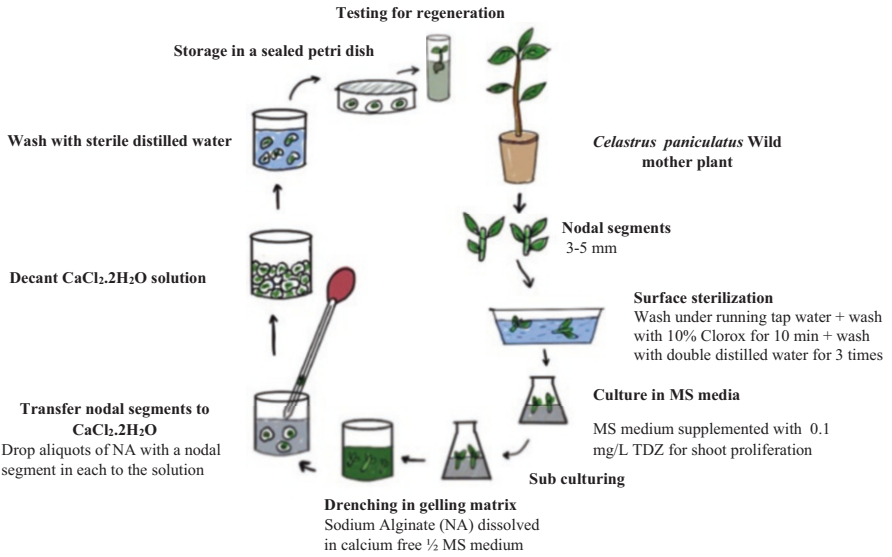

Fig. 7 Schematic diagram to represent the synthetic seed production of *Celastrus paniculatus* Willd. using nodal segments as explants. (Adapted from Fonseka et al. 2019)

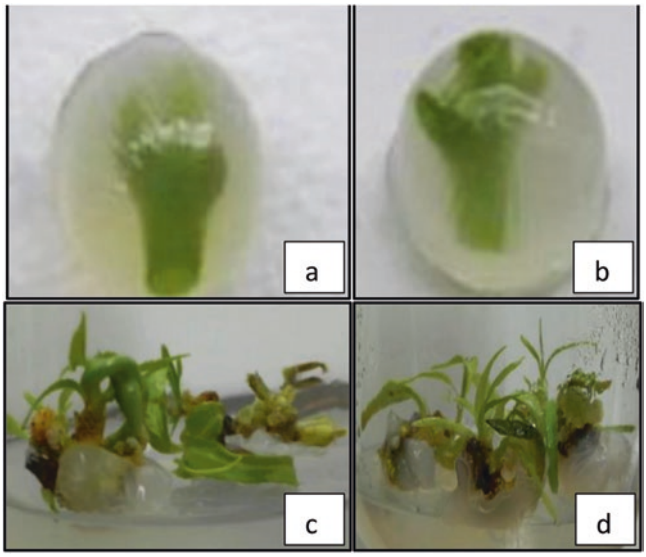

Fig. 8 (**a**, **b**) Encapsulated in vitro-derived nodal segments of *C. paniculatus* Willd.; (**c**, **d**) in vitro-germinated *C. paniculatus* Willd. Seeds (Fonseka et al. 2019).

2 M glycerol, and 0.4 M sucrose. Bead formation was done by dropping the micro-shoots into 0.1 M $CaCl_2$ solution with 2 M glycerol and 0.4 M sucrose (Nirmal Babu et al. 1999).

3.3.2 In Vitro Propagation of MPs to Produce Secondary Metabolites

The pharmaceutical industry values medicinal plants because they contain a variety of secondary metabolites that can be utilized directly or indirectly. Each plant has its own specific set of secondary metabolites produced by interacting with its environment (Rao and Ravishankar 2002). A lot of these substances are employed as therapeutic remedies for significant diseases such as cancer, fever, blood pressure, malaria, etc. (Moraes et al. 2021). Presently, a significant number of secondary metabolites are obtained from intact plants that are grown in a natural manner. However, prolonged wild harvesting has pushed certain species to the brink of extinction. In this context, the development of a complementary strategy for whole-plant extraction is a matter with great socioeconomic importance. Therefore, plant cell, tissue, and organ culture (shoot and root culture) play a vital role in the large-scale production of secondary metabolites of industrial importance (Rao and Ravishankar 2002). The extraction of pharmaceuticals through their in vitro propagation carries numerous advantages; the method is more

dependable, straightforward, swift, and effective in comparison to the extraction from the whole plant and can avoid unnecessary compounds produce in field-grown plants, and instead, a set of defined phytochemicals can be produced in large scale without the interference of geographical variations and other environmental factors all year around. Considering these factors, culturing of plant cells, tissues, and organs becomes a more desirable option for producing new metabolites while preserving the natural plant resources (Singha and Pradhan 2015). Apart from cell culture techniques, micropropagation of endangered medicinal plants and high-yielding varieties, development of transgenic plants/organisms, and utilization of newer sources such as algae and other photosynthetic marine forms are some other aspects of the production of biotechnological approaches to plant-derived metabolites (Rao and Ravishankar 2002). Plant cell cultures represent potential renewable source of valuable medicinal compounds, flavors, fragrances, and colorants, which cannot be produced by microbial cell or chemical synthesis. These systems are continuous source of natural products. These systems are continuous source of natural products.

3.3.3 Hairy Root Culture (*Agrobacterium Rhizogenes*-Mediated Transformation)

The culture of hairy roots refers to a plant tissue culture technique in which roots are grown that have transformed and exhibit hairlike projections on their exterior. This method is achieved by introducing a bacteria *Agrobacterium rhizogenes* into the plant tissue, which causes the plant cells to undergo a process called "hairy root induction." It results in the formation of new roots that have a higher growth rate and metabolic activity compared to normal roots in non-transgenic mother plants. It's an effective method for producing plant-derived secondary metabolites and valuable compounds. The transformed roots have a rapid growth rate and high metabolic activity, which enables hairy root cultures to generate substantial quantities of secondary metabolites in a short span. Moreover, they are employed for investigating the progression and maturation of plants and for determining the genes related to secondary metabolism. Samaddar et al. (2019) successfully establish hairy root cultures of *Swertia chirayita* Roxb. via *Agrobacterium rhizogenes*-mediated transformation. Leaf explants of *Gentiana scabra* L. were infected with *Agrobacterium rhizogenes* and hairy roots were successfully induced. Quantitative analysis of hairy root cultures for loganic acid, zeatin, and gentiopicroside proved higher concentrations than roots cultivated in green houses (Huang et al. 2014). *Echinacea purpurea* (Moench) inoculated with *Agrobacterium rhizogenes* induced hairy roots to produce chicoric acid (Liu et al. 2006). This innovative method is highly regarded for its capability to generate specific biomass with ecological purity, regardless of the season, climate, and weather circumstances.

3.3.4 Bioprocess Technology for the Production of MP Materials and Secondary Metabolites

Bioprocess technology refers to the use of biological systems, such as microorganisms or plant cells, to produce a desired product; in this case, plant secondary metabolites. Plant cell culture systems are used in plant bioprocess technology to manufacture secondary metabolites. Through in vitro methods, this includes the use of bioreactors, as well as other equipment and techniques for the cultivation and maintenance of plant cells and tissues. Secondary metabolite production, purity, and yield can be scaled up using bioprocess technology. Additionally, it is used to manipulate the metabolic pathways of plant cells to produce desired secondary metabolites that may not be found in the wild. Automation of micropropagation in a bioreactor by organogenesis or somatic embryogenesis is suggested as a cost-cutting measure (Paek et al. 2005). Recent breakthroughs in plant biotechnology research have demonstrated that adventitious root bioreactor cultivation is an appealing and substitute method to whole plant, cell, or hairy root culture for biomass and bioactive compound production (Baque et al. 2012).

Bioreactors are equipment used in in vitro plant metabolite production through tissue culture. They provide a controlled environment for the development of plant cells, tissues, or organs. Organogenic explants are actively cultured in bioreactors with the purpose of producing transplants for large-scale production. This method outperforms than conventional tissue culture systems because it manipulates various physical and chemical parameters such as temperature, pH, humidity, light, and nutrient levels to optimize growth and metabolite production (Baque et al. 2012). They can be of different types, such as fluidized bed, airlift, or stirred-tank bioreactors, each with its own advantages and disadvantages depending on the application. There are three types of bioreactors: those producing biomass such as cells, shoots, or roots and embryogenic propagules; those producing metabolites such as alkaloids, flavonoids, and terpenoids and enzymes; and those used for biotransformation of externally supplemented metabolites (Paek et al. 2005). Adventitious root culture of *Echinacea purpurea* L. in bioreactors with 1/2 strength MS medium increased the root dry weight and secondary metabolite production by tenfold after 1 month of culture (Wu et al. 2007). According to Cui et al. (2010), bioreactor-based adventitious root culture for *Hypericum perforatum* L. is successful when 1/2 strength MS medium is supplemented with 0.1 mg L^{-1} kinetin with 1 mg L^{-1} IBA and 30 g L^{-1} sucrose. Meanwhile, some studies report of using bioreactors for shoot biomass of MPs. The balloon-type bubble bioreactor system was discovered to be beneficial for increasing *Bacopa monnieri* L. shoot biomass ensuring a continuous supply (Sharma et al. 2019).

3.3.5 In Vitro Propagation as a Conservation Strategy of MPs

In vitro propagation of MPs is an effective biotechnological approach for ex situ plant conservation strategies. Because of the renewed interest in herbal medicine for healthcare, conservation is receiving increased attention. MPs are preserved in two ways using in vitro propagation. First, it is used as a multiplication tactic for species with reproductive concerns or for species with extremely low populations. Secondly, it is a storage method for certain species with recalcitrant seeds when long-term seed conservation is impossible (Sharma N, Pandey 2015a). Sometimes, endangered MPs may have specific growth requirements with special requirements of modified techniques for in vitro culture. Biotechnology advancements have enabled the use of in vitro methods for the gathering, preservation, and utilization of plant resources. Tissue culture concepts for in vitro conservation of MPs have been widely adapted in slow growth cultures and cryopreservation protocols (Rajasekharan and Wani 2020).

Cryopreservation is a method of preserving biological samples, such as cells or tissues, at freezing temperatures (−196 °C) using liquid nitrogen. The low temperature slows down metabolic processes and reduces the chance of damage to the sample. This method is used to preserve the integrity of the plant material and to prevent deterioration or damage due to environmental factors such as heat, light, pathogens, or genetic drift. It can be used to preserve many different types of plant materials, including seeds, embryos, shoots, buds, and even mature plants. It is a useful tool for preserving rare or endangered MP species or for maintaining germplasm collections of MPs. By preserving their genetic diversity, cryopreservation helps to ensure their long-standing conservation and availability for continued studies and breeding. Slow growth cultures, on the other hand, refer to growing cells at a slower rate than the optimal growth conditions. This is often done to study the effects of slower growth on the cells or to preserve the cells for a prolonged period.

In summary, the production of medicinal plant materials has evolved over time, from traditional cultivation methods to more modern techniques. These include cultivation inside protected houses using new production technologies to advance the quality and quantity of the crops while avoiding negative impacts from the environment and extraction of secondary metabolites from in vitro-generated plant cells, tissues, or organs in a tissue culture flask or bioreactor, rather than in the field. Both conventional and modern medicinal plant production and propagation methods are important for different reasons. Modern methods produce standardized extracts with consistent chemical profiles and produce genetically modified plants with overexpression of desirable secondary metabolites of MPs compared to conventional propagation techniques. Conventional methods, such as wild harvesting and traditional cultivation techniques, are important for maintaining biodiversity and preserving traditional knowledge. They also provide a long-term supply of raw materials for traditional medicine. Both traditional and modern methods have a role to play in ensuring a sustainable and reliable supply of medicinal plants for future generations (Fig. 9).

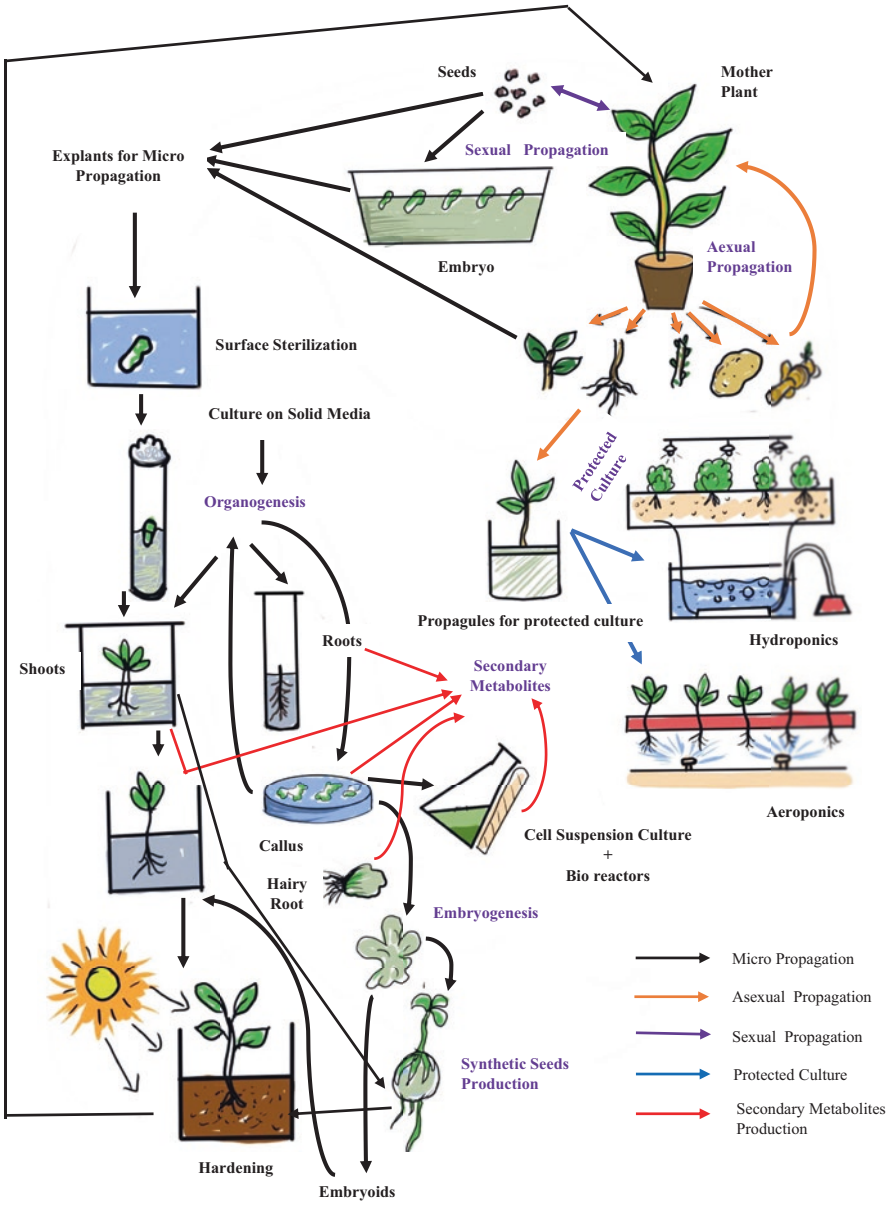

Fig. 9 Schematic representation for the production of MP materials with ex vitro and in vitro techniques

Acknowledgments The authors acknowledge Mr. Navodh Waduwawala for designing schematic diagrams for this chapter.

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Hydroponic System for Cultivation of Medicinal Plants

Leila Mehdizadeh and Mohammad Moghaddam

Abstract Hydroponics is the soilless culture in which nutrients are gained from formulated nutrient solution, and it is a quick way to produce crops. Hydroponics is a proper system for urban areas and is highly productive. Hydroponics is regarded as an alternative technique to routine cultivation systems to decrease water necessity. There are different methods including various water or container cultures. Several studies are available related to medicinal plants cultivated in hydroponic conditions. This part investigates some reports about medicinal plants under hydroponic culture. The effects of hydroponics on different characteristics of medicinal plants were evaluated in the previous research. Hydroponics can be used to produce industrial crops of medicinal plants with high qualities and high amount of specific secondary metabolites like essential oil and phenolic acids.

Keywords Bioactive compounds · Herbal medicine · Nutrient solution · Plant growth · Soilless culture

1 Introduction

Soil and land claim for producing plants is probably increasing with a fast-growing worldwide population. New and modified agricultural system development is needed due to the limitation of arable land and challenges like water scarcity, soil degradation, and urban area (Lal 2013). Nowadays, traditional agriculture faces many problems because of poor soil fertility, weeds and pest presence, pesticide use, etc., as well as common climate changes. Alternative crop production systems such as hydroponic systems that need finite soil, water, and land which play a great role in future agriculture of urban areas can be improved (Maucieri et al. 2019).

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N. Kumar, R. S. Singh (eds.), *Biosynthesis of Bioactive Compounds in Medicinal and Aromatic Plants*, Food Bioactive Ingredients,
https://doi.org/10.1007/978-3-031-35221-8_10

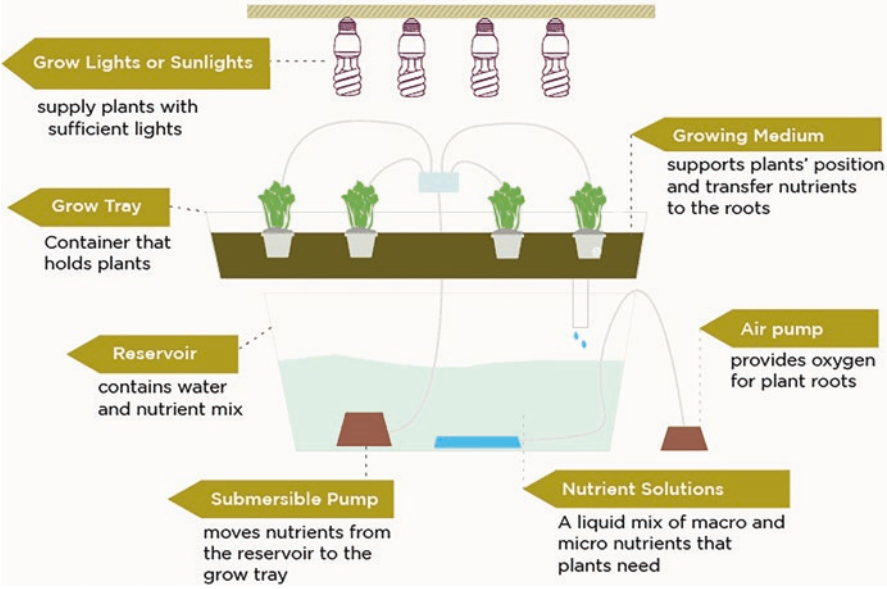

Fig. 1 Basic hydroponic parts

Hydroponics is a water-based growing system, and its nutrients are provided from formulated fertilizer; it is a fast way for plants to grow due to the nutrients absorbed by the roots directly from the nutrient solution, and the crops are never under water stress. In addition, hydroponics is appropriate for urban areas and highly productive. It can describe the land deficiency in connection to a growing requirement production of the food (Liang and Chien 2013; Medina et al. 2016). Hydroponics is a subset of hydroculture (the plant's growth in an aquatic-based or soilless medium). All the plant nutrients are dispersed through water, and hydroponics is a method of plant growth that allows mineral nutrient solutions to be absorbed from water without the requirement for soil medium) (Fig. 1).

The plants grow in the mineral nutrient solution with their roots (liquid hydroponic systems) submerged in water or an inactive medium (Son et al. 2020). In hydroponics, substrates are re-largely divided into organic (unique or blends of bark, peat moss, or coconut husk) and inorganic (perlite, rock wool, mineral wool, urethane sponge, gravel, sand, expanded clay pebbles) materials (Son et al. 2020).

2 Hydroponic Advantages

Hydroponics increases plant production in comparison with field area and a controlled environment without disease, and pest problems are provided (Putra and Henry 2015). The maximum yield and high nutrient and water-use efficiency can be gained in hydroponic culture due to the climate control methods, drip irrigation,

plant protection, and fertilization (Pardossi et al. 2004; Surendran et al. 2016). Furthermore, environmental pollution by high agrochemical application is minimized in hydroponic systems because only the required quantity of these materials is used (Sonmez and Kaplan 2004).

Briefly, the advantages of the hydroponic culture include:

- No soil is required.
- Less water requirement. In the system, the water remains, so it can be used again and higher water competence.
- Less space necessity.
- Lower nutrition necessity, no nutrition pollution, nutrition control possibility, and equal supply of nutrient solution may causes homogeneous crop.
- Decrease in the time of adjusting solution compared with traditional agriculture.
- Healthier plants without pests and soilborne diseases, with higher yields.
- Energy efficient, early growth, and easy harvesting (Son et al. 2020).

3 Hydroponic Systems

Soilless culture systems supply plant management under controlled conditions, including water and nutrient solutions, with or without the medium. Generally, hydroponic systems are soilless production systems. Soilless cultivation includes a system with a solid, a liquid, and aerated mediums (Son et al. 2020; Koriesh and Abo El-Soud 2020). To create a matrix in advanced hydroponics, some kind of substrate is used to form the root zone. So, in all hydroponic systems, no soil is used (Koriesh and Abo El-Soud 2020). Different methods can be applied for soilless cultivation. Modern agricultural systems include hydroponics, aeroponics, and aquaponics, which use nutrient-rich water more than soil (Koriesh and Abo El-Soud 2020).

4 Types of Hydroponics

Various techniques, such as different water cultures or container cultures in artificial substrate, are used for hydroponic culture (Pardossi et al. 2004) (Fig. 2). The most important techniques include the following:

4.1 Water Culture

The simplest dynamic hydroponic system is water culture. This type of hydroponic system is a very cheap and is preferred for quick-growing and water-loving plants, such as leaf lettuce. A stand made of Styrofoam fastens the plant, which directly

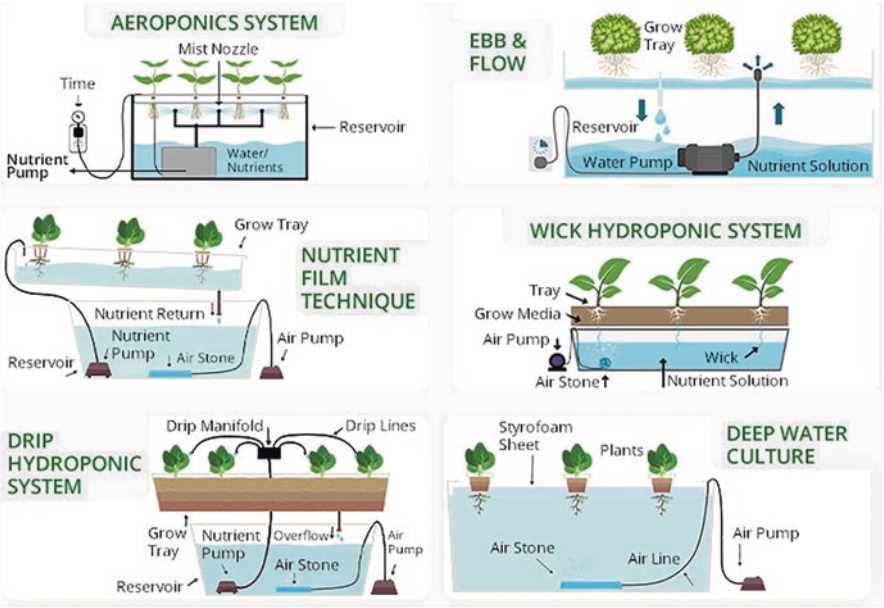

Fig. 2 Types of hydroponic systems

floats on the nutrient solutions. Foodstuff air is created by an air pump to the air stone and the nutrient solutions and oxygen bubble to the roots of the plant (Koriesh and Abo El-Soud 2020) (Fig. 3).

4.2 Water Stream Hydroponics

In the water stream hydroponic system, the polymeric film is used. The advantages of this system are a further automated system, little cost, and a beforehand instructed program (Mairapetyan et al. 2018; Koriesh and Abo El-Soud 2020).

4.3 Wick System

It is the most comfortable hydroponic system without an electrical system. Inside the pot, the plants are placed which is stuffed with medium-like coco peat which the plant roots are connected to the nutrient solution container with a nylon wick. This hydroponic system is suitable for small plants, like some spices or herbs (Eldridge et al. 2020; Kannan et al. 2022) (Fig. 4).

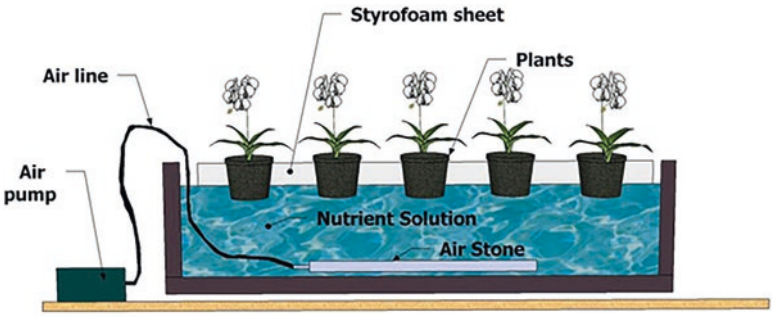

Fig. 3 Water culture system

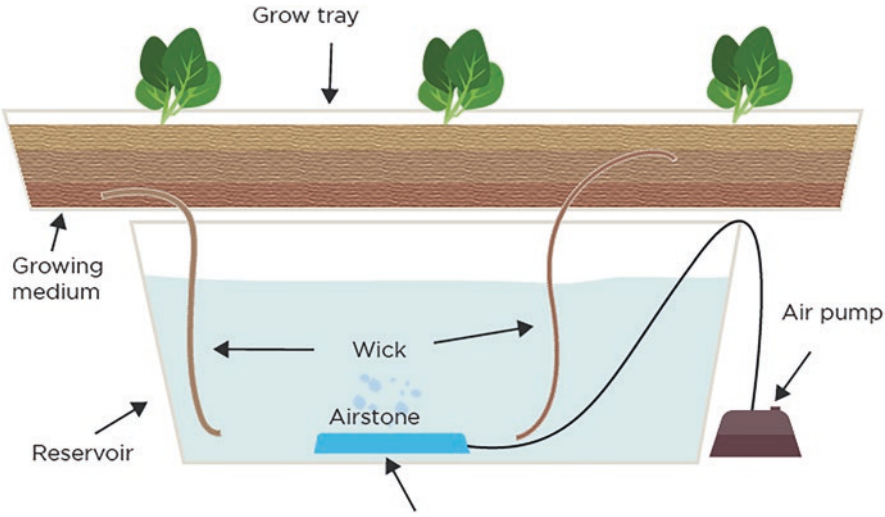

Fig. 4 Wick system

4.4 Drip System

In this system, the roots of plants are generally dripped into a nutrient solution. An electric pump supplies adequate nutrient solution from the main reservoir. It is known for its economic and simple advantages compared with other systems. By using this system, various plants can be grown in a systematic fashion with more water conservation (Gentry 2019) (Fig. 5).

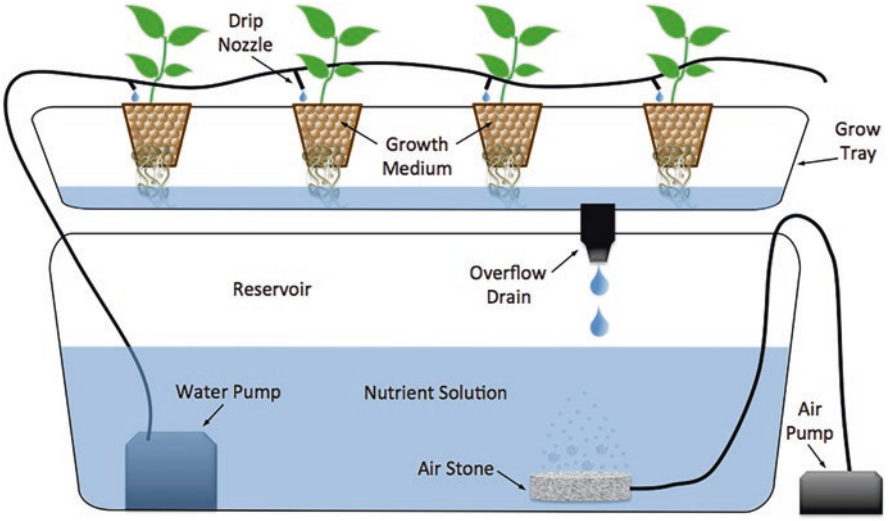

Fig. 5 Drip system

4.5 Nutrient Film Technique (NFT) System

In NFT systems, the roots of the plants that are placed in a plastic basket are suspended in the nutrient solutions. Generally no growing medium applied except air in this system which keeps the cost of returning the growing medium following every plant. They have a continuous flow of the nutrient solutions due to the submarine pump without a timer. The nutrient solutions are pumped toward the inside of the growing tube or tray and smoothly moved together with the plants roots and after that drain back into the container (Koriesh and Abo El-Soud 2020) (Fig. 6).

4.6 Deep Flow Technique (DFT) System

In this system, nutrient solutions for the plants are rewarded when in the culture bed the water level lower than the set value, and they recirculate and supply to the bare roots of plants, at constant time intervals (Son et al. 2020) (Fig. 6).

4.7 Ebb and Flow (Flood and Drain) System

In this system, the grow tray is temporarily flooded with nutrient solutions, and then it is drained back into the container with a submerged pump linked to a timer. This adaptable system can be applied with various growing mediums. Gravel, rocks, or granular rock wool is used to fill the grow tray (Koriesh and Abo El-Soud 2020) (Fig. 7).

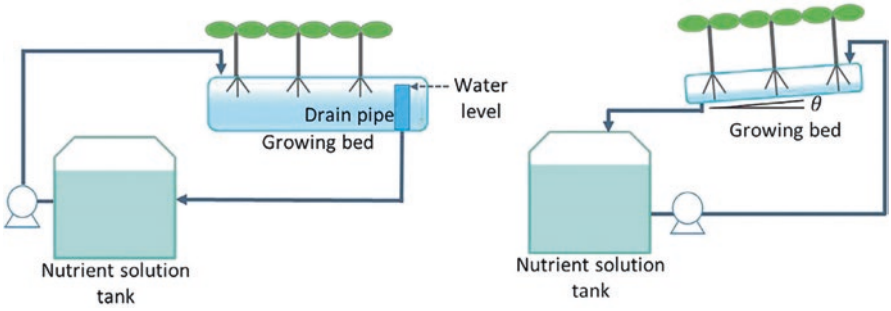

Fig. 6 DFT (**a**) and NFT (**b**) hydroponic systems

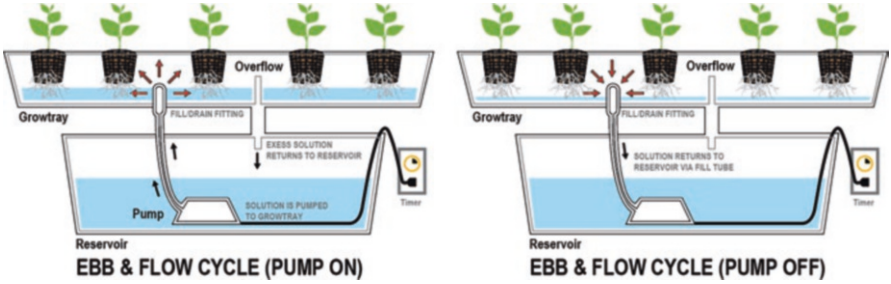

Fig. 7 Ebb and flow system

4.8 Continuous Flow Hydroponic Systems

In this hydroponic system, the nutrient solution is pumped to the root systems of plants. In order to supply the roots with oxygen, the plants can be oxygenated by an air pump in the tank. The ends of the roots are suspended and immersed in the nutrient solution, and the roots absorb the nutrients; the main root is exposed to air and oxygenated directly (Koriesh and Abo El-Soud 2020).

4.9 Floating Raft System (FRS)

Floating raft system needs comparatively low investing expenses; this method is usually applied for high-density and short-cycle greenhouse cultures of leafy vegetables which are marketed as fresh-cut crops (Maggini et al. 2011, 2012) (Fig. 8).

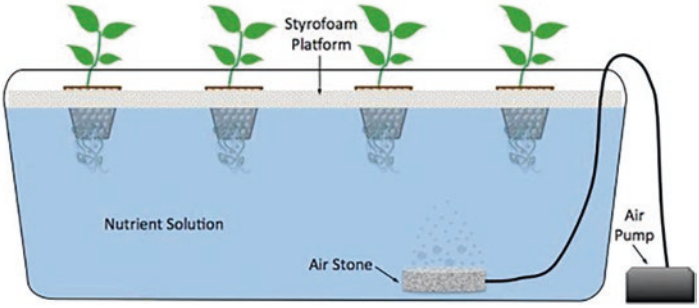

Fig. 8 Floating raft system

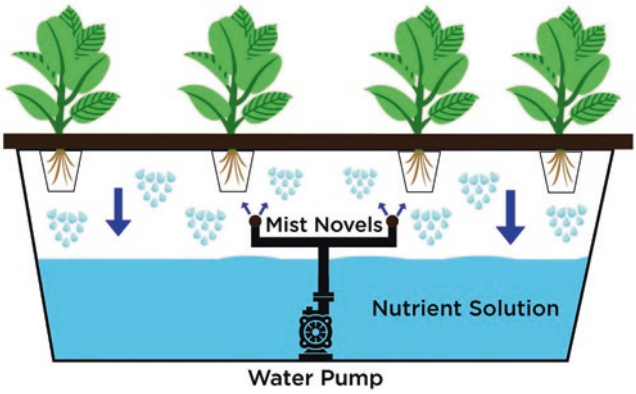

Fig. 9 Aeroponic system

4.10 Aeroponic System

The aeroponic system is a more high-tech system of hydroponics. It is the procedure of plant growth in an air or mist environment with no use of soil or an aggregate medium. The aeroponic system is composed of a growing chamber, pump, and nozzles. In this system, water is applied to transmit nutrients as well. The plant roots are suspended in the air and misted with nutrient solutions. The nutrient solutions are sprayed into the roots of the plants directly (Koriesh and Abo El-Soud 2020). The aeroponic method can decrease water usage by 98%, fertilizer application by 60%, and application of pesticides by 100%, thus maximizing the crop yields by 45–75% (Son et al. 2020; Koriesh and Abo El-Soud 2020) (Fig. 9).

4.11 Aquaponic System

Aquaponics is a merge of hydroponics and aquaculture (growing of aquatic animals, plants, or organisms in an intended water) where in a soilless water-based system plants and aquatic species can be grown together (Rakocy et al. 2004). Aquatic animals such as fish function as the nutrition base for plants. The nutrients released from the excreta of fish are utilized by plants (Roosta and Hamidpour 2011). So, the aquaponic systems is an advantageous symbiotic system, where the plant and aquatic species benefit each other (Koriesh and Abo El-Soud 2020). Several advantages of aquaponics consist of reducing the need for formulated fertilizers, the possible elimination of agricultural runoff, and reducing the water cleaning through biofilter treatments (Rakocy et al. 2004). Moreover, it is a closed system and was not obliged to flow in one pipe and out of another (Koriesh and Abo El-Soud 2020) (Fig. 10).

5 Nutrient Management Systems

The hydroponics is a method of growing plants in which mineral nutrients are incorporated in water with no soil. Hydroponic solutions contain different nutrients that are necessary for growth of the plants. These nutrients are usually transported to the plants in different ionic forms, through a combination of root interruption and distribution. To make nutrient solutions, essential nutrient elements are dissolved in water, which are mainly in ionic and inorganic forms. The nutrients are supplied to the plants via their roots in the form of a solution. In total, 17 nutrients with the proper concentration and comparative ratios are required for normal and suitable plant growth (Lauria et al. 2009; Son et al. 2020). The most basic of them include H, C, and O, and the others are classified as follows:

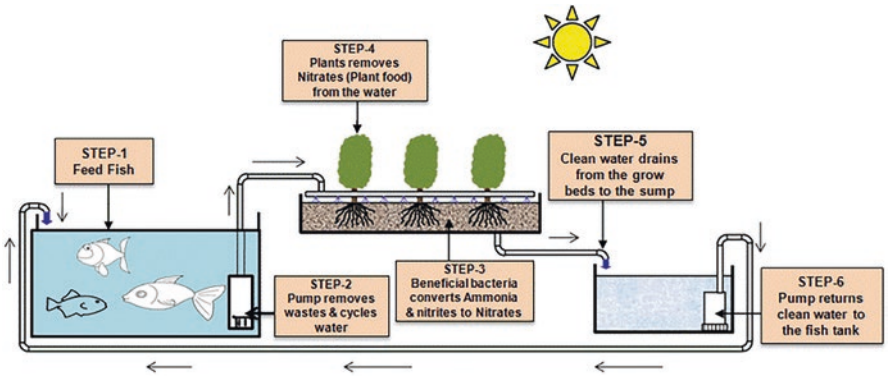

Fig. 10 Aquaponic system

Micro: Fe, Mn, Cu, Zn, B, Ch, Mo, Ni
Macro: N, P, K, Ca, Mg, S

The complete nutrient solution contains 210.1 mg N, 31 mg P, 234.6 mg K, 200.4 mg Ca, 48.6 mg Mg, 64.2 mg S, 648 μg Cl, 500 μg B, 502 μg Mn, 50 μg Zn, 11 μg Mo, 20 μg Cu, and 5.022 μg Fe (Hoagland and Arnon 1950).

In the nutrient solutions, ionic concentration is modified with time, and afterward, nutrient imbalance takes place in the closed hydroponic system (Son et al. 2020).

Macro- and micronutrients play important roles in the growth and development of plants through their involvement in various physiological procedures, such as photosynthesis, respiration, and cell wall formation. Moreover, these nutrients supply the required cofactors for several enzymes of primary and secondary metabolism, and the limitation or lack of an element causes modification in the biosynthetic and/or catabolic capacity of a plant (Figueiredo et al. 2008). Nutrient management is important for medicinal plant cultivation because their curative properties are connected to the presence of some micronutrients such as Zn, Fe, Cu, etc. (Parejo et al. 2002).

Through irrigation to the substrate, a nutrient solution is supplied by using a tube or needle or by revealing plant roots directly to the nutrient solution in an excellent manner and appropriate doses such as NFT, DFT, and aeroponics (van Os et al. 2019). Due to the fact that hydroponic cultivation systems utilize nutrient solutions, the water and nutrient supply, mineral element concentration, and composition are exactly controlled and well-adjusted based on the plant requirements, which can enable rapid growth, improve plant productivity and biomass production, and prevent the crop rotation need (Garlet and Dos Santos 2008; Palermo et al. 2012; Santos et al. 2013; Huo et al. 2020). In addition, the buffer capacity of the nutrient solution is low. The pH and mineral composition of the solution are easily modified (Saha et al. 2016). Electrical conductivity (EC) of hydroponics suffers from imbalance of nutrient (Ahn and Son 2011). In addition, to balance the nutrient solutions, periodic analysis and nutrient ratio adjustment were managed (Ko et al. 2013). Therefore, hydroponic systems are applied in industrial cultivation for the mass production of high-quality plants.

6 pH and EC Management

For most hydroponic crops, the ideal EC range is between 1.5 and 2.5 dS/m. A higher EC value will prevent nutrient absorption by plant roots due to osmotic pressure, and a lower EC will severely influence the health and yield of the crop. To determine the essential plant element presentation in a nutrient solution, pH value is applied. The most effective pH range for the best plant growth and development is 5.5–6.5. Therefore, to improve plant growth, health, and yield, maintaining EC and pH can be demonstrated as profitable (Kannan et al. 2022).

7 Irrigation Systems in Hydroponics

Open, closed, and semi-closed systems are irrigation systems of hydroponics. Hydroponics with no substrate such as NFT, ebb and flow, and aeroponics are closed systems (Giurgiu et al. 2014, 2017; Waller and Yitayew 2016; Chow et al. 2017; Maucieri et al. 2018).

7.1 Open Systems

This hydroponic system requires no recirculation of drainage water with low water-use efficiency (Khan et al. 2018). Nutrient solutions in this recirculation system that are not absorbed by plants does not return to the nutrient tank (Fig. 12).

7.2 Closed Systems

In closed systems, the drainage solution is reused, and this causes the increase of nutrients and ions, so the nutrient ratio is changed (Maucieri et al. 2018). This modification is important to influence the nutrient concentrations in the reused solution to renew the optimal composition of the nutrient solution because extended reuse of drainage water may cause to accumulate some nutrients and the nutrient ratios were modified change in the nutrient ratios (Gutierrez et al. 2007) (Figs. 11 and 12).

7.3 Semi-closed Systems

This system is an exactly closed system that is open sometimes to flush out the nutrient solution in the drainage or modify the solution in the system (Koriesh and Abo El-Soud 2020).

8 Hydroponic System for Cultivation of Medicinal Plants

In recent years, the demand for medicinal plants as raw materials in agro-foods, pharmaceuticals, perfumes, and natural cosmetics has been increasing. Medicinal plant quality is influenced by their genetic characteristics and plant biomass with consistent and higher secondary metabolite concentration (Kozai 2005). The rate of medicinal plant growth and natural product yield can be significantly influenced by the environmental conditions of the cultivation place of the plants (Gil et al. 2002).

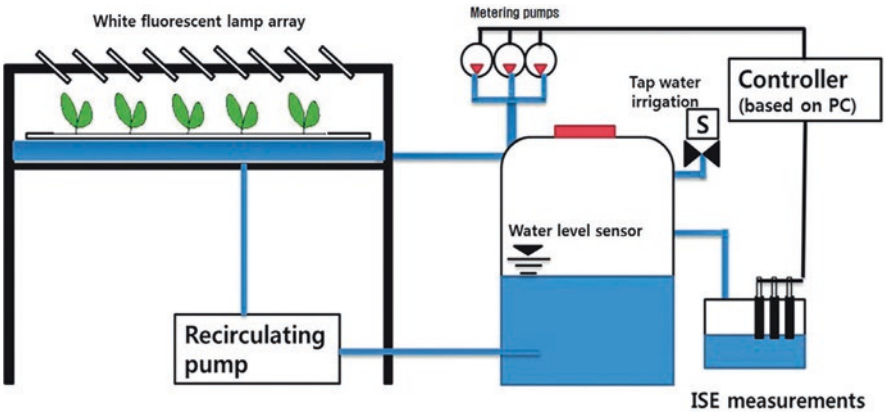

Fig. 11 Closed hydroponic system

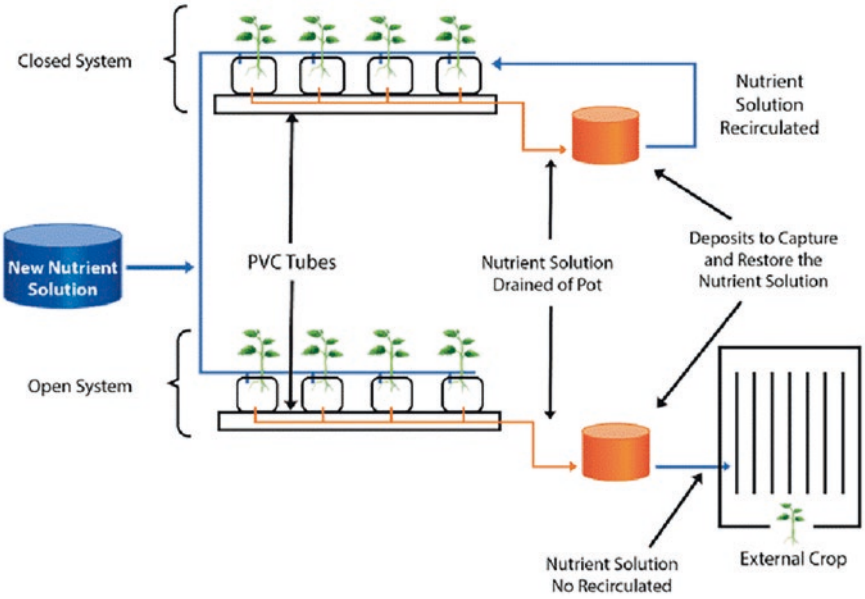

Fig. 12 Closed (left) and open (right) hydroponic systems

There is growing concern in medicinal plant cultivation in a soilless culture which supplies the probability of secondary metabolism regulation by proper control of the nutrient solution nourished to the plants (Zheng et al. 2006a, b). Specific factors controlling the environment such as temperature, relative humidity, CO_2 concentration, nutritional factors (composition of nutrient medium and use of supports), and

artificial light can produce high-quality herbs. Therefore, limited time and space in this system can produce contaminant-free herbal medicines (Kozai 2005).

Recently, increasing interest in growing medicinal plants in sand culture is noted due to the good results in this hydroponic systems. Mainly, there are plants which are used in the food industries, such as herbaceous plants, herbs, or others, cultivated in this kind of hydroponics (Giurgiu et al. 2014; Koriesh and Abo El-Soud 2020). Climate change and the irregularity of weather events cause variability of plant active compounds. Hydroponic production or soilless culture of medicinal plants is a new method in agricultural systems, particularly in organic agriculture (Dorais et al. 2001). Hydroponic systems in defended horticulture can suggest a maintainable solution for cultivation of medicinal plants (Son et al. 2020).

The hydroponic system is an exact way of controlling the temperature, irrigation, light, humidity, and plant fertilization for plants to gain exceptional growth in an inactive substrate connected with a superior quality of the plant and high bioactive substance (Giurgiu et al. 2014; Koriesh and Abo El-Soud 2020). The possibility of medicinal plant cultivation in hydroponics has been investigated, and it has been indicated that bioactive substance accumulation in soilless culture can influence plant production in the highly concentrated plant production system too (Resh 2012; Koriesh and Abo El-Soud 2020). Medicinal plants may be cultivated in hydroponics with less water and space and hydroponics may produce high-quality product with the more quantity of secondary metabolite accumulation versus field-grown ones (Grewal et al. 2011). In addition, essential oil accumulation and composition in aromatic plants are affected by cultivation methods like soilless culture compared with soil-based ones (Al-Tawaha et al. 2013).

Medicinal plants can be cultivated in hydroponics with low water usage. Based on the majority of the studies, the plants which are grown hydroponically have higher secondary metabolite content and better yield due to the application of specific growth and nutrient solution that induces the conditions which improve the plant quality by increasing stress-responsive compound accumulation. Also, mineral composition plays an important role in the medicinal values of the plants and their therapeutic effects on health and in treating diseases (Jakovljević et al. 2019).

Greenhouse hydroponics or soilless culture may release industrial necessity for medicinal plants. There are several advantages of hydroponic cultivation of medicinal plants over field cultivation, including faster crop growth, process standardization, huge economic advantage, high-quality standard of raw material, year-round production, multiple harvesting, clean and easy to process because of minimal contamination from microorganisms or pollutants, as well as stimulation of active metabolism by suitable nutrient nutrition manipulation (Giurgiu et al. 2014; Koriesh and Abo El-Soud 2020).

On the other point of view, medicinal plant cultivation in hydroponics leads to produce uniform yield plants with high percentage of bioactive materials for economic purposes as well as defend spontaneous flora and the species diversity (Maggini et al. 2011; Koriesh and Abo El-Soud 2020). Ecological problems and low germination rates are the risks of medicinal plant cultivation in hydroponics, which are defeated by controlling the excellent characteristics to achieve the best climate

for germination. Also it needs unique nutrient solutions (Azarmi et al. 2012; Koriesh and Abo El-Soud 2020).

Nutrient availability serves as a key factor in affecting the content of secondary metabolite biosynthesis and accumulation (Verpoorte et al. 2002). Nowadays, in a consequence of the comprehensive cultivation and commercialization of medicinal and aromatic plants and intensive growing methods such as hydroponics, medicinal plants need to control manner for plant growth and appropriate fertilization (Koriesh and Abo El-Soud 2020) in controlled climates medicinal herb and root production provides chances for enhancing the quality, bioactivity, purity, consistency, and biomass of the raw material (Koriesh and Abo El-Soud 2020). The float system is successfully applied for a short cultivation period of leafy vegetable production, such as spinach, lettuce, and endive, and is also favorable for aromatic plant and herb cultivation. Generally, the main hydroponic systems used for growing leafy vegetables are the DFT and NFT systems (Son et al. 2020).

In FRS system, bare-rooted plants are grown slowly or standing recirculating nutrient solution. Thus, FRS seems moderately appropriate for medicinal plants cultivated for the root production. Previous reports suggested that FRS could be beneficially used in medicinal plant cultivation (Dorais et al. 2001; Letchamo et al. 2002), such as *E. angustifolia* (Zheng et al. 2006a, b). For the soilless production of different medicinal and aromatic plants, stream hydroponics was established in Armenia by using polymeric film. Its varieties are cylindrical and continuous hydroponics (Mairapetyan et al. 2018).

9 Growing Medicinal Plants in Hydroponic Systems in Previous Research

To obtain high water-use efficiency and higher productivity in an environmentally sustainable way, the development of a controlled environment for agricultural methods such as hydroponics as an alternative method is considerable (Surendran et al. 2015).

The reports connected to medicinal plants are limited. There are several studies that are available related to medicinal plants cultivated in hydroponic conditions. In this section, we evaluated some investigations about this subject and indicated to understand the effect of hydroponics on different characteristics of medicinal plants.

Both basil and mint plant species are suitable crops for hydroponic or soilless culture, where various growing media compositions can be applied (Corrado et al. 2020; Avdouli et al. 2021; Khater et al. 2021). There are several reports about basil cultivation in aquaponic or hydroponic systems (Roosta 2014; Mangmang et al. 2016; Saha et al. 2016). In basil, under soilless systems, the better yield was obtained than soil culture. For instance, aquaponic basil showed higher yield, increased height, fresher product, and more dry weight, in comparison with hydroponic basil (Rakocy et al. 2004).

In the previous research, *Salvia officinalis* L., *Bidens tripartita* L., *Mentha piperita* L., *Leonurus quinquelobatus* Gilib., and *Ocimum basilicum* L. (Mairapetyan

et al. 2016) were cultivated under hydroponic systems. In addition, *Mentha piperita* L. is cultivated in different hydroponic systems such as classical, continuous, and cylindrical. Continuous hydroponic system motivated the extractive substance accumulation in *Salvia officinalis* L., *Leonurus quinquelobatus* Gilib., *Ocimum basilicum* L., and *Mentha piperita* L. (Mairapetyan et al. 2018).

The results of one study in soilless culture indicated that different nitrogen concentrations affected the plant growth traits, photosynthetic pigments, micro- and macronutrient uptake, total phenolic content, EO yield and compositions of *Salvia officinalis*, as well as antioxidant activity (Abbasi Khammar et al. 2021).

In addition, based on the results of the preceding study, *Mentha piperita* which is cultivated in different hydroponic methods surpasses the soil culture with more dry weight. Furthermore, during the third cut of the plants cultivated in classical and cylindrical hydroponic systems essential oil synthesis was extra concentrated, and qualitative indices such as high content of menthol were observed in cylindrical, classical, and soil cultures. But the high content of isomenthone was noticed in continuously alternates (Daryadar 2015).

To determine the variations between spearmint (*Mentha spicata* L.) and Japanese mint (*M. arvensis* L. var. piperascens Malinv.) cultivated in either soil or nutrient solution using the DFT, an experiment was conducted previously. The results showed that spearmint and Japanese mint cultivation in nutrient solution using DFT system are an effective method to produce crops with an earlier harvest period and higher quantity of essential oil content (Vimolmangkang et al. 2010). In another study, spearmint growth and essential oil production were influenced by phosphorus content in the nutrient solution under the hydroponic system (Chrysargyris et al. 2019). Nutrient solution management can be considered as a way to produce high-quality and valuable crops with high content of bioactive compounds and essential oil composition (Chrysargyris et al. 2019).

In one study, *Mentha spicata* is cultivated under hydroponics, and the yield, plant productivity, and biochemical traits are compared with soil-grown plants. The results of the experiment indicated that the yield, plant productivity, enzyme activity, antioxidant activity, and the active compounds of the plant extracts were higher in hydroponically grown plants in comparison with soil-grown plants. Thus, it is suggested that medicinal plant cultivation under hydroponic conditions is a viable alternative in urban areas and prevents the problems of the conventional characteristics of cultivation of these plants (Surendran et al. 2016).

The floating raft growing system for the greenhouse cultivation of *Ocimum basilicum* L. and *Echinacea angustifolia* DC. which are typically cultivated for their leaves and roots was performed (Maggini et al. 2011) and showed that both species grew healthy, quickened, and accumulated large biomass with lower contamination in this system. In addition, an acceptable rosmarinic acid content was obtained in basil, which was cultivated in a floating raft growing system (Kiferle et al. 2011). On the other hand, in sweet basil, which is grown hydroponically, higher total of phenolic and rosmarinic acid was obtained compared to soil-cultured plants (Sgherri et al. 2010).

The cylindrical hydroponic system elevates the qualitative indices of medicinal plants and increase secondary metabolites. This system increases peppermint, sage, and basil yield as well as total flavonoids, essential oils, and tannin of sage NFT hydroponics (Mairapetyan et al. 2016, 2018).

The use of hydroponic cultivation of *Melissa officinalis* L. was studied before to clarify the best production conditions for the aromatic plants in peat (Manukyan 2013; Manukyan and Schnitzler 2006) or sand (Safari et al. 2019, 2020) culture. Lemon balm (*Melissa officinalis* L.) was cultivated in three hydroponic systems (artificial soil bed, perlite bed, and aeroponics). Based on the results, the soilless culture system showed lowered total phenolic acid content and antioxidant capacity due to a reduction in rosmarinic acid and lithospermic acid as the major phenolic compounds in this plant. On the contrary, in the soilless culture system, the caffeic acid and methyl rosmarinate content are higher than in soil-based conditions (Son et al. 2021).

Herbal material obtained in the hydroponic culture of *Catharanthus roseus* showed a considerable concentration of alkaloids in different plant parts (Buchwald et al. 2007). In another study, thyme, lavender, and *Hypericum perforatum* were cultivated in a hydroponic system, and *Hypericum perforatum* indicated the best effects with a shorter time and excellent growth compared with the plants cultivated in soil (Jakovljević et al. 2019). In a research greenhouse, the volatile oil concentration of *Lippia citriodora* var. *verbena* and *Valeriana officinalis* var. *common* in different soilless cultures was evaluated before (Azarmi et al. 2012). The findings of this study indicated that floating and aeroponic systems produced higher volatile oil content due to the higher leaf fresh weight compared to the plants that were cultivated in media and soil systems. The root size of valerian in the floating and aeroponics was higher and there is increased total essential oil content. But different systems had no influential effect on essential oil concentration in lemon verbena (Azarmi et al. 2012).

In a controlled greenhouse, the constant growing condition can produce medicinal plants with more concentrations of active constituents, which is important for the phytopharmaceutical industries. Most hydroponic systems are organized for plants with fibrous roots or rhizomatous producing plants such as ginger as well as the medicinal plants which produce leaves or fruits.

One research in aeroponics on ginger was performed in a controlled greenhouse (Hayden et al. 2004a, b). Aeroponic cultivation of medicinal plants has indicated extra ability for root crop production which is uniform, earlier maturing, and cleaner (Pagliarulo and Hayden 2002). Cultivation of ginseng in hydroponic systems and mineral nutrient solutions produces the plants without accumulation of heavy metals, pesticides, or infectious diseases from soil such as damping-off and root rot which are common in soil-cultivated plants (Noh et al. 2016). Moreover, hydroponics can shorten the growth period of ginseng, provide higher productivity per unit area, and reduce the cost of production (Kim et al. 2010; Ministry of Agriculture, Food and Rural Affairs 2017). Total polyphenol content was highest in shoots and roots of hydroponic ginseng, followed by soil-cultured ginseng. Also, hydroponic ginseng may have the potential for utilization as an alternative to soil-cultured

ginseng, because of its superior antioxidant and anti-inflammatory properties (Hwang et al. 2019).

In one preceding experiment, basil was cultivated under three soilless systems including aeroponic, hydroponic, and peat moss slab systems. The findings showed that at the end of the growth period, the shoot lengths of basil plants were higher for aeroponic, hydroponic, and peat moss slabs plants, respectively. Moreover, the highest value of root height was obtained for the aeroponic system (Khater et al. 2021). In one experiment, the differences were noticed between the leaf metabolite profile of hydroponic and field-grown *Moringa oleifera* Lam. plants. In the hydroponic plants, ferulic acid, chlorogenic acid, wogonin, and vanillic acid were higher in comparison with field-grown plants (Managa et al. 2021). Previously, hydroponic cultivation of *Echinacea angustifolia* and *Ocimum basilicum* L. displayed healthy and rapid growth of plants with higher ferulic acid, caffeic acid, and chlorogenic acid (Maggini et al. 2011). Furthermore, the hydroponic production of *Urtica dioica*, *Arctium lappa*, and *Anemopsis californica* was high-quality and very clean herbs (Hayden 2006).

The findings of previous studies on cultivating medicinal plants in soilless conditions indicated that a possible alternative production method can be adopted to optimize and enhance the bioactive compound productivity of herbal medicine. In addition, the concentration level of bioactive compounds changes due to cultivation systems (Managa et al. 2021). Therefore, hydroponic culture systems can produce high-standard plant compounds by controlling the growth conditions. Also, proper manipulation of mineral nutrient stimulates secondary metabolite production (Singh et al. 2017). Moreover, in hydroponic systems, by using fewer agrochemicals, a wide range of safer medicinal plants are produced. In spite of the fact that shade net structure hydroponics does not take control above the climatic factors, it indicated to be a more useful system to grow a diversified species of medicinal and ornamental plants (Abul-Soud et al. 2014; Lefever et al. 2014). In conclusion, the hydroponic systems, as an eco-friendly and sustainable agro-practice, could be used for the industrial production of medicinal plants with high yields and qualities. In addition, hydroponic systems could be applied to produce medicinal plants with high specific bioactive compound content such as phenolic acids and essential oils.

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Application of Recent Advanced Technologies for the Improvement of Medicinal and Aromatic Plants

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Abstract Medicinal and aromatic plants are important natural sources of active substances that are not only used as a primary source of medicines but also as phytochemical raw materials for the production of new drugs. The increasing demand of various industries for medicinal plants and their effective ingredients requires increased crop yield and improvement of the quality of their secondary metabolites. Considering that the medicinal plant quantity and quality are mainly determined by environmental factors, genetics, and their interaction effects, several factors can apply to change the yield and profiles of secondary metabolites. Recently, controlling the climatic condition and applying different implemented fertilizers and biotechnology approaches are needed in improving the medicinal and aromatic plant production with interest quality. In this chapter, we investigated the effects of bio-fertilizers, environmental elicitors, tissue culture techniques, and polyploidy induction on improving the yield and modifying the biological compounds of medicinal and aromatic plants.

Keywords Bio-fertilizer · Elicitors · In vitro culture · Polyploidy · Secondary metabolites

1 Introduction

Plants are essential due to their ornamental value and their role in the production of oxygen, food, beverages, and perfumes, as well as industrial uses. A group of plants, in addition to their nutritional value, have significant therapeutic properties, which are classified as medicinal and aromatic plants. A medicinal plant is any plant that,

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N. Kumar, R. S. Singh (eds.), *Biosynthesis of Bioactive Compounds in Medicinal and Aromatic Plants*, Food Bioactive Ingredients,
https://doi.org/10.1007/978-3-031-35221-8_11

in one or more of its parts, contains active substances that can be used for therapeutic purposes or which are precursors for the synthesis of valuable drugs. So, the value of medicinal plants is related to the presence of high concentrations of secondary metabolites (alkaloids, flavonoids, terpenoids, and phenolics) compared to other plants, which leads to widespread use of medicinal and aromatic plants in the pharmaceutical, food, cosmetic, and health industries (Hussein and El-Anssary 2018). Also, medicinal plants have a crucial role in the development of different societies through the treatment and prevention of different diseases. Recently, adverse side effects of chemical medicines and drugs and their pharmacokinetic interactions have led to increasing day-by-day use of medicinal plants due to their beneficial effects, cheap cost, no side effects, and compatibility with the environment. According to reports from the International Union for Conservation of Nature and the World Wildlife Fund, more than 80,000 flowering plant species are considered herbal medicines (Chen et al. 2016). Nowadays, over 80 and 25% of required medicines in developing and developed countries are derived from medicinal and aromatic herbs, respectively (Zhao et al. 2022).

The increasing demand for medicinal and aromatic plants necessitates high production of these plants with appropriate quantity and quality of secondary metabolites. Despite the high economic value of medicinal plants, most of them are collected from natural habitats, and only a limited number of these herbs are cultivated. A comprehensive research has been conducted to improve medicinal plant yield through the application of different approaches. In the present study, the applied techniques to solve the low efficiency encountered in medicinal plant production were investigated.

2 Bio-fertilizers Improve the Quantity and Quality of Medicinal and Aromatic Plants

According to the increment in population and food needs, it is estimated that by 2050, food production should be increased by 70%. This issue requires the immediate adaptation of the applications of biological sciences in the different stages of crop production, from preparing inputs to planting and harvesting in agriculture systems. The use of pesticides and chemical fertilizers, besides imposing high costs on farmers, negatively influences the ecosystem and underground water and consequently threats human health. In this regard, biological fertilizers, due to environment-friendly properties, can be an excellent alternative to inorganic/chemical fertilizers (Mitter et al. 2021; Moghaddam et al. 2022). More than a century has passed since bio-fertilizer application in agriculture, and nowadays, bio-fertilizers are one of the best modern tools for sustainable agriculture, which are recommended for increasing production efficiency and quality as well as improving soil fertility in the long term (Mehdizadeh et al. 2021). Bio-fertilizers are plant growth-promoting microorganisms composed of one strain or several strains of

microorganisms that increase plant growth by increasing the availability and absorption of nutrients (Singh et al. 2019).

In natural conditions, plants are associated with several beneficial endophytic or symbiotic fungi that significantly improve the growth, development, and productivity of plants as well as have a vital role in the regulating of primary and secondary metabolite pathways (de Vries et al. 2020; Compant et al. 2021). The coexistence of plants with the soil microorganisms such as arbuscular mycorrhizal (AM) fungi is a valuable solution for increasing the soil organic matter; strengthening microbial communities; increasing the efficiency of agricultural inputs, especially irrigation water; and ultimately improving the quantitative and qualitative performance of plants (Gujre et al. 2021). Mycorrhizal fungi as obligate biotrophs coexist with more than 70% of plant species to complete their life cycle, which decreases the consumption of chemical fertilizers by 50% (Hussain et al. 2021). The plant roots are the target sites for these fungi to produce hyphae and establish a symbiotic relationship (Ferrol et al. 2019). The produced hyphae, depending on the rhizosphere condition and host plant, varies between 10 and 22 m, which acts as additional absorptive surface area for plants to increase and facilitate the nutrients and water uptake (Selvaraj et al. 2020).

The formation symbiotic relations depend on transferring of signaling molecules between the plant and fungi. Figure 1a depicts the root colonization by mycorrhizal fungi. In this process, strigolactones as plant exudates are perceived by the fungi and in return, chito and lipooligosaccharides act as fungi signaling compounds (Salvioli di Fossalunga and Novero 2019) that is concurrent with the activation of involved genes in the signaling pathway of symbiotic (corresponding cells and nuclei are showed in green). Calcium ion is the principal secondary messenger in this pathway that significantly increases in the nuclear region of root hairs (Ca^{2+} spiking) due to fungal exudates. These processes facilitate direct contact between the symbionts. This contact induced the aggregation of cytoplasm known as the pre-penetration apparatus (PPA, as shown in yellow) in the contacted regions in the epidermal cell. Subsequently, intracellular colonization occurs through the epidermis to the inner cortex and then branches along the root axis to form a broad surface for

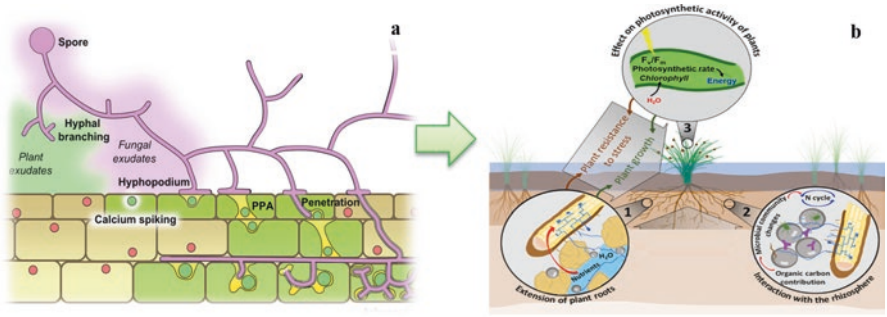

Fig. 1 The plant root colonization (**a**), growth and yield improvement mechanisms (**b**) by mycorrhizal fungi. (Bonfante and Genre 2010)

nutrient and water exchange (Bonfante and Genre 2010). The mycorrhizal fungi produce a branched structure of hyphae within the cortical root cells of host plants that are called arbuscular. Arbuscular are the bidirectional sites of nutrient element transfer from the fungal cell to the plant cell, and in the opposite direction, the required carbon compounds of fungi are provided by plants (Brundrett and Tedersoo 2018).

The spread fungi hyphae around the rhizosphere of inoculated plants with AM comprise 10–60% of the total soil microbial population. The formed mycelium network by fungi hyphae through modifying the soil structure (Chen et al. 2018) and enhancing the soil fertility (Thirkell et al. 2017) improves the availability of water and mineral nutrients for the roots of host plant that results in the increment of biomass (Bowles et al. 2016) and quality of products (Noceto et al. 2021) as well as increasing the plant resistance to unfavorable conditions including biotic (pests, diseases) and abiotic stresses (salinity, drought, temperatures, heavy metals) (Salam et al. 2017). The high uptake of nitrogen and magnesium in inoculated plants improves chlorophyll and protein biosynthesis which resulted in the high efficiency of Photosystems I and II as well as the photosynthesis rate (Pellegrino and Bedini 2014). Also, the increase of cytokine concentration in the plant cells in response to symbiotic relationships improves photo-assimilation and consequently increases the soluble sugar content (Ganjeali et al. 2018). So the mycorrhizal symbiotic improves plant growth and biomass by increasing the required constituent structures for plant development (Pellegrino and Bedini 2014) (Fig. 1b).

Mycorrhizal inoculation changes the plant's physiological and biochemical behavior, which affects the quantity and quality of produced compounds in the secondary metabolism pathways (Salam et al. 2017; Kaur and Suseela 2020). It was confirmed that in the cultivation of medicinal and aromatic plants, mycorrhizal fungi are a good opportunity to crop production with high yield and quality. The ability of symbiotic inoculation on the increment of different secondary metabolites was previously reported by Kheyri et al. (2022) in *Calendula officinalis* (phenolic compounds), Duc et al. (2021) in *Eclipta prostrata* (polyphenols), Thokchom et al. (2020) in *Ocimum tenuiflorum* (terpenoids), Gheisari Zardak et al. (2017) in *Foeniculum vulgare* (essential oil), Lazzara et al. (2017) in *Hypericum perforatum* (hypericin and pseudohypericin), Xie et al. (2018) in *Glycyrrhiza uralensis* (flavonoid), Pandey et al. (2014) in *Gloriosa superba* (alkaloids), as well as in the other several plant species.

The variations of phytohormones and signaling mechanisms in inoculated plants also affect the quantity and quality of produced active metabolites (Mandal et al. 2015). Shaul-Keinan et al. (2002) reported that mycorrhizal fungi affect the secondary metabolism of host plants by controlling the indigenous concentrations of different phytohormones, especially cytokinins, jasmonic acid, and gibberellic acid. In *Artemisia annua*, jasmonic acid plays a crucial role in the expression of involved genes in the sesquiterpenoid synthesis pathway (Maes et al. 2011). According to reports of these authors, the essential oil increase in the inoculated *A. annua* plant compared with non-treated plants was attributed to the high expression of the allene oxidase synthase gene that encodes the key enzymes in the jasmonic acid biosynthesis pathway.

The enhancement of essential oil content in the inoculated *Ocimum basilicum* (Copetta et al. 2006), *Artemisia annua* (Kapoor et al. 2007), and *Origanum vulgare* (Morone-Fortunato and Avato 2008) was attributed to increasing the trichome density of leaves in response to applied AM fungi. The formation of glandular trichomes as the main sites of biosynthesis and accumulation of terpenoids are regulated by gibberellic acid and 6-benzyl amino purine. It was reported that the induced phytohormones by mycorrhizal fungi directly or indirectly determine the content and constituents of produced essential oils in trichomes of aromatic plants by influencing the structure and density of these secretory structures (Covello et al. 2007). Also, it was stated that symbiotic fungi, through inducing the gene expression of key involved enzymes in terpenoids pathways such as terpene synthase, change the composition profile of produced essential oil (Zouari et al. 2014). According to the findings of Walter et al. (2002), mycorrhizal colonization elevates the transcript levels of 1-deoxy-D-xylulose 5-phosphate reductoisomerase (DXR) that regulates the first step in the methylerythritol phosphate (MEP) pathway in the biosynthesis of many isoprenoid compounds.

The changes in phenolic biosynthesis in the host plants of symbiotic relationship are related to activation levels of L-phenylalanine ammonia lyase (PAL) and chalcone synthase (CHS) that regulate by mycorrhizal fungi-induced signaling molecules such as nitric oxide, salicylic acid, and hydrogen peroxide (Battini et al. 2016). Also, mycorrhizal fungi induced the accumulation of glycyrrhizin in the inoculated *Glycyrrhiza uralensis* plant by induction of signaling molecules in the related ascending pathway of glycyrrhizin biosynthesis (Xie et al. 2018). A literature survey showed that the effects of AM on plant yield and secondary metabolites vary depending on the species of fungi and plant (Kapoor et al. 2007; Johny et al. 2021). The plant yield in the inoculated *Cynara cardunculus* was higher than in the non-inoculated plants, but the phenolic compounds were reduced in response to symbiotic relationships (Colonna et al. 2016). In the marjoram plants, the inoculation with *Glomus mosseae* decreased the phenolic compounds; however, the flavonoid content in colonized plants with *Glomus claroideum* was higher than in the non-inoculated plants (Hristozkova et al. 2015). Generally, it seems that AMF regulates the biosynthesis of secondary metabolites through two mechanisms: (I) by increasing the production of required precursors in the secondary metabolic pathways (Dos Santos et al. 2021) and (II) by induction of key involved genes and enzymes in these pathways (Dos Santos et al. 2021).

3 Induction of Secondary Metabolite Production through Controlled Environmental Stresses

Medicinal and aromatic plants such as other plant species are faced with different limiting factors of climatic conditions during their growth and development stages. Different environmental stresses, such as water deficit, salinity, un-normal

temperature, carbon dioxide, lighting, and soil fertility, considerably change the plants' physiological and biochemical processes. Consequently, secondary metabolites of medicinal plants are affected in response to climatic conditions (Kleinwächter and Selmar 2015). The variations in secondary metabolism and production of active substances with different quantities and quality are essential plant mechanisms to cope with unfavorable conditions (Zhang et al. 2017). A literature survey revealed that environmental effects on the synthesis and accumulation of secondary metabolites comprise a complex network that not only is species dependent process but also varies within the species (Moghaddam et al. 2020). The environmental factors act as abiotic elicitors on plant metabolism that result in increment or decrement of secondary metabolite production up to 50%. There are two suggested mechanisms to explain environmental effects on secondary metabolite biosynthesis: (I) "passive shift," which leads to the consumption of plant energy to the biosynthesis of active substances as defense compounds to protect plant survival, which is observed in growth reduction (Kleinwächter and Selmar 2015), and (II) "active," which is related to up-regulation of the involved enzymes in secondary metabolism (Yahyazadeh et al. 2018). The molecular mechanism of environmental stress effects as abiotic elicitors on the secondary metabolite biosynthesis is shown in Fig. 2. This cycle is initiated by the activation of plasma membrane receptors. Receiving the elicitor message by receptors causes to start a cascade of reactions, including fluxes of Ca^{2+} ion, acidification of cytoplasm through K^+/Cl^- effluxes and H^+ influxes, and generation of reactive oxygen species (ROS) through activation of NADPH oxidase, followed by activation of G-protein and mitogen-activated protein kinase phosphorylation (MAPK) (Seybold et al. 2014). These reactions activate the plant defense signaling compounds (salicylic acid, methyl jasmonate, etc.) that influence the transcription factors, gene expression, and enzyme activity in plant secondary metabolism and consequently determine the biosynthesis of active substances (Shakya et al. 2017).

The assay of environmental conditions on medicinal and aromatic plants can aid in the simulation of the appropriate climatic and ecologic situation with controlled factors to obtain the high quality and quantity of secondary metabolites (Pant et al. 2021). Moghaddam and Mehdizadeh (2015) reported a great variation of rosmarinic acid content ranged from 0.1 to 9.9 mg mL^{-1} among different accessions of *Ocimum ciliatum* due to significant effects of environmental conditions. Similar environmental effects on essential oil content and composition of *Ocimum ciliatum* were reported by Moghaddam et al. (2017). The variation of essential oil quantity and quality of *Origanum vulgare* was observed under different environmental conditions (Mehdizadeh et al. 2018). The temperature and rainfall effects on essential oil content and antioxidant potential of *Achillea millefolium* were also reported by Farhadi et al. (2020). The essential composition of *Cuminum cyminum* as regards to ϒ-terpinene, cumin aldehyde, cumin alcohol and ß-pinene was significantly influenced by climatic condition (Moghaddam and Ghasemi Pirbalouti 2017). Therefore, it is important to investigate the effects of each limited factor of environmental condition on the active substance profile of medicinal plants to know the adaptability mechanism of a specific plant to newly introduced grown conditions.

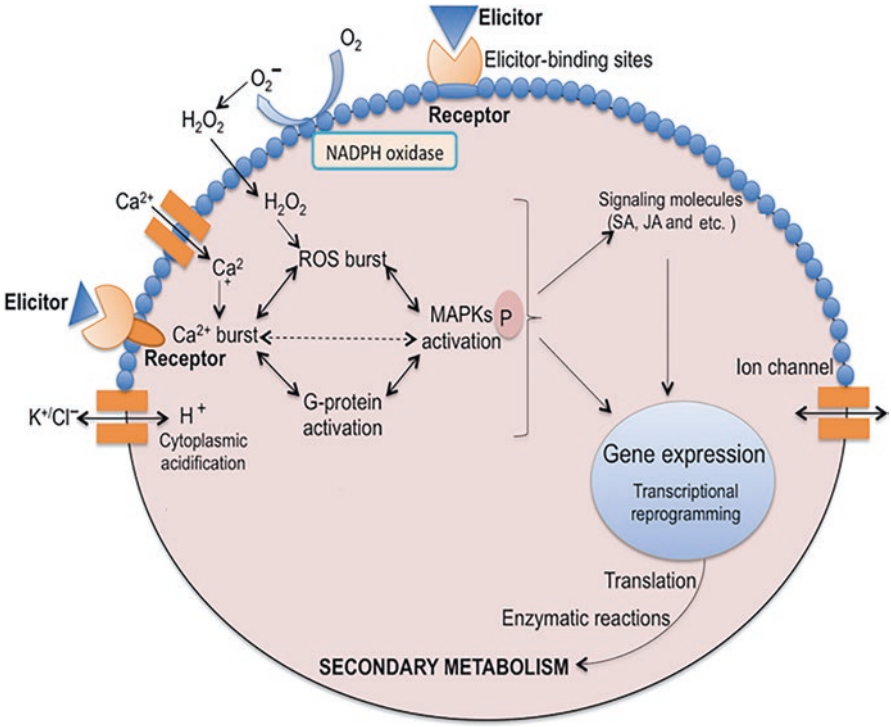

Fig. 2 The molecular mechanism of environmental stress effects as abiotic elicitors on secondary metabolite biosynthesis in medicinal and aromatic plants. (Shakya et al. 2017)

In this part, the effect of the three main environmental factors (water deficit, salinity, and temperature) that can regulate increasing secondary metabolite production was investigated.

3.1 Water Shortage

Drought stress occurs when the available water of soil due to low rainfall and high transpiration and evaporation decreases. Drought stress negatively affects plant growth and biomass; however, the increment of secondary metabolite accumulation in the drought-exposed plant was reported in several plant species (de Abreu and Mazzafera 2005; Hosseini et al. 2018; Alhaithloul et al. 2020). It is noticeable that the secondary metabolism influenced by water deficit varies between and within species depending on the climatic condition and plant growth stage (Kleinwächter and Selmar 2015). It has been reported that although the content of active substances rises in response to water stress, the yield of these compounds not always increases but also may remain unchanged or decrease under severe drought stress (Mahajan

et al. 2020). So that, to obtain the high content and yield of secondary metabolites in drought-stressed plants, the determination of appropriate intensity and duration of water limitation is necessary that vary from one plant species to another. Different phenolic compounds and betulinic acid of *Hypericum brasiliense* plants had increment trends with increasing drought intensity (de Abreu and Mazzafera 2005). In *Prunella vulgaris*, the highest ursolic and rosmarinic acid was reported at moderate drought stress conditions (Chen et al. 2011). Hosseini et al. (2018) reported the induction of glycyrrhizin synthesis in *Glycyrrhiza glabra* under mild and moderate water stress that was attributed to stimulating the expression and activity of involved enzymes in the glycyrrhizin biosynthesis pathway. In the exposed *Mentha piperita* and *Catharanthus roseus* to drought stress, the total phenols, flavonoids, and saponins of both plants were decreased; however, the essential oil content of *M. piperita* and alkaloid content of *C. roseus* increased under water shortage conditions (Alhaithloul et al. 2020). The saponin content of *Stellaria dichotoma* was increased at first and then decreased with the elongation of the drought stress period. In this plant, to increasing of saponin accumulation and its yield, applied water stress at moderate intensity was suggested (Zhang et al. 2017). The diallylthiosulfinate allicin content in drought-stressed *Allium hirtifolium* was higher than in the non-stressed plant (Ghassemi-Golezani et al. 2018). According to Pant et al. (2021), the variations of endogenous hormones in response to drought stress considerably influence the biosynthesis of different secondary metabolites.

3.2 Salinity

Salinity stress, as one of the growth-limiting factors, significantly affects the biosynthesis of different active substances of medicinal plants. The growth reduction under saline conditions is due to nutritional and osmotic imbalances and consequently reduction of water uptake and photosynthesis efficiency (Moghaddam et al. 2020). Ghassemi-Golezani and Farhadi (2022) reported that salinity stress increased the essential oil content of pennyroyal (*Mentha pulegium*) up to a moderate level. Also, the results of these authors showed that salinity changed the essential oil quality of *M. pulegium* by induction of new constituents, such as myrcene, α-thujene, isophorone, and germacrene D. A partial enhancement of essential oil biosynthesis under salinity stress is related to increasing of oil gland density of trichomes (Karray-Bouraoui et al. 2009). Salinity stress at a mild level increased the diterpene content in *Stevia rebaudiana* up to 8.25%; however, further salinity intensity decreased these compounds by 4.2% (Aghighi Shahverdi et al. 2019). Saline conditions at any level increased the phenolic compounds in stressed *Plantago ovata* plants through induction of the high activity of phenylalanine ammonia lyase as the key enzyme in the phenylpropanoid pathway (Verma and Shukla 2015). However, the total phenol content in salinity-stressed *Salvia macrosiphon* was lower than in the non-stressed plant (Valifard et al. 2017). The flavonoid rutin content in

treated *Fagopyrum esculentum* plant by salinity stress at 75 mM NaCl concentration was three times more than non-stressed plants (Farhadi et al. 2022a). Salinity stress differentially affected the essential oil quantity and quality of two basil cultivars (Talebi et al. 2018). These results confirm that the salinity effects on the production of secondary metabolites considerably vary depending on plant species (Pant et al. 2021). During the plant growth and development stages, saline condition differentially affects the assimilate partitioning in different plant species, and these differences are revealed as changes in secondary metabolite production with various qualities (Mahajan et al. 2020).

3.3 Temperature

Temperature variation as a major weather variable induces the signaling pathway to activate the defense responses and secondary metabolite production by influencing the physiological processes and primary metabolism (Al Jaouni et al. 2018). Temperature changes the composition of produced metabolites due to regulating photosynthesis efficiency, disruption of photo-assimilates, and vegetative growth as well as reproductive organs (Rahimi and Hasanloo 2016). Controlling the temperature range can accelerate or postulate the growing season of specific species according to the condition of the cultivation area. The lower temperature (4 °C) induces high production and accumulation of scopolamine alkaloids in *Duboisia myoporoides* (Ullrich et al. 2017). However, the increasing temperature had a significant effect on improving the secondary metabolite profile of *Salvia miltiorrhiza* (Zhang et al. 2019). The optimum temperature for maximum biosynthesis of phenolic compounds in *Tithonia diversifolia* was 22 °C during vegetative and flower induction periods, and then, these compounds showed a reduction trend in this temperature (Sampaio et al. 2016). Moghaddam and Farhadi (2015) reported the significant and positive correlation of temperature with essential oil content and its constituents in different populations of *Ferula assa-foetida.* The monitoring of responsible genes of the jasmonic biosynthesis pathway in *Camellia japonica* by Li et al. (2016) revealed the expression of studied genes, which was decreased at a low temperature of the growing site. The biosynthesis of silymarin in the *Silybum marianum* plant is a temperature-dependent process, and high temperature has a negative effect on the accumulation of this compound (Rahimi and Hasanloo 2016). Yuan et al. (2020) assayed the alkaloid and flavonoid content in *Dendrobium officinale* under three different ecological situations in the wild, bionic, and greenhouse. The highest content of measured metabolites was obtained in grown plants in the wild, followed by bionic and greenhouse conditions, which attributed to the shade lover intrinsic of this plant. The finding of these authors indicated that the alkaloid and flavonoid contents in *Dendrobium officinale* plant decrease with increasing temperature. The observed differences in the content of extracted oil and its fatty acid profile from *Ricinus communis* seeds at different

sowing dates were attributed to temperature variations during the studied dates (Farhadi et al. 2013). Al Jaouni et al. (2018) by studying the effect of different climatic conditions on essential oil production in *Ocimum basilicum* and *Mentha piperita* reported that the temperature effect on each metabolite is not consistent and varies in species-specific status.

4 In Vitro Techniques to Production of Secondary Metabolites

The pharmaceutical industries for preparation of their natural products widely depends on the extracted secondary metabolites from plants as raw substances. However, the most required compounds are synthesized in low quantities in medicinal plants. In addition, the laboratory synthesis of these naturally active substances due to their complex structure is difficult (Hussein and El-Anssary 2018). Nowadays, the sufficient supply of secondary metabolites for the market demand of different related industries is the main challenge. As reported in the previous parts, different methods are applied to increase the production efficiency of natural plant metabolites. The cell, organ, and tissue culture of medicinal and aromatic plants under in vitro conditions is a sustainable procedure for the mass production of biologically active compounds (Fazili et al. 2022).

Due to the high ability of tissue culture to large-scale production of plant samples under in vitro conditions, the use of this technique can provide enough plant materials for breeding programs, comprehensive studying of secondary metabolite pathways, and investigating the phytochemical changes of the medicinal plants during different growth stages as well as in response to exogenously applied growth regulators and stresses (Ayaz and Memon 2021). Also, tissue and cell cultures have a suitable performance for the mass production of plant bioactive compounds with high-speed and low-cost independence from environmental conditions (Ho et al. 2020). The obtained secondary metabolites under in vitro tissue culture are easily purified due to the absence or low quantity of pigments and simple extraction processes that considerably decrease the cost of production and processing costs of these compounds (Grigoriadou et al. 2019). So the research in this field has flourished beyond expectations.

As shown in Fig. 3 under plant tissue culture systems, two main procedures are available for the production of medicinal plant bioactive compounds, namely, (I) organogenesis and (II) callogenesis. In the organogenesis process, the homogenous plants are directly regenerated from meristems of different cultured organs and explants (such as fragmented leave, nodal, and root) or indirectly produced from callus through cell dedifferentiation. The obtained plants can be reproduced for mass production under fields or greenhouse conditions and consequently used to extraction of their biologically active substances (Espinosa-Leal et al. 2018).

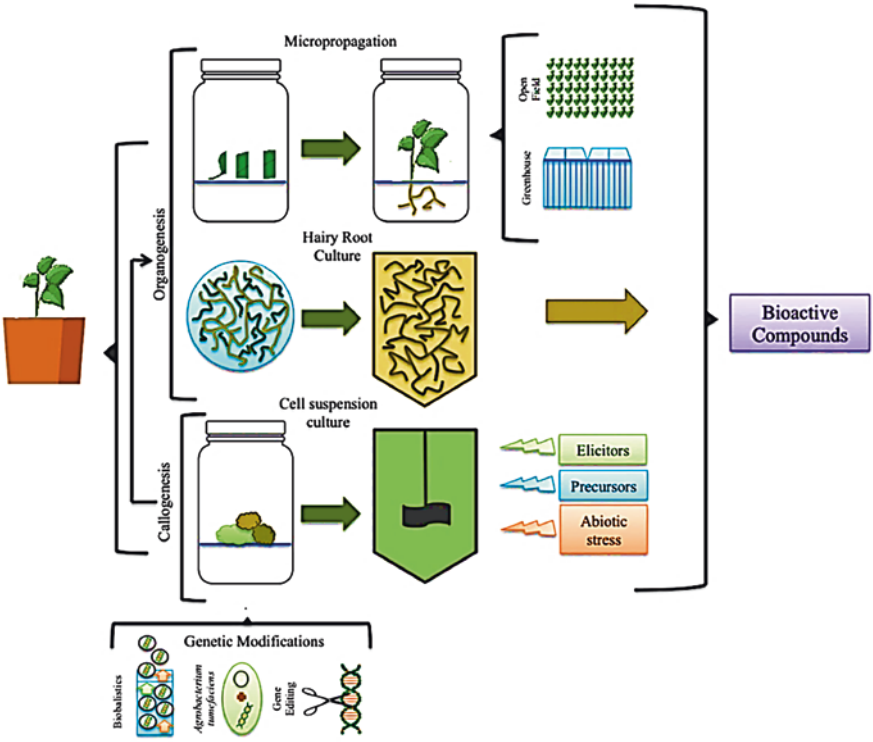

Fig. 3 The applied methods for the mass production of secondary metabolites under in vitro condition. (Espinosa-Leal et al. 2018)

4.1 Hairy Root Culture

In the organ culture, the root culture is the common and important method for the production of secondary compounds, especially root-produced metabolites, through the induction of hairy roots. Hairy roots are induced by *Agrobacterium rhizogenes* by transferring T-DNA into the plant host. The hairy root culture is low-cost technology with a high growth rate and constant regard to genetics and the potential of phytochemical production (Bahramnejad et al. 2019). Hairy roots as differentiated tissues produce a wide array of metabolites at comparable content with native roots. In this case, the species that naturally is the primary source of considered compounds selected for hairy root induction. The successful production of flavonoids, terpenoids (Wawrosch and Zotchev 2021), alkaloids, and anthocyanin (Barba-Espin et al. 2020) has been reported in bioreactors of hair root cultures. In addition, the hairy root culture can be used in the production of protein-based medicines such as therapeutic proteins (Cardon et al. 2019), vaccines (Massa et al. 2019), and antibodies (Donini and Marusic 2019).

It is noticeable that in vitro cultures are sensitive to elicitors (chemical compounds and environmental stresses) and growth conditions (light intensity, pH, carbon source, aeration), which provides a desirable chance to increasing of specific bioactive compounds by applying effective elicitors and appropriate culture medium composition (Winson et al. 2021). Wang et al. (2016) reported that the applied UV-B radiation and methyl jasmonate in hairy root cultures of *Salvia miltiorrhiza* were effective in the expression of related genes in the tanshinone biosynthetic pathway. The cultures of hairy roots also provide a convenient system for genetic investigation, including the assay of gene expression, induced transformation, and studying the metabolite pathways; and the aim of all these attempts is the increasing phytochemical production (Morey and Peebles 2022). Singh et al. (2021) reported the production of a high yield of curcumin from the transformed hairy roots of *Atropa belladonna.* Fu et al. (2020) increased the total phenol production of *Salvia miltiorrhiza* in hairy root systems by transforming hairy roots with the main involved genes in the activity of rosmarinic acid synthase (RAS).

4.2 Callogenesis

Callogenesis refers to the process of callus production that induces exposed explants to growth regulators, especially auxins (Farhadi et al. 2017). The obtained callus can be used for two goals, including whole plant regeneration and proliferation in cell suspension cultures for mass production of biologically active substances. The recent one is the more important and common aim in the callogenesis process (Wu et al. 2021). The massive production of paclitaxel as an important anticancer compound from the cell culture of *Taxus* spp. is a typical example of metabolite production under in vitro conditions (Escrich et al. 2021). The genetic variation due to methylation of DNA, changes of transposable elements, induction of polyploidy, and, consequently, occurrences of somaclonal variations are a common phenomenon in cell cultures. So this system is an unreliable method for homogenous plant propagation (Govindaraju and Arulselvi 2018). However, somaclonal variations sometimes result in the identification of elite lines with the superior potential of the specific phytochemical compound. Also, these variations provide the facility for genetic engineering and manipulation in chromosome numbers in regard to industrial points to the production of phytochemicals (Martínez-Estrada et al. 2017). Chee et al. (2017) reported the successful production of vanillin as an expensive spice at a high concentration by genetic engineering under cell culture.

To establish a cell suspension culture, the selected explant is exposed to callus induction conditions in solid media, then the obtained calli are transferred to liquid media in shaking flasks for more proliferation. On the industrial scale, liquid-phase bioreactors are used for cell suspension and, consequently, biological compound production. The production of secondary metabolites through the cell cultures is

genotype dependent, followed by the selection of appropriate species and suitable tissue with the highest concentration of interest compounds (Ochoa-Villarreal et al. 2016). The used bioreactors are usually complemented with an optimized condition for the enhancing of the production efficiency of metabolites. In these bioreactors, appropriate growth regulators, elicitors, and precursors are added to the induction of specific compound biosynthesis (Winson et al. 2021). The selection of an appropriate elicitor for the induction of active compounds in cell cultures depends on plant species and metabolite kind. The placed receptors on the cell surface or at the intracellular level are responsible for the identification of elicitors that lead to the activation of a signal transduction cascade to stimulate high biosynthesis of bioactive compounds (Rakesh and Praveen 2022). The biosynthesis and accumulation of anthraquinones in cell cultures of *Oldenlandia umbellata* were significantly elicited by adding pectin and xylan as elicitors (Saranya Krishnan and Siril 2018). The supplement cell suspension bioreactors with intermediate compounds in secondary metabolism pathways also improve and accelerate the production of unique biological compounds. The selection of the best precursor for cell cultures needs a complete understanding of the biosynthesis pathway of target metabolite, involved enzymes, and side products of this pathway. It is noticeable that the selected precursors in the cell culture systems for elicitation of interest compounds must be cheap and available (Chee et al. 2017). In cell cultures of *Antrodia cinnamomea*, the exogenous application of stigmasterol as a precursor in mevalonic acid and methylerythritol 4-phosphate pathways resulted in a significant increase in terpene content (Chen et al. 2016). Cholesterol, sodium acetate, squalene, and isopentenyl pyrophosphate are the intermediate compounds in the azadirachtin biosynthesis pathway. The finding of Srivastava and Srivastava (2014) showed that cholesterol is the best precursor for incrementing azadirachtin biosynthesis in the cell culture of *Azadirachta indica.* Parra et al. (2017) reported that glycerol is an essential intermediate in triglyceride biosynthesis in cacao (*Theobroma cacao*) that can be as suggested as a precursor in the cell suspension of this plant.

5 Induced Tetraploid Plants with High Secondary Metabolite Content

Polyploidy is a common phenomenon in the plant evolution process that polyploids have more adaptive potential than their diploid ancestors. The expression of novel and multiple phenotypes in polyploidy individuals is another aspect of polyploidy. Polyploidy species show drastically reduced fertility due to abnormal meiosis, which leads to unbalanced chromosomal distribution to gametes (Castro et al. 2018). The most common effect of polyploidy is increasing of nuclear and cell volume, which could affect all the plant traits, including morphology, physiology,

and, consequently, primary and secondary metabolisms (Galán-Ávila et al. 2020). Due to reduced sexual fertility and increased vegetative growth in autopolyploids, polyploidy induction in breeding programs is usually restricted to cultivated crops for their vegetative organs and those with vegetative propagation. The size of leaves, flowers, fruits, and seeds is often increased in response to polyploidization (Madani et al. 2021). Polyploidization is associated with an increase in vigor and adaptation of the newly formed polyploidy to novel conditions. It has been reported that polyploid plants are more tolerant to different stresses, such as water deficit, temperature, nutrient deficiency, pests, and pathogens (Denaeghe et al. 2018). Enhancement of biomass and stress tolerance of polyploids leads to an increased interest in using artificial polyploidy induction in crop breeding programs, especially in the cultivated crops for their vegetative organs (Lin et al. 2011). Several methods have been used to induce the production of unreduced gametes in the last decades, which included temperature, nitrous oxide (N_2O), antimitosis agents (such as trifluralin, colchicine, and oryzalin), and ethyl methane sulfonate (EMS), as well as gene silencing by RNA interference (RNAi) and virus-induced gene silencing (VIGS) (Cui et al. 2017).

Polyploidy induction affects the biologically active compounds depending on species resulting in the increment or decrement of specific compound biosynthesis (Julião et al. 2020). Polyploid breeding has been successfully increased plant biomass and production of secondary metabolites in different medicinal and aromatic plants. The enhancement in secondary metabolite biosynthesis in polyploid plants is attributed to increased expression of involved genes in the metabolism pathway of the target compound (Madani et al. 2021). The cell surface considerably affects the physiological and biochemical processes by influencing the alteration and rearrangement of genes in cell nuclear (Doyle and Coate 2019). So the variations of secondary metabolism in polyploid plants are the result of a high ratio of chromatin content to the cell membrane (1.5–2 times more than the diploid cells) that leads to high contact of plant genetic materials with the membrane of the nucleus and high expression of genes (Chung et al. 2017). Increasing the ploidy level in *Linum album* enhanced the podophyllotoxin biosynthetic by increasing the expression of involved genes in the activity of phenylalanine ammonia lyase, cinnamyl-alcohol dehydrogenase, and pinoresinol-lariciresinol reductase (Javadian et al. 2017). The results of Farhadi et al. (2022b) revealed that the artificially induced tetraploid plants of *Allium hirtifolium* under in vitro conditions, besides larger bulbs (as shown in Fig. 4), had higher phenolic compounds and antioxidant potential. Interestingly, these authors reported that tetraploid *A. hirtifolium* with high allicin content in bulbs had different genotypes from the parental diploid plant. A considerable increment of artemisinin biosynthesis was reported in tetraploid *Artemisia annua* compared with diploid (Lin et al. 2011). Morphine content in tetraploid *Papaver somniferum* significantly was higher than in the diploid plants (Mishra et al. 2010).

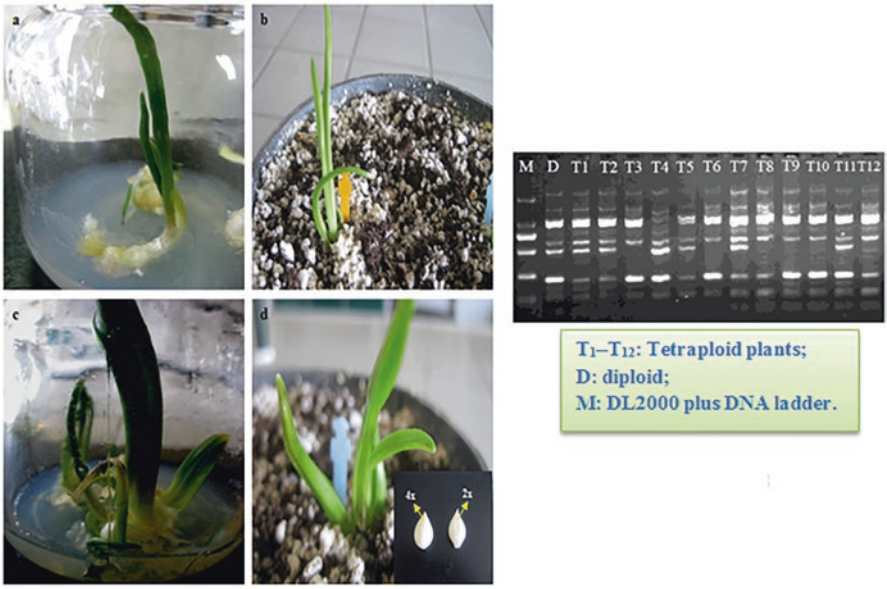

Fig. 4 The changes of growth vigor, bulb size, and genetic variation of tetraploid (**c**, **d**) *Allium hirtifolium* plants compared with diploid plants (**a**, **b**). (Farhadi et al. 2022b)

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Medicinal Flora of the Trans-Himalayan Cold Desert in Ladakh, India

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Abstract The extreme and unique trans-Himalayan ecosystem of the cold desert region in NW India sustains several important medicinal plants, representative to the region. Ethnomedicinal knowledge based on plants validates to be functional in this harsh and secluded topography, where proper medicinal facilities are still wanted. Simultaneously, large-scale extraction of medicinal plant resources, to meet the industry demand, has resulted in devastation of natural populations of a good number of medicinal plants. Habitat destruction, livestock grazing, unscientific harvesting of native plants, increased tourism, and natural disasters have further added in threatening the status of these trans-Himalayan medicinal plants. The characteristic scattered vegetation in the region, coupled with the threat status of medicinal plants, warrants effective strategies that include sustainable consumption and management and effective conservation. Proper identification, followed by documentation of medicinal plants along with their traditional knowledge, and the development of scientific application will therefore attest to be highly serviceable in sustaining the medicinal plant diversity in the region.

Keywords Trans-Himalaya · Cold desert · Ethnomedicinal knowledge · Sustainable management

1 Introduction

> *"Cold" and "desert" may be two words that you never thought could appear together. But it is not temperature that defines a desert, but a very low average annual rainfall, which makes places like frigid Antarctica or Asia's Gobi Desert qualify as deserts. Cold deserts have soils similar to hot deserts, with soil types ranging from salty to sandy to rocky.*
>
> –Tallulah Philange

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N. Kumar, R. S. Singh (eds.), *Biosynthesis of Bioactive Compounds in Medicinal and Aromatic Plants*, Food Bioactive Ingredients,
https://doi.org/10.1007/978-3-031-35221-8_12

Major cold desert areas across the world are located in the Asian continent that includes Gobi Desert of northern China and southern Mongolia, Iranian desert (including parts of Afghanistan and Pakistan), Taklamakan Desert of northwest China, Turkestan desert in parts of the Middle East and southwest Russia, and the trans-Himalayan desert (parts of Afghanistan, Pakistan, India, and China) (Cressey 1960; Jishtu et al. 2003; Chauhan et al. 2020). Ladakh Union Territory, along with Lahaul–Spiti, Pooh subdivision of Kinnaur district, and Pangi area of Himachal Pradesh account for more than 90% area of the Indian trans-Himalayan cold desert with the remaining 10% area falling in the higher regions of Uttarakhand and the arid North Sikkim (Jishtu et al. 2003).

The trans-Himalayan cold deserts of India have been formed primarily due to the rain shadow effect of the Greater Himalaya. It has been recognized with distinct biogeography, distinguished by an extremely harsh cold – arid climate, stark seasonality, low primary productivity, and unique assemblage of biodiversity. Depending on latitude, longitude, elevation, and orographic conditions, there is enormous variation in thermal and moisture regimes within the region. There are clear differences into two distinct biogeographic provinces of the western rugged high mountains and the eastern flatter plains. These trans-Himalayan cold deserts stand out for having the world's largest glaciers in highlands, the world's highest motorable road, and the highest permanent settlements (4000–4500 m) in the world, source of water, feeding several rivers, which are crucial for livelihood of millions of people, besides the unique culture, biodiversity, and ecosystem.

The Ladakh region forms the major part of the cold desert in India, and the name itself means "Land of High Passes," which is apt because of the multitude of its towering mountain ranges, river valleys, and high plateaus. The towering mountain peaks capped with permanent snow, and the rugged mountainsides with steep gorges draining their bottoms, represent major part of the landscape. The Karakoram range separates its northern border, which holds the highest peak of Saser Kangri (7672 m) in Ladakh part. Along the southern and eastern border lies the Himalayan range, which holds the twin peaks of Nun and Kun (*ca* 7000 m). The Stok Kangri (6121 m) in the Stok range across Leh and the Kang Yatse (6401 m) in the Zanskar Range form other important peaks (Singh 2009). Routes over these high passes of Ladakh were established centuries ago by the caravan traders of the silk route and the local people themselves. It boasts in some of the world's highest motorable roads, from Leh to Nubra over the Khardung La (5602 m) and the road from Kargil to Padum in the Zanskar Valley, over the Fentse La (4450 m) (Bhasin 1992). Within Ladakh, there are clear physiographical and ecological differences between the eastern broader highland plateau (Rupshu) and the remainder of the cold deserts (especially Ladakh). Thus, physiographically, Ladakh is distinguished into Nubra (northernmost region, bounded by the Karakoram range to the north and the Kailash ranges on the south), Ladakh (central part), Zanskar (with Ladakh to the north, Rupshu in the east, and Lahaul south), and with Warle (to the west), Rupshu (most elevated region, bounded by Tibet in the east and Spiti to the south) and, the Dras-Suru region (west of Zanskar).

2 Rivers

The Indus River is the westernmost river system in the country that originates from the Bokhar Chu glacier in the northern slopes of Kailash Mountains in Tibet – China – and enters Ladakh in the eastern part near Hanle. Indus flows westward across the Ladakh landscape into Kashmir, before entering northern Pakistan, which then flows south to the Arabian Sea. Indus river forms the main broader valley of habitation, between the Ladakh and Stok Mountain ranges, before it receives the fast-flowing Zanskar River, a little short of Nimu. The main tributaries of the Zanskar River are the Stod and Tsarap, arising between the Zanskar and Himalayan ranges. Shyok River originates from the Rimo glacier, and along with its main tributary, the Nubra River flows in a southeasterly direction, between the Ladakh and Karakoram ranges, forming the lifeline of Nubra Valley. In the western Ladakh region of Kargil, it is the Suru River that originates from the Panzella glacier and flows in to join the Drass River at Kargil, before flowing into northern Jammu and Kashmir.

3 Lakes

The high plateau of Ladakh, especially the eastern region, contains a number of large to very large brackish water lakes. The largest is the Pangong Tso lake, which extends into the Tibet landscape. The Rupshu plains to the south contain the lakes, Tso Moriri and Tso Kar in the Changthang region, and being part of the wetland reserve under Ramsar site. The basin of the Tso Kar coupled with the adjoining More Plains constitutes one of the most important habitats of the kiang, Tibetan gazelles, Tibetan wolves and foxes, and steppe marmots in the higher reaches of the catchments. Pangong Tso is an endorheic lake at 4350 m, a large water body, extending from India to Tibet, with approximately 40% in India and the remainder in China – Tibet. The lake is in the process of being identified under the Ramsar Convention as a wetland of international importance that would place it as the first trans-boundary wetland in Southern Asia.

4 Soil

Depending upon the topography, terrain, parent rock, and the nature and type of vegetation, the cold desert region of Ladakh has great variations in the soil. The principal soil types encountered here are red and black soils (light to dark brown), red soil (ferruginous), brown soil, mountain and hill soil, high-altitude meadow, and alpine soils. Physically, these soils are coarse (gravelly loamy sand) to fine (silty clay loam) and shallow to deep and with moderate nutrient contents. A high degree

of variability in soil properties is however evident from valley to valley. In general, much of cold desert of Asia has soil erosion as a major threat and factor limiting productivity. To sum up, the soils of the cold desert region, in general, have poor physical characteristics due to the coarse texture and poor water retention characteristics (Sharma and Sharma 2011).

5 Climate

The cold deserts of Ladakh have a short mild summer, but long harsh winters. Snowfall occurs during the prolonged winters (October–March), while annual rainfall being less than 150 mm occurs during the short summers (July–August). Temperature ranges between −20 and around 35 °C, with mean temperatures between 9 and 11 °C, and the annual mean soil temperature less than 8 °C. During summer, the daytime temperatures may at times reach 40 °C, and in winter, the nighttime temperatures may reach as low as −40 °C. The region also experiences permafrost and fast-blowing gusty winds across its vast open rolling plains.

6 Fauna

Ladakh is home to numerous magnificent and endangered faunal elements, being home to the elusive snow leopard (*Uncia uncia*) and the rare Tibetan antelope (*Pantholops hodgsonii*). With its barren plateaus and uplands, Ladakh has a distinct faunal variety which includes the blue sheep, yaks, marmots, Tibetan hare, ibex, kiangs, bharal, etc. Despite its harsh climate, surprisingly, a good number of birds can be spotted in Ladakh, which include the black-necked crane, bar-headed geese, woodpeckers, ducks, partridges, barbets, kingfishers, parakeets, swift eagles, and owls, to name a few. During September, 2021, the Union Territory (UT) of Ladakh declared the iconic black-necked crane (*Grus nigricollis*) and snow leopard as its state bird and animal, respectively. However, today, this complex and unique fauna is threatened by habitat loss, rising tourism, and a number of other anthropogenic pressures.

7 Flora

The physiographic uniqueness and ensuing harsh climatic conditions of the cold deserts have led to the emergence of unique plant diversity. In general, the flora of Ladakh differs remarkably from the rest of the Himalaya, as it is influenced broadly by the climatic factors and, more significantly, with poor soil conditions, drainage pattern, and region-specific microclimate. Though relatively poor in species

diversity, this flora has significant biological interest, illustrating, with its diversity of origin and endemic species, a high adaptability to extreme climatic conditions. The affinity of these plants bears close similarity to that of northern Iran, Mongolia, Afghanistan, China, Siberia, and the Tibetan plateau. Tree line is almost nonexistent; however, it is mainly perennial herbs, followed by stunted, spinescent shrubs and bushes that dominate the flora. Earliest records of flora of Ladakh and western Tibet have been compiled by Stewart (1916–1917) and later by Kachroo et al. (1977). Thereafter, for a long period of time, the region remained secluded and politically closed, mainly it being unapproachable (Chandra 2006), and its strategic border positioning with Pakistan and China also put restrictions on travel by outsiders. The dominant plant families are Asteraceae, Fabaceae, Brassicaceae, Poaceae, Lamiaceae, and Ranunculaceae, while the important genera include *Astragalus*, *Polygonum*, *Carex*, *Poa*, *Nepeta*, *Arnebia*, *Potentilla*, *Corydalis*, *Pedicularis*, *Artemisia*, *Lonicera*, *Hippophae*, *Saussurea*, and *Caragana* (Jafri 1973; Kachroo et al. 1977; Aswal and Mehrotra 1994; Murti 2001; Klimeš and Bernhard 2005; Chaurasia et al. 2007; Kumar et al. 2011; Srivastava and Shukla 2013; Gurmet and Stobgais 2016; Jishtu and Goraya 2020). The plant richness, diversity, and abundance of floral diversity are reported to decrease significantly with rise in elevation (Behera et al. 2014; Wani et al. 2022).

8 Medicinal Plants

Indigenous knowledge stands crucial to the people living in harsh regions, as it brings about their betterment of life. Thus, the medicinal plant wealth has been the mainstay of healthcare in the isolated region of Ladakh (Chauhan et al. 2020). The use of plants in medicine is deeply rooted in Ladakhi culture, which resulted in the establishment of their own rich medicinal system, known as the *Amchi*. For centuries, local communities in Ladakh have relied on this highly revered *Amchi* medicinal system for their health and well-being. This *Amchi* system of medicine also referred to as the Tibetan medicine system is very popular in Ladakh (Chaurasia and Singh 1996; Ballabh and Chaurasia 2007; Jishtu et al. 2021). The *Amchi* system is fully dependent upon the native plant diversity for their collection of medicinal plants and their parts. These medicinal plants are found in unique diverse habitats, such as the mesophytic valley plains, xerophytic rocky terrain, dry slopes, and moist alpine pastures, among moraines and boulders in high craggy mountain landscapes.

A number of studies on medicinal plants have been conducted across the Himalaya. However, in case of Ladakh, only a few references are available that are too fragmentary (Bhattacharyya 1991; Kaul 1997; Buth and Navchoo 1988; Gurmet et al. 1998; Kala 2000, 2002, 2005, 2006; Jishtu et al. 2003, 2021; Anonymous 2005; Ballabh and Chaurasia 2007; Ballabh et al. 2008; Chaurasia and Khatoon, 2008; Kumar et al. 2009; Chaurasia and Ballabh 2011; Gairola et al. 2014; Namtak and Sharma 2018; Rigzin et al. 2019; Jishtu and Goraya 2020; Chauhan et al. 2020; Konchok and Phuntsog 2021; Dawa et al. 2022). Besides, in Ladakh, the Defense

Institute of High-Altitude Research (DIHAR) and the National Institute of Sowa Rigpa (NISR) have been working on the conservation practices of medicinal and aromatic plants (MAPs). Of late, some NGOs have started to show interest on encouraging medicinal plant cultivation, particularly in the Nubra, Indus, and Zanskar valleys. It is very much evident that the studies on medicinal plants started toward the end of the last century and picked up in the past two decades only. However, it is still in its early stages and requires more focused research, especially in the field of taxonomy, chemistry, propagation, augmentation, marketing, sustainable utilization, and more importantly its conservation.

9 Medicinal Plant Conservation

The review of various studies reveals that Ladakh is a treasure house of rare and threatened medicinal flora, being largely endemic to the region. The rich traditional knowledge of plants is highlighted by their *Amchi* system of medicine and healthcare. The extraction of these plant resources, largely as a result of industry demand, has resulted in the degradation of their natural populations. With time, local communities have significantly contributed to the conservation of native plant diversity and, as such, have in the process come up with unique indigenous knowledge regarding their potential value, in particular to the medicinal usage. Thus, conservation, especially in situ, needs to be promoted to ensure that the onus remain in the hand of local communities, who will surely manage the native medicinal plant diversity in a sustainable manner. Moreover, the scientific development with research inputs and sustainable utilization will surely auger well for the conservation of medicinal plants in Ladakh.

10 Elucidation of Medicinal Plants of Cold Desert Along with Their Medicinal Use

The medicinal plants listed below are a result of over a decade of field surveys and interactions with village communities, *Amchi*'s, local scholars, and the detailed literature reviews of studies conducted in the Ladakh landscape. The nomenclature followed is in accordance with the Plants of the World Online (https://powo.science.kew.org/), an online database published by the Royal Botanic Gardens, Kew. A total of 133 plants have been enlisted below belonging to 45 families and 101 genera. Asteraceae (21) is the most dominant family, whereas Fabaceae (9), Lamiaceae (8), Polygonaceae (12), Ranunculaceae (9), Apiaceae (4), Crassulaceae (4), Orobanchaceae (4), Papaveraceae (4), Rosaceae (5), and Solanaceae (5) were among other dominant families (Fig. 1). *Arnebia*, *Artemisia*, *Corydalis*, *Pedicularis*, *Potentilla*, and *Rhodiola* were among common genera from the region (Fig. 2).

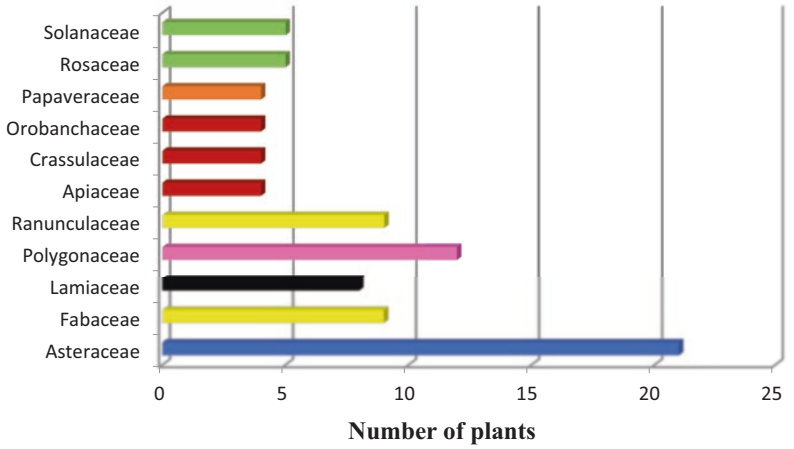

Fig. 1 Dominant families from the study area

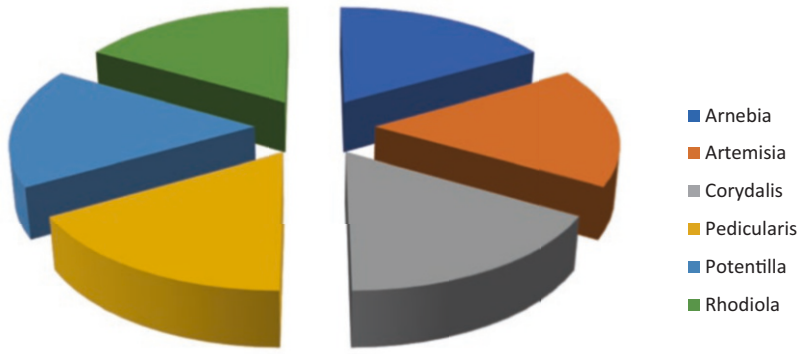

Fig. 2 Common genera from the study area

- ***Acantholimon lycopodioides* (Girard) Boiss.** [Plumbaginaceae]

  Ladakhi name: Longze, Long-zeh.
  Habitat: Found on high-altitude open rocky slopes above 3000 m, in compact tufts.
  Medicinal use: Plant ash is administered with milk for cardiac disorders, including cardiac arrest.

- ***Achillea millefolium* L.** [Asteraceae]

  Ladakhi name: Chuang.
  Habitat: Moist meadows and near cultivated areas.

Medicinal use: The leaves chewed to treat toothache and inflammation in gums. The tea prepared from the entire plant is known to treat cold, fevers, gastritis, and bile and liver troubles. Poultice prepared from the plant is applied to cure some skin infections.

- ***Aconitum heterophyllum* Wall. ex Royle** [Ranunculaceae]

  Ladakhi name: Bong-kar, Sho-Bonga, Bong-dkar.
  Habitat: Restricted distribution, mainly in moist alpine meadows.
  Medicinal use: The root tubers expel intestinal worms and treat arthritis, gout, swelling pain, inflammation, body pain, and lymph fluid diseases. Useful in treating stomach ailments and high fever. In powdered form, the tuber is put in tooth cavities to prevent toothache, and when mixed with honey, it is given to children suffering from cough, fever, and vomiting.

- ***Aconitum violaceum* Jacquem. ex Stapf** [Ranunculaceae]

  Ladakhi name: Bong-nak, Kyno-Bunga.
  Habitat: Found in moist open meadows and near water bodies in alpine zones.
  Medicinal use: Used to treat kidney pain, rheumatism, and high fever. Fresh root powder is taken with hot water in case of fever in severe cough and cold conditions.

- ***Allardia glabra* Decne.** [Asteraceae]

  Ladakhi name: Phillu.
  Habitat: Common in alpine slopes and in moist glacial moraines.
  Medicinal use: The entire plant is used to cure septic wounds. Besides, it is also used locally in various household remedies.

- ***Allardia tomentosa* Decne.** [Asteraceae]

  Ladakhi name: Palu, Solokarpo.
  Habitat: Found on higher-altitude drier slopes or rocky moraines.
  Medicinal use: The entire plant is used to cure rheumatism. Used as incense along with *A. glabra*.

- ***Allium przewalskianum* Regel** [Amaryllidaceae]

  Ladakhi name: Koche, Skiche, Scotche.
  Habitat: Dry rocky slopes in alpine meadows.
  Medicinal use: Juvenile leaves are used for flavor and are believed to be a good remedy for curing joint pains. Leaves are collected during summers, then dried, and stored for winter use.

- ***Anaphalis busua* (Buch. -Ham.) DC**. [Asteraceae]

  Ladakhi name: Prag-yung.
  Habitat: Found growing on moderate, moist slopes.
  Medicinal use: The entire plant is used as an antidote against poisons and also to treat swellings and bleeding.

- ***Anaphalis triplinervis* (Sims) C.B. Clarke** [Asteraceae]

  Ladakhi name: Spra-rgod, Ta-wa.
  Habitat: Common on dry rocky high alpine slopes.
  Medicinal uses: The entire plant heals wounds by checking bleeding and swellings and is very effective in epidemic fever. It is also an antidote against poisons, while the flowers are collected for decoration during religious ceremonies and for keeping away insects from damaging woolen items.

- ***Aquilegia fragrans* Benth.** [Ranunculaceae]

  Ladakhi name: Cho-Cho.
  Habitat: Found growing in moist places near cultivated fields and grasslands.
  Medicinal use: The plant is cooked as vegetable and considered to boost immunity. The leaves are added to buttermilk, to prepare "*Dantur*," which is widely used to improve digestion.

- ***Arctium lappa* L.** [Asteraceae]

  Ladakhi name: Shikling, Pizums, Byi-bzyung.
  Habitat: Found on open barren slopes and near habitations.
  Medicinal use: The entire plant is used in the preparation of a paste, which is applied on blisters, pimples, and as a remedy for burns. It also treats kidney diseases, urinary bladder cysts, and nerve disorders. The roots are especially used to cure cancer and tumors.

- ***Arnebia benthamii* (Wall. ex G. Don) I.M.Johnst.** [Boraginaceae]
- ***A. euchroma* (Royle ex Benth.) I.M.Johnst.**
- ***A. guttata* Bunge**

  Ladakhi name: Balchar, Demok.
  Habitat: Found in moist subalpine slopes; open sandy and rocky slopes between 3000 and 4500 m.
  Medicinal use: Plants are considered as expectorant and used for cardiac ailments. The leaves and flowers are used against fever and as blood purifier. Roots are used to treat pulmonary diseases and in cases of blood vomit. The root is used as a hair tonic, to rejuvenate and strengthen hair. Pinkish–red dye from the roots is used for dying milk products like butter and cheese.

- ***Artemisia brevifolia* Wall. ex DC.** [Asteraceae]

  Ladakhi name: Khamchu, Mkhan-dkar.
  Habitat: Common on drier rocky as well as sandy slopes and along the roadsides.
  Medicinal uses: Foliage is used for the treatment of worms, both thread and round, and also against fever. The leaf extract is effective against indigestion and other stomach complaints. The seeds are considered useful against obesity, as they are said to reduce the fat deposition and also fed to horses with hay, especially during winters, when they are weak due to cold.

- ***Artemisia dracunculus* L.** [Asteraceae]

  Ladakhi name: Brama, Bur-tse, Tsar-bong.
  Habitat: Prominent along roadsides, and drier areas, mostly in association with *A. brevifolia*.
  Medicinal use: The aerial parts, especially leaves and inflorescence, are beneficial against stomach worms and other stomach complaints. Besides, it finds use in toothaches and is also used to control the female menstrual cycle.

- ***Artemisia parviflora* Roxb. ex D.Don** [Asteraceae]

  Ladakhi name: Garprek.
  Habitat: Common on dry sandy slopes, stony waste places, and roadsides.
  Medicinal use: The whole plant is used for rheumatism, joint pain, and skin infections. The root is used to treat throat infections.

- ***Aster flaccidus* Bunge** [Asteraceae]

  Ladakhi name: Lukmik.
  Habitat: Found growing on moist alpine grasslands and open highland pastures.
  Medicinal use: Popular use for the treatment of cough and cold. The flowers are collected during morning hours and thereafter dried in the open sun, later boiled with water, and then administered for effective treatment.

- ***Astragalus rhizanthus* Benth.** [Fabaceae]

  Ladakhi name: Sarma, Krelseng.
  Habitat: Common on dry riverbeds, among boulders and drier rocky slopes
  Medicinal use: Decoction of the plant is used for the treatment of skin diseases. Roots are also used as a heart stimulant.

- ***Berberis ulicina* Hook.f. & Thomson** [Berberidaceae]

  Ladakhi name: Sin-sking-nama, Shinner.
  Habitat: Restricted mainly to Khardung area, on alpine arid slopes, forming thick tufts.
  Medicinal use: The root and dried fruit are used for the treatment of cough and fever and fruits given orally for ringworm cure. The plant is also used in the preparation of eye drops.

- ***Bergenia stracheyi* (Hook.f. & Thomson) Engl.** [Saxifragaceae]

  Ladakhi name: Gatikpa.
  Habitat: Alpine glacial moraines and moist rocky slopes.
  Medicinal use: The leaves and roots are useful to treat kidney stones. Root paste is applied to ulcers, cuts, and wounds.

- ***Betula utilis* D. Don** [Betulaceae]

  Ladakhi name: Stakpa.

Habitat: Found at a single location in Shakar–Chiktan region near moist stream.
Medicinal use: The bark and leaves are used for jaundice, burns, and wound healing. The paste prepared from its bark is applied on the vaginal wall to expel the placenta.

- ***Biebersteinia odora* Stephan** [Biebersteiniaceae]

  Ladakhi name: Khardung.
  Habitat: Found on higher-altitude rocky open slopes.
  Medicinal use: The upper parts of the plant are used to treat septic wounds. An aromatic oil is also prepared; besides, the plant is used in religious ceremonies.

- ***Bistorta affinis* (D.Don) Greene** [Polygonaceae]

  Ladakhi name: Samtsong.
  Habitat: Common on moist higher-altitude slopes and meadows.
  Medicinal use: The plant is used in diabetes and rheumatism and is also a good source of vitamin E. The decoction prepared from the plant is given in bleeding piles and also for checking profuse menses.

- ***Bistorta vivipara* (L.) Delarbre** [Polygonaceae]

  Ladakhi name: Mikchay.
  Habitat: Found growing in moist humid places and along water streams.
  Medicinal use: The roots and seeds of the plant are boiled with milk and consumed orally to cure back pains.

- ***Blitum virgatum* L.** [Amaranthaceae]

  Ladakhi name: Parangh
  Habitat: Common on sandy riverbeds, roadsides and other wastelands, and cultivated areas.
  Medicinal use: The leaves are boiled with milk and taken as a health tonic.

- ***Caltha palustris* L.** [Ranunculaceae]

  Ladakhi name: Mamiri, Horgul, Pipling-tsa.
  Habitat: Found in damp places and glacial moraines, especially after snowmelt in early spring.
  Medicinal use: Locally applied, the green leaves are used to cleanse the hands. Powdered leaves are used to keep out maggots in cattle.

- ***Capparis spinosa* subsp. *himalayensis* (Jafri) Fici** [Capparaceae]

  Ladakhi name: Kabra.
  Habitat: Commonly found on slopes in drier valleys.
  Medicinal use: It is used to treat rheumatism, paralysis, and toothaches. The root bark is used as tonic, expectorant, and diuretic, while the fruit is useful against scurvy.

- ***Capsella bursa-pastoris* (L.) Medik.** [Brassicaceae]

Ladakhi name: Medikus, Shamsoh, Soka-pa.
Habitat: Common weed in agricultural fields, homesteads, and wastelands.
Medicinal uses: Leaves are used as vegetables for health supplement and are also used as an immunity booster. The fruits are used to check vomiting and restore kidney functions. The fruits are also helpful in bronchitis, nerve disorders, obstruction of urine, and to stop bleeding.

- ***Caragana brevifolia* Kom.** [Fabaceae]

  Ladakhi name: Brama.
  Habitat: Found growing on open, dry slopes.
  Medicinal use: It is useful for treatment of the muscles and for soothing nerves.

- ***Carum carvi* L.** [Apiaceae]

  Ladakhi name: Kosniyot, Go-Snyod, Kajnut.
  Habitat: Common in grassy meadows, slopes, forest undergrowth, and in cultivated fields.
  Medicinal uses: The fruit and seeds are useful for the treatment of amenorrhea, rheumatism, weak eyesight, intermittent fever, and worm infestations. The seeds are fried with sand in mustard oil and then applied to the spine and other joints for pain relief. The tea brewed from its flowers is used to effectively treat fever and various skin eruptions. The fruits and seeds are also used as febrifuge, to improve eye vision, and as a digestive.

- ***Cicer microphyllum* Royle ex Benth.** [Fabaceae]

  Ladakhi name: Sarri.
  Habitat: Common on dry to moist sandy slopes and nearby irrigated land.
  Medicinal use: The aerial parts of the plant are used to treat tongue infections. It is more commonly used for the treatment of mouth sores in cattle.

- ***Clematis tibetana* Kuntze** [Ranunculaceae]

  Ladakhi name: Zakzic, Bisho.
  Habitat: Found commonly in shrubberies, along riverbanks, and boundaries of cultivated fields.
  Medicinal uses: The root and stems are collected and boiled in water, for use against skin itching. The leaves and flowers also find good use as laxative, besides treating indigestion and loss of appetite. Also helpful for the cure of tumors and pus-related issues.

- ***Codonopsis clematidea* (Schrenk) C.B. Clarke** [Campanulaceae]

  Ladakhi name: Phak-phakmo.
  Habitat: Common near cultivated fields and homesteads.
  Medicinal uses: The roots are used to treat stomachache and to enhance digestion. The flowers taste sweet and thus are eaten raw. Used in the treatment of arthritis, gout, rheumatism, and other joint pains. Besides, use in nerve

disorders and planetary diseases. It is favored among the communities, to cure diseases supposedly, caused by evil spirits.

- ***Colutea nepalensis* Sims.** [Fabaceae]

  Ladakhi name: Braa.
  Habitat: Shrubberies and rocky dry slopes.
  Medicinal use: The leaves find use as local purgative, while the seeds too are emetic.

- ***Convolvulus arvensis* L.** [Convolvulaceae]

  Ladakhi name: Harangi, Grachi.
  Habitat: Common in cultivated fields, shrubberies, dry open slopes, and wastelands.
  Medicinal use: The aerial parts are sometimes used as a light purgative.

- ***Corydalis flabellata* Edgew.** [Papaveraceae]

  Ladakhi name: Maqshang.
  Habitat: Common on dry slopes and alongside seasonal water streams.
  Medicinal use: The plant is known to purify blood and also helpful in treating muscular pains.

- ***Corydalis govaniana* Wall.** [Papaveraceae]

  Ladakhi name: Nakpo.
  Habitat: Common in moist alpine meadows, glacial moraines, rocky crevices, and open slopes.
  Medicinal use: The root is used as tonic, diuretic, alternative, and antiperiodic. The root paste is applied externally to reduce inflammation.

- ***Corydalis rutifolia* (Sm.) DC.** [Papaveraceae]

  Ladakhi name: Nakpo.
  Habitat: Found in moist and shady slopes of higher alpine meadows.
  Medicinal use: The plant extract is used for curing various skin diseases and infections. The root is considered tonic and also finds use as a diuretic.

- ***Cremanthodium ellisii* (Hook.f.) Kitam.** [Asteraceae]

  Ladakhi name: Mingchan-nagpo.
  Habitat: Found growing on high-altitude passes in moist locations.
  Medicinal uses: Mainly, the leaves and flowers are used for diphtheria and infectious diseases including cold. It treats some type of swellings and muscular tissue inflammations and as an antidote for poisoning. Also used traditionally to keep away evil spirits from their house.

- ***Dactylorhiza hatagirea* (D.Don) Soo** [Orchidaceae]

  Ladakhi name: Ang-bolakpa, Sanchu, Wang-bolakpa.

Habitat: Grows in moist grassy meadows of alpine area and in marshy locations.
Medicinal uses: Mucilage from tubers is taken with water as a nerve tonic, which is nutritious and useful in treatment of diarrhea, dysentery, and chronic fevers. It is very effective in the treatment of kidney complaints and against urinary troubles.

- ***Datura stramonium* L.** [Solanaceae]

  Ladakhi Name: Dha-dhu-ra.
  Habitat: Near village habitations, wastelands, and cultivated fields.
  Medicinal uses: The leaves and seeds are narcotic and sometimes used as an effective poison. Leaf poultice is applied to boils and sores. The flower and seeds possess sedative and analgesic properties. It is helpful in treating various kinds of pathogenic diseases like sinusitis, toothaches, and any other diseases associated with bacteria and viruses. Commonly used for treating decayed teeth in elders.

- ***Delphinium brunonianum* Royle** [Ranunculaceae]

  Ladakhi name: Byar-god-spos, Mokhoto, Chargo-sposz.
  Habitat: Found in alpine meadows and moist rocky slopes.
  Medicinal use: The people use the decoction of the plant in fever and in diarrhea. It is also used for certain neural pain.

- ***Delphinium cashmerianum* Royle** [Ranunculaceae]

  Ladakhi name: Byar-kang, Chargosposz, Lunde-kaown
  Habitat: Found in rocky crevices and moist alpine slopes.
  Medicinal use: Local people use the roots to get relief from cold, cough, and fever.

- ***Dolomiaea costus* (Falc.) Kasana & A.K. Pandey** [Asteraceae]

  Ladakhi name: Kooth.
  Habitat: Moist alpine slopes at altitudes of 2500–4200 m; also found in irrigated fields in high altitudes.
  Medicinal use: The roots have medicinal value and are useful for treating asthma, bronchitis, coughs, dental problems, diarrhea, dysentery, fevers, flatulence, headaches, and menstrual troubles. It is also used as an antiseptic and disinfectant.

- ***Dolomiaea macrocephala* DC. ex Royle** [Asteraceae]

  Ladakhi name: Dhup.
  Habitat: Found in alpine pastures, glacial moraines, and rocky crevices between 3400 and 4500 m.
  Medicinal use: It is considered a stimulant and given to treat fever after childbirth; root oil is useful in gout and rheumatism. Locally, the bruised roots are applied to skin eruptions.

- ***Dracocephalum heterophyllum* Benth.** [Lamiaceae]

  Ladakhi name: Zinkzer, Zipche, Chip-che.
  Habitat: Alpine meadows and the high-altitude plateau.
  Medicinal use: The aerial parts of plant are used to disinfect the eye. The decoction prepared from the dried leaves and floral portion is used to cure cough as well as headache.

- ***Dysphania botrys* (L.) Mosyakin & Clemants** [Amaranthaceae]

  Ladakhi name: Sokann.
  Habitat: Common in wastelands and cultivated fields.
  Medicinal use: Anthelmintic, diuretic, and laxative. It is used in the treatment of stomach and liver diseases, for headache, and for relief from gall bladder deformities.

- ***Echinops cornigerus* DC.** [Asteraceae]

  Ladakhi name: Ekzema, Akjenia
  Habitat: Found in grazing grounds and on rocky slopes.
  Medicinal use: The water extract of the plant is used to get rid of skin eruptions. The entire plant is used to treat general weakness, cold, cough, and fever. The leaf paste prepared is applied to septic wounds, and the powdered leaves are taken to cure jaundice.

- ***Elaeagnus angustifolia* L.** [Elaeagnaceae]

  Ladakhi name: Sarsing.
  Habitat: Found growing on moist areas.
  Medicinal use: Oil is extracted from roots and is used as hair tonic. Fruits are edible.

- ***Ephedra gerardiana* Wall. ex Klotzsch & Garcke** [Ephedraceae]
- ***Ephedra intermedia* Schrenk & C.A. Mey.**

  Ladakhi name: Chepat.
  Habitat: Both plants are found in the alpine, arid rocky slopes, and moraines.
  Medicinal use: Locally, the decoction of stem and roots is used as remedy for rheumatism and syphilis. The fresh twigs are utilized by local people as toothbrush. Dried stem and sometimes roots are used to prepare the drug "ephedrine," a cure for bronchial asthma, cold, hay fever, and allergic rashes.
  Medicinal use: The stem and the roots are known to treat asthma and fever.

- ***Epilobium angustifolium* L.** [Onagraceae]

  Ladakhi name: Utpal-wambo.
  Habitat: Frequent among stones and boulders nearer to the alpine zone, between 3000 and 4200 m.
  Medicinal use: The leaves are used for relieving abdominal pain and for hepatic, renal, and intestinal diseases.

- ***Epilobium angustifolium* subsp. *angustifolium* L.** [Onagraceae]

  Ladakhi name: Byarpan- Chutse.
  Habitat: Found growing on high-altitude damp slopes.
  Medicinal use: It treats disorders like dropsy and obstruction in urine passing.

- ***Eriocapitella rivularis* (Buch. -Ham. ex DC.) Christenh & Byng** [Ranunculaceae]

  Ladakhi name: Zukpa.
  Habitat: Common on moist scree slopes and near snow-fed streams.
  Medicinal use: The plant shoots are sun-dried and made into fine powder, which is consumed with honey to cure diabetes. It is also used to treat severe headaches.

- ***Eriophyton tibeticum* (Vatke) Ryding** [Lamiaceae]

  Ladakhi name: Yakzas.
  Habitat: Common along pathways and wastelands.
  Medicinal use: Tea made out of this plant reduces headache.

- ***Euphrasia officinalis* L.** [Orobanchaceae]

  Ladakhi name: Sulai, Kangchun.
  Habitat: Found in the grassy or alpine, open slopes.
  Medicinal use: The whole plant is used to treat heart-burning sensation. Infusion of dried herb has soothing effect on eyes in conjunctivitis.

- ***Fagopyrum esculentum*** Moench [Polygonaceae]

  Ladakhi name: Brahbo.
  Habitat: Found growing on shady and moist places.
  Medicinal use: "Chang," i.e., local Ladakhi beer is brewed from this plant which is also known to be effective against indigestion.

- ***Ferula jaeschkeana* Vatke** [Apiaceae]

  Ladakhi name: Bakhyot.
  Habitat: Found growing on grassy or alpine open slopes from 2000 to 3000 m.
  Medicinal use: The roots and fruit are collected for extraction of an essential oil that has a good market value. The gum resin extracted is applied to cuts and wounds by the local people.

- ***Galium aparine* L.** [Rubiaceae]

  Ladakhi name: Zangsi-karpo.
  Habitat: Common on moist places and near cultivated fields.
  Medicinal use: The plant treats diseases like jaundice and treats external wounds.

- ***Geranium pratense* L.** [Geraniaceae]

  Ladakhi name: Gugchuk, Porlo.
  Habitat: Prominent along rocky slopes and graveled terraces and on moist shady places.
  Medicinal use: The decoction of leaves is used to check diarrhea and other stomach trouble by the local healers. The boiled roots are applied as poultice on bruises.

- ***Geranium wallichianum* D. Don ex Sweet** [Geraniaceae]

  Ladakhi name: Polo, Le-gha-dur.
  Habitat: Frequent in shrubberies and moist open slopes, from temperate zones to alpine meadows.
  Medicinal uses: The roots are used as astringent, in toothaches and eye problems and on cuts and bruises to stop bleeding and heal wounds. The root paste is given in stomach disorders of infants. Also, treats contagious fever, lung fever, and poisonous situations and also to reduce pain and inflammation from the swollen limbs.

- ***Heracleum lanatum* Michx.** [Apiaceae]

  Ladakhi name: Tu-dkar.
  Habitat: Common on open slopes, grassy meadows, and in drier areas, up to 4300 m.
  Medicinal uses: The sweet-smelling roots are used to treat bleeding, skin diseases, tumors, inflammation, and pain caused by vulnerable fever and for treating internal cancers and leprosy. Also beneficial in the treatment of abdominal cramps caused by intestinal worms. The seed in particular is beneficial for treating wind-related disorders and for relieving pain. Commercially, the roots are being used in the preparation of various suntan lotions, besides, providing a highly efficacious chemical for the treatment of leukoderma and psoriasis.

- ***Herminium monorchis* (L.) R. Br.** [Orchidaceae]

  Ladakhi name: Paliksket.
  Habitat: Frequent on moist slopes, marshy ground in June–Aug.; 3000–4200 m alt.
  Medicinal use: The tubers are used as antiseptic on open wounds. The paste prepared from the bulb is used for the treatment of kidney complaints.

- ***Hippolytia longifolia* (Rech.f.) C.Shih** [Asteraceae]

  Ladakhi name: Dhoop, Seig-manlo.
  Habitat: Found along rocky slopes in alpine regions.
  Medicinal use: The dried leaves and flowers are used against intestinal worms. The leaf is rolled to a pill and swallowed with water to provide relief in stomach pain and indigestion.

- ***Hippophae rhamnoides* L. subsp. *turkestanica* Rousi** [Elaeagnaceae]

  Ladakhi name: Dhurchuk, Almich, Chharma, Sarla, Tirku, Gartsak, Tsarmang.
  Habitat: Common along riverbanks, glacial streams, and sandy soils, often planted as hedge in irrigated areas.
  Medicinal uses: The fruit is considered as a rich source of vitamin C and is used for the preparation of tea, jam, jelly, and syrup and for the treatment of various kinds of pulmonary diseases. It is also used as an emollient for wrinkle prevention around the eyes. It is also effective for curing high-altitude sickness and in some blood-related problems.

- ***Hippophae salicifolia* D.Don** [Elaeagnaceae]

  Ladakhi name: Sasta-lulu, Chharma.
  Habitat: Common along water streams and wastelands.
  Medicinal use: Oil obtained from the plant is applied on skin burns. It finds similar use to that of *H. rhamnoides* subsp. *turkestanica*.

- ***Hylotelephium ewersii* (Ledeb) H.Ohba** [Crassulaceae]

  Ladakhi name: Dachungpa.
  Habitat: Found growing on dry and open slopes.
  Medicinal use: The plant treats external injuries and toothache.

- ***Hymenolaena candollei* DC.** [Apiaceae]

  Ladakhi name: Rtsad-rgod.
  Habitat: Found growing on shady, rocky, and sandy places.
  Medicinal use: It is effective in treating meat poisoning and treats constipation.

- ***Hyoscyamus niger* L.** [Solanaceae]

  Ladakhi name: Gyalamthang.
  Habitat: Found in wastelands and cultivated areas and forest clearings.
  Medicinal use: Dried leaves and flowers smoked for hallucination. The leaves and seeds are used as a sedative, for whooping cough and asthma, as an astringent, and for tooth infections.

- ***Hyssopus officinalis* L.** [Lamiaceae]

  Ladakhi name: Tengu, Tyangu, Zufah.
  Habitat: Found in the dry arid slopes at high altitudes.
  Medicinal use: The tea prepared from the plant is said to be effective in nervous disorders, toothaches, and in treating pulmonary, digestive, and urinary problems. It is also used as a gargle for throat inflammations and in chronic bronchitis and asthma.

- ***Inula racemosa* Hook.f.** [Asteraceae]

  Ladakhi name: Kuth, Mano, Manu, Poshkar.

Habitat: Cultivated as a substitute for *Saussurea costus*.
Medicinal uses: The roots are collected in large quantities, stored for its aroma, and used as a substitute for the traditionally grown "kuth" (*Saussurea lappa*). The roots are also used for cough, cold, and chest pain (chronic bronchitis).

- ***Inula rhizocephala* Schrenk ex Fisch. & C.A. Mey.** [Asteraceae]

  Ladakhi name: Tikta.
  Habitat: Common on alpine meadows and alongside water streams.
  Medicinal use: The plant is known to purify blood and treats muscular pains.

- ***Iris lactea* Pall.** [Iridaceae]

  Ladakhi name: Kricksma-mendok.
  Habitat: Found growing on moist habitat and open slopes.
  Medicinal use: The plant is known to treat sore throat.

- ***Juniperus semiglobosa* Regel** [Cupressaceae]

  Ladakhi name: Shukpa.
  Habitat: Dry river valleys, gregarious, forming open forests; 2300–4300 m.
  Medicinal use: The twigs and berries are used in stomach cramps, cough and pectoral infections, diarrhea, impotency, indigestion, leukorrhea, paralysis, piles, and skin diseases. Berries are used in ayurvedic formulations under the name "Hauber." Poultice of needles and twigs is used to treat wounds.

- ***Koenigia rumicifolia* (Royle ex Bab.) T.M. Schust. & Reveal** [Polygonaceae]

  Ladakhi name: Choarh, Goronthu, Kanthala, Nyello.
  Habitat: Found in moist alpine meadows and humid slopes; 3000–3800 m.
  Medicinal use: The tubers are used to treat skin diseases and joint pain. The root is an antidote for aconite poisoning, snakebites, and scorpion stings.

- ***Koenigia tortuosa* (D.Don) T.M. Schust. & Reveal** [Polygonaceae]

  Ladakhi name: Nyalo, Snya-lo, Serpa-lulu.
  Habitat: Found on high-altitude open moist scree slopes over 4000 m.
  Medicinal uses: The roots are used to treat dysentery, especially in case of diarrhea with blood flow. Some people also reported the use of leaves and stems for inflammation and pain in the joints and muscles (rheumatism). The complete plant is also used in the treatment of painful urine discharge (micturition).

- ***Lancea tibetica* Hook.f. & Thomson** [Mazaceae]

  Ladakhi name: Payak-tsava, Spayak-rtsa-ba, Raikche.
  Habitat: Found growing on damp places and open marshy grasslands.
  Medicinal uses: The roots, flowers, leaves, and fruits used in the treatment of various kinds of pulmonary diseases (diphtheria, lung inflammation), besides,

cardiac diseases, large intestine tumors, and wounds. Extract prepared from the plant is used as a tonic.

- ***Lepidium latifolium* L.** [Brassicaceae]

  Ladakhi name: Hallo, Shang-shu, Seoji.
  Habitat: Frequent on open stony slopes and wastelands.
  Medicinal use: The entire plant is crushed and made into a paste and then applied as poultice for cure of rheumatism. The seeds are used for joint pains and as an ointment for wounds and abscesses.

- ***Lonicera spinosa* (Decne.) Jacquem. ex Walp.** [Caprifoliaceae]

  Ladakhi name: Brama, Phang-ma.
  Habitat: Found along cultivated fields and pathways.
  Medicinal use: The fruits are rubbed on cheeks and applied as lotion on skin.

- ***Lycium ruthenicum* Murray** [Solanaceae]

  Ladakhi name: Umila, khizer.
  Habitat: Found growing on riverine scree and dry rocky open valley flatlands and slopes.
  Medicinal use: It is known to treat urine-related problems.

- ***Malva neglecta* Wallr.** [Malvaceae]

  Ladakhi name: Khubasi, Sonchala.
  Habitat: Common along roadsides, moist places, cultivated area, and wastelands.
  Medicinal use: Decoction prepared from it is a good cure for malaria. Dried plant is used in bladder and kidney disorders. The seeds are also used for bronchitis and coughs.

- ***Malva verticillata* L.** [Malvaceae]

  Ladakhi name: Guchha-pushp, Mradu-patra.
  Habitat: Typically found in moist, shady places and in cultivated fields.
  Medicinal use: The root is used to treat whooping cough; the leaves and stems are given to women in advanced stages of pregnancy. Ash of dried leaves is used in preparation of a drink given to treat scabies.

- ***Meconopsis aculeata* Royle** [Papaveraceae]

  Ladakhi name: Achat-sermum, Landre-mentok.
  Habitat: Occasionally found in alpine zones of rocky crevices, slopes, and among boulders at an altitude ranging from 2400 to 4700 m.
  Medicinal use: The entire plant is used as a tonic in folk medicine. It is dried and administered with other herbs too, for the cure of lung disorders and liver inflammation. Water extract of whole herb, including flowers, is used to wash wounds.

- ***Medicago lupulina* L.** [Fabaceae]

  Ladakhi name: Buk-shuk, ole.
  Habitat: Common along cultivated fields.
  Medicinal use: Used to treat cold and fever and is often cooked as vegetable.

- ***Melilotus officinalis* (L.) Lam.** [Fabaceae]

  Ladakhi name: Gyasposd-manpa.
  Habitat: Found in the ground along roadsides, pathways, and cultivated fields.
  Medicinal use: It is known to treat bacterial diseases and used to treat body swelling.

- ***Mentha longifolia* (L.) L.** [Lamiaceae]

  Ladakhi name: Pholo-ling, Takchi.
  Habitat: Common in marshy places and along glacial streams and irrigation channels.
  Medicinal uses: The astringent leaves are used for rheumatic pains and indigestion. The decoction prepared from the plant is used in fever and sun stroke. The leaves soaked in water for sometime provides an infusion, which is used as a cooling medicine. The root yields an essential oil that helps in treating dysentery.

- ***Morina longifolia* Wall. ex DC.** [Caprifoliaceae]

  Ladakhi name: Pyang-tsher.
  Habitat: Found growing along roadside and dry mountain slopes.
  Medicinal use: Used in the treatment of body swellings and indigestion.

- ***Myrtama elegans* (Royle) Ovcz. & Kinzik.** [Tamaricaceae]

  Ladakhi name: Umbu, Umbuk, Humbu.
  Habitat: Common on stony river and stream banks and in moist sandy soils.
  Medicinal use: The plant is known to be effective as blood purifier. Also find use in stomach-related ailments like diarrhea and in severe headaches. Some use it in the cure of arthritis.

- ***Oxyria digyna* (L.) Hill** [Polygonaceae]

  Ladakhi name: Chu-cha, Laman-chu, Chum-cha.
  Habitat: Common along water sources and in meadows and glacial moraines.
  Medicinal use: The entire plant is used as an effective laxative. The leaves are cooked as vegetable to increase appetite.

- ***Paraquilegia microphylla* (Royle) J.R. Drumm & Hutch.** [Ranunculaceae]

  Ladakhi name: Yumo-deujin.
  Habitat: Found growing on rock crevices, open rocky slopes, and high alpine scree.
  Medicinal use: Used to treat blood and gynecological disorders.

- ***Parnassia cabulica* Planch. ex C.B. Clarke** [Celastraceae]

  Ladakhi name: Dnyul-tik.
  Habitat: Found growing on moist areas and along water streams.
  Medicinal use: The plant is used to treat the side effects caused by the use of improper medication.

- ***Pedicularis bicornuta* Klotzsch** [Orobanchaceae]

  Ladakhi name: Kyang-shogpa.
  Habitat: Found on moist alpine slopes, grasslands, and marshes.
  Medicinal uses: The upper parts of the plant are known to heal wounds and treat urine-related disorders, especially urine obstruction. It is also used to entice vomiting in patients.

- ***Pedicularis longiflora* Rudolph** [Orobanchaceae]

  Ladakhi name: Lugru-serpo.
  Habitat: Common on high altitude moist plains and along moist riversides and streams.
  Medicinal use: It is known to treat urine obstruction diseases, disturbance in breathing and weakness.

- ***Pedicularis punctata* Decne.** [Orobanchaceae]

  Ladakhi name: Lugru-mug-po.
  Habitat: Found growing on high-altitude open meadows.
  Medicinal use: The plant treats infections caused by microorganisms and pathogens and reduces inflammation.

- ***Peganum harmala* L.** [Nitrariaceae]

  Ladakhi name: Gandhya, Sepan.
  Habitat: Common in dry places, wastelands, and along the roadsides.
  Medicinal use: The whole plant is used as an aphrodisiac, abortifacient, and in syphilis. Dried seeds of the plant are narcotic and constitute the drug "Harmal" which is used in asthma, fever, and rheumatism.

- ***Physochlaina praealta* (Decne.) Miers** [Solanaceae]

  Ladakhi name: Sholar, Lal-tang.
  Habitat: Arid fields, wastelands, between stones and boulders at altitudes between 2700 and 4300 m.
  Medicinal uses: Poisonous herb is applied externally to treat boils, besides its use for ulcers. The seeds treat bacterial and viral infections and diphtheria and reduce severe pain and are also used against body inflammations. The plant yields "atropine" and "hyoscyamine" which are used as antidote in various modern formulations.

- ***Picrorhiza kurroa* Royle ex Benth.** [Plantaginaceae]

  Ladakhi name: Karu.
  Habitat: Found in alpine, glacial moraines, and moist slopes at elevations of 3300–4500 m.
  Medicinal use: It is used as an appetizer, blood purifier, blood pressure reducer, cardiac expectorant, and febrifuge; also useful in asthma, cold, cough, bile trouble, jaundice, leprosy, constipation, and stomach troubles.

- ***Podophyllum hexandrum* Royle** [Berberidaceae]

  Ladakhi name: Denmo-Tenumoo-Kusso, Ol-mose.
  Habitat: Occurs rarely in moist clearings and open slopes at altitude 3000–4200 m.
  Medicinal uses: Locally, the entire plant is used for gynecological diseases, like menstrual irregularity and disease of the uterus, and improves blood circulation, and helps in delivery of the baby and placental removal. The roots are used as hepatic stimulant and against skin problems, while the young and ripe fruits are edible and are useful against high-altitude mountain sickness. In industry, the rhizomes and roots constitute the main source of the medicinal resin called "*Podophyllum*" or "podophyllin," which is used for developing anticancer drugs.

- ***Potentilla atrosanguinea* G.Lodd.** [Rosaceae]

  Ladakhi name: Gyumkhris-mugpo.
  Habitat: Common on moist alpine grasslands.
  Medicinal use: It is known to treat common cold and epidemic diseases.

- ***Potentilla fulgens* Wall. ex Sims** [Rosaceae]

  Habitat: Found in temperate and alpine zones from 2500 to 4000 m alt.
  Medicinal use: The paste prepared from its leaves is used in curing stomachache, cough cold, sore throat, and sometimes ulcer. The root is effective in strengthening the teeth and gums. Commercially, the industry uses it for the manufacture of Ayurvedic toothpaste and tooth powder.

- ***Potentilla multifida* L.** [Rosaceae]

  Ladakhi name: Thakto.
  Habitat: Found in alpine grasslands
  Medicinal use: The entire plant is known to treat insomnia and restlessness.

- ***Primula macrophylla* D.Don** [Primulaceae]

  Ladakhi name: Shangdril-nagpo.
  Habitat: Found growing on high-altitude open slopes and moist grounds.
  Medicinal use: The plant is used to treat cough and fever.

- ***Prunella vulgaris* L.** [Lamiaceae]

  Ladakhi name: Austa-khaddus, Syan-gara.
  Habitat: It occurs on moist shady slopes between 2000 and 3800 m.
  Medicinal use: An infusion of leaves and flowers is used as a gargle and to obtain relief from throat irritations. It also finds use to treat diarrhea, hemorrhages, bleeding piles, and colic and for relieving stomach gas.

- ***Prunus armeniaca* L.** [Rosaceae]

  Ladakhi name: Chuli.
  Habitat: Found growing on cultivated fields and homesteads.
  Medicinal use: The plant is known to treat cough and fever. Oil is applied on the body to treat muscular cramps and as a defensive mechanism against cold dry winter winds.

- ***Rheum australe* D. Don** [Polygonaceae]
- ***R. moorcroftianum* Royle**
- ***R. spiciforme* Royle**
- ***R. webbianum* Royle**

  Ladakhi name: All species go by the name Lachu.
  Habitat: Found in alpine meadows at altitudes ranging between 3000 and 4200 m.
  Medicinal use: The roots are used as purgative and in diarrhea and also in high-altitude sickness. The paste made with water is applied to cuts, open wounds, rashes, and inflammations. In the indigenous system of medicine, it goes by the name "Revand chini" and "Rhubarb" and is extracted by local people for sale and is known to treat swelling, wounds, and internal injuries, with good results.

- ***Rhodiola imbricate* Edgew.**
- ***Rhodiola tibetica* (Hook.f. & Thomson) S.H. Fu** [Crassulaceae]
- ***Rhodiola wallichiana* (Hook.) S.H. Fu**

  Ladakhi name: Shrolo is the common name for all *Rhodiola* species.
  Habitat: Found on open rocky and high mountain moist slopes.
  Medicinal use: The roots are used to treat various pulmonary diseases like cold and cough. Shoot portion is known to treat toothache and fever. Health tonic is prepared from the plant and it is known to treat various mouth disorders and cough infections. Very effective against high-altitude sickness and as tonic for energy boost. Dried foliage is crushed and used with curd as a diuretic and is also effective to reduce obesity.

- ***Ribes orientale* Desf.** [Grossulariaceae]

  Ladakhi name: Askuta, Ser-god.
  Habitat: Found on dry and rocky slopes and along river valleys.
  Medicinal use: The fruits are edible and a good source of vitamin C and are known to treat food poisoning and epidemic fever.

- ***Rosa webbiana* Wall. ex Royle** [Rosaceae]

  Ladakhi name: Mentouck, Siah, Marpo.
  Habitat: Common in open sandy, dry slopes, and river valleys.
  Medicinal use: The fruits are edible and a rich source of vitamin C. The petals are used to cure nasal bleeding and swelling and against liver diseases like hepatitis and jaundice. Stem bark is burnt and the residue is then applied on skin rashes.

- ***Rumex acetosa* L.** [Polygonaceae]

  Ladakhi name: Lung-sho.
  Habitat: Common on field margins and moist locations near homesteads.
  Medicinal use: The plant is used to treat dermatological diseases and blisters.

- ***Rumex patientia* L.** [Polygonaceae]

  Ladakhi name: Shoma.
  Habitat: Common on field margins and water-logged areas.
  Medicinal use: The plant is used to treat skin infections and in healing cuts and wounds.

- ***Salsola kali* L.** [Amaranthaceae]

  Habitat: Of common occurrence in the drier flatlands of valley plains.
  Medicinal use: When in full bloom, the plants are used for decreasing water retention in the body, by increasing urine, and also during menstrual flow. It is also used against hypertension.

- ***Salvia abrotanoides* (Kar.) Sytsma** [Lamiaceae]

  Ladakhi name: Iskilling.
  Habitat: Found growing on open dry boundary slopes.
  Medicinal use: The plant is used in case of fever, cough, and headache and for burning sensations.

- ***Salvia moorcroftiana* Wall. ex Benth.** [Lamiaceae]

  Ladakhi name: Halu, Shrematus, Shobri, Thuth.
  Habitat: Open slopes and wastelands.
  Medicinal use: The leaves are applied as poultice for wounds and chronic affections of the skin. Roots are used to treat coughs, colds, and stomach pain.

- ***Saussurea bracteata* Decne.** [Asteraceae]

  Ladakhi name: Span-rtsa- dobo, Jar-bag.
  Habitat: In arid rocky and glacial slopes at higher altitudes of 5000 m.
  Medicinal use: The flowering buds are collected and made into paste before applying on boils and other skin eruptions. The roots are used in preparations that are applied to wounds and cuts. Floral bracts are boiled in water for use as fermentation.

- ***Saussurea laniceps* Hand. -Mazz.** [Asteraceae]

  Ladakhi name: Loskarch.
  Habitat: Moist alpine slopes at altitudes 2500–4200 m; also found in irrigated fields in higher altitudes.
  Medicinal use: The woolly tomentum of the herb is applied to fresh cuts and to seal the wound and it also helps to heal it quickly.

- ***Solanum nigrum* L.** [Solanaceae]

  Ladakhi name: Makoh.
  Habitat: Common in waste places, roadsides, and cultivated fields.
  Medicinal use: Young shoots are used to treat skin diseases and psoriasis. Leaf extract is used for skin eruptions. Freshly prepared extract of plant is considered useful in treating cirrhosis of the liver, and boiled leaves and tender shoots are recommended to patients suffering from dropsy. Ripe fruit is edible and used to treat fevers, diarrhea, eye problems, and hydrophobia.

- ***Swertia petiolata* D.Don** [Gentianaceae]

  Ladakhi name: Chagstik, Zantik.
  Habitat: Found growing in high-altitude marshy meadows and nearby streams.
  Medicinal use: Decoction of the whole plant in milk helps to treat body inflammation and aches, wounds, common cold, and cough.

- ***Taraxacum* sect. *Taraxacum* F.H. Wigg.** [Asteraceae]

  Ladakhi name: Han.
  Habitat: Grasslands, roadsides, cultivated fields, and other waste places.
  Medicinal use: The leaves are used to treat loss of appetite and for upset stomach. Useful remedy for chronic disorders of the kidney and liver; also used in jaundice, gallstones, muscular rheumatism, and constipation. Tea prepared by boiling flowers is used to treat heart trouble.

- ***Thermopsis barbata* Benth.** [Fabaceae]

  Ladakhi name: Ghlaba-sadma.
  Habitat: Grows on open sunny slopes, grasslands, along roadside, and alpine forests.
  Medicinal use: The roots are known to treat hypertension and flowers and fruits treat dog bite poison.

- ***Thermopsis inflata* Cambess.** [Fabaceae]

  Ladakhi name: Llamo.
  Habitat: Open moist slopes in high mountain areas.
  Medicinal use: The plant is collected in whole and used to remove excess water from the body. Fruits are collected and used to reduce swellings and pain.

- ***Thymus linearis* Benth.** [Lamiaceae]

  Ladakhi name: Tumburk.
  Habitat: Common on meadows and slopes, from low to high elevations.
  Medicinal use: Taken in the form of an infusion for irritating coughs. The crushed leaves are smelt during cold weather to keep off cold.

- ***Tribulus terrestris* L.** [Zygophyllaceae]

  Ladakhi name: Gokhru, Zama, Trikuta.
  Habitat: Found in dry and waste places up to 3200 m.
  Medicinal use: The fruits are used to treat coughs and skin diseases. Leaf paste is used for treatment of stones in the bladder. Leaf decoction is useful as a gargle for mouth problems. The leaves also help to cure gonorrhea, by increasing the menstrual flow.

- ***Trifolium pratense* L.** [Fabaceae]

  Ladakhi name: Darbai-mentok, Glober.
  Habitat: Cultivated as forage crop; often seen as an escape in grassy meadows.
  Medicinal use: Flowers are used as an effective sedative, while the mature plant is used to treat whooping cough, liver ailments, digestive disorders, skin sores, and ulcers.

- ***Urtica hyperborean* Jacquem. ex Wedd.** [Urticaceae]

  Ladakhi name: Zatsod.
  Habitat: Common along pathways and rocky slopes
  Medicinal use: Treats joint pain, cough, fever, throat pain, and increased body temperature. Locally, it also used to make *thukpa* (vegetable soup). The young leaves are relished as vegetable during summers and also dried and stocked for use during the winter months, to keep the body warm.

- ***Verbascum thapsus* L.** [Scrophulariaceae]

  Ladakhi name: Sman-moshing.
  Habitat: Found in dry wastelands, roadsides, and open slopes up to 3800 m.
  Medicinal use: Matured leaves, flowers, and roots are used as medicine. Leaves are smoked for relief in asthma and sore throat. Infusion of plant is given orally as cure for insect bite including snakebite. Oil is extracted from the flowers and is used to treat earache.

- ***Vitis vinifera* L.** [Vitaceae]

  Ladakhi name: Gunbhrum.
  Habitat: Cultivated in fields of Aryan Valley.
  Medicinal use: It is used to treat constipation and pulmonary disorders, especially in children.

- ***Xanthium strumarium* L.** [Asteraceae]

  Ladakhi name: Bisher, Byis-tsher.
  Habitat: Common on pastures, roadsides, and fields.
  Medicinal use: It is used as an anti-inflammatory and as diuretic. It is also helpful in treating epidemic cough and fever.

Acknowledgments The authors are grateful to the Ministry of AYUSH and National Medicinal Plants Board (NMPB), New Delhi, for providing funds for the research project. The authors are also grateful to Brij Bhushan, Pankaj Kumar, Monica Chauhan, and Bhanu Verma (research staff from HFRI) and the local inhabitants of Ladakh for sharing their valuable traditional knowledge on ethnobotany used by them and also for their kind cooperation during collection of ethnobotanical data and related field work. We also take this opportunity to thank Dr. Padma Gurmet and Dr. Sonam Dawa, from the National Institute of Sowa Rigpa (NISR), Leh for their valuable inputs.

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Novel Secondary Metabolites in Tea and Their Biological Role in Communicable and Noncommunicable Human Diseases

Krishnaraj Thirugnanasambantham, Sam Nirmala Nisha, and Abul Kalam Azad Mandal

Abstract Tea is regarded as a functional food because, in addition to its nutritional benefits, it can provide other advantages via its application in the treatment of several illnesses including both communicable and noncommunicable human diseases. Tea and its metabolites are well known to inhibit the growth of the virus, bacteria, fungi, and different parasites which plays a major role in various communicable diseases. In addition, they also hold both preventive and therapeutic potential over different noncommunicable diseases that include but are not limited to cancer, arthritis, cardiovascular diseases, respiratory disorders, and diabetes, etc. Green tea metabolites including catechins and polyphenols have a limited bioavailability, and there haven't been enough clinical trials of them to go very far, even though most preclinical research has revealed a correlation between increasing green tea consumption and a decreased risk of human diseases. Thus, new formulations of green tea metabolites are urgently needed to overcome their low bioavailability and increase their beneficial effects.

Keywords Communicable diseases · Noncommunicable diseases · Tea · Catechin · EGCG · Polyphenol

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N. Kumar, R. S. Singh (eds.), *Biosynthesis of Bioactive Compounds in Medicinal and Aromatic Plants*, Food Bioactive Ingredients,
https://doi.org/10.1007/978-3-031-35221-8_13

1 Introduction

Tea (*Camellia sinensis* L.), which is most commonly drank as green, black, or oolong tea but can also be found as red or white tea, is the second most accepted beverage in the world, right behind water. The tea plant harvested as the "flush," or top leaves attached to the bud and part of the stem, is what is used to make tea (Botwright 1997). All three types of tea including black, green, and oolong tea are manufactured from the leaves of the tea plant, but they are processed and prepared differently. Oolong tea comprises both fermented and non-fermented leaves, whereas black tea is entirely fermented and green tea does not experience fermentation. When green tea is steeped in water that is almost boiling, a wealth of polyphenolic compounds, including anthocyanins, catechins, and phenolic acids, as well as theanine, caffeine, tannins, vitamins, and trace elements, are released into the solution. In contrast to black and oolong tea, which experience greater oxidation and consequently lower polyphenol concentrations, green tea has less oxidation of its primary ingredients (polyphenols), resulting in higher quantities of polyphenols (Johnson et al. 2010). Green tea has undergone the most extensive research on health advantages, including chemopreventive efficacy. Additionally, tea polyphenols are believed to help prevent a range of degenerative illnesses, including cancer and cardiovascular disease.

Tea is regarded as a functional food because, in addition to its nutritional benefits, it can provide other physiological advantages. Due to its antioxidant properties, it is a key mediator in controlling free radicals, making it useful in the healthcare industry (Hayat et al. 2015). In ancient Asian folk medicine, tea was thought to be a good treatment for several illnesses, including both communicable and noncommunicable human diseases. Also, for many years, people have enjoyed drinking tea, and several diseases and malignancies have been examined to see if tea has any potential as a preventative measure. Epidemiological, cell culture, animal, and clinical research provide strong evidence for green tea's ability to prevent cancer. Several biological pathways may be affected by green tea polyphenols, which can also cause cell cycle arrest and apoptosis in cancer cells but not in respective normal cell counterparts (Yu et al. 2014). In addition, green tea treatment has been shown in numerous animal studies to reduce the occurrence and intensification of tumors in a variety of organ sites, including the skin, mammary gland, liver, lung, stomach, and colon. Phase I and II clinical trials have recently been conducted to examine the anticancer properties of green tea in people (Gee et al. 2017; Scholl et al. 2018). According to extensive research on both green tea and EGCG, which was done as part of epidemiological studies to assess the impact of green tea consumption on human health, this compound exhibits cardioprotective, neuroprotective, renal protective, osteoprotective, anticancer properties, and the capacity to regulate obesity-associated metabolic syndrome and type 2 diabetes (Afzal et al. 2015). Numerous studies have recommended that drinking green tea may have a small favorable effect on lowering blood cholesterol levels and preventing atherosclerosis. One possible reason for this is enhanced expression of the low-density lipoprotein receptor

(LDLR) and elucidated as a mechanism in the avoidance of cardiovascular diseases (Kuhn et al. 2004). The majority of studies have discovered a connection between growing green tea consumption and a reduced risk of human diseases. Preclinical research on the components of green tea strongly suggests that green tea catechins have intra- and extracellular actions that could have positive health benefits. Tea and its metabolites have demonstrated potential health advantages despite the low bioavailability of green tea catechins and polyphenols and the insufficiency of well-controlled clinical trials of green tea components too far. The green tea metabolite's low bioavailability may be overcome by new formulations, which would also increase the benefit-risk ratio.

2 Effect of Tea Extract and Its Components on Communicable Diseases

2.1 Bacterial Infections/Diseases

The biggest threat to global health is the rise of various antibiotic-resistant bacterial diseases. There arises a vital need for the identification of new antibiotics or alternative therapeutic strategies for combating the situation. The antibacterial potency of tea is attributed to the polyphenolic catechins particularly (−)-epigallocatechin (EGC), (−)-epicatechin gallate (ECG) (−), and epigallocatechin gallate (EGCG) (Taylor et al. 2005). The effect is profound on multidrug-resistant (MDR) strains that include both gram-positive and gram-negative bacteria. The antibacterial ability of catechins is not by one but varied strategies that ultimately inhibit the growth of many of the pathogenic bacteria (Wu and Brown 2021) and are enlisted below with the research reported to date.

2.1.1 Damage to the Cell Membrane

The ability of catechins to partition and disrupt the bacterial lipid bilayers is widely exploited. It is believed that this property of catechins decreases lipid packing and increases membrane expansion, causing a decrease in the membrane's capability to act as a barrier (Sun et al. 2009). EGCG effectively inhibited the growth and biofilm formation of *Streptococcus mutans* by causing irreversible cell membrane damage (BAI et al. 2016). Growth of *Escherichia coli* and *Pseudomonas aeruginosa* collected from skin wounds were potentially inhibited by catechin. ECGg was reported to inhibit the growth of antibiotic-resistant *E.coli* and *P. aeruginosa*, which suggested its use as an antibacterial agent (Jeon et al. 2014). A gene expression study in *E. coli* revealed that the antimicrobial effect of polyphenols from green tea was mediated by bacterial cell membrane damage (Cho et al. 2007).

2.1.2 Reactive Oxygen Species (ROS) and Hydrogen Peroxide (H_2O_2)

In another study, green tea extract (GTE) showed bactericidal potency against multidrug-resistant (MDR) gram-positive *S. aureus* but not against MDR gram-negative *E. coli*, which might be due to their cell wall component variation. The same study found that the purified compound EGCG exhibited bactericidal activity against both the bacterial species, which could be due to the generation of ROS and H_2O_2 (Parvez et al. 2019). The lesser effect of the tea catechins on the gram-negative bacterial cell membranes was thought to be due to the negatively charged lipopolysaccharide outer membrane (Ikigai et al. 1993). According to susceptibility tests, green tea extracts are more effective in eradicating MDR *Salmonella typhi* and susceptible *S. paratyphi* A. *Staphylococcus aureus*, *Micrococcus*, *Streptococcus pneumonia*, and *Bacillus subtilis* have all been proven to be highly susceptible to the effects of green tea (Farooqui et al. 2015).

To understand the mode of antibacterial effect of EGCG against gram-positive bacteria, a method to detect the localization of EGCG on the cell surface of *B. subtilis* was devised. The function of several membrane proteins, including the oligopeptide ABC transporter (Oppa), PTS system transporter, phosphate ABC transporter, and PBP5 in *B. subtilis*, was reported to be inhibited by EGCG (Nakayama et al. 2015). A study by Arakawa et al. demonstrated the production of H_2O_2 in the culture by catechins reacting with oxygen (Arakawa et al. 2004). The relationship between the concentrations of H_2O_2 and the viability of bacteria was demonstrated by adding catalase which decomposes the accumulated H_2O_2. A decrease in the ability to reduce bacterial population was observed with increasing concentrations of catalase in the culture which supported the role of hydrogen peroxide in conferring the antibacterial ability of catechins (Arakawa et al. 2004). In a study to determine the mechanism by which ECGg causes antibacterial activity, it was found that EGCG increased endogenous oxidative stress in *E. coli*, which prevented it from growing (Xiong et al. 2017).

2.1.3 Inhibition of Cell Adherence

Inhibiting bacterial species' ability to adhere to host cells is one more method used by GTE components to regulate bacterial infection. The GTE-treated bacterial pathogens, including *Fusobacterium nucleatum*, *S. epidermidis*, and *Helicobacter pylori*, were reported to display lower cell adhesion in human and animal cell line investigations, which validated this finding (Reygaert 2018). The effect of the green tea particles suspended in the green tea extracts from commercial green tea bag infusions against live human oral bacterial samples indicated size-dependent antibacterial activity (Gopal et al. 2016).

2.1.4 Effect on the Virulence Factors/Toxins

Interest has grown in employing substances that are effectively proven to display anti-virulence action to inhibit the activity of bacterial virulence factors as a therapy option instead of using antibiotics (Mühlen and Dersch 2015). Catechins have also been discovered to have a range of anti-virulence characteristics, mostly through the suppression of toxins, in addition to their antimicrobial property. These are often achieved at very low concentrations when compared to that required for antibacterial potential. Some of the virulence factor targets of catechins are listed in Table 1.

2.1.5 Synergism with Antibiotics

Catechins are known to boost bacterial susceptibility of MDR bacteria to conventional antibiotics through several mechanisms, such as altering cell membrane permeability to cause an increase in antibiotic uptake and/or by inhibiting efflux pumps to reduce drug export out of the bacterial cell (Wu and Brown 2021). It has been well established that catechins can work in synergy with penicillin to treat

Table 1 Tea and their virulence factor targets in bacteria

Bacteria	Virulence factor/toxin	Molecule	Mode of action/effect	References
Aggregatibacter actinomycetemcomitans	Leukotoxin (LtxA)	ECg and EGCG	Inhibit the ability to bind to membrane	Chang et al. (2019)
P. aeruginosa	FapC fibrils	EGCG	Inhibition of fibrillation and remodeling of fibril structure	Stenvang et al. (2016)
P. gingivalis	Phenylacetic acid	EGCG	Inhibition of production and cytotoxicity	Sakanaka and Okada (2004)
V. cholerae	Cholera toxin (CT)	EGCG	Inhibit subunit A activity	Cherubin et al. (2016)
E.coli	Shiga-like toxin (Stx1)	GCg and EGCG	Inhibits cytotoxic effects	Miyamoto et al. (2014)
P. gingivalis	Fimbriae	EGCG	Inhibits expression of gene fimA, encoding for major subunit	Fournier-Larente et al. (2016)
M. morganii	Acyl homoserine lactones (AHLs)	Crude green tea extract	Inhibits synthesis and blocks AHL-mediated quorum sensing	Guzman et al. (2020)
C. violaceum	Violacein	Green tea ethyl acetate fraction	Inhibits synthesis and changes receptor binding affinity	Qais et al. (2019)
P. aeruginosa	Pyocyanin and pyoverdin			
S. marcescens	Prodigiosin			

methicillin-resistant *Staphylococcus aureus* (MRSA). These antibiotics work by attaching to a class of proteins called penicillin-binding proteins (PBPs), which are in charge of polymerizing and cross-linking peptidoglycan (Yam 1998). A synergistic relationship between gentamycin and a green tea metabolite was discovered using the fractional inhibitory concentration index (FICI) method. Combination of EGCG and gentamicin induced cell membrane damage and inhibition of DNA supercoiling (Namita et al. 2012).

2.1.6 Effect on Dental Infections and Disorders

High-catechin-gallate concentrations, especially EGCG, have been shown to suppress the development of specific periodontal bacteria, lowering the indicators of gingivitis (Sakanaka et al. 1989). EGCG also successfully neutralizes the protein tyrosine phosphatase and gingipains linked to periodontal disease, which are toxic end products of bacteria (Okamoto et al. 2003). At quantities similar to those found in brewed tea, catechins have been identified as mild inhibitors of the cariogenic bacterial agents *S. mutans* and *S. sobrinus* that cause caries (Sakanaka et al. 1989). It is found that they can prevent the attachment of oral streptococci, including *S. mutans*, to host cell surfaces (Melok et al. 2018). EGCG and ECg, as well as the catechin portion of tea, inhibit the streptococcal enzyme known to catalyze the production of the glycocalyx, a virulence factor crucial for adhesion (Hattori et al. 1990; Hairul Islam et al. 2020). It is also claimed that tea extracts can lessen the activity of the enzyme α-amylase in human saliva, which prevents the breakdown of starch, the substrate for the synthesis of the glycocalyx, and prevents the build-up of cariogenic streptococci on tooth surfaces (Zhang and Kashket 1998). GTE and EGCG modulate adherence of *Porphyromonas gingivalis* to oral epithelial cells by downregulation of expression of genes involved in tissue destruction (rgpA, kgp), host colonization (fimA, hagA, hagB), and heme acquisition (hem) and upregulation of expression of the stress protein htrA gene (Zhang and Kashket 1998).

2.1.7 Effect Against Urinary Infections

E. coli are the infective agent for 80–90% of all urinary tract infections (UTIs). It has already been documented that EGC has antimicrobial activity against *E. coli* and also that it is excreted in the urine in concentrations higher than the concentrations to exhibit antimicrobial activity; this study suggests that the intake of green tea could potentially inhibit or reduce the risks of developing UTIs (Reygaert and Jusufi 2013). Green tea has a synergistic impact with amoxicillin, azithromycin, chloramphenicol, ciprofloxacin, and cefodizime, while black tea has a synergistic effect with chloramphenicol, gentamycin, cefodizim, and tobramycin (Passat 2012).

2.1.8 Effect on Bacterial Skin Infections

The GTE prevented adherence of pathogenic *B. subtilis*, *M. luteus*, *P. fluorescens*, *S. epidermidis*, and *B. Linens* to mammalian cells without any cytotoxicity (Sharma et al. 2012). EGCG and GTE have been suggested as alternative or adjunct topical antimicrobial agents for infections caused by pathogens resistant to traditional antibiotic therapy (Jeon et al. 2014).

2.1.9 Effect on Other Bacterial Infections

The EGCG's potential antioxidative and antibacterial effects prevented *H. pylori*-induced gastritis (Trompezinski et al. 2003). EGCG was able to successfully protect gastric mucosal cells from *H. pylori*-induced DNA damage and apoptosis, and its treatment accelerated the proliferation of gastric epithelial cells (Lee et al. 2004). *Acinetobacter baumannii* is a most important gram-negative human pathogen involved in hospital-acquired infections. Lee et al. evaluated the synergistic antibacterial activity of EGCG with conventional antibiotics against carbapenem-associated multidrug-resistant strains of *A. baumannii* (Lee et al. 2017). The antibacterial potential of EGCG was confirmed in carbapenem- and polymyxin-associated multidrug-resistant clinical isolates of *A. baumannii* (Lee et al. 2017).

EGCG inhibited the intracellular growth of *Listeria monocytogenes* in macrophages by attenuating the hemolytic and cholesterol-binding activity of the pathogen, which is responsible for damage in the phagosomal membrane (Kohda et al. 2008). EGCG's antibacterial activity concerning the opportunistic pathogen *P. aeruginosa* interferes with quorum sensing (QS) by altering the structure of amyloid fibrils (Stenvang et al. 2016). The various effects of GTE and its components on bacterial pathogens are illustrated in Fig. 1 (Spina et al. 2008; Chinnam et al. 2010; Noormandi and Dabaghzadeh 2015).

2.2 Fungal Infections

Exploring new drugs that can combat the development of resistance has been of interest in recent years due to the nonavailability of highly efficient antifungal agents and also due to the emergence of resistance to the existing drugs (Rajeshkumar and Sundararaman 2012). Natural products from plants like flavonoids have been of high scientific interest for their antifungal properties (Park et al. 2011a). Among the human fungal pathogens, *Candida* and *Aspergillus* are the most common with an increased number of invasive infections. With the availability of numerous antifungal drugs like azoles and echinocandins, there are still reports showing the decreased susceptibility of *Candida* species to azole (Pfaller et al. 2012). Catechins' antifungal action against *C. albicans* was pH-dependent, and in acidic environments, EGCG activity was reduced. In comparison to catechol catechins (EC, ECg, C, and

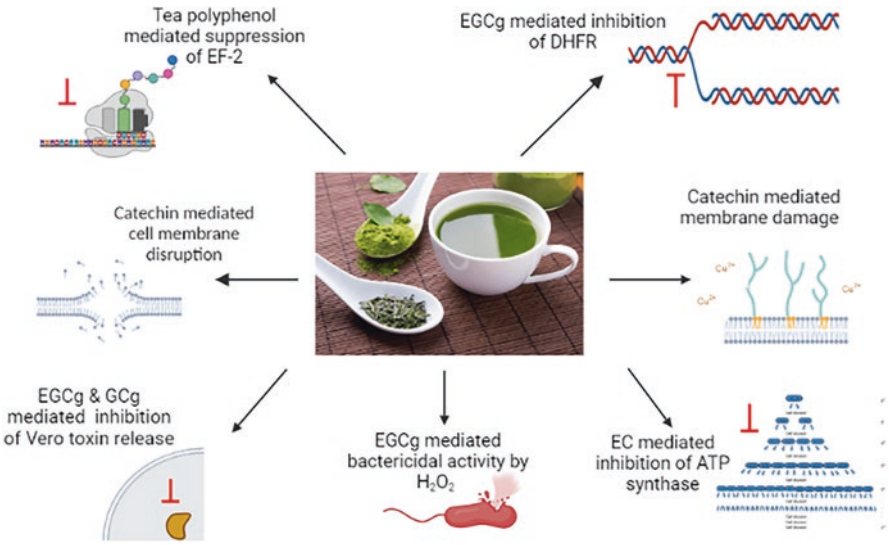

Fig. 1 An illustration of diverse biological application of tea extract and its metabolites

Cg), pyrogallol catechins (EGCG, EGC, GC, and GCg) were more efficient against *C. albicans*. The combination of EGCG and amphotericin B also had synergistic antifungal efficacy, where amphotericin B's stimulated catechin uptake into the cell and functioned as a fungicidal agent (Hirasawa 2004). Catechins and theaflavins are primarily responsible for the anti-*Candida* activity of tea polyphenols against different *Candida* sp., including *C. albicans*, *C. glabrata*, *C. krusei*, *C. parapsilosis*, and *C.* tropicalis, by inducing cell wall damage (Sitheeque et al. 2009). The antifungal activity of EGCG was found to be strain-specific as *C. albicans* and *C. parapsilosis* were less susceptible, with no inhibition in growth. Synergism was observed between EGCG and conventional antimycotics (amphotericin B, fluconazole, or miconazole) in both planktonic and biofilm forms of *Candida* species (Ning et al. 2015). Green tea extracts, specifically ECG and EGCG, were found to exhibit broader antimycotic activity toward different fungal pathogens (Turchetti et al. 2005), and inhibition of ergosterol synthesis was reported as the mechanism of action against *C. albicans* (Navarro-Martinez 2006). Furthermore, the antifungal susceptibility of 21 clinical isolates toward flucytosine and fluconazole was synergically reduced by EGCG (Park et al. 2006). In a study using a mouse model of *C. albicans*-induced disseminated candidiasis, it was shown that treatment with EGCG prevented the yeast form of the fungus from forming hyphae, hence preventing proliferation. In the study's later stages, synergism with amphotericin B was noted (Han 2007).

2.3 Viral Infections

Various viruses have been known to cause numerous types of infections in humans and are life-threatening in most cases. They are known to cause not only acute infections but also diseases that are chronic, affecting the quality of life. Effective treatment is crucial to have control over chronic viral infections. Numerous studies have demonstrated the inhibitory activities of green tea catechins (GTCs) against many human viruses (Xu et al. 2017). Peripheral blood cells were cultured with one of the two HIV strains and increased EGCG concentrations to ascertain the impact of EGCG on HIV infection. Reverse transcriptase and p24 assays on cell supernatants showed that both the two HIV strains were severely suppressed, supporting the idea that EGCG inhibits HIV reproduction (Fassina et al. 2002). Semen-derived enhancer of virus infection (SEVI), a significant infectivity factor during sexual transmission of HIV, was known to be synergistically degraded by antiretroviral microbicides supplemented with EGCG (Hauber et al. 2009). Hepatitis C virus (HCV) infection worldwide has been related to the development of chronic liver diseases and hepatocellular carcinoma. Epicatechin isomers (+)-epicatechin and (2)-epicatechin's ability to limit HCV replication was shown using in vitro cell-based HCV replicon and JFH-1 infectious systems. This impact was correlated with the inhibition and downregulation of virus-induced cyclooxygenase-2 (COX-2) (Lin et al. 2013). Further, EGCG was reported to inhibit the attachment of HCV to host cells (Calland et al. 2012).

The EGCG effectively suppressed HBV antigen secretion in a dose- and time-dependent manner in HBV-infected HepG2 cells (Pang et al. 2014). The EGCG was able to interact competitively with the virion surface proteins, thus inhibiting the HSV-1's attachment to heparan sulphate (Colpitts and Schang 2014).

The use of catechin/theanine for 5 months had a significant preventative impact on clinically diagnosed influenza infection, according to a randomized, double-blind, placebo-controlled trial (Matsumoto et al. 2011). To ascertain the relationship between green tea drinking and the prevalence of influenza infection, a survey of schoolchildren was conducted. In Kikugawa City (a tea plantation area), Japan, questionnaire surveys were conducted twice during the influenza season in all primary schools. The study's findings revealed that consuming green tea daily in amounts of one to five cups could protect kids from getting the flu (Park et al. 2011b). A randomized, double-blind, placebo-controlled study conducted among healthy adults 18–70 years old for the investigation of the anti-flu effects of green tea showed that consumption of a formulation of *Camellia sinensis* (CSF) capsules twice a day for 3 months significantly reduced the symptoms and overall flu-related illness. γδT cells from subjects taking CSF were found to proliferate more, and also the secretion of IFN-γ in response to the antigens for the γδ T cell increased (Rowe et al. 2007).

The SARS-CoV-2 global epidemic has put the world's public health systems under strain. Understanding the immunological modulation caused by the SARS-CoV-2 virus, which will hold the key to the creation of vaccines and therapies, is

essential for successful control (Deshpande et al. 2020). Initial studies on SARS-CoV infections provided pieces of evidence that the modified and worsened/dysregulated host immune response to the virus and not the virus itself is the factor responsible for the serious and intense damage to the host body at pulmonary and organ levels, leading to grievous outcomes (Channappanavar et al. 2014). Impairment of innate immunity disrupts various signaling pathways that may lead to an increase in proinflammatory cytokines, decrease and deplete interferons and natural killer cells, and activate the production of ROS which later has a detrimental consequence on the body's capability to combat infection contributing to disease progression. An effective therapeutic should meet both the antiviral and immune-modulatory approaches in controlling the infection and also the adaptation of multiple approaches. Ways by which the immunity of the healthy and infected population can be approached through nutrition should also be the focus in addition to other treatment strategies. The role of tea constituents, especially EGCG and theaflavin (TF) in regulating valuable innate immune responses, has been supported in various research findings. Since tea is an acidophilic perennial plant that accumulates various micronutrients from soil, micronutrients are a crucial component of the innate immune response and their occurrence in tea infusions has been well established (Chowdhury and Barooah 2020).

The potential role of the GTC in combating different approaches to the SARS-CoV-2 virus is discussed by Tallei et al. (2021). It could be due to any of the below said mechanisms or their combinations that the GTC is thought to be a potential therapeutic agent: the ability to modulate immune responses leading to preserved immune homeostasis, anti-inflammatory activity by suppression of NF-κβ and MAPK, antioxidant activity to prevent cell damage, anti-fibrotic potential through the reduction of inflammation and mucin secretion, and inhibition of the synthesis of main protease (M^{pro}) of SARS-CoV-2 (Tallei et al. 2021). Anti-inflammatory drugs that are directed to control cytokine production as in the case of autoimmune diseases are the most effective drugs for the control of severe COVID-19 infections. EGCG from green tea leaves has been efficient in counteracting the increased cytokine production for many autoimmune diseases, and its efficacy to control or reduce the severity of COVID-19 can be exploited as one of the treatment therapeutics. Signal transducer and activator of transcription (STAT)1/3 and nuclear factor kappa-light-chain-enhancer of activated B cells (NF-B), which are essential components of most downstream pro-inflammatory signaling pathways, have both been shown to be inhibited by EGCG. EGCG is a viable and safe supplement to counteract dysregulated inflammation in COVID-19 due to its antiviral, antisepsis, anti-fibrotic, and capacity to downregulate most inflammatory chemicals capabilities (Menegazzi et al. 2020). A pilot scale revealed GTE-based treatment strategies against COVID-19 syndrome (Bettuzzi et al. 2021). The green tea beverage (GTB) and its catechins were reported to inhibit infection of live SARS-CoV-2 and three newly emerged variants (UK-B.1.1.7, SA-B.1.351, and CA-B.1.429) (Liu et al. 2021). The EGCG and theaflavin, which are the most important active ingredients of both green and black tea, inhibited the 3CL-protease of SARS-CoV-2 in a dose-dependent manner (Jang et al. 2020).

2.4 Parasite Infections

A significant category of pathogens, endo, and ectoparasites can induce yet frequently go unnoticed infectious agents that result in serious health issues with high morbidity and death. There have been limited vaccines because the transmission of these parasites is by multiple routes and they are also known to have a complex life cycle involving multiple morphological stages. These factors lead to the search and development of new antiparasitic compounds (Garcia 2020). Through various research findings, it has been reported that the main effect exerted by the GTCs on different parasite infections is to cause a decline in growth and ultimately parasite number. This was thought to be due to the effects it had on causing fragmentation of parasite DNA and reduced fatty acid synthesis (Reygaert 2018). A study was carried out to investigate the green tea components' activity against *Leishmania infantum*. Catechin, EC, ECG, and EGCG have antileishmanial activity, and EGCG and ECG were the most effective against *L. infantum* promastigotes (Khademvatan et al. 2019).

Oral administration effectively acted against *L. braziliensis* infection in BALB/c mice, by increasing ROS generation and decreasing mitochondrial membrane potential/intracellular ATP concentrations without any serological changes (Inacio et al. 2014). Since iron is a vital element that all living things need, iron chelators are frequently employed to treat parasite infection at concentrations that are safe for mammalian cells. It was discovered that GTE displayed antimalarial efficacy by preventing the uptake of external iron or by reducing the cytoplasmic labile iron reserve in malaria parasites, which inhibited proliferation (Thipubon et al. 2015). EGCG was discovered to be a medication for the treatment of babesiosis by the EGCG-mediated decrease in the proliferation of the bovine *Babesia* parasite and the mouse-adapted rodent babesia, *B. Microti* (ABOULAILA et al. 2010). The EGCG decreased the virulence of *Trypanosoma brucei* in an acute animal model by increasing the phosphorylation of acetyl-CoA carboxylase (ACC), an enzyme that catalyzes the initial step of FA production by activating AMP-dependent protein kinase. The results indicate that even while EGCG has a limited ability to treat infections, it might be a useful component to investigate how ACC is regulated (Vigueira et al. 2012). The kinetoplastid protozoan *Trypanosoma cruzi* causes chronic Chagas' disease. The disease does not have either a total eradication measure or an effective treatment strategy. The trypanocidal activity of green tea catechins against the trypomastigote and amastigote stages of *Trypanosoma cruzi* revealed that GCG and EGCG were the most potent tea metabolites (Paveto et al. 2004).

3 Effect of Tea Extract and Its Components on Noncommunicable Diseases

3.1 Cardiovascular Disease

The most significant and frequently curable risk factor for cardiovascular disease (CVD), one of the important causes of fatality worldwide, is hypertension. According to scientific data, reducing blood pressure (BP), especially in hypertensive individuals, lowers the risk of developing and dying from cardiovascular disease (CVD) (Iellamo and Volterrani 2010). Polyphenols in both green and black tea reduced blood pressure (BP) elevations in stroke-prone hypertensive rats through their antioxidant capabilities, indicating that frequent use of them may be protective against hypertension (HTN) in people (Negishi et al. 2004). Later research revealed that while green tea consumption had no impact on systolic, diastolic, or heart rate, it had a positive effect on mean arterial blood pressure and the response to an acute resistance exercise (Arazi et al. 2014). The advancement of cardiovascular illnesses is associated with an increase in body fat, which is favorably correlated with C-reactive protein (CRP) concentrations (Ellulu et al. 2017). After hepatic beta-oxidation, ketone bodies have been found to lower inflammatory indicators such as CRP, indicating a reduction in cardiovascular risk (Gershuni et al. 2018). When the antioxidant EGCG is given to multiple sclerosis patients, the blood ketone body level rises and the risk of heart disease is reduced (Benlloch et al. 2020). A rare condition known as amyloid light chain (AL) amyloidosis, which has a poor prognosis and few treatment options, has been related to an increased risk of cardiac-involved death. Under current conventional care, daily ingestion of EGCG lowered left ventricular wall thickness and mass and improved left ventricular ejection fraction in patients with AL amyloidosis (Mereles et al. 2010). The anti-inflammatory impact of EGCG reduced neuronal apoptosis and reversed anxiety-like behavior, suggesting that it may be an effective treatment for anxiety-like behavior following myocardial infarction (Wang et al. 2020a).

Cardiac fibrosis, a pathological characteristic of many cardiac illnesses, is characterized by an excessive synthesis and accumulation of myocardial extracellular matrix, including collagen and fibronectin, as well as interstitial fibroblast proliferation. Heart failure patients' cardiac tissue was shown to have elevated amounts of the connective tissue growth factor (CTGF) protein, and the region stained with CTGF was found to be correlated with the level of myocardial fibrosis (Koitabashi et al. 2007). The therapeutic effects of EGCG for the treatment of cardiac fibrosis in people with pressure load hypertrophy are shown by its ability to suppress NF-B activation and the subsequent generation of CTGF (Cai et al. 2013). In the apolipoprotein E-null mice, EGCG decreased developing atherosclerotic lesions without affecting established atherosclerosis (Chyu et al. 2004). Administration of theaflavin-enriched green tea extract in adjuvant to low saturated fat diet once a day reduced low-density lipoprotein-cholesterol (LDL-C) in hypercholesterolemic human subjects (Maron et al. 2003). By altering the proteasome/nicotinamide mononucleotide

adenylyltransferase/SIRT6-dependent signaling cascade, EGCG reduced the cardiac hypertrophy caused by Ang II (Cai et al. 2021). Continuous consumption of a green tea extract rich in catechins reduced body fat, systolic blood pressure, and LDL-C, indicating that doing so lowers the risk of obesity and cardiovascular disease (Nagao et al. 2007).

Due to the rising prevalence of peripheral artery disease (PAD) among older persons, which is brought on by partial or total occlusion of the arteries in the hindlimb, PAD has attracted a lot of attention. In a mouse model of hindlimb ischemia, a copper metal-polyphenol capsule containing EGCG (Cu-EGCG) restored the inflammatory microenvironment of ischemic limbs by removing ROS, reducing the inflammatory reaction, and recovering angiogenesis by inducing the expression of VEGF, PCNA, and CD31 (Duan et al. 2020). One of the main factors contributing to the development of heart hypertrophy is persistently high blood pressure. EGCG reduced left ventricular end-diastolic and systolic dimensions and increased cardiac performance in addition to preventing pressure overload cardiac hypertrophy (Hao et al. 2007). Green tea catechins and EGCG exhibit antithrombotic properties, and their mechanisms of action may be related to their antiplatelet rather than anticoagulant properties (Kang et al. 1999). The elevated autophagy in the myocardium associated with diabetes-related cardiac mitochondrial insufficiency and dysfunction suggests that EGCG may be a viable drug for preventing and treating diabetic myocardial diseases (Liu et al. 2014a). In H9c2 cardiomyoblasts, excessive glucose was found to promote autophagy through the acetylated form of FoxO1. In H9c2 cardiomyoblasts and related cardiac diseases, EGCG reversed acetylated FoxO1-mediated autophagy and decreased the high-glucose-induced oxidative stress (Liu et al. 2014b).

The astounding effect that EGCG has on the protection against isoproterenol (ISO)-induced myocardial infarction in rats provided evidence that EGCG can sustain the redox balance and demonstrate an anti-apoptotic function, via which EGCG protected myocardial infarction and heart failure (Othman et al. 2017). Heart failure is a side effect of the cardiac muscle condition known as hypertrophic cardiomyopathy (HCM), which is characterized by diminished diastolic function. Increased myofilament Ca2+ sensitivity was shown to be the key functional defect initiating the pathogenesis of HCM, and recent genetic studies indicated the importance of troponin (Ahmad et al. 2005). One of EGCG's main targets in the myofilament is cardiac troponin C (cTnC), and it has been proposed that this interaction can stop the growth of cardiac hypertrophy (Robertson et al. 2009). The pharmacological substances to potentially improve diastolic dysfunction of HCM, at least in part, are EGCG and ECG due to their direct Ca2 + −desensitizing actions on cardiac myofilament (Tadano et al. 2010). Because cardiac myofilaments' enhanced Ca2+ sensitivity cause hypertrophic cardiomyopathy, tea metabolites are advantageous substances for the creation of therapeutic medicines. Globally, deep venous thrombosis (DVT) represents a significant threat to public health. Injury to endothelial cells causes inflammatory and oxidative reactions that aid in the development of thrombi. By modifying HIF-1 and VEGF through the PI3K/AKT and ERK1/2 signaling pathways, EGCG in combination with warfarin prevents DVT in rabbits (Li et al. 2022).

3.2 Cancer

3.2.1 Lung Cancer

Lung cancer is a significant source of morbidity and mortality, particularly for men, as well as around the world, and its burden is anticipated to increase further. Usually, lung cancer is diagnosed at an advanced stage of the disease, and there is a delay in treatment. Tobacco use and air pollution are the primary risk factors for lung cancer. Chemotherapy has been accepted as a potential treatment strategy for lung cancer in advanced stages, but its potential toxicity during maintenance therapy is a serious worry. Melanoma growth and lung metastases were significantly suppressed by a combination of dacarbazine and EGCG treatment. The inhibition of cell migration, cell-extracellular matrix, cell-cell interactions, MMP-9, and FAK activities are all related to how EGCG works (Liu et al. 2001). Previous research demonstrated that Lewis lung carcinoma and the ability of mouse melanoma cells to metastasize to the lungs was decreased by the peroral injection of a green tea infusion or EGCG (Taniguchi et al. 1992; Sazuka et al. 1995). A combination of EGCG with docetaxel may be a viable tactic to help boost the effectiveness of docetaxel in reducing metastasis in lung cancer cells, as EGCG-mediated decrease in MMP-2 mRNA expression through JNK signaling revealed. Additionally, by triggering G2/M arrest in CL1–5 cells, EGCG reduced cell growth (Deng and Lin 2011). Human lung adenocarcinoma A549 cells are observed to be noticeably resistant to apoptosis induction by EGCG, although EGCG has been known to exert antiproliferative and proapoptotic actions in many cancer cells. It has been observed that overexpressing Nrf2-mediated HO-1 confers resistance to EGCG's induction of apoptosis (Kweon et al. 2006). Copper transporter 1 (CTR1), a copper influx transporter, was responsible for significant cisplatin internalization in tumor cells. EGCG treatment in vitro and in vivo revealed that long noncoding RNA (lncRNA) named nuclear-enriched abundant transcript 1 (NEAT1) appears to increase CTR1 gene expression by competing for the binding of hsa-mir-98-5p to its target CTR1 (Fig. 2) (Jiang et al. 2016). Later, EGCG-induced raise in ROS level in the A549 xenograft mice model was reported to be responsible for the upregulation of CTR1 expression through the ERK1/2/NEAT1 signaling pathway (Chen et al. 2020a). In cisplatin-resistant A549 cells, EGCG is a potent anticancer agent that increases miR-485 expression in a dose-dependent manner. EGCG-induced expression of miR-485 restrained CSC-like characteristics in cisplatin-resistant A549 via decreasing the expression of CD44, a putative target of miR-485 (Jiang et al. 2018). The CLOCK which is a circadian rhythm protein controls key aspects of a range of diseases including cancer stem-like cells in cancer. EGCG also decreased the CD133+ cells in the sphere formation assay and repressed CLOCK expression in both H1299 and A549 cells. Additional research using xenograft models showed that EGCG decreased lung cancer stem-like cells' capacity to self-renew by targeting CLOCK and so repressed the CSC-like properties of the cancer cells (Jiang et al. 2020). The synergistic benefits of combining green tea polyphenols and chemopreventive drugs are anticipated

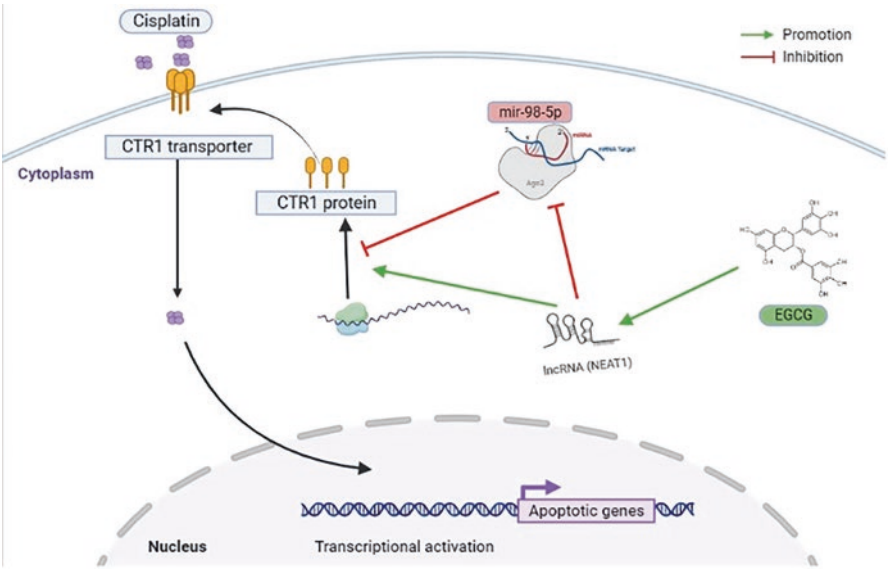

Fig. 2 Mode of action of tea extract and its metabolites-mediated sensitivity to cisplatin in lung cancer

to conquer the side effects of chemopreventive medications. EGCG is the most promising of green tea polyphenols due to the development of synergistic combinations with commercially available anti-tumor drugs to boost the efficiency of cancer therapy and also to minimize potential side effects of conventional antineoplastic medications (Yamauchi et al. 2009).

3.2.2 Liver Cancer

One of the most common types of cancer in people, liver cancer, also known as hepatocellular carcinoma (HCC), is diagnosed commonly at an advanced stage and has a high incidence and mortality rate that are both increasing annually. Surgery is the first line of treatment for HCC, followed by chemotherapy and adjuvant radiotherapy. However, because of their harmful side effects and the most widespread multiple drug resistance, the prognosis overall remains frustratingly dismal. Natural compounds with superior efficacy and lesser toxicity on HCC were the subject of numerous investigations. Since tobacco contains several carcinogenic substances, such as benzo[a]pyrene and N-nitrosamines, chronic use of tobacco has been identified as one of the primary etiological factors linked to malignancies in several organs, including but not limited to the liver. Whereas oral administration of EGCG and theaflavin (TF) restricted N-nitrosodiethylamine (NDEA) induced liver cancer by downregulation of cyclin D1, cMyc, and EGFR associated with self-renewal Wnt/β-catenin along with upregulation of E-cadherin and decreased the prevalence

of CD44-positive population (Sur et al. 2016). Mobilization of endogenous copper and the following prooxidant properties of plant-derived polyphenols has been proposed as the mechanism of its anticancer activity (Hadi et al. 2000). Later, in vivo study revealed that EGCG-mediated accumulation of copper in a prooxidant pattern is a leading mechanism of anticancer potential against hepatocellular carcinoma (Farhan et al. 2015). Invasion is known to be vital for the metastatic progression in HCC; EGCG was reported to inhibit thrombin-induced cancer cell invasion in Hep-3B and primary cancer cells by inhibiting the p42/p44 MAPKinases phosphorylation (Kaufmann 2009). The multifunctional protein known as stress-inducible glucose-regulated protein 78 (GRP78) is responsible for cell proliferation, invasion, survival, and metastasis in a variety of cancer cells. It was proposed that EGCG, a natural GRP78 inhibitor, sensitized hepatoma cells to chrysin in the course of caspase-mediated apoptosis in hepatoma cells and that their combination would be a new chemoprevention and treatment regimen for liver cancer (Sun et al. 2011). The high molecular weight of oolong tea polysaccharides and its polyphenol synergistically inhibited tumor growth of hepatocellular carcinoma; in addition, they increased antioxidative and immune function in H22 tumor-bearing mice model (Wang et al. 2017). By promoting autophagy in hepatoma cells through boosting lysosomal acidification, EGCG effectively suppressed HBV replication and was unfavorable for HBV replication (Zhong et al. 2015). Chronic HBV infection can lead to the development of end-stage liver conditions such as hepatocellular cancer and liver fibrosis. The mechanism by which EGCG prevents HBV transcriptional activity and replication has been identified as ERK1/2 activation and HNF4α downregulation (Wang et al. 2020b).

3.2.3 Stomach Cancer

One of the most common cancers and the leading source of malignant-associated mortality worldwide is gastric cancer. In underdeveloped nations, the prevalence of stomach cancer is particularly high. Patients with locally advanced gastric cancer continue to get treatment primarily by gastrectomy. Despite significant improvements in diagnosis and care, the overall 5-year survival rate following curative gastrectomy is only around 30%. Therefore, in creating innovative methods for preventing and reducing stomach cancer, cell proliferation is crucial for developing potent treatments (Yang et al. 2016). Green tea, and its metabolite specifically EGCG induced tumor cell apoptosis, repressed formation of fresh blood vessels and subsequent colon tumor growth in vivo by downregulation of VEGF (Jung et al. 2001). In a human stomach cancer cell line, okadaic acid-induced TNF-induced gene expression and TNF-release were reduced by EGCG and other tea polyphenols, whereas AP-1 and NF-B transcriptional factors were activated (Okabe et al. 1999). Additionally, it has been observed that EGCG induces apoptosis in gastric cancer cells via suppressing survivin expression in a p73-dependent manner (Onoda et al. 2011). The p68 DEAD-box RNA helicase directly upregulates proto-on genes, which aids in the development and spread of cancer (cyclin D1, c-Jun, c-Myc, and

Fra-1). In fact, p68 is regarded as a possible cancer marker and a key molecular target for cancer-prevention drugs (Mooney et al. 2010; Fuller-Pace and Moore 2011). Later studies provide new light on the molecular mechanism of EGCG activity, showing that EGCG inhibits the growth of human stomach cancer cells (AZ521) by inhibiting β-catenin oncogenic signaling through direct interaction with the proteasome (Tanaka et al. 2011). Despite the earlier research that suggests consumption of green tea extract (GTE) and EGCG may help prevent N-ethyl N'nitro-N-nitrosoguanidine (ENNG)-induced gastrointestinal carcinogenesis, subsequent epidemiological studies have failed to definitively confirm or deny a green tea consumption's ability to prevent cancer (Yamane et al. 1996; Yuan 2013). Epigastric discomfort and sleeplessness caused by caffeine have been proposed as minor adverse effects of GTE, and further decaffeinated GTE has been suggested to overcome the above adverse effects (Yamane et al. 1996).

3.2.4 Prostate Cancer

Diet and nutrients are thought to be major environmental risk factors in candidates diagnosed with prostate cancer. It has been hypothesized that the prevalence rate of prostate cancer in East Asian countries more than in Western countries can be attributed to their greater consumption of green tea polyphenols. Tea polyphenols have been proven to be responsible for the suppression of prostate carcinogenesis (Chen et al. 2019). Expression of p21 and Bax are necessary for EGCG-induced apoptosis in the p53-dependent pathway. Bax expression was eliminated by siRNA transfection, which prevented EGCG-induced apoptosis but had no effect on the expansion of cells in the G1 phase. However, in PC3 cells that express the p53 protein, ablation of the p21 protein blocked EGCG-mediated G1 arrest and apoptosis (Hastak et al. 2005). The low lipophilicity of EGCG reduced its physiological activities and limited its bioavailability in vivo. While enzymatic esterification of EGCG with lauric acid to produce lipophilized EGCG derivative (LEGCG) was demonstrated to increase its bioactivity. LEGCG has been shown to have anti-proliferation properties on DU145 prostate cancer cells via the p53/p21 activation pathway, similar to EGCG (Chen et al. 2019). Additionally, EGCG nano-formulated on chitosan is released slowly in simulated gastric juice with an acidic pH and quickly in simulated intestinal fluid. In addition, when compared to EGCG and control groups, Chit-nanoEGCG dramatically reduced prostate-specific antigen levels and tumor growth in a prostate cancer xenograft model (Khan et al. 2014). The HGF-induced activation of cellular mesenchymal-epithelial transcription factor (c-Met) located in lipid rafts in prostate tumor cells was inhibited by green tea polyphenol (EGCG) by altering the structure of lipid rafts (Duhon et al. 2010). High levels of growth factors, like vascular endothelial growth factor (VEGF) and hepatocyte growth factor (HGF), are secreted by myofibroblasts. These growth factors can bind to specific receptors on tumor epithelial cells to promote proliferation, migration, and invasion (Orimo et al. 2005). Combinations of EGCG and luteolin-reduced TGF-β synergistically produced myofibroblast characteristics in prostate fibroblast cell lines,

suggesting that these natural substances inhibit the progression of cancer by focusing on the tumor microenvironment (Gray et al. 2014). In androgen-sensitive (LNCaP) and androgen-insensitive (PC-3) human prostate cancer cell lines, EGCG specifically induced COX-2 without affecting the expression of COX-1 mRNA or protein levels, and this suggested using chemotherapeutic drugs in conjunction with EGCG as an improved approach for the prevention and cure of prostate cancer (Hussain et al. 2005). Matrix metalloproteinases (MMPs) and their binding associate, tissue inhibitors of MMPs (TIMPs), which are implicated in metastatic prostate cancer, are regulated primarily by epigenetic mechanisms. Patients undergoing prostatectomy who consumed EGCG (polyphenon E) along with grade-matched controls showed an increase in plasma TIMP3 levels. TIMP3 activation was identified by epigenetic research as a crucial epigenetic process that green tea altered to restore the MMP: TIMP balance, which slowed down the progression of prostate cancer (Deb et al. 2019). The emergence of drug resistance restricts its potential use of apoptosis-inducing ligands (TRAIL/Apo2L). But a combination of EGCG and Apo2L/TRAIL sensitized TRAIL-resistant LNCaP cells to TRAIL-mediated apoptosis by upregulating the expression of TIMP1 (Siddiqui et al. 2008). Treatment of metastatic prostate and initial cancers originating from tumor-initiating cells has been suggested using localized delivery of a combination of high-dosage EGCG and low amounts of doxorubicin (Stearns et al. 2010).

3.2.5 Breast Cancer

The most prevalent cancer in women worldwide is breast cancer, with a sixfold difference between high-risk locations (such as North America and Europe) and low-risk regions in terms of incidence (e.g., Asia) (Chang et al. 2003). Similar to prostate cancer, regional variance in the incidence of breast cancer is sometimes related to a diet high in foods and beverages that contain antioxidants, such as green tea. A negative relationship between green tea drinking and breast cancer incidence was found in a meta-analysis that included 5617 cases of breast cancer (Ogunleye et al. 2010). Raman spectroscopy imaging revealed EGCG treatment increased intracellular saturated fatty acids accumulation and decreased mitochondrial membrane potential, thereby triggering cytochrome c release and apoptosis in triple-negative breast cancer cells (MDA-MB-231) (Mignolet et al. 2018). A well-defined combination of decaffeinated green tea catechins of pharmaceutical grade called polyphenon E (Poly E) has 65% of its contents as EGCG (Chang et al. 2003). During a 6-month phase, IB multicenter clinical experiment in women with a history of stage I–III hormone receptor-negative breast cancer, the most tolerated dose of an oral green tea extract using polyphenon E (Poly E) was discovered to be 600 mg twice a day (Crew et al. 2011). Although 5-aza-20-deoxycytidine (5-aza 20 dC), a DNA demethylating drug, has demonstrated clinically effective in treating acute myeloid leukemia, it was a modest success in treating breast cancer but has significant damage to normal cells, whereas EGCG, a green tea polyphenol, possesses DNA demethylating and anticancer capabilities without being particularly harmful to

normal cells (Tyagi et al. 2015). Treatments with both EGCG and a pro-drug of EGCG (pEGCG) inhibited the proliferation of human breast cancer MCF-7 (ER + ve) and MDA-MB-231 (ER-ve) cells without affecting MCF10A cells. Inhibition of activities of histone acetyltransferase and DNA methyltransferase and subsequent hypomethylation of human telomerase reverse transcriptase (hTERT) promoter has been postulated as an epigenetic mechanism associated with cellular apoptosis (Meeran et al. 2011).

EGCG was also reported to inhibit the growth, survival, and invasive properties of aggressive inflammatory breast cancer (IBC) cells (SUM-149,190). In addition, EGCG-treated decreased VEGF-D secretion in IBC cells and tube formation and endothelial cell migration in the human telomerase reverse transcriptase-human dermal lymphatic endothelial cells model (Mineva et al. 2013). Obese subjects have an increased risk of developing hormone receptor-positive breast cancer by obesity-independent regulation of adipocyte fatty-acid-binding protein (Li et al. 2019a). Breast cancer cells can proliferate, advance, invade, and migrate when dysfunctional adipocytes emit cytokines, adipokines, and metabolic substrates. Adipocyte secretion can prevent apoptosis, cause inflammation and hypoxia, and change the gene expression profile (Chu et al. 2019). Inhibiting adipogenesis and altering the secretome profile of adipocytes with EGCG prevented the beginning of an obesogenic environment that favored the development of breast cancer (Suarez et al. 2021). Additionally, EGCG's antitumor effects were mediated through the inhibition of crucial glycolytic pathway enzymes and the regulation of glucose metabolism (Wei et al. 2018). Reduced MMP-9 in radiotherapy patients who received EGCG was a factor in NF-B level reduction and suppression of breast cancer cell growth (Zhang et al. 2012). Chemoprevention, adjuvant therapy, and the treatment of metastatic breast cancer appear to benefit from the synergistic interaction between green tea catechins and selective estrogen receptor modulators (SERMS) in the treatment of estrogen receptor (ER)-positive and ER-negative breast cancer (Yiannakopoulou 2014).

3.2.6 Cervical Cancer

The third leading cause of death from cancer among women worldwide each year is cervical cancer. Although cervical cancer incidence and mortality have reduced in developed nations, this disease still poses a severe threat due to its high estimated incidence and mortality in developing and underdeveloped countries. Cervical cancer is often treatable if diagnosed early and can be prevented by vaccination. The treatments for metastatic cervical cancer are ineffective and have substantial side effects. Furthermore, cervical adenocarcinoma is considered to be less responsive to chemotherapy and radiation than squamous cell cancer. Therefore, there is a need for study on natural substances; numerous dietary components have been shown to have therapeutic and preventative properties in the development of cancer. Because dietary chemicals have a variety of biological actions and are less toxic and have fewer adverse effects than conventional chemotherapeutic medicines, they may play

a considerable role in the prevention and cure of cancer. The fact that EGCG causes telomere fragmentation in HeLa cells may be important for understanding why it causes apoptosis in malignant cells but not in normal cells, MRC-5 fibroblasts (Li et al. 2005). Premalignant lesions and adenocarcinoma of the cervix that is thought to be less sensitive to radiation and anticancer medications were also successfully prevented and treated by EGCG. The reduction of telomerase activity, the induction of apoptosis, and cell cycle dysregulation were found to be the antitumor actions of EGCG (Noguchi et al. 2006). Genes implicated in the stimulation of proliferation, adhesion, motility, and invasion activities were downregulated, whereas genes producing proteins that antagonize the above effects were upregulated by EGCG administration. The above trend suggests EGCG may play a significant role in cervical cancer treatment as an anti-angiogenic strategy (Tudoran et al. 2012). In the cervical cancer cell line HeLa, EGCG's capacity to depolymerize microtubules through tubulin binding has been linked to its antiproliferative action (Chakrabarty et al. 2015). Within 2 years, 10% of HPV (+) women will have high-grade cervical dysplasia. The prevalence of precancerous cervical lesions worldwide is predicted to have sharply increased based on the number of women who are HPV-positive globally. The HPV vaccines are efficient at preventing disease, but they have no benefits for women who already have the virus (Khan et al. 2005). TriCurin, a novel combination of curcumin, EGCG, and resveratrol, has been described as effective against HPV (+) cells, and its microemulsion-based cream formulation is a promising medication for cervical cancer prevention and treatment (Einbond et al. 2021). IGF-IR kinase activity has been linked to the pathophysiology of cancer, and interference with IGF-IR signaling has been shown to have anticancer effects. As a result, a molecule that inhibits IGF-IR kinase activity may have therapeutic potential in the treatment of cancer. By competing with ATP to bind with active IGF-IR, EGCG reduced cell growth and transformation and prevented IGF-IR downstream signaling, subsequently confirming the chemopreventive effect of EGCG on cancer cell line HeLa (Li et al. 2007). In a mouse model of the HeLa tumor, photothermal-chemotherapy using zeolitic imidazolate framework-8 (ZIF-8) coated with polydopamine (PDA) and dual-loaded with doxorubicin and EGCG was shown to be a promising approach to the problems associated with the use of synergistic chemotherapy and photothermal therapy in the treatment of cervical cancer (Chen et al. 2020b).

3.3 Chronic Respiratory Disease

Chronic obstructive pulmonary disease (COPD) is thought to be primarily brought on by smoking, with the accumulation of oxidant load and airway mucus hypersecretion playing significant roles in the pathogenesis of COPD. Although other types of tea displayed comparable nonsignificant trends of connections, green tea seemed to be the most potent. In Asian groups including Korea, drinking tea was linked to decreased COPD incidence rates (Oh et al. 2018; Ng et al. 2021). According to

reports, stimulation of the epidermal growth factor receptor (EGFR) regulates mucin synthesis and secretion in the airways. Through the inhibition of the EGFR signaling pathway, EGCG treatment reduced oxidative stress, abnormal airway mucus production, and chronic airway inflammation caused by cigarette smoke (Liang et al. 2017). The diverse disease of asthma is influenced by both environment and heredity. Worldwide, environmental pollution is getting worse due to the country's rapid urbanization and industrial growth. Airborne fine dust particles (FDPs) and gaseous pollutants make up ambient air pollution. In BEAS-2B cells, flavonols, catechins, and polysaccharides from green tea all had protective effects both individually and collectively against FDP-induced cellular damage (Kim et al. 2021). Through its inhibition of ROS generation, EGCG and gallocatechin gallate protected against acute lung injury and the progression of acute lung injury brought on by air pollutants brought on by urban aerosols (Tanaka et al. 2022). In asthmatic rats out in the open to fine particulate matter (PM2.5), EGCG significantly reduced levels of inflammatory cell infiltration and inflammatory factor. Additionally, EGCG reduced lung injury by inhibiting the expression of high mobility group box protein 1 (HMGB1) and receptor for advanced glycation end products (RAGE) in bronchial epithelial cells and alveolar epithelial cells, respectively (Li et al. 2019b). Additionally, because of its capacity to bind to the IgE receptor (Fc-epsilon RI) on mast cells, IgE plays a significant role in asthma and allergic reactions. Green tea extract can reduce IgE production without causing cell death or apoptosis in B cells (Hassanain et al. 2010).

The dangerous and fatal lung condition pulmonary arterial hypertension (PAH), which frequently results in right ventricular heart failure, has a high morbidity and fatality rate. It is well recognized that MMP-2 predominantly contributes to the expansion of several lung disorders, including PAH (Tan et al. 2012). The galloyl group in EGCG and ECG appears to play a crucial role in the interplay with pro−/active forms of Matrix metalloproteinases (MMP-2) and inhibited MMP-2 activity, according to bioinformatics research using molecular docking and gelatin zymogram analysis. This finding suggests catechins may be beneficial as therapeutic drugs in preventing pulmonary hypertension (Chowdhury et al. 2017). Thoracic irradiation for lung cancer is connected with a serious consequence known as radiation-induced pulmonary fibrosis. Steroids or nonsteroidal anti-inflammatory drugs are part of the current clinical treatment for pulmonary fibrosis. In a rat model of radiation-induced lung fibrosis, EGCG treatment, but not dexamethasone, stimulated Nrf-2 and its associated antioxidant enzymes HO-1 and NQO-1 (YOU et al. 2014). In a lung inflammatory model, oral administration of green tea extract suppressed ROS production, reduced elastase activity, apoptosis, and chemokine-induced neutrophil chemotaxis, therefore considerably lowering pulmonary fibrosis (Donà et al. 2003). Together, these findings make it abundantly evident that using green tea extract and its constituents as a treatment for lung injury has a protective impact and provides a cutting-edge therapeutic strategy.

3.4 Diabetes

A severe global health issue, diabetes affected 537 million adults (20–79 years) in 2021, and it was expected that number will climb to 783 million by 2045. Among the several forms of diabetes, type 2 diabetes is typically encountered in middle-aged and older persons and is rare in teenagers and young adults. Type 2 diabetes is thought of as a metabolic condition (Xie et al. 2022). According to theories, tea polyphenolics can increase insulin sensitivity and inhibit sugar transporters in the small intestine to lower blood sugar levels. Gallated catechins decreased GLUT2 expression both at the gene and protein levels as compared to non-gallate catechins, indicating they can prevent postprandial hyperglycemia in people, including those with or at risk for type 2 diabetes (Ni et al. 2020). While CG and EGCG reduced insulin-induced translocation of GLUT4 by the insulin signaling route in 3 T3-L1 cells, EC and EGC increased the translocation of GLUT4 by activation of phosphatidylinositol 3′-kinase (PI3K) (Ueda et al. 2010). In L6 skeletal muscle cells, EGCG was discovered to improve glucose uptake and stimulate GLUT4 translocation to the plasma membrane via PI3K/AKT signaling pathway. EGCG also inhibited α-glucosidase in a reversible and noncompetitive manner (Xu et al. 2019). The small intestine may not absorb too much glucose if alpha-glucosidase is inhibited. Alpha-glucosidase inhibitors including acarbose, miglitol, and voglibose are the most frequently prescribed medication for people with type 2 diabetes (T2DM). The alpha-glucosidase activity was found to be inhibited by tea polyphenol (EGCG), with activity better than that of a typical amylase inhibitor (acarbose). By slowing down the process of intestinal starch digestion, functional foods containing EGCG may delay the onset of diabetes (Dai et al. 2020). Optimized preparation of corn starch with 0.5% tea polyphenols or 0.5% EGCG with greater inhibitory effects on amylase and amyloglucosidase was identified as a special dietary or functional food for improving beneficial postprandial glucose levels in diabetic patients (Zhang et al. 2018). In addition to α-amylase inhibition, EGCG improved insulin sensitivity and reduced blood glucose content via activation of drug-responsive receptors, constitutive androstane receptor (CAR)/pregnane X receptor (PXR), and subsequently prevented diabetes (Li et al. 2018). Diabetes and cardiovascular disease are both at risk due to insulin resistance, a defining characteristic of metabolic illnesses. Insulin resistance is a result of impaired vascular endothelium insulin response. Supplementation of EGCG improved glucose tolerance, insulin sensitivity, glucose metabolism, and endothelial function modulating the inflammatory response of HFD-induced insulin resistance (Jang et al. 2013). In humans, elevated intracellular cortisol levels can lead to fat formation, reduce insulin sensitivity in adipose tissues, increase glucose production in the liver, and increase the risk for metabolic syndrome including diabetes. Glucocorticoid receptor-inert cortisone is converted to receptor-active cortisol by the microsomal enzyme 11-hydroxysteroid dehydrogenase type 1 (11-HSD1). The greatest suppression of 11-HSD1 activity was found in treatment with tea extract, and the main polyphenolic ingredient EGCG is found in it (Hintzpeter et al. 2014). The skeletal muscle plays a role in energy control and

homeostasis and is the main location for insulin-stimulated glucose uptake. Elevated plasma concentrations of free fatty acids (FFAs) are linked to severe resistance to insulin in skeletal muscle and may be a major factor in both obesity and its associated type 2 diabetes mellitus, which are both conditions characterized by increased insulin resistance. By inhibiting IRS-1 Ser307 phosphorylation and inducing Akt, ERK1/2, p38 MAPK, and AMP-activated protein kinase activation, tea polyphenol, particularly EGCG, restored palmitate-mediated insulin resistance in C2C12 skeletal muscle cells of mouse origin. Additionally, EGCG enhanced glucose absorption in C2C12 cells, prevented intracellular lipid buildup, and suppressed acetyl-CoA carboxylase activity (Deng et al. 2012).

One crucial metabolic issue in the emergence of type 2 diabetes is pancreatic cell failure. Reduced viability and malfunction of cells would hasten the development of diabetes, which is linked to greater mortality. Chronic excessive glucose exposure may cause pancreatic cells to gradually become stressed as a result of the overloaded metabolism, which may lead to the development of glucotoxicity. Through the activation of IRS2 and AMPK signaling, EGCG and Rutin synergistically decreased the glucotoxicity effects. This suppressed lipogenic enzymes and attenuated type 2 diabetes by protecting pancreatic cells (Cai and Lin 2009). The death of pancreatic beta-cells by autoimmunity causes type 1 diabetes (T1D), which results in inadequate insulin production. In nonobese diabetic (NOD) mice, a model for type 1 diabetes, epigallocatechin-3-gallate (EGCG) increased the protein levels of peroxiredoxin 6 (PRDX6), a key antioxidant defense protein, which delayed and avoided the onset of autoimmunity (Dickinson et al. 2014). In comparison to the control animals, the NOD mice supplied with EGCG exhibited significantly greater levels of the anti-inflammatory cytokine (IL-10), insulin, and survival rate, but decreased HbA1C level (Fu et al. 2011). When normal islets were exposed to IFN-γ, pancreatic and duodenal homeobox (Pdx-1) transcription factor's nuclear localization was reduced. These findings imply that decreased nuclear localization of Pdx-1 mediates IFN-induced cell dysfunction. Pretreatment with the tea polyphenol, EGCG restored Pdx-1's nuclear localization (Pondugala et al. 2015).

Diabetic nephropathy (DN) is one of the acute vascular consequences of diabetes. The prevention of the shift of brain microglia toward the proinflammatory M1 phenotype depends critically on insulin-like growth factor 1 (IGF-1). Treatment with EGCG reduced the pain-like behaviors brought on by diabetes, decreased neuroinflammation, stopped the polarization of M1 microglia, and increased IGF-1 expression in the microglia (Chen et al. 2022b). Additionally, EGCG provided a potential therapeutic approach to improve the symptoms of DN by inhibiting ER stress by attenuating the production of GRP78, pPERK, and caspase-12 protein and protecting against high glucose-induced podocyte death (Xiang et al. 2017). Albumin excretion in diabetic nephropathy (DN) is a serious vascular consequence of diabetes that results in glomerular filtration failure and necessitates dialysis treatment owing to uremia. Another known cause of DN is abnormal protein kinase C (cPKC) activation. De novo production of diacylglycerol (DAG) increases in hyperglycemia, and because DAG activates cPKC, this aberrant activation causes diabetic vascular problems, including DN. The lipid kinase, diacylglycerol kinase (DGK),

which phosphorylates DAG and generates phosphatidic acid, can inhibit cPKC activation by lowering the concentration of DAG. As a novel approach to treating DN, EGCG induced DGKα translocation and ameliorated albuminuria under high-glucose circumstances (Hayashi et al. 2020). Supplementing with EGCG (40 mg/kg and 80 mg/kg) in two diabetic rats improved hyperlipidemia, hyperglycemia, and renal histopathological dysfunction and suppressed NLRP3 inflammasome and renal ER stress. It clearly showed how the EGCG treatment suppressed the ER stress-induced NLRP3 inflammasome to have renoprotective effects (Yang et al. 2022).

Maternal diabetes raises the incidence of neural tube defects in kids, and hyperglycemia is a factor that causes the malformation of an embryo. Neural tube defects are widespread complicated congenital anomalies of the central nervous system that arise during embryogenesis. EGCG inhibits the expression and activities of DNA methyltransferases, which suppresses DNA hypermethylation and restores the expression of crucial genes for neural tube closure (Grhl3, Pax3, and Tulp3). This, in turn, prevents the development of maternal diabetes-induced neural tube abnormalities (Zhong et al. 2016). Under a high-glucose setting, cleaved-cas3 upregulation induces death in retinal Muller cells. EGCG prevents apoptosis by activating autophagy, and it is an appealing therapeutic strategy for diabetic retinopathy (Wang et al. 2019). By accelerating reepithelialization and angiogenesis by boosting Ki-67 and CD31, respectively, and by improving the cellular rearrangement of granulation tissue by inducing the activity of myofibroblasts, collagen sponge combined with EGCG improved wound healing in diabetic mice (Kim et al. 2008).

3.5 Neurological Disease

Memory loss and cognitive impairment brought on by cholinergic system dysfunction; a buildup of insoluble intracellular (iA) and extracellular (eA), primarily eA-42; intracellular aggregation of the microtubule protein TAU (tubulin-associated unit) in neurofibrillary tangles; synaptic dysfunction; and neuroinflammation are all symptoms of Alzheimer's disease (AD), a progressive multifaceted neurodegenerative disorder. Tau oligomers can be converted to unfolded monomers by EGCG, which also inhibits tau aggregation and restrains tau phosphorylation (Guéroux et al. 2017). To enhance the antioxidant system and learning and memory function in AD-rats, EGCG administration reduced the hyperphosphorylation of the tau protein, decreased β-site APP-cleaving enzyme 1 (BACE1) expression, and upregulated Aβ1–42 expression (Nan et al. 2021). In the transgenic AD mice model, the combination of an α-secretase activator (EGCG) and a β-secretase modulator (ferulic acid) reduced neural inflammation and oxidative stress and facilitated the development of alternative approaches, such as the use of naturally occurring dietary substances with anti-AD therapeutic potential (Mori et al. 2019). One of the main causes of the clinical characteristics of Down syndrome (DS) is thought to be the dual-specificity tyrosine phosphorylation-regulated kinase 1A (DYRK1A) gene,

which was located on chromosome 21. Additionally, EGCG has been suggested as a potential nutraceutical candidate for the treatment of several DS phenotypic characteristics because it is a potent and secure DYRK1A inhibitor (BAIN et al. 2003). Recent research found that supplementing EGCG with fish oil rich in omega-3 s corrected mitochondrial respiratory chain complex activities without enhancing developmental performance in DS children aged 1 to 8 years (Scala et al. 2021).

Obesity and T2DM have been suggested as possible modifiable risk factors for late-onset AD (LOAD). Therefore, medications that control glucose levels and reduce amyloid β (Aβ) can block or delay cognitive decline through central insulin changes (Clarke et al. 2018). In high-fat-diet-fed APP/PS1 mice, EGCG administration enhanced the insulin signaling pathway in the liver and hippocampus tissues. By raising levels of α-secretase, EGCG significantly reduced brain Aβ accumulation and plaque burden. Treatment with attenuated neuroinflammation by reducing astrocyte activation and toll-like receptor 4 (TLR4) levels. Together, EGCG had a significant impact on metabolic and neurobiological mechanisms of obesity-induced AD, by which it protects against cognitive decline, raising the possibility that this substance may be a promising disease-modifying therapy for neurodegenerative diseases (Ettcheto et al. 2020). Protein carbonyls, hydroxynonenol, and nitrosylate-modified proteins can be found in the brain and CSF of patients with HIV infection, where they tend to be associated with the severity of neurocognitive impairment. Trans-activator of transcription (Tat) and envelope glycoprotein (Gp120) are HIV proteins that have been linked to the development of neurotoxicity, either directly by affecting neurons or indirectly by triggering macrophages and glial cells. The family of catechins (EC and EGCG) was found to be a class of compounds with strong neuroprotective properties in neurodegenerative diseases, including HIV-associated neurocognitive disorders, during the screening of neuroprotective molecules using 3-nitropropionic acid cytotoxic effects in primary neuronal cells (Nath et al. 2012). Mesenchymal stem cells generated from adipose tissue (ADSC) have anti-inflammatory and neuroprotective properties. By reactivating RA-induced suppression of the PI3K/Akt survival pathway, EGCG and ADSCs jointly boosted the neuroprotective potential to suppress the detrimental effects of rheumatoid arthritis (RA) on the brain (Chen et al. 2022a). A point mutation of the human plasma protein transthyretin (TTR), which binds to and facilitates the transport of thyroxine, has been implicated as the cause of the genetic disease named familial amyloid polyneuropathy (FAP). The structural investigation of the interaction between EGCG and TTR showed that EGCG binds to TTR, inhibits the development of TTR amyloid fibrils, and raised the potential that EGCG would be a candidate substance for FAP therapy (Miyata et al. 2010).

The neurodegenerative disease Parkinson's disease (PD) is the second highest prevalent, and EGCG has been shown to have positive effects on PD sufferers. In a PD model of *Drosophila melanogaster* with PINK1 (PTEN-induced putative kinase 1) mutations, EGCG restored an abundance of gut microbiota; further transcriptomic analysis revealed gene encoding Turandot M protein as the central gene responding to EGCG or microbial manipulations (Xu et al. 2020).

In Western nations, black tea is far more popular than green tea. Just as effective as EGCG in theaflavins prevented and reversed the production of amyloid, and both have a comparable mechanism of action. Theaflavins, which are present in high concentrations in black tea, is thus suggested to be capable "nutraceuticals" that may postpone the development of age-related amyloid disorders (Grelle et al. 2011). Following oral treatment of EGCG (100 mg), it was demonstrated that EGCG could not be discovered in the brain tissues of young control rats but was present in the brain tissues of naturally aged rats with cognitive deficits (CI). This alteration in blood-brain barrier (BBB) permeability serves as the physiological structural underpinning for EGCG treatment to enhance learning and memory, offering strong support for the druggability of EGCG in the anti-AD therapeutic sector (Wei et al. 2019).

3.6 Arthritis

An inflammatory condition called rheumatoid arthritis (RA) causes ongoing synovial joint inflammation, which eventually leads to the gradual deterioration of bone and cartilage. Though the underlying mechanisms of RA are not well understood, it is becoming more and clearer that the pathogenesis of RA is significantly influenced by the upregulation of IL-17 production and Th17 response. Another specialized subgroup of T cells called Tregs cells controls immune system homeostasis, inhibits immune system activation, and promotes tolerance toward self-antigens. The local cytokine environment or the stimuli to which the cells are exposed determine whether uncommitted CD4+ T cells differentiate into Th17 cells or Tregs. By inducing NRF-2, HO-1, and inhibiting STAT-3 activation, EGCG treatment reduced arthritis symptoms, decreased osteoclastogenesis, and T helper 17 cell activation; raised the number of regulatory T cells; and prolonged the antiarthritic effects (Lee et al. 2016). In later stages of RA, myeloperoxidase (MPO), which is highly present in neutrophils, is believed to contribute to cartilage damage, and hypochlorous acid (HOCl), an MPO-specific oxidant, has some anti-inflammatory properties. By restoring the HOCl-production of MPO, constant oral administration of EGCG reduced both the acute and chronic phases of RA (Leichsenring et al. 2016). By increasing the expression of the microRNA has-miR199a-3p in interleukin (IL)-1-stimulated human osteoarthritis (OA) model, EGCG reduced COX-2 or prostaglandin E2 (PGE 2) synthesis in chondrocytes (Rasheed et al. 2016). In various autoimmune diseases, including RA, adipose tissue-derived mesenchymal stem cells (ADSC) have neuroprotective and anti-inflammatory properties. By reactivating the PI3K/Akt survival pathway that RA suppressed, EGCG synergistically increases the neuroprotective capacity of ADSCs to suppress the harmful effects of RA on the brain of Wistar rats (Chen et al. 2022a). Theaflavin-3,3′-gallate (TFDG) decreased osteoclast differentiation more potently than EGCG, despite both tea polyphenols, EGCG and TFDG, inhibiting osteoclast production and differentiation via inhibiting MMP-2 and MMP-9 activities (Oka et al. 2012).

By controlling the catalytic activity of cyclooxygenase-2 (COX-2) and inducible nitric oxide synthase (iNOS), respectively, EGCG protects IL-1β-induced generation of catabolic mediators (NO and PGE(2)) in human chondrocytes. This suggested that EGCG might be a useful therapeutic compound for preventing cartilage resorption in joints with arthritis (Ahmed et al. 2002). An important step in the pro-inflammatory cytokine-induced signaling cascade in chondrocytes and synoviocytes that results in the creation of various mediators of cartilage destruction in an arthritic joint is the activation of mitogen-activated protein kinases (MAPK). The EGCG-inhibited IL-1β-induced phosphorylation efficiency of c-Jun N-terminal kinase (JNK) isoforms, an abundance of phospho-c-Jun, and DNA-binding potential of AP-1 in osteoarthritis chondrocytes without influencing the phosphorylation of p38-MAPK suggested advantages of EGCG in inhibiting IL-1beta-induced catabolic effects in OA (Singh et al. 2003). Along with the aforementioned effects, EGCG also prevented NF-kappaB from activating and translocating to the nucleus by preventing the breakdown of inhibitor of nuclear factor kappa B (IkappaB), in the cytoplasm of IL-1beta-stimulated chondrocytes (Singh et al. 2002). Inflammation may be a connecting factor between RA and cardiovascular dysfunction because synovial inflammation in RA extends systemically and silently alters chronic inflammation, which is characterized by increased cytokine (IL-6 and TNF) release and abnormally elevated levels of C-reactive protein (CRP) (Riegsecker et al. 2013). EGCG's positive benefits in controlling these two pathologies through the same driving cause support the idea that it might be a potential molecule that could act against CV and RA (Fig. 3).

3.7 Inflammatory Bowel Disease (IBD)

IBD stands for idiopathic intestinal chronic inflammatory disease and has two main conditions: ulcerative colitis (UC) and Crohn's disease (CD). It is characterized by the remission and aggravation of clinical syndromes that are marked by diarrhea and intestinal bleeding, disrupting the epithelial barrier and resulting in epithelial ulceration. Immune cells including but not limited to lymphocytes, monocytes, macrophages, and neutrophils are the primary players in IBD. The chemokines, cytokines, and adhesion molecules are necessary for their migration into the inflamed tissue and their activation there. With their well-known anti-inflammatory, antioxidant, and antibacterial activity, tea metabolites have a significant potential for use in treating IBD and related aberrant disorders. Increased intestinal barrier permeability is thought to be a main issue in the development/pathogenesis of IBD. IBD etiology is heavily influenced by TNF-α, which is partly responsible for tight junction (TJ) barrier disruption. Epicatechin (EC) inhibited NADPH oxidase (NOX)-mediated NFκ-B activation and downstream TJ disruption, which helped to prevent TNF-α-mediated Caco-2 cell barrier permeabilization. Diets high in EC may help to reduce the increased intestinal permeability brought on by IBD (Contreras et al. 2015). Chronic administration of nonsteroidal anti-inflammatory

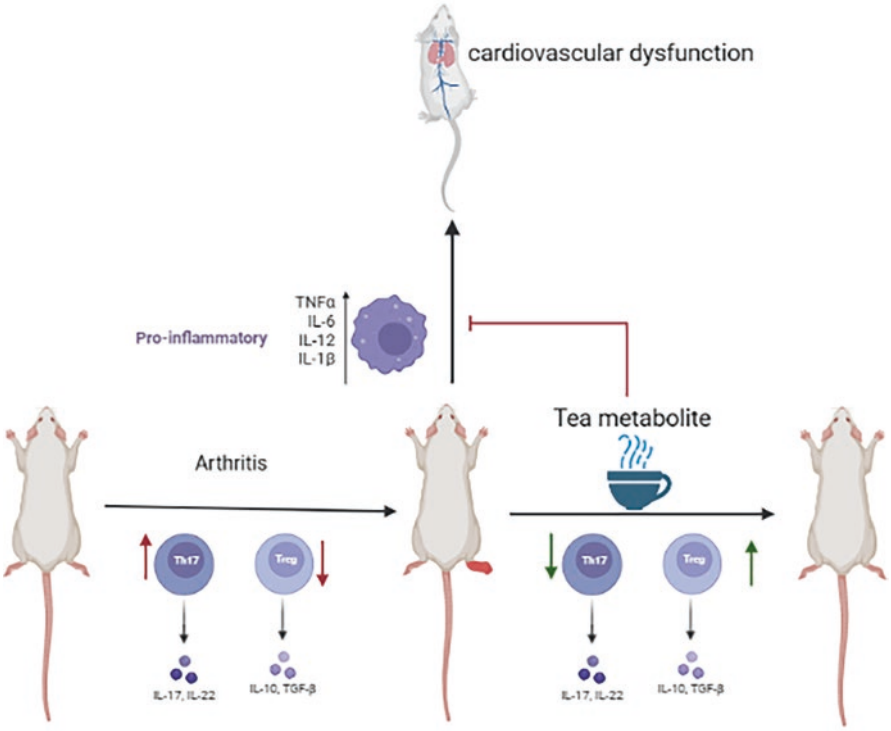

Fig. 3 Mode of action tea extract and its metabolite in treatment of arthritis and associated cardiovascular dysfunction

drugs (NSAIDs) called indomethacin (INDO) strongly affected the integrity of the gastrointestinal (GI) barrier function and is associated with adverse effects on GI mucosa (Gotteland et al. 2001). The green tea-derived polyphenol EGCG revealed beneficial effects on INDO-induced acute gastric ulceration by inducing expression of the cyclooxygenase (COX) isoforms and prostaglandin (PG) synthesis (Adhikary et al. 2011). By controlling cell gap junctions and enhancing TJ expression in the epithelium, tea metabolites may prevent gastrointestinal lesions from progressing into cancer. Inflammation in IBD may benefit from the regulation of inflammation. Finally, flavonoids from tea may potentially promote IBD recovery by stabilizing gut flora.

Even though the majority of IBD patients react to traditional treatments, a considerable portion stops responding or develops severe clinical colitis. Where colon resection is thought to be a curative method. Ileal pouch-anal anastomosis (IPAA) restores anal continence, but patients may experience acute or chronic pouch inflammation (pouchitis). The EGCG decreased the frequency of bowel movements and the amount of blood in the stool in patients who underwent IPAA, demonstrating its efficacy against the pouchitis-related disease of IBD (Mehta et al. 2018). The DSS

model of colitis benefits from the anti-inflammatory characteristics of EGCG, a result that may be mediated by its robust antioxidative effects. EGCG and piperine synergistically improved body weight, clinical course, and overall survival of DSS-induced colitis model by reducing neutrophils accumulation, malondialdehyde, and myeloperoxidase in the colon tissue (Brückner et al. 2012). Green tea polyphenol and EGCG both increased antioxidant levels and reduced colitis severity like sulfasalazine, but only EGCG decreased leptin levels in DSS-induced ulcerative colitis model (Oz et al. 2013). However, there are relatively few studies that could support the biological activities of tea metabolites, either in IBD patients or in animal models of the disease. More research is required to fully understand the precise actions and conceivable mechanisms, even at the molecular level.

3.8 Obesity

Globally, obesity and its associated metabolic disorders are a major public health issue that raises the risk of chronic illnesses like diabetes, cardiovascular disease, and cancer. A high-fat diet is a significant risk factor for developing obesity and related metabolic syndrome. Epidemiological research has demonstrated a connection between obesity and diet. Increasing physical activity and/or lowering energy consumption as part of a lifestyle change have successfully lowered body weight; nevertheless, different measures are needed to control body weight because, for many people, these changes are quite challenging. Although advanced surgical and pharmaceutical treatments have been created to address obesity, these procedures can be expensive and come with a risk of side effects. Therefore, natural plant-derived chemicals have increasingly been viewed as a great alternative approach for creating anti-obesity medications that are efficient, secure, and affordable. Green tea extract (GTE) was reported to have potent hypolipidemic activity and reduced lipid content in the high-fat-induced obesity model of zebrafish (Xiao et al. 2019). The anti-obesity impact of GTE varies depending on the kinds of lipids or fatty acids that are present in high-fat diets and may be diminished by saturated fatty acids. Therefore, drinking green tea and substituting vegetable oils high in unsaturated fatty acids, such as olive oil, for saturated fatty acids could be a helpful nutritional habit to prevent obesity (Yamashita et al. 2018). Obesity causes a persistent inflammatory state, which has been linked to dyslipidemia, a major risk factor for atherosclerosis and cardiovascular disease, and has been demonstrated to play a significant role in its development. Tumor necrosis factor (TNF) synthesized by macrophages is known to cause dyslipidemia. The synthesis of the inflammatory cytokine TNF is first triggered by the toll-like receptor 4 (TLR4), which is linked to obesity. EGCG dramatically decreased TLR4 expression by upregulating E3 ubiquitin-protein ligase RNF216, alleviated inflammation brought on by the high-fat/high-sucrose diet, and counteracted its concomitant hypolipidemic impact (Kumazoe et al. 2017).

Combinations of soybean genistein, green tea EGCG, and/or grape resveratrol dramatically reduced the ability of 3 T3-L1 pre-adipocyte cells to differentiate by reducing the expression of peroxisome proliferator-activated receptor gamma (PPAR-γ) and CCAAT binding proteins alpha (C/EBP-α) proteins (Ahmed et al. 2017). Cyclic guanosine monophosphate (cGMP), the second messenger, mediates the anti-inflammatory effects of EGCG-induced 67-kDa laminin receptor (67LR)-dependent signaling. Consuming citrus polyphenols and glucosyl-hesperidin (gH) enhanced the cGMP-inducing anti-obesity properties of green tea (GT) (Kumazoe et al. 2021). A randomized placebo-controlled clinical trial revealed the weight loss and anti-obesity effect of GT-gH (Yoshitomi et al. 2021). Supplementation with EGCG and resveratrol (RES) suppressed genes implicated in adipogenesis, apoptosis/autophagy, and inflammation in adipose tissue of obese human subjects without altering the shape, lipolysis, or insulin sensitivity of adipose tissue(Most et al. 2018). Male and female fecal microbiota in obese human subjects differs, with EGCG+RES supplementation particularly reducing *Bacteroidetes* abundance in obese men without having any impact on female obesity (Most et al. 2018).

Acknowledgments KT acknowledges the Pondicherry Centre for Biological Science and Educational Trust, Puducherry, India, for support; SNN acknowledges the management of Vel Tech Rangarajan Dr. Sagunthala R&D Institute of Science and Technology, Chennai, India; and AKAM acknowledges the management of Vellore Institute of Technology, Vellore, India, for support in preparing the book chapter.

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The Medicinal Potential and Application of In Vitro Techniques for Improvement of *Galega officinalis* L.

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Abstract It has been verified throughout the history of medicine that nature is a reliable and excellent source of new drugs. Compared to chemical drugs, herbal remedies are either more effective or have slight side effects in many cases. Medicinal plants are being used to extract the active components used in various drug, cosmetic, and healthcare product synthesis. *Galega officinalis* L., commonly known as Galega, is an herbaceous plant of the Leguminosae family. It contains various secondary metabolites such as alkaloids, saponins, flavonoids, tannins, fatty acids, and phytoestrogens used in traditional medicine to increase breast milk and as an antidiabetic product. The chemical content, pharmaceutical properties, and medicinal potential of the plant were reviewed in this chapter. In addition, the application of biotechnological techniques, such as cell and tissue cultures and transformed hairy roots, for valuable pharmaceutical production, particularly galegine, was discussed. We argued that *G. officinalis* having beneficial medicinal effects needs intensive research for better usage in industrial applications.

Keywords Alkaloid · Antidiabetic · *Galega officinalis* · Galegine · Medicinal plants

1 Introduction

Over the past 50 years, research on active components of plants has led to the extraction of many valuable pharmaceuticals to treat many diseases (Dar et al. 2017). Leaves, flowers, seeds, and many other parts of medicinal plants having active compounds can be used for meeting the medical needs of more than 80% of the world's

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N. Kumar, R. S. Singh (eds.), *Biosynthesis of Bioactive Compounds in Medicinal and Aromatic Plants*, Food Bioactive Ingredients,
https://doi.org/10.1007/978-3-031-35221-8_14

population (Savithramma and Rao 2011), which are readily available to people at affordable prices (Singh et al. 2020). They also generate income for the local population (Roberson 2008). Herbal medicines have fewer side effects, and it looks like they may usher in a new medical system for disease treatment (Shakya 2016). Medicinal plants have an imperative role in the synthesis of various medicines (Hassan 2012) containing antioxidant, antiviral, anticancer, antimicrobial, antifungal, and antiparasitic activities and molecules that scavenge free radicals, including flavonoids, phenols, anthocyanins, and vitamins (Chopra and Doiphode 2002). Several herbal medicines are used as crucial drugs in the treatment of human cardiovascular, liver-kidney, diabetes, and cancer diseases (Modak et al. 2007; Shakya 2016).

Galega officinalis L., or goat's rue, from the Faboideae subfamily, is widely cultivated as a fodder crop, ornamental, honey bee-favored plant, and green fertilizer. The name Galega is derived from gale (milk) and ega (to bring) and was used as a galactogenic agent (milk bringer) for small domestic animals, which is why it is also called "goat's rue" (Luka and Omoniva 2012). It is also named Galega, catgut, common milk pea, Italian fitch, and professor weed (Darbyshire et al. 2021). It thrives in and along watercourses and wetlands and is considered a critical weed in wet grasslands (Darbyshire et al. 2021). It is a well-known plant because of its medicinal value, especially for its blood sugar-lowering effects and its use to treat diabetes (Palit et al. 1999). It appears that its oral value is low; due to having the quinazoline alkaloid vasicine, with a bitter taste (Klugh 1998).

Given the history of human consumption of Galega, its health, safety, and side effects are widely known. Currently, industrial products from the plant, such as pills, powders, and oral drops, are available for lowering blood sugar, weight loss, and lactation. Because of their low cost and availability, they can be a good substitute for treating chronic and costly diseases such as diabetes. If taking a specific dose of medicine, no side effects have been reported for this plant, but more than the dose should be avoided. Also, in the case of taking blood glucose-lowering drugs, their dosage should be reduced.

2 Botany of *G. officinalis*

G. officinalis is an herbaceous diploid ($2n = 2x = 16$) plant (Izmaiłow 1990) in the Leguminosae family and Faboideae subfamily. It is grown in southeastern Europe, the Mediterranean region, Western Asia, and some regions in the United States, South America, and China (Lasseigne 2003).

G. officinalis is a bushy plant with branched and oval long stems and bilateral leaves with a height of 0.6–1.5 m. It has many purple to pink or purple-white flowers (Fig. 1) (Luka and Omoniva, 2012) and forms dense crowns that can regenerate over several years (Klugh 1998). It spreads by seeds. The Galega pods are 2.5 cm in length, each containing one to nine seeds (Whitson et al. 2000). Seeds are about 2.5

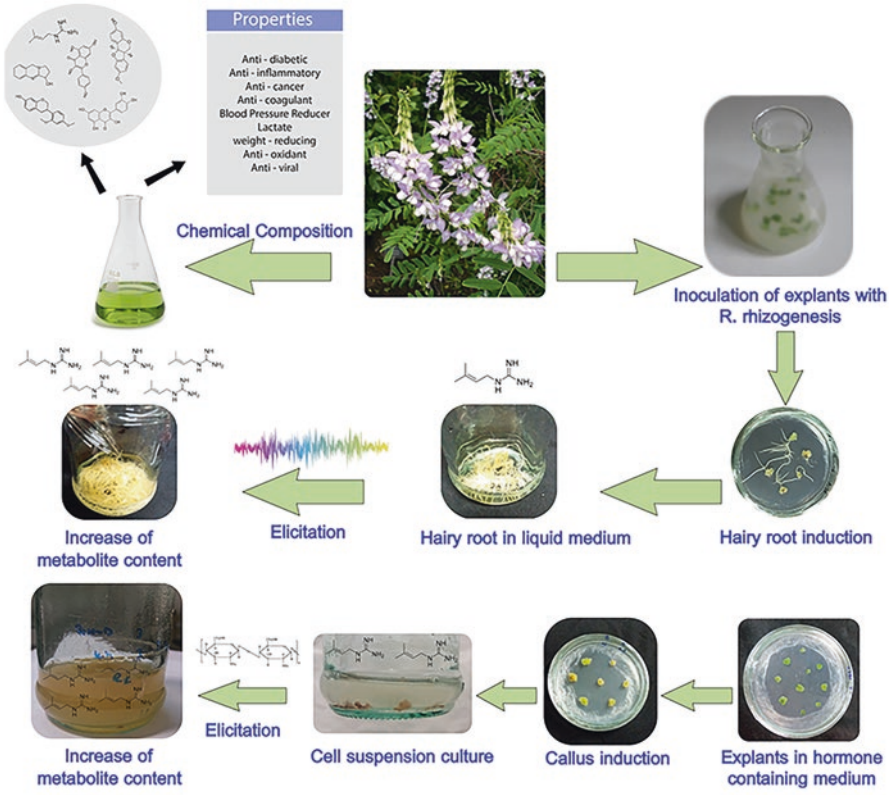

Fig. 1 The presence of secondary metabolites such as alkaloids, tannins, terpenoids, flavonoids, and phenols leads to the properties of antioxidant, antiviral, anticancer, antimicrobial, antifungal, and antiparasitic activities in *Galega officinalis*. The use of biotechnological techniques, such as cell and tissue culture, hairy roots, and the application of elicitors, helps to increase the production of secondary metabolites in this medicinal plant

times the size of alfalfa seeds (Evans et al. 1997; Oldham and Ransom, 2009). They are mustard yellowish and are thought to be able to survive in the soil for 15 years (Evans et al. 1997). Goat's rue grows on summer days and in moist soil. It thrives in and around waterways and moisture areas. Although it tolerates light shade, it prefers sunny days (Klugh 1998; Darbyshire et al. 2021). Detailed morphological characteristics of *G. officinalis* can be found in CABI (2019).

G. officinalis, like other legumes, transforms atmospheric N_2 in the species-specific symbiosis with *Rhizobium galegae* symbiovar *officinalis* (Österman et al. 2011). Therefore, in non-native soils, seed inoculation with the most effective strains of *R. galegae* sv. *officinalis* is necessary (Egamberdieva et al. 2013).

3 Biochemical Content of *G. officinalis*

Plants are crucial sources for novel medicine development because their secondary metabolites are beneficial to human health, which are formed from primary metabolites in plant organs at various growth stages (Halder et al. 2019; Naik and Al-Khayri 2016; Neumann et al. 2009). The production processes of these compounds should be well understood to enhance the synthesis of these advantageous molecules in plants (Savithramma and Rao 2011). As shown in Table 1, *G. officinalis* contains some important alkaloids, saponins, flavonoids (Le Bail et al. 2000; Bednarska et al. 2020), phytoestrogens (Champavier et al. 1999), tannins (Chevallier 1998), fatty acids (Peiretti and Gai 2006), glycosides, phenols, resins, terpenes, and steroids (Okhale et al. 2010).

Recently, tricyclic quinazoline alkaloid; guanidine; flavonoid; hydroxycinnamic acid (HCAs); mono-, di-, and triglycosylated flavonols; and monocaffeoylhexaric acid presence in Galega extract have been reported by Bednarska et al. (2020). They indicated that flavonols and HCAs had powerful antiradical (lowering reactive carbonyl species), antioxidative, and methylglyoxal (MGO, a precursor in the glycation and oxidation of proteins) trapping properties; among them guanidines (galegine and hydroxygalegine) and flavonoids (mostly flavonols) are more effective for MGO trapping, and also in the avoidance of diabetes vascular impediments; however, HCA esters and quinazoline alkaloids showed negligible effects.

Moreover, it can be said that the anticancer activity in Galega extracts is due to guanidines (galegine) or flavonoids (sativan, medicarpin, quercetin, and kaempferol) as its dominant compound (Karakaş et al. 2016b). Medicarpin and sativan flavonoids, extracted from the leaves of goat's rue, had cytotoxic effects on cancerous cell lines (Le Bail et al. 2000). According to Karakaş et al. (2016b), different plant extracts (MeOH, dichloromethane (DCM), hexane, and aqueous extracts) may contain diverse amounts of secondary metabolites due to the polarity differences among the extraction solvents.

4 Galegine: The Dominant Ingredient in *G. officinalis*

Galegine is a guanidine derivative that was first isolated from *G. officinalis*, and the biguanides such as metformin and phenformin were made from it (Bailey and Day 2004; Mooney et al. 2008). As a result, this plant is the source of metformin, a synthetic version of galegine that lowers blood glucose levels. It is an active molecule in most antidiabetic drugs (Oubré et al. 1997; Vuksan and Sievenpiper 2005).

Galegine decreases blood sugar levels (Oubré et al. 1997; Vuksan and Sievenpiper 2005). It has anticoagulant effects (Vermaak et al. 2011) and lowers blood pressure (Fabricant et al. 2005). In addition, it increases milk in mammals (González-Andrés et al. 2004). Galegine, metformin, and phenformin stimulate 5′-adenosine monophosphate-activated protein kinase (AMPK) (Watt et al. 2006; Zhou et al.

Table 1 Chemical composition and medicinal properties of *Galega officinalis* L.

Group	Components	Properties	References
Guanidine	Galegine, hydroxygalegine	Antidiabetic, weight-reducing, anticancer	Le Bail et al. (2000) and Bednarska et al. (2020)
Quinazoline alkaloids	Vasicine (peganine), hydroxyvasicine (vasicinol), vasicine-O-hexoside, vasicinone	Antioxidant, anti-inflammatory, bronchodilatory activity	Bednarska et al. (2020)
Flavonoids	Kaempferol, quercetin, isorhamnetin, medicarpin, sativan, taxifolin glycoside, taxifolin-3-O-hexoside, quercetin-3-O-dideoxyhexosyl-hexoside isomers, rutin, hyperoside, quercitrin, kaempferol-3-O-rutinoside (nicotiflorin), kaempferol-3-O-robinoside, astragalin, kaempferol-3-O-α-rhamnoside (kaempferin), isorhamnetin-3-O-rutinoside (narcissin), mauritianin, kaempferol-3-O-rutinoside (nicotiflorin), kaempferol-7-O-neohesperidoside, quercetin-3-O-rutinoside (rutin), quercetin-3-O-β-glucoside (isoquercitrin), quercetin-3-O-β-galactoside (hyperoside), quercetin-3-O-α-rhamnoside (quercitrin)	Antioxidant, anti-inflammatory, anticancer, antibacterial	Le Bail et al. (2000), Okhale et al. (2010), and Bednarska et al. (2020)
Phenolic acids	Vanillic acid, apigenin, caffeic acid, ferulic acid, p-coumaric acid, coumaric acid O-hexoside, monocaffeoylhexaric acid, chlorogenic acid	Antioxidant; anti-inflammatory; anticancer; antibacterial; neuro-, cardio-, and hepato-protective effects, anti-collagenase	Karakas et al. (2016a), Okhale et al. (2010), and Bednarska et al. (2020)
Saponins		Anticarcinogenic, antimutagenic, antioxidative, decrease blood lipids, lowering blood glucose	Champavier et al. (1999), Peiretti and Gai (2006), and Le Bail et al. (2000)
Tannins	Tannic acid	Antioxidant properties, Carcinogenic, Reduction in blood pressure	Chevallier (1998)
Fatty acids	α-Linolenic acid, palmitic acid, linoleic acid		Peiretti and Gai (2006)

2001). Biguanides' capability to the inhibition of complex I at the respiratory chain and increase the cellular AMP:ATP ratio may further contribute to their influence on AMPK (Mooney et al. 2008). The effects of galegine, such as increased glucose uptake in skeletal muscle and inhibition of acetyl-CoA carboxylase, which in turn

constrains fatty acids synthesis, may be explained by its AMPK activation property. This could influence how galegine affects body weight in vivo (Mooney et al. 2008).

Goat's rue has varying amounts of galegine depending on the plant organ and growth stage. Its concentration is the highest in reproductive tissues, leaves, and stem tissues. The amount of galegine rises from its lowest point in the primary growth stage to its peak at the immature pod stage, and then declines at the mature seed stage. The distribution of nutrients necessary for the development and maturity of reproductive structures may be the main reason for the drop in galegine content throughout the flowering stage. At the mature stage, galegine may disintegrate or be converted to other compounds (Alpert et al. 1985; Boege and Marquis 2005).

5 Pharmaceutical Properties

Several studies are available on various properties of *G. officinalis*, such as a diuretic (Lemus et al. 1999; Chan et al. 2010), antibacterial (Ertürk 2010), antidiabetic (Jung et al. 2006), antipyretic, anti-inflammatory (Karakaş et al. 2016b), weight reducing (Palit et al. 1999; Shojaee et al. 2013), anticancer, antioxidant (Karakaş et al. 2016b), mutation inhibiting (Le Bail et al. 2000), antiviral (Le Bail et al. 2000), nematicidal activity (Insunza et al. 2001), antihyperglycemic (Shojaee et al. 2013), antimicrobial (Pundarikakshudu et al. 2001), and anti-aggregate (Atanasov and Spasov 1999, 2000), protective effect on streptozotocin-induced kidney damage (Seyd-Hosein et al. 2017), and lactate-forming effects (González-Andrés et al. 2004). The main properties of this plant are as follows:

5.1 Antidiabetic

The most prevalent form of the endocrine disease is diabetes mellitus (DM), known as hyperglycemia (Tripathi and Chandra 2010). Nowadays, diabetes is treated with insulin and other synthetic medications. Despite their cost, they have specific side effects when used for prolonged. Much work has recently gone into researching the potential applications of medicinal plants and traditional medicine. There have been numerous reports of medicinal plants and their preparations having blood sugar lowering and DM prevention abilities (Shojaee et al. 2013). *G. officinalis* is believed to have been utilized to treat type 2 diabetic symptoms (Bailey and Day 2004). Galegine, the guanidine derivative of *G. officinalis*, was used to make metformin, a familiar, safe, and affordable medication in 1950 (Chan et al. 2010; Yang 2011). Clinical studies have demonstrated that therapies containing saponins (Chang et al. 2011; Lu et al. 2008), glycosides (Cherian and Augusti 1995), and alkaloids (Wadkar et al. 2007) can lower blood glucose levels and reduce clinical symptoms of type 2 diabetes.

Damage to apoptosis in both pancreatic and immune system cells is the underlying cause of diabetes. Programmed cell death controls the immune system's response to antigenic provocations, response timing, and immunological tolerance (Hetts 1998). Consuming Galega over a prolonged period may cause diabetes to reverse, due to the inhibition of apoptotic cell death in Langerhans β-cells (Sabeva et al. 2004). According to Mooney et al. (2008), AMPK activation is the main reason for the galegine effects in enhancing glucose uptake and lowering body weight, like metformin and phenformin.

5.2 Lactogenic Activity

Increased milk production in livestock is one of Galega's properties. According to González-Andrés et al. (2004), in sheep, a regulated daily dosage of 2 g dry weight per kg body weight from 30 to 60 days postpartum increased milk production by 16.9%. Additionally, they proposed that Galega phytoestrogens might cause the development of estrogenic receptors, which would have biochemical consequences and increase milk production. Previously, Le Bail et al. (2000) discovered that several phytoestrogens, including flavonol triglycosides, kaempferol, and quercetin, are present in methanolic extracts of *G. officinalis* (Peiretti and Gai 2006).

5.3 Antimicrobial and Antiplatelet Activity

Antibacterial activities of Galega have been detected against Gram-positive and Gram-negative bacteria (Atanasov and Spasov 2000; Pundarikakshudu et al. 2001). Gram-positive and Gram-negative bacteria are significantly inhibited by the ethanolic (60%) plant extract; its ethanolic extract also helps the skin recover more quickly following the surgery (Pundarikakshudu et al. 2001). Also, it has been reported that the leaf and shoot extract of *G. officinalis* were moderately effective on fungi (Karakaş et al. 2012) and inhibited platelet aggregation (Atanasov and Spasov 2000).

5.4 Anti-inflammatory Activity

In traditional medicine, aerial parts of *G. officinalis* have great use in inflammatory disease healing (Chevallier 1998). A high level of nitric oxide (NO) causes inflammatory disorders. Therefore, preventing NO production in cells is an excellent strategy in the treatment of inflammatory infections (Marletta 1993). *G. officinalis* DCM extract is a potent inhibitor of NO secretion (Karakas et al. 2016b). The

alkaloid or flavonoid components of the plant are responsible for this powerful anti-inflammatory effect of DCM extract (Le Bail et al. 2000).

5.5 *Anticancer Effects*

Cancer is dramatically increasing worldwide, earning its epidemic status (Abudawood 2019). A lower incidence of cancer was reported in diabetic patients who took metformin, which led to using it as an anticancer drug (Bo et al. 2012; Cazzaniga et al. 2009; Libby et al. 2009). Metformin, for its inhibiting effect on mitochondrial respiration, was selected as an attractive drug candidate for cancer treatment (Boukalova et al. 2016; Christodoulou and Scorilas 2017; Palma et al. 2021). According to reports, metformin suppresses the mitochondrial respiratory complex I chain, increases the AMP:ATP ratio by reducing ATP production (Foretz et al. 2010; Hardie et al. 2012; Owen et al. 2000), and then suppresses gluconeogenesis even in the lack of AMPK through the disruption of the of dihydroxyacetone phosphate production and inhibiting mitochondrial glycerophosphate dehydrogenase (mGPDH) (Madiraju et al. 2014). It has been shown that metformin can inhibit the transforming growth factor-β (TGF-β) receptor, thereby reducing TGF-β oncogenic signal transduction (Xiao et al. 2016). The leaves and flowers of *G. officinalis* indicated extensive anticancer effects against human cancer cells (Karakaş et al. 2012, 2016b; Pundarikakshudu et al. 2001).

In addition, it has been demonstrated that guanidine compounds such as galegine and phenformin are authentic tumor disruptors (Arjmand et al. 2022, García Rubiño et al. 2019). Galegine inhibited the growth of the mouse melanoma B16F1 (Lee et al. 2012) and induced cytotoxicity and apoptosis in a concentration-dependent manner with IC50 of 630 μM and 3300 μM in DFW and SK-MEL-5 melanoma cell lines, respectively (Arjmand et al. 2022). Also, the galegine showed an inhibitory effect on mitochondrial respiration (Lotina et al. 1973). Phenformin is estimated to be more effective than metformin, particularly for cancer treatment (Jiang et al. 2016; Yuan et al. 2013). Thus, the adoption of galegine as a more potent guanidine compound for cancer treatment is also worthy of consideration because it possesses relatively lower toxicity and might be more acceptable for cancer treatment.

5.6 *Other Uses*

Due to their significance in nutritional value and nitrogen fixation, forage crops from the legume family (Leguminosae) have a great potential for efficient and sustainable agriculture (Karakaş et al. 2012, 2016b). Galega is utilized as green and winter food for various animals because of its high organic material content (Adamovich 2000). To produce high-quality forage, it is better to harvest it at the shooting stage, before flowering, when it is most edible and nutritive and its

poisonous ingredients are low (Peiretti and Gai, 2006; Oldham et al. 2011). In addition, *G. officinalis* has been used as a highly productive green manure for soil enrichment and potential bioremediation of soils polluted with hydrocarbons (Našinec and Němcová 1990; González-Andrés et al. 2004).

6 Application of In Vitro Techniques to the Improvement of *G. officinalis*

As previously indicated, harvesting medicinal plants for secondary metabolites from natural habitats has led to these plants being endangered (Gantait and Mukherjee 2020). The Earth loses at least 1 potentially significant medicinal plant every 2 years, 100 to 1000 times more than the expected rate (Pimm et al. 1995). Cell and tissue culture and molecular biotechnological methods have made it possible to increase the effectiveness of medicinal plants production.

Additionally, in vitro cultivation aids in improving wild stocks, lowering prices, and reducing medicinal plant natural harvesting (Chen et al. 2016; Larsen and Olsen 2006; Schipmann et al. 2003). So, using plant cell or tissue culture in laboratories and bioreactors is possible year-round, stable, and high-yield production of therapeutic chemicals under sterile controlled conditions (Krol et al. 2021). Some in vitro techniques used to improve Galega's yield and production, including cell suspension culture, hairy root induction, and elicitor application, are discussed.

6.1 Cell Suspension Culture

Under appropriate conditions, plant cells have the biochemical potential to synthesize similar phytochemicals to their parental plants. However, the balance between primary and secondary metabolisms is generally affected by cell growth stage and tissue differentiation (Collin 2001). Secondary metabolites are mainly biosynthesized in various tissues or organs and different growth stages (Luka and Omoniva 2012). Cell suspension culture has many advantages over many other plant systems, including a short cell cycle, independence from environmental factors (like weather, soil quality, season, and day length), high biological safety, the impossibility of gene escape through pollen grains (Xu et al. 2011), and the possibility biosynthesis of secondary metabolites (Tulecke and Nickell 1959). Moreover, when the metabolites produced by the cell suspension are secreted into the culture medium, their purification is much easier because the cells do not need to be collected, destroyed, and homogenized (Shi et al. 2009). Plant cell cultures also offer the opportunity to conduct research under carefully regulated conditions for studying gene expression involved in the biosynthesis of secondary metabolites (Cesarino et al. 2013). Plant cell cultures are now utilized for novel chemical metabolite production, cell cycle

regulation, and other cellular processes due to the advantages and opportunities indicated above (De Schepper et al. 2004).

A prerequisite to the plant's secondary metabolite production under in vitro conditions is a successful cell suspension culture establishment after a friable callus culture. A friable callus is obtained by excising proper explants and growing them onto a semi-solidified culture medium containing macronutrients, micronutrients, a carbon source, and several phytohormones or plant growth regulators. After cell culture establishment, growth parameters, including cell viability, settled cell volume (SCV), packed cell volume (PCV), fresh weight, or dry weight of each cell line, are measured at time intervals to measure cell growth and determine the best cell line(s) (González-Cabrero et al. 2018).

Studies on *G. officinalis* cell suspension culture are scarce. Karakaş et al. (2016a) showed that callus induction was seen in stem, root, petiole, and leaf explants of *G. officinalis* (Karakaş et al. 2016a). Furthermore, Asghari Zakaria et al. (2021) used *G. officinalis* leaf, root, hypocotyl, and nodule explants in MS (Murasnige and Skoog 1962) medium containing various concentrations of 2,4-D, BAP, or Kin for callus induction. They revealed that 2,4-D combined with Kin was very efficient in callus induction and resulted in 100% callus induction at most of the used concentrations. In the nodule explant, the MS medium supplemented with 0.5 mg L^{-1} 2,4-D and 5 mg L^{-1} Kin generated the most callus development. To regeneration, they also employed leaf, cotyledon, and nodule explants in an MS medium that contained various concentrations of BAP and Kin along with NAA. They found only the nodule explants were regenerated (Asghari Zakaria et al. 2021). Karakaş et al. (2016a) also noted that only nodule explants triggered regenerated shoots.

6.2 Hairy Root Culture

While undifferentiated cultures such as callus and cell suspensions are extensively used for secondary metabolite production in many species, as cultivation time increases, genetic instability in cells brought on by somaclonal alterations, which can diminish or completely stop product formation (Georgiev et al. 2009). The system must be stable for a prolonged time to scale up the production of secondary metabolites. Organized and differentiated cultures such as shoot, root, or hairy roots cultures are frequently utilized to avoid instability and poor synthesis capacity of cell suspension cultures (Roychowdhury et al. 2013; Bayesteh et al. 2021).

Hairy roots were produced by *Rhizobium rhizogenes* (formerly *Agrobacterium rhizogenes*), a soil-borne bacteria recognized in 1934 as a plant syndrome (Mehrotra et al. 2015; Gutierrez-Valdes et al. 2020). These rod-shaped and Gram-negative bacteria belonging to the *Rhizobiaceae* family are now one of the most well-known species in the genus *Rhizobium* (Rogowska and Szakiel 2021). *R. rhizogenes* is not currently considered a pathogenic bacterium because it has multiple hosts and a plasmid used as a vector for gene transfer and is of value for the genetic engineering of medicinal plants. Factors such as bacterial strain (Crane et al. 2006), explant type

and age (Kim et al. 2004), and incubation time play a role in T-DNA transference from *R. rhizogenes* to a host (Barik et al. 2005). *R. rhizogenes* has been shown to infect and genetically modify various plant tissues and organs, leading to the development of hairy roots (Giri et al. 2001; Han et al. 1993). Thus, the bacterial strain and plant cell play an essential role in increasing the probability of T-DNA transfer to the plant cell. Compared to undifferentiated cultures, hairy root cultures often accumulate phytochemicals to a greater extent (Georgiev et al. 2012; Halder et al. 2019; Mehrotra et al. 2015; Ono and Tian 2011). Moreover, hairy root cultures appear to be the most capable for the production of secondary metabolites at the industrial scale, as they outperform whole plants, cell suspensions, and traditional root cultures in many ways, including a high growth rate in a hormone-free medium and a higher genetic and biosynthetic performance (Halder and Jha 2021). Many studies have shown that hairy root cultures can be successfully established and used to produce specific phytochemicals in different plant species (Karuppusamy 2009; Mehrotra et al. 2015; Roychowdhury et al. 2013, 2017; Vaghari et al. 2017). Khezri et al. (2022b) used three strains (A13, A4, and 15,834) and two types of explants (leaf and cotyledon) to induce in vitro hairy roots of *G. officinalis*. They observed that the highest induction rate is in leaf explants inoculated with the A4 strain. Also, Ghanbari Namin et al. (2022) reported that hairy roots were induced in *G. officinalis* by leaf, cotyledon, and hypocotyl explants inoculation with *R. rhizogenes* strain A4. They indicated significant differences between different explants for the average number of induced roots. The highest amount of root induction after 5–10 days was related to the leaf explant, with 5 hairy roots per explant; the lowest amount was related to the hypocotyl explant, with 0.8 roots per explant (Ghanbari Namin et al. 2022).

6.3 Elicitation

Due to the inadequate production of secondary metabolites in the cell culture, elicitation is used as one of the best biotechnological methods for the enhancement of secondary metabolite in plant cells and tissue culture (Wang and Wu 2013, Thakur et al. 2019). The kind and treatment duration with elicitors are very influential factors in achieving satisfactory results in elicitation application strategies. Also, the selection of the best-growing cell lines along with the best growth stage of cells plays a crucial role in this regard (Khezri et al. 2022b; Kubes et al. 2019). Elicitors cause a signaling cascade, which, in turn, alters the expression level of related genes and transcriptional factors and increases the production of secondary metabolites (Mishra et al. 2012; Wang and Wu 2013; Zhai et al. 2017).

The most appropriate and widely accepted classification of elicitors is grouping them into two types: abiotic and biotic elicitors (Rogowska and Szakiel 2021). Biotic elicitors are compounds generated from plants or pathogens (fungi, bacteria, and yeasts) such as polysaccharides, glycoproteins, chitosan, pectin, yeast, and fungal extracts, etc. that are either widely purified or crude extracts (Vasconsuelo

and Boland 2007). In contrast, ultrasonic and UV waves, heavy metal (Ag, Cd, Cu, VO, Ni, and Se) salts, and other chemical and physical stresses are examples of abiotic elicitors (Wang and Wu 2013). Jasmonic acid (JA), salicylic acid (SA), and their derivatives are examples of compounds that some researchers label as biotic elicitors, while others categorize them as abiotic elicitors (Halder et al. 2019).

Various elicitors such as MeJA, SA, yeast extract, chitosan, $AgNO_3$, and $CdCl_2$ have all been demonstrated to be the most successful elicitors in cell suspension culture (Açıkgöz 2020; Du et al., 2020; Mahendran et al. 2021). Salicylic acid (SA) activates genes implicated in the plant defense system and enhances secondary metabolites (Liu et al. 2018). It is used to increase Psoralen in *Cullen corylifolium* (L.) Medik (Singh et al. 2020), total polysaccharides, phenols, and flavonoids in *Orostachys cartilaginous* (Wen et al. 2019), and camphor in *Achillea gypsicola* cell cultures (Açıkgöz et al. 2019).

In *G. officinalis* cell suspension culture, the application of salicylic acid and chitosan for 3 days in the most used concentrations increased the content of phenol, flavonoid, and galegine compared with the untreated cells, while the use of this elicitor for 6 days in all concentrations decreased the production of the abovementioned compounds compared with the control. This shows the elicitors' long-term toxicity on plant cells (Khezri et al. 2022a). Chitosan as a potent biotic elicitor improved the vinblastine and vincristine contents in the cell suspension culture of *Catharanthus roseus* L. (Pliankong et al. 2018), silymarin in the callus culture of *Silybum marianum* (Shah et al. 2021), lepidine in the callus culture of *Lepidium sativum* L. (Bakhtiari and Golkar 2021), triterpenoid and saponins in *Calendula officinalis* L. (Alsoufi et al. 2019), and *Psammosilene tunicoides* hairy root cultures (Qiu et al. 2021).

Ultrasonic waves at high-energy levels destroy cell membranes and inactivate proteins, enzymes, and DNA, however, at low-intensity cause membrane penetrability and increase the passage of substances from cells into the medium (Joersbo and Brunstedt 1992). Due to the mechanical stress caused by low-intensity ultrasound waves, cells release enzymes that lead to the formation of secondary metabolites (Sales and Resurreccion 2010). Low-energy ultrasound waves can be used as abiotic elicitors to stimulate secondary metabolite production in plant cell cultures (Liu et al. 2003; Wu and Ge 2004; Khezri et al. 2022a, b). It was reported that the highest content of galegine and total flavonoid was obtained 2 days after elicitation of *G. officinalis* hairy root culture with ultrasonic waves for 4 min. In addition, elicitation resulted in a significant increase in total phenol, hydrogen peroxide (H_2O_2), and malondialdehyde (MDA) contents compared with the control (Khezri et al. 2022b). Ultrasound treatment of hazelnut cells dramatically increased the phenolic content (Rezaei et al. 2011). Also, 20-second sonication of *Papaver bracteatum* cell suspension culture 6 days after treatment significantly increased thebaine yield (Zare et al. 2014).

Metallic nanoparticles can be used as effective elicitors for increasing secondary metabolite production (Zhang et al. 2013). Silver and silicon nanoparticles induced dose-dependent changes in galegine, phenol, and flavonoid content in cell suspension and hairy root cultures of *G. officinalis* (Ghanbari Namin et al. 2022; Minaei et al.

2022). The highest content of galegine was obtained by the treatment of hairy roots of *G. officinalis* with 10 or 20 mg L^{-1} silver and 100 mg L^{-1} silicon nanoparticles for 36 h (Ghanbari Namin et al. 2022). Also, it has been reported that the highest galegine content was achieved in *G. officinalis* cells treated with 5 and 10 mg L^{-1} AgNPs for 48 h. Also treatment with 100 mg L^{-1} iron and 60 mg L^{-1} molybdenum nanoparticles increased galegine content in *G. officinalis* cell suspension culture (Minaei et al. 2022). Also the increase in papaverine, thebaine, and codeine in cell suspension of *Papaver somniferum* L. by application of copper, iron, and silicon NPs (Bondarian et al. 2013) and a 12-fold increase of tanshinones by application of silver nanoparticles in *Perovskia abrotanoides* have been reported (Bayesteh et al. 2021).

7 Prospects

The medicinal importance of this plant encourages the metabolic profiling of different populations of this species and the evaluation of genetic diversity among its various populations based on molecular markers, selection, and crossing of suitable parents to start a breeding program. In addition, the induction of transformed hairy roots, the application of biotechnological techniques such as cell and tissue cultures, and the development of optimal bioreactors for producing valuable active pharmaceutical ingredients, especially the galegine, is another crucial step toward the exploitation of this plant. Overall, in this review, we argued that *G. officinalis*, as a valuable medicinal plant species, needs intensive research for better utilization in industrial applications.

Acknowledgments This work was supported by the Iran National Science Foundation (INSF) (grant number: 96013039) and the Vice-Chancellor for Research and Technology of the University of Mohaghegh Ardabili.

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A Review on the Golden Plant Turmeric and Its Bioactive Compound Curcumin

Dipa Mahato and Harishankar Mahto

Abstract Golden plant (Turmeric) has succulent, religious views with numerous pharmaceutical values. Due to its pharmaceutical values, it has been a research center for long years. Many bioactive compounds like curcuminoid, identified in turmeric, are rich in therapeutics. In the last 10 years, research interests have concentrated on bioactive curcuminoid compounds (curcumin, demethoxy curcumin, and bisdemethoxy curcumin). Lipophilic polyphenol, curcumin ((1E,6E)-(1,7-bis-(4-hydroxy-3-methoxyphenyl)-hepta-1,6-diene-3,5-dione;4-hydroxynaphthalene-1,2-dione)), was found to have maximum amounts in *Curcuma longa* as curcuminoids. In recent studies, it has been found to play a very effective role against diseases such as cancer, biotic, inflammatory, and aging. The present study summarizes the pharmaceutical usages of turmeric with distinct reference to its polyphenolic compound curcumin.

Keywords *Curcuma longa* · Nutraceutical · Phytochemistry · Curcuminoids · Pharmaceutical and disease

1 Introduction

Natural plant products have been used by humans for a variety of purposes throughout history. The plant root, leaves, fruits, etc. that are the source of many natural goods date back billions of years. Higher plants synthesize a number of secondary metabolites which shows natural defense over infectious diseases and illness. Most of the secondary metabolites contain pharmacological or medicinal properties that can be used in the development of therapeutic drugs. Plant-based medicines have

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N. Kumar, R. S. Singh (eds.), *Biosynthesis of Bioactive Compounds in Medicinal and Aromatic Plants*, Food Bioactive Ingredients,
https://doi.org/10.1007/978-3-031-35221-8_15

been essential to the health care of many civilizations, both ancient and modern (Newman et al. 2003; Butler 2004; Balunas and Kinghorn 2005; Gurib-Fakim 2006; Newman and Cragg 2007). Plant-based medications or formulations have been used to treat a variety of infectious diseases and illnesses including cancer in Ayurveda, an Indian holistic medical approach. The majority (61%) of the 877 or so small-molecule medications that were made available globally between 1981 and 2002 may be attributed to herbal products (Newman and Cragg 2007). Even though a number of synthetic medications are created using combinatorial chemistry, herb-based pharmaceuticals are better appropriate for human usage, at least in terms of biochemistry. However, neither has modern medicine promoted nor placed a high value on using natural items for medical purposes.

A plant known as turmeric has been used medicinally for around thousands of years. The root of turmeric is utilized in Southeast Asia both as a spice and as a part of religious ceremonies. This plant is sometimes known as "Indian saffron" because of its attractive yellow color (Goel and Aggarwal 2010). More than enough number of research papers on turmeric have been published in the last few years, and nowadays, current medicine has started to understand its significance. This review discusses turmeric and its bioactive compound curcumin, and there pharmacology is also addressed.

2 Nomenclature, History, and Cultivation of Turmeric

The ginger family, Zingiberaceae, includes turmeric (*Curcuma longa*). The Persian word "kirkum," which means "saffron," is the source of its Latin name, which alludes to the rhizome's vivid yellow-orange color. It is a native of Southeast Asia (Velayudhan et al. 2012), but has been grown and used for a very long time in India. For optimum growth, the turmeric plant requires 20–30 °C temperature as well as a substantial rainfall yearly. The plants have elongated, oblong leaves and its height is about 1 m. These plants are harvested yearly for their rhizomes, and parts of such rhizomes are used to reseed by the next coming season. Rhizomes of the plant are tuberous and have a coarse, segmented layer. The rhizomes develop beneath the ground and leaves are above the ground. The rhizome has dull orange inside and yellowish-brown cover color outside. The main part of rhizome is 2.5–7.0 cm in length and 2.5 cm in diameter, with minor tubers branching out. It is also elongated and tapered at the distal end (Prasad and Aggarwal 2011). The plant is herbaceous perennial (Garg et al. 2011) with tufts of bulky wider lanceolate, which arises from rhizome, long acuminate, bright green leaves tapered at both the ends. These plant raises up to 60–90 cm in height; shoots are leafy and erect, sheathing petiole forming a pseudo stem bearing around 6–10 leaves. The ligule remains a minor lobe, and ciliate margins are found in the sheath near the ligule (Kaliyadasa and Samarasinghe 2019).

The plant grows in diverse tropical climates, but most favorable condition for growth of the plant remains at 1500 m above sea level, with annual rainfall of 1500 mm or more, and temperature range of 2035 °C. It blooms finest in consistently

moist soil or sandy soils with an alkalinity around of 4.5–7.5. In India the highest turmeric-producing states are south Indian states (Andhra Pradesh, Karnataka, Tamil Nadu, Orissa), West Bengal, Gujarat, Meghalaya, Maharashtra, and Assam (Yadav and Tarun).

3 International and National Scenario

The exact origin of turmeric is generally considered to be from the countries of Southeast Asia (Vietnam, China, and Western India) (Sopher 1950). Major producer, consumer, and exporters are India, Thailand, Vietnam, China, Taiwan, and the United States. World turmeric production is approximately 11 lakh tons per year. India dominates the global production case with a 78% contribution, followed by China (8%), Myanmar (4%), and Nigeria and Bangladesh which together contribute 6% of the global production. The United Arab Emirates (UAE) is India's largest importer of turmeric, representing 18% of total exports, followed by the United States (USA) with 8%. Other major importers include Bangladesh, Japan, Sri Lanka, the UK, Malaysia, South Africa, the Netherlands, and Saudi Arabia. Together they account for 75% of the world's imports, and Asian countries are the world's main suppliers. The remaining 25% is covered by Europe, North America, and American countries. The United States imports 97% of their turmeric requirements from India and the rest of the Pacific Islands and Thailand. Of the total world production, the United Arab Emirates accounts for 18% of imports, followed by the United States (11%), Japan (9%), Sri Lanka, United Kingdom, and Malaysia (Fig. 1).

The best turmeric in the world is thought to originate from India because of it consist high curcumin value. Among the world, India stands the biggest producer and exporter of the spice crop curcuma (Angles et al. 2011). India exports 65% turmeric to Japan, Sri Lanka, Malaysia, UAE, the Unites States, and UK. The Western institutional sector buys turmeric and oleoresins, whereas dry turmeric is preferred by industry (Fig. 2).

In India, turmeric is farmed from February to May and from August to October. Alleppey finger (Kerala) and Erode (Tamil Nadu), Salem (Tamil Nadu), Rajapore (Maharashtra), Sangli (Maharashtra), and Nizamabad bulb (Andhra Pradesh) are some of the several types of turmeric that are marketed in India (Sasikumar 2012). Nizamabad, Duggirala in Andhra Pradesh, Sangli in Maharashtra, and Salem, Erode, Dharmapuri, and Coimbatore in Tamil Nadu are the hubs for turmeric (Table 1).

4 Traditional Medicine Turmeric

Over the years, turmeric has been employed in various sections of the world's traditional medicine in medicinal concoctions. Turmeric is said to provide a variety of medical benefits in Ayurvedic traditions, including boosting bodily energy and

Fig. 1 Worldwide geographic distribution of curcuma. (Content available from Evidence-Based Complementary and Alternative Medicine)

enhancing digestion. Turmeric is applied on a piece of burned cloth and placed over a wound in Pakistan and Afghanistan to clean the wound and speed healing (Araujo and Leon 2001). Turmeric is regarded as a bitter digestive aid and a carminative in both traditional Chinese and Ayurvedic medicines. Turmeric is also used by Unani practitioners to remove phlegm or kapha and to widen blood vessels to enhance blood flow. To enhance digestion and lessen gas and bloating, it may be added to foods like rice and bean dishes. It is cholagogue which increases bile output through the gallbladder and liver and enhances the body's capacity to digest fats. Turmeric is occasionally used to treat digestive issues as well as colds and sore throats by combining it with milk or water.

5 Consumption and Medicinal Value of Turmeric

Uses for turmeric include food, cosmetics, and medicinal. It is a common spice in Middle Eastern and South Asian cuisine. Curry's unique yellow shade and flavor come from it. It used in milk products such as cheese and butter and other foods as a coloring agent (Govindarajan and Stahl 1980; Ammon and Wahl 1991). Turmeric has been incorporated in Ethiopians food as a result of Indian impact. Turmeric has

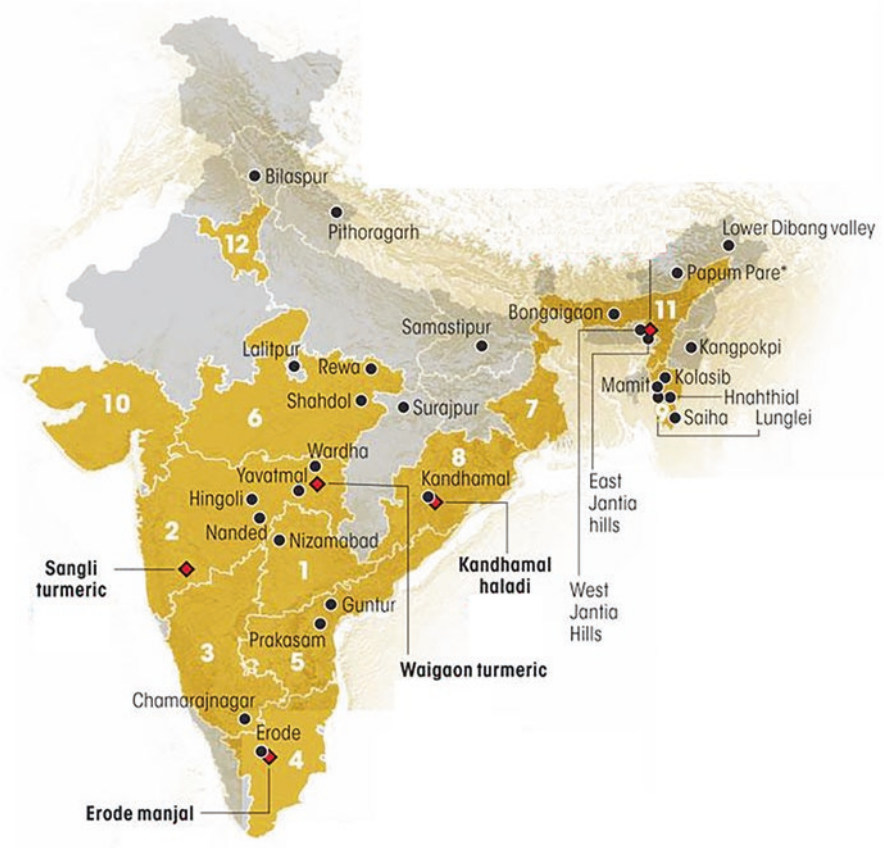

Fig. 2 Major turmeric-growing states of India with varieties. (Source: http://www.indianspices.com/indianspices/sites/default/files/majorspicestatewise2021.pdf)

long been used in Africa to add a golden color when cooking white rice. Additionally, turmeric is utilized in processed foods including gelatins, canned beverages, baked goods, dairy products, ice cream, cakes, orange juice, biscuits, popcorn, candies, and cake frostings. It is a key component in the majority of commercial curry powders. Asian cuisine use turmeric in a variety of ways. It is a common ingredient in Eastern delicacies like fresh turmeric pickles and is used in both savory and sweet recipes. Human intake of turmeric in Asian countries is from 200 to 1000 milligrams per day (Thimmayamma et al. 1983; Polasa et al. 1991), about 160 to 440 gram per person per year (Krishnaswamy 1996). Urban regions have a lower intake (200 mg per day) compared to rural areas (600 mg per day per person) (Thimmayamma et al. 1983).

Some estimates indicate that 800 billion rupees are spent in a year on alternative therapies. Botanical supplements cost over 5.3 million and are used to medicate chronic inflammatory conditions such as rheumatoid arthritis (RA), chronic

Table 1 State-wise area, production, and productivity of turmeric in India (2020–2021)

State	Area (ha)	Production (tons)	Productivity (tons/ha)
Telangana	49,000	818,000	16.70
Andhra Pradesh	30,518	73,244	02.40
Tamil Nadu	20,894	86,513	04.14
Orissa	27,867	43,611	01.56
West Bengal	17,749	45,698	02.57
Assam	16,359	20,885	01.28
Maharashtra	57,669	226,714	03.93
Karnataka	21,496	130,928	06.09
Haryana	1831	8009	04.37
Gujarat	7653	29,510	03.86
Madhya Pradesh	17,053	60,097	03.52
Mizoram	7653	29,510	03.86
Total	275,742	1,572,719	05.70

Source: http://www.indianspices.com/indianspices/sites/default/files/majorspicestatewise2021.pdf

obstructive pulmonary disease (COPD), and asthma. In traditional medicine, including Ayurveda, Chinese, Japanese, and Egyptian medicine, botanical supplements have been utilized for millennia. Several of the conventionally prescribed herbal medications contain anti-inflammatory activity (Aggarwal et al. 2006; Garodia et al. 2007); another similar herb is turmeric.

Many more infectious diseases can also be treated with turmeric as a natural remedy (Dixit et al. 1988). It is also used to treat digestive issues and menstruation problems as well as stomach discomfort and distension (Bundy et al. 2004) as well as for dyspeptic diseases including loss of appetite, postprandial symptoms of sluggishness, and complaints of the liver and gallbladder. Turmeric powder possesses astringent, choleretic, antibacterial, and anti-inflammatory properties (Mills and Bone 2000). Turmeric's primary therapeutic objectives include the gastrointestinal tract, where it is used to treat conditions including adenomatous polyposis (Cruz-Correa et al. 2006), inflammatory bowel disease in the intestines (Hanai and Sugimoto 2009), and colon cancer (Naganuma et al. 2006).

Doses of 8–60 g of garden-fresh turmeric root thrice per day have been suggested for treating autoimmune disorder like arthritis (Fetrow and Avila 1999) and 1.3–3.0 g suggested for dyspepsia. But still the monographs of the German regulatory body, Commission E, have not documented any known medication interactions with turmeric (Blumenthal et al. 2000).

6 Phytochemistry of Turmeric

The chemistry of turmeric comprises of carbohydrates, proteins, oils, fats, minerals, curcuminoids, and bit amounts of vitamins. Among these biomolecules and minerals curcuminoids and curcumin were found in abundant quantity (Kotha and Luthria 2019).

The plant contains numerous bioactive compounds, amongst them pharmaceutical significance mainly because of the curcumin; it has been well-known from years back; however the capacity to pinpoint the precise processes of action and identify the bioactive ingredients has only lately been studied. The polyphenol curcumin has been demonstrated to target a number of system signaling molecules and also exhibit action at the cellular level, supporting its numerous health advantages (Ashraf 2017).

Curcumin was isolated for very first time by Vogel and Pelletier from the rhizome of the plant *C. longa* (Vogel and Pelletier 1815). After a number of decades, Lampe et al. defined the structure of curcumin such as diferuloylmethane or 1,6-heptadiene-3,5-dione-1,7-bis(4-hydroxy-3-methoxyphenyl)-(1E,6E) (Lampe et al. 1910) (Fig. 3).

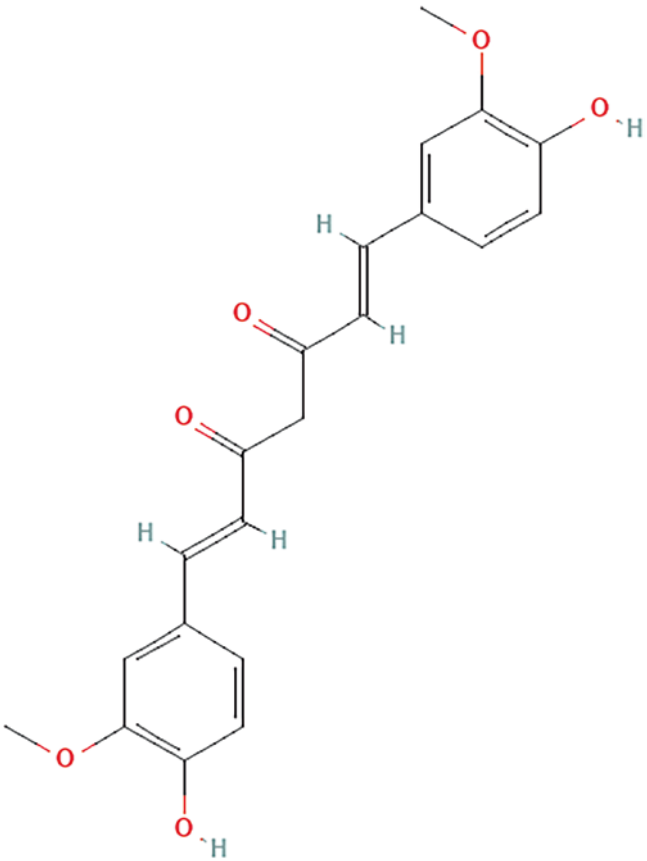

Fig. 3 Two-dimensional structure of curcumin. (Source: https://pubchem.ncbi.nlm.nih.gov/compound/969516#section=2D-Structure&fullscreen=true)

Three years later, in 1913, Lampe et al. again reported a process for synthesizing curcumin (Lampe and Milobedzka 1913). Srinivasan briefed the characterization as well as quantification of curcumin constituents by chromatography (Srinivasan 1953).

At neutral and acidic pH levels, curcumin is virtually insoluble in aqueous solutions at ambient temperature. But since it has a log P value of less than 3.0 and is lipophilic, it is soluble in organic solvents including methanol, ethanol, acetone, and dimethyl sulfoxide. The ketone form is still predominant at neutral and acidic pH levels, while the enol tautomer is only present under alkaline settings. This is explained by the intramolecular hydrogen bond in the enol form (Kotha and Luthria 2019).

7 Nutraceutical Implication

Curcuma longa consist of more than 100 components and rich source of carbohydrates and fiber. The polyphenolic compounds, found from its rhizome are promise for a variety of impairment in human health (Kurien et al. 2017). In addition, curcumin have certain proteins and fats, but it does not comprise cholesterol. The plant also has pyridoxine, vitamin C, and a number of minerals such as potassium, calcium, magnesium, and phosphorus in adequate quantities. With the presence of such nutrients, the plant is said to be nutritionally rich food product (Ahmad et al. 2020). Ikpeama et al. publicized that the turmeric plant had 0.05% iron, 0.20% calcium, 0.63% phosphorus, 0.16% riboflavin, 2.30% niacin, and 0.89% thiamine (Ikpeama et al. 2014). Because turmeric powder contains thiamine (0.59%), riboflavin (0.16%), potassium (0.46%), and iron (0.05%), it may be crucial in maintaining healthy bones, muscular tightening and relaxation, and blood pressure and clotting control. In addition to contributing to blood coagulation, muscular contraction, and relaxation, calcium is essential for maintaining healthy bones. Potassium and magnesium are also known to lower blood pressure. Additionally, potassium regulates the transmission of nerve impulses and the tightening of skeletal muscles. High-calcium and high-potassium diets are frequently advised to patients with soft bone issues (Kubmarawa et al. 2007). The extract's iron concentration can aid in hemoglobin synthesis, making it recommended for iron deficiency anemia. The fact that different minerals work as co-enzymes in several biochemical processes in the body highlights the significance of plants in metabolic processes (Ikpeama et al. 2014) (Table 2).

8 Pharmacology of Turmeric

Long before the ancient era, people employed plants for medical purposes. Chinese texts, Egyptian papyrus, and ancient Unani scrolls all discussed the usage of plants (Pan et al. 2014). There is evidence that around thousands years ago, Unani Hakims,

Table 2 Nutraceuticals of *Curcuma longa*

Nutrient	Value	Nutrient	Value
Energy	312 Kcal	Phosphorous	299 mg
Fat	3.25 g	Zinc	4.5 mg
Carbohydrate	67.1 g	Sodium	27 mg
Protein	9.68 g	Copper	1.3 mg
Total dietary fiber	22.7 g	Manganese	19.8 mg
Glucose	0.38 g	Vitamin C	0.7 mg
Sucrose	2.38 g	Selenium	6.2 µg
Fructose	0.45 g	Riboflavin	0.15 mg
Magnesium	208 mg	Niacin	1.35 mg
Iron	55 mg	Thiamin	0.058 mg
Potassium	2080 mg	Vitamin B6	0.107 mg

Note: Nutrients in 100 grams of *Curcuma longa* root (Fabianowska-Majewska et al. 2021)

Indian Vaids, and cultures from the Mediterranean and Europe used plants as medicine. Herbs were employed in healing rituals by indigenous societies in Rome, Egypt, Iran, Africa, and America. Other cultures created traditional medical systems like Unani, Ayurveda, and Chinese Medicine that systematically utilized herbal remedies (Patwardhan et al. 2005). There are still many people who practice traditional medical methods. The use of plant materials as a source of medicines for a wide range of human ailments has received more attention as a result of factors including population growth, insufficient drug supply, prohibitive cost of treatments, side effects of several synthetic drugs, and development of resistance to currently used drugs for infectious diseases (Sofowora et al. 2013).

According to estimates from the World Health Organization (WHO), 80% of people worldwide rely on herbal remedies for some of their basic medical requirements (Hom 2021), and around 21,000 plant species have the potential to be utilized as medical plants. A lot of health-related issues and disorders were solely treated using herbs, according to ancient experts. This is the reason why herbal medicine is becoming more and more well-liked worldwide. These therapeutic plants offer logical techniques for treating several interior ailments that are normally thought to be difficult to treat (Oliveira et al. 2012).

More than 75% of the world's population is primarily dependent on plants (Abelson 1990) and plant extracts (Jeney et al. 2015) for their health care needs. Over 30% of all plant species have been utilized for medical reasons at some point. According to estimates, plant-based medications account for up to 25% of all pharmaceuticals used in industrialized nations like the United States, while they account for up to 80% of all drugs consumed in rapidly growing nations like India and China. As a result, countries like India place a considerably greater value on medicinal plants economically than the rest of the globe. The health care system for the rural population depends on indigenous systems of medicine, and these nations contribute two thirds of the plants utilized in contemporary medicine (Kassaye et al. 2006). Although the use of herbal medicine has significantly increased over the past 20 years, there is still a dearth of research data in this area. As a result, three

volumes of WHO monographs on specific medicinal plants have been released since 1999 (World Health 1999). The majority of people in poor nations still rely on herbal remedies (Khan and Ahmad 2019). Tens of thousands of plant compounds continue to be created as secondary metabolites as a kind of protection against infection and illness. Plant-based medicines have been essential to the health care of numerous societies, both ancient and modern (Suntar 2020). In Veda's many plants mentioned are abundant in the country for treatment of various diseases. Among them numerous exist spice plants used in day-to-day life. Turmeric is one of most popular spices with tremendous medicinal properties (Kataki et al. 2019). Due to its high-iron content, turmeric is helpful for anemia. In order to cure this ailment, every day, 1 teaspoon of fresh turmeric juice and honey are ingested. Measles can be treated with turmeric. The sun-dried turmeric roots are pounded into a fine powder. This can be consumed by diseased with the condition when it is mixed with a little honey and the juice of a few bitter gourd leaves. Due to its antibacterial potentials, turmeric is a helpful treatment for dogged cough and throat irritations. In these cases, a mixture of 30 ml of warm milk and half a teaspoon of fresh turmeric powder works miracles. To make this, milk is poured onto a heated ladle that has been infused with turmeric and cooked over (Patwardhan 2000). The four main systems of indigenous medicine are Ayurveda, Unani, Siddha, and Folk (tribal) remedies. Ayurveda and Unani medicine are the two most established and popular systems in India (Sen et al. 2011). Many people have now begun planting this plant and other medicinal plants in their backyard gardens after learning about the plant's use in medicine. Turmeric is regarded as a rich source of ingredients that can be used in the creation of pharmaceuticals. In addition, this plant is essential for the growth of human cultures all over the world (Altman et al. 2022). The use of medicinal plants is thought to be very safe because there are rarely any negative side effects. The biggest benefit is that these treatments work in harmony with nature. The use of herbal remedies can benefit people of all ages and genders, which is a key fact (Lynch and Berry 2007). These days' pharmaceutical manufacture relies heavily on medicinal plants. While turmeric is also used as an ancient spice (Yadav and Tarun 2017), among Southeast Asian native, it has been used as a condiment and tincture for centuries. Due to its natural, unprocessed, and affordable qualities, it is quiet utilized in Hindu rituals as a dye for holy clothing. In fact, one of the least expensive spices is turmeric. Although it is used similarly to saffron as a color, the two spices' culinary applications should not be confused, and they should never take the place of saffron in food preparations. It was employed as a food spice and had some religious importance in the Vedic civilization of India, where it goes back over years (Prasad and Aggarwal 2011).

9 Antioxidant and Anti-inflammatory Action

Turmeric has long been utilized as traditional remedy for its anti-inflammatory properties, which have received substantial scientific validation. Curcumin has extracted from the oleoresin of turmeric which is also the reserve material for the

extraction of certain oil and resin. Throughout a laboratory study, several portions of turmeric oil showed remarkable antimutagenic and antioxidant activity (Prasad et al. 2014). The metabolism of arachidonic acid, cyclooxygenase, lipoxygenase, cytokines (tumor necrosis factor and interleukin-6), nuclear factor-B, and steroid hormones have all been shown to be inhibited by curcumin (Jobin et al. 1999). In addition to stabilizing the lysosomal membrane and causing the decoupling of oxidative phosphorylation, curcumin has also been linked to high-oxygen radical scavenging activity, which is what gives it its anti-inflammatory properties (Mullaicharam and Maheswaran 2012).

10 Antimicrobial

Turmeric could be an alternative antimicrobial mediator alongside fatal bacterial infections. Various types of curcumoids have been reported to have antifungal activities against some major plant pathogenic microbes (Mahato and Sharma 2018). Epigallocatechin gallate (EGCG) greatly improved the antibacterial activity of curcumin against the multidrug-resistant *Acinetobacter baumannii*. Consequently, the pharmaceutical combination of EGCG and curcumin can be utilized to treat *Acinetobacter baumannii* infections (Betts and Wareham 2014). It also has broad-ranging antiviral activity (Zorofchian Moghadamtousi et al. 2014).

11 Hepatoprotective Effects

When administered to ducklings afflicted with *Aspergillus parasiticus*, turmeric extract can 90% prevent the formation of the fungus that causes aflatoxin. Additionally, curcumin and turmeric corrected lipid alterations, necrosis, and biliary hyperplasia brought on by the synthesis of aflatoxin. (Akram et al. 2010). Curcumin has antioxidant activity, which leads to drop in structural alternation of the liver and total bilirubin; it also increases in serum proteins (Khan et al. 2019). It was effective in treating cholestasis, hepatic fibrosis, and liver cancer (Garcia-Nio and Pedraza-Chaverri 2014).

12 Pharmacology of Curcumin

12.1 Anticancer

Cancer, one of the most common health diseases today, impacts people of all age groups of the world. On malignant tumors, a potential clinical trial of curcumin was undertaken. Its anti-inflammatory properties may have the ability to prevent cancer

on some ventricular arrhythmias (Wongcharoen and Phrommintikul 2009). Curcumin has now been used in numerous clinical investigations, and its capacity to modulate multiple objectives has allowed it to apply considerable efficacy alongside various forms of cancer in clinical trials (Hatcher et al. 2008).

One of the most effective chemopreventive and anticancer medications is curcumin (Singh and Khar 2006). It has been shown to have anticancer activity in addition to having a variety of biological properties, including antioxidant, anti-inflammatory, and angiogenesis inhibition. In-depth investigation into the molecular mechanisms behind its wide range of cellular activities has shown interactions between it and several distinct macromolecules and cell targets. The anti-angiogenic properties of curcumin have been demonstrated, and its angioinhibitory effects are brought on by the downregulation of proangiogenic genes including VEGF and angiopoietin, as well as a reduction in endothelial cell motility and invasion (Wongcharoen and Phrommintikul 2009).

12.2 Anticardiovascular

Curcumin contains anti-inflammatory and antioxidant properties; therefore it can help to prevent cardiovascular problems (Ahmad et al. 2020). Antioxidant properties of curcumin have been proven to reduce adriamycin-induced cardiotoxicity and can help in diabetes-induced cardiovascular problems. Curcumin's antithrombotic and antiproliferative activities, as well as its capability to reduce serum cholesterol expressions, can defend pathological alterations associated with atherosclerosis. Curcumin restricts the p300-HAT inhibitory actions which have been established in animal models to increase the development of ventricular hypertrophy and heart failure.

12.3 Role in Hyperlipidemia

Curcumin controls cholesterol metabolism by acting as a regulator of lipid expression. It has been demonstrated to be effective against atherosclerosis in both humans and animals in clinical tests. Curcumin has been demonstrated in animal experiments to lower LDLc and triglycerides while boosting HDLc (Yadollahi and Zargaran 2019).

12.4 Antidiabetes

Curcumin's antidiabetic properties have been linked to its capacity to reduce oxidative stress and inflammation, according to research. It may also help to prevent the negative effects of diabetes (Kubmarawa et al. 2007). It is a natural anti-inflammatory

and antidiabetic substance that is a safe and cost-effective alternative for diabetic condition medication, while it is still required to know the correct dose. Hoda et al. discovered that daily administration of 1500 mg of curcumin pulls down fasting blood glucose and weight in type 2 diabetes patients (Hoda et al. 2019).

12.5 Arthritis

Arthritis has a chronic and acute disease with a thoughtful psychosocial and economic effect (Bardwell et al. 2002). Rheumatoid arthritis is a chronic systemic autoimmune illness that affects more women than men, with older women being the most commonly affected (Guo et al. 2018). Both rheumatoid arthritis and osteoarthritis haven't any cure; however, there are a number of pharmacological therapy choices, but many of them are expensive and have unfavorable side effects. As a result, interest in complementary therapies has grown. It comprises herbal preparations and dietary supplements. Numerous investigations have revealed that curcumin has anti-arthritic properties in people with rheumatoid arthritis and osteoarthritis (Hewlings and Kalman 2017).

12.6 Immunity Enhancement

Curcumin is potent immunomodulatory substance that has the ability to control the activation of dendritic cells, natural killer cells, macrophages, and lymphocytes. It has been discovered that curcumin influences the development and cellular response of many immune system cell types (Jagetia and Aggarwal 2007). It is useful at preventing and treating a number of human diseases, such as cancer, cardiovascular, inflammatory, metabolic, neurological, and skin disorders, according to a number of preclinical and clinical studies (Kunnumakkara et al. 2017). Furthermore, it has many diverse qualities, but one of the most researched is its anti-inflammatory profile, which may be helpful in both acute and chronic inflammation. In recent decades, proof for curcumin as a possible medicinal and nutraceutical agent has grown (Dulak 2005; Košťálová et al. 2013; Kotha and Luthria 2019); the development of a vast various curcumin compounds demonstrates this. The growing interest has prompted more in vivo and in vitro clinical trials to assess curcumin and curcuminoids' bio-efficacy. Curcumin's medicinal usage is inadequate due to its lowly water solubility, bioavailability (Ravichandran 2013), and pharmacokinetic characteristics. Numerous curcumin preparations have been established to treat these concerns. However, inaccurate assessment of bioactivities and their clinical efficacy is frequently hampered by inefficient sample preparation and analytical procedures. The evaluation of alternative preparations and biological actions of curcuminoids will be considerably improved by using ideal sample preparation, chromatographic partitioning, and finding procedure (Kotha and Luthria 2019).

Numerous studies have suggested that turmeric may be effective in treating a variety of diseases. However, when you read news headlines regarding the therapeutic benefits of turmeric, it's crucial to keep a limited things in mind. First, the herb may not function as effectively in people as it does in test tubes and animals, where several studies have been conducted. Second, curcumin, turmeric's key ingredient, has been administered intravenously in several experiments. Finally, several of the investigations present contradictory data. However, turmeric may show promise in the treatment of digestive issues, the prevention of certain malignancies and infections, and the reduction of inflammation (Singletary 2020).

13 Conclusion

The usual method for obtaining turmeric's health benefits is through long-term, low-dose food ingestion. Understanding the precise dosage, safety, and mechanism of action of turmeric is required for its logical application in the treatment of human ailments. More clinical research is needed if turmeric is to be used to meet human requirements and improve wellness. Turmeric has a variety of beneficial effects, including those that are antibacterial, antiviral, anti-inflammatory, antitumor, antioxidant, antiseptic, cardioprotective, hepatoprotective, nephroprotective, radioprotective, and digestive. Numerous additional components of turmeric found via phytochemical study, including curcumin, volatile oil, and curcuminoids have been shown to have potent pharmacological effects. Curcumin can now alter a wide range of cell signaling pathways and interact with a large number of molecular targets. As a result, it could be able to treat a wide range of illnesses. It can be applied for treatment. Therefore, alternative formulations and biological activities of curcumin require to access by which we can resolve the complications of such diseases.

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Phytochemical Diversity and Biological Activity of Basil (*Ocimum* L.) Secondary Metabolites Produced In Vitro

Dragana Jakovljević, Edyta Skrzypek, Milan Stanković, and Marzena Warchoł

Abstract The increased need for natural products leads to the expansion of new techniques and creates the necessity for the implementation of these techniques to adequately respond to the increased interests. Plants of the genus *Ocimum* L. (basil) are natural sources of biologically active products with widespread use and wide utilization both traditionally and industrially. Numerous basil plants have been used for decades mainly because of their aromaticity, significant amount of essential oils, and biologically active phenolic compounds. Additionally, recent research indicates that isolated compounds from extracts of different basil cultivars possess significant biological effects important for human health. The in vitro plant culture (culture of plant cells, tissues, and organs) includes methods that are in recent years commonly used to produce secondary metabolites, primarily due to the efficient production of particular metabolites at significantly higher concentrations than ex vitro. The present chapter aims to summarize the crucial publications on the genus *Ocimum*, with a focus on research related to the production and diversity of biologically active secondary metabolites under tissue culture conditions. With a high content of biologically active secondary metabolites and with a multidisciplinary approach, different varieties of basil provide significant opportunities for in vitro manipulation and offer great opportunities for practical application.

Keywords Antioxidant activity · Callus · Cell suspension · *Ocimum* · Phenolic compounds · Tissue culture

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N. Kumar, R. S. Singh (eds.), *Biosynthesis of Bioactive Compounds in Medicinal and Aromatic Plants*, Food Bioactive Ingredients,
https://doi.org/10.1007/978-3-031-35221-8_16

1 Introduction

The *Ocimum* (basil) genus includes the most cultivated species of the family Lamiaceae together with species of the genera *Rosmarinus* L., *Thymus* L., *Salvia* L., etc. The main characteristics of all representatives are the quadrangular stem, oppositely placed leaves, distinctly zygomorphic flowers, and the presence of highly aromatic essential oils. The first taxonomic description of the genus was done in 1753 by Carl von Linné, who included five species within the genus *Ocimum*, but since then the classification of this genus has changed several times. Pushpangadan (1974) is the author of the infrageneric classification according to which two groups are distinguished: the *Basilicum* group (herbaceous annual, rarely perennial plants with strongly mucinous black, ellipsoid seeds, and the basic number of chromosomes $x = 12$) and the *Sanctum* group (bushy perennial forms with spherical, brown seeds, without or with very little mucus and basic number of chromosomes $x = 8$). Based on this classification, the section *Ocimum* and subsection *Ocimum* together with the most widespread taxa (var. *basilicum*, var. *difforme*, var. *purpurascens*) are included in the *Basilicum* group, while all other representatives are classified in the *Sanctum* group. Although this type of classification is most often used in scientific and popular literature, the main problem with this type of infrageneric classification is noncompliance with the standards of the International Code of Botanical Nomenclature (Paton et al. 1999; Carović-Stanko et al. 2011). Today, the genus *Ocimum* includes about 150 annual and perennial species of herbaceous plants, rarely shrubs, native to subtropical and tropical parts of Asia, central parts of South America, and Africa (Labra et al. 2004). The most numerous are annual, shallow-rooted herbaceous plants, and less often representatives have a solid bushy structure. They are characterized by branched quadrangular stems on which are oppositely arranged leaves on long stalks, egg-shaped, with a flat or toothed edge. The inflorescences (with flowers located on a short flower stem) are at the top of the stem.

The cropping of economically significant representatives of the genus *Ocimum* has intensified worldwide, primarily due to their nutritional and pharmaceutical importance, and their adaptability to different climatic conditions and various soil types. Long-term traditional use together with wide distribution, selection, and breeding has significantly contributed to the variability among the genotypes. Nowadays agronomists mainly distinguish different basil genotypes based on the color of the leaves and leaf area (green to dark purple; small to large leaf), flower color (white to purple), and other characteristics such as shoot shape, flowering period, height, and aroma (Carović-Stanko et al. 2010). However, the criteria for the characterization of taxa within the genus *Ocimum* are far more complex. Characteristics used for decades in the taxonomic classification of representatives of the genus *Ocimum* because the intensive cultivation, intraspecific hybridization, and polyploidy show a high degree of variability, including chemical composition and characteristics such as the color, shape, and size of the flower, leaf, or stem (Patel et al. 2016). Recent taxonomic studies, in order to achieve better intra- and interspecies classification, are based on different approaches, including

geographical origin, morphology, karyotype, and chemical composition. Based on the geographical origin, Hiltunen and Holm (2003) classified basil chemotaxonomically into four chemotypes: reunion (Egyptian) chemotype with methyl chavicol (about 80%) as the main component; European chemotype with the highest-quality aroma and high content of linalool (from 35% to 50%) and methyl chavicol (from 15% to 25%); tropical chemotype where methyl cinnamate is the main component; Java (eugenol) chemotype with a high content of eugenol. According to Grayer et al. (1996), basil chemotype is defined by the component that has more than 20% of the total essential oils content, and by applying this classification system, methyl chavicol, linalool, methyl eugenol, eugenol, and geraniol type can be distinguished within the most cultivated basil species *Ocimum basilicum* L. Nevertheless, the classification based on chemotypes has its weaknesses, considering that one taxon can include two or more components that are almost equally represented. This is why various plant breeding, tissue culture, and metabolic and transgenic engineering strategies are being investigated to improve specific chemotype profiles and essential oils yields in *Ocimum* species (Gurav et al. 2021). Additionally, it has been shown that the quantitative-qualitative content of basil essential oils varies depending on numerous abiotic factors (Daneshian et al. 2009). According to Pushpangadan and Bradu (1995), the *Ocimum* genus counts about 160 species; however, about 65 species are native to *Ocimum*, while the rest should be taken into account as synonyms (Chowdhury et al. 2017). Sipos et al. (2021) pointed to seven different morphotypes including tall type, bush (dwarf) type, large-leafed (Italian) type, compact (Thai) type, purple and purpurascens types, and flavored citriodorum type. The standardized list for the identification of basil cultivars based on morphological characteristics is developed by the International Union for the Protection of New Varieties of Plants (UPOV). Today, more than 70 basil taxa possess accepted scientific names (www.theplantlist.org). Considerable progress has been made in basil classification using DNA analyses that allow phylogenetic and taxonomic studies and cultivar identification by comparing genotypes independently of phenotypes (Labra et al. 2004). Therefore, to better identify different *Ocimum* genotypes, existing evaluation techniques and chemotaxonomy should be integrated with environmentally independent molecular markers (Chowdhury et al. 2017). No matter of classification and geographical origin *O. basilicum* var. *basilicum* (cultivars 'sweet basil' and 'Genovese'), *O. basilicum* var. *purpurascens* (cultivars 'purple ruffle' and 'dark opal'), *O. basilicum* var. *thyrsiflorum*, *O. basilicum* var. *difforme*, *O. minimum* (syn. *O. basilicum* var. *minimum*), *O. gratisimum*, and *O. sanctum* (syn. *O. tenuiflorum*) are most commonly grown worldwide. Figure 1 shows the most cultivated *Ocimum* taxa where different morphology of aboveground plant parts can be seen.

In recent decades basil cultivation has increased globally, mainly because of the fragrance and nutritional properties, but at the same time also due to the high harvest index and profitability margin (Polyakova et al. 2015). For instance, it is estimated that the market-available essential oils from basil originating from India typically cost between 40 and 45 euros per 1 kg (Lubbe and Verpoorte 2011). Sipos et al. (2021) pointed out that according to the research report of the global basil leaves market growth is expected from 57 million USD to 62 million USD due to

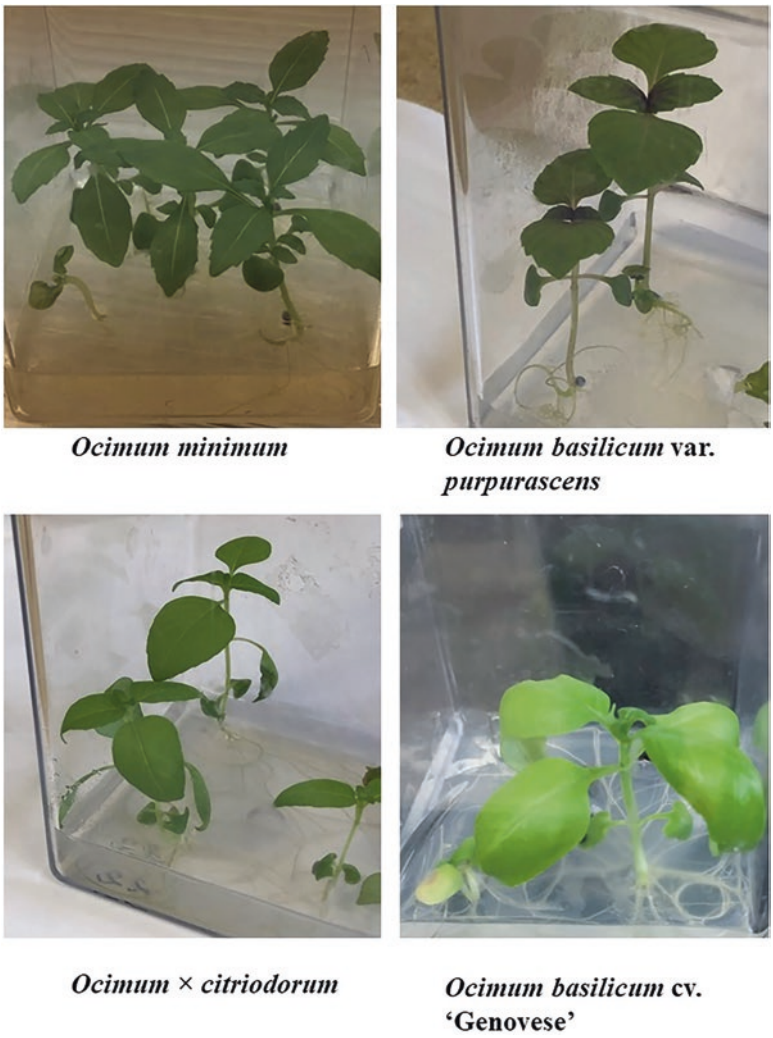


Fig. 1 Morphology of selected *Ocimum* taxa cultivated in vitro

the worldwide increasing consumption of basil. Basil plants are mostly produced in an open field under the traditional production method as fragrant plants for fresh herbs. However, it is impossible to manage environmental factors such as light or temperature under these cultivation conditions (Bączek et al. 2019). Additionally, it has been demonstrated that better basil production can be obtained under soilless systems compared to traditional ones, with two times reduced production costs (Saha et al. 2016; Khater et al. 2021). In the food industry, basil plants are commonly used fresh for culinary purposes but also can be preserved by freezing, drying, or extracting (Raimondi et al. 2006). In recent decades cultivation of basil

plants is enhanced due to increased demand for biologically active natural compounds since various basil genotypes are significant sources of phenolic compounds (PC) and essential oils with antimicrobial, antioxidant, anticancer, cytotoxic, antiproliferative, bioherbicidal, and insecticidal activities (Dudai et al. 1999; Grayer et al. 2004; Nguyen and Niemeyer 2008; Telci et al. 2009; Carović-Stanko et al. 2010; Imen et al. 2012; Tang et al. 2013; Zabka et al. 2014; Flanigan and Niemeyer 2014; Elansary and Mahmoud 2015; Jakovljević et al. 2019, 2021; Zagoto et al. 2021).

2 Secondary Metabolism in Basil Plants

By reviewing earlier studies that include different varieties of basil, it can be seen that the majority of research was conducted to examine morphological and anatomical differences, as well as differences in biological properties of herbs originating from different genotypes of basil. When it comes to secondary metabolism, investigations have mainly included differences in terms of the quantity of essential oils or identifications of chemotypes based on the qualitative characteristics of essential oils (Grayer et al. 1996; Lee et al. 2005; Anandjiwala et al. 2006; Politeo et al. 2007; Hussain et al. 2016). The obtained data indicate significant variations in the quantity and quality of secondary metabolites, primarily concerning the origin of the tested samples but also due to cultivation conditions and environmental factors. Basil, like most representatives of the Lamiaceae family, is characterized by a high concentration of secondary metabolites, particularly from the PC group, but their composition and characteristics are variable depending on numerous factors (Jakovljević et al. 2019). It was shown that extracts from different varieties of basil are characterized by the presence of various PC from the group of phenolic acids (primarily rosmarinic, caffeic, caftaric, and cichoric acids), which significantly contribute to antioxidant properties. In addition to phenolic acids, PC from the flavonoid group are also presented (Javanmardi et al. 2002; Lee and Scagel 2009; Kwee and Niemeyer 2011; Flanigan and Niemeyer 2014; Ghasemzadeh et al. 2016; Jakovljević et al. 2022).

Aromatic amino acid phenylalanine overall represents the central molecules of plant metabolism because, in addition to being an essential component in protein building, it is a significant precursor for a wide range of secondary metabolites, in particular aromatic metabolites with numerous biological activities. The synthesis of phenylalanine takes place through the shikimic acid pathway and is restricted exclusively to the plastid stroma, and the complexity of this process was explored by Tegeder and Weber (2006) and Vogt (2010). The first step in the synthesis of phenylalanine is catalyzed by the enzyme chorismite-mutase, which converts chorismate to prephenate. Through further transamination, arogenate is produced, while the removal of hydroxyl and carboxyl groups by arogenate-dehydrogenase leads to the production of phenylalanine. The phenylalanine ammonia-lyase (PAL) catalyzes non-oxidative deamination of phenylalanine to trans-cinnamate (Tzin and Galili 2010; Fraser and Chapple 2011). The produced trans-cinnamate is further

transformed into numerous PC. Phenylpropanoid metabolism is a necessary metabolic pathway ending with different aromatic products and building components essential for structural support and vascular integrity and plant defense against various biotic and abiotic factors. The expression of the genes which are responsible for PAL enzyme activity is controlled developmentally and spatially, and changes in activity can additionally occur due to abiotic and biotic factors (Vogt 2010; Fraser and Chapple 2011; Zhang and Liu 2015).

For the initial steps catalyzed by PAL, cinnamate 4-hydroxylase (C4H) and 4-coumarate-coenzyme A ligase (4CL) are necessary components creating the basis of further metabolite synthesis of various aromatic components, i.e., polyphenols (Fig. 2). About 8000 aromatic metabolites are formed in this way, and these metabolites are further classified into different subclasses (or classes), including phenolic acids, flavonoids, anthocyanins, coumarins, stilbenes, lignins, and others. During the process of growth and development, PAL activity is induced, but the activity can also be induced by many environmental factors such as UV radiation, pathogen attack, lack of nutrients, and other processes in which synthetized aromatic components protect plants (Baque et al. 2010; Jakovljević et al. 2019). Control of PAL activity takes place through various mechanisms, including transcriptional and translational regulation, product inhibition, posttranslational inactivation, subcellular compartmentation, and metabolic feedback regulation. Understanding the mechanisms of synthesis of secondary metabolites and their quantity and quality under various environmental conditions continues to be a research priority (Lattanzio et al. 2009; Zhang and Liu 2015).

Although the PAL enzyme cannot be regarded as a component of the plant antioxidant system, the compounds synthesized through the activity of this enzyme significantly contribute to the overall antioxidant capacity of plants. Considering that PAL enzyme activity is induced during the process of growth and development but also during irradiation, nutritional deprivation, and many other processes during which aromatic components protect plant compartments (Baque et al. 2010; Jakovljević et al. 2017, 2019), understanding the mechanisms that control quantitative and qualitative traits of synthesized compounds have become a priority in recent investigations of secondary metabolites (Lattanzio et al. 2009; Zhang and Liu 2015). Besides the fact that the identification of metabolic differences between organs, tissues, and cells is an important component of understanding plant metabolism (de Miguel et al. 2016), there is obscure information about the secondary metabolite content and PAL enzyme activity correlation in plant cells and tissues of different basil genotypes. In a recent study conducted to investigate the possibilities of induced synthesis of secondary metabolites in different basil cultivars under tissue culture conditions (Jakovljević et al. 2019), it was demonstrated that the increased activity of PAL and accordingly high content of PC and better antioxidant capacity of three basil cultivars (‘Genovese’, ‘small leaved’, and ‘lemon basil’) can be obtained under nutrient deficiency conditions. Additionally, for the purple basil cultivar ‘dark opal’, reduced PAL activity was correlated with the lower amount of PC under nutrient deprivation. These types of research involving PAL enzyme activity may be of particular interest when considering the instability in the production of

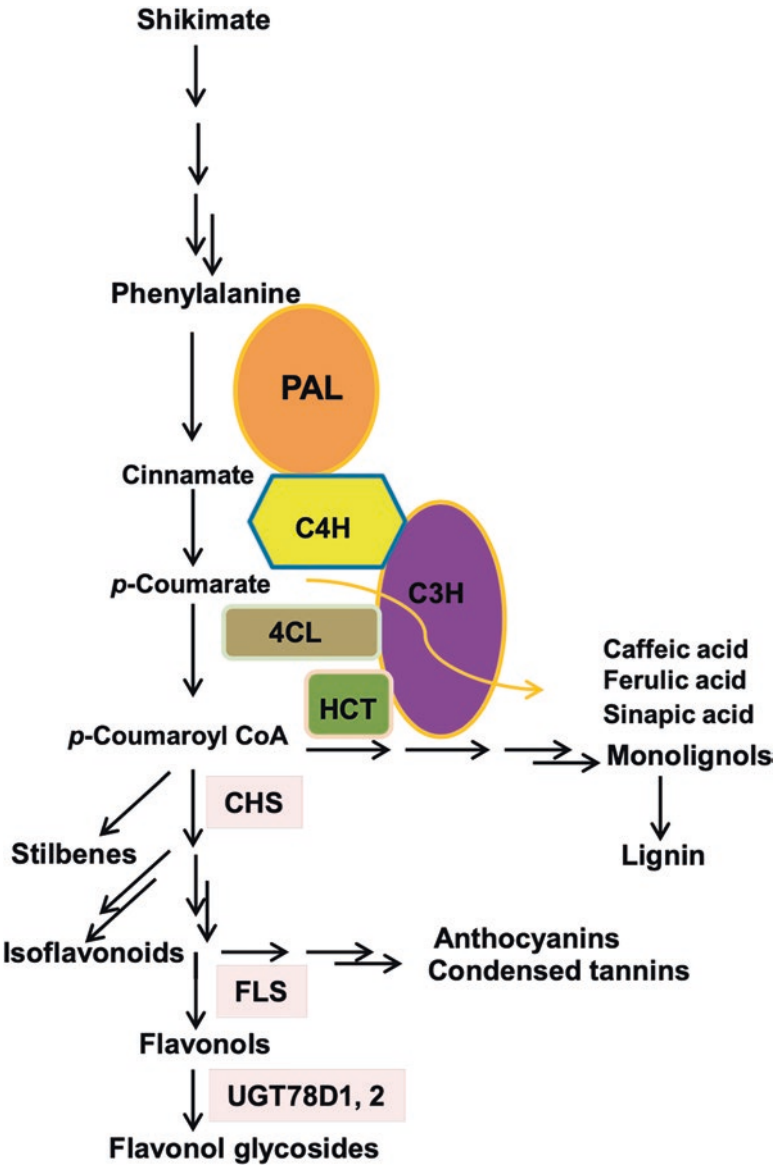


Fig. 2 Pathway of phenylpropanoid biosynthesis and phenylalanine ammonia-lyase (PAL) as an entry point enzyme of the general phenylpropanoid pathway (according to Zhang and Liu (2015) with minor modifications). *4CL* 4-coumarate-coenzyme A ligase, *C4H* cinnamate 4-hydroxylase, *CHS* chalcone synthase, *FLS* flavonol synthase, *HCT* hydroxycinnamoyl- coenzyme A:shikimate hydroxycinnamoyltransferase, *UGT78D1* flavonol 3-*O*-rhamnosyltransferase, *UGT78D2* flavonoid 3-*O*-glucosyl- transferase

secondary metabolites of certain basil varieties. For example, the dark purple color of the *O. basilicum* var. *purpurascens* originates from anthocyanins, which are localized in the epidermal layer in the leaves and flowers, while in the stem, they are also present in the inner layers. Although the purple color and the presence of anthocyanin increase the utility value of this variety, the instability of the cultivars of this basil variety in the amount of anthocyanin is one of the main problems during cultivation. The genes responsible for anthocyanin synthesis are extremely unstable, so even vegetatively propagated shoots can lose their purple color. In the 'dark opal', depigmentation is accompanied by a uniform loss of anthocyanins throughout the shoot, while in the 'purple ruffles', the loss of pigments occurs in individual parts of the shoot (Phippen and Simon 2000). Therefore, investigations of PAL activity and synthesis of basil PC under controlled conditions can have significant applications in the future.

3 Basil Tissue Culture

The plant cell, tissue, and organ culture, i.e., aseptic culture of plants in vitro, mean the cultivation and multiplication of cells, tissues, or organs of plants on a liquid or solid medium, under strictly controlled conditions. Based on the totipotency, i.e., the principle of pluripotency of plant cells, in vitro culture can be applied for almost any plant species, and it is commonly used for mass production of uniform plant material, preservation of germplasm, studies of growth and development regulation, synthesis of metabolites, etc. The tissue culture method can provide pathogen-free plants, rapid preservation, and cloning of the genotypes of interest, and all this can be achieved in a short period of time. Additionally, for certain plant species, conventional techniques of propagation may require large financial investments and a longer period. Therefore, for these plant species, plant cell, tissue, or organ culture are of multiple importance (Máthé et al. 2015; da Silva et al. 2017). Today, in vitro culture of plants is established routinely under aseptic conditions, and in recent years it has been intensified for medicinal and aromatic plants, mainly because of the efficient production of secondary metabolites under in vitro controlled conditions. An additional advantage of the established in vitro system in comparison to the plantation method of plant growing is the use of aseptic media with a clearly defined composition, without the need for nutritional supplements, and with less physical space. All these advantages enable easier material processing with an effective screening of the obtained plant material. However, difficulties and failures may arise in the establishment of tissue cultures with the aim of the production of secondary metabolites mainly due to the insufficiently clarified pathways of the synthesis of particular metabolites (Karuppusamy 2009; Srivastava et al. 2014).

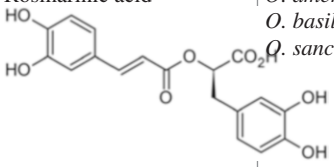
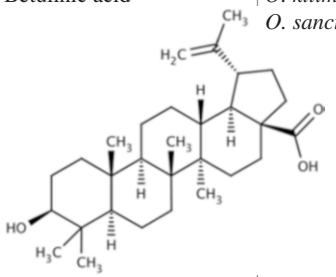
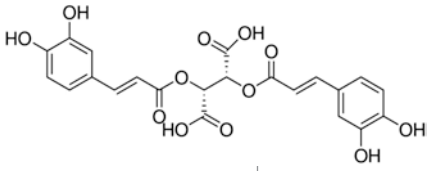
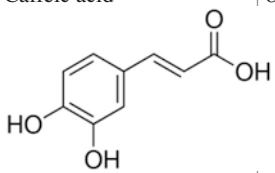
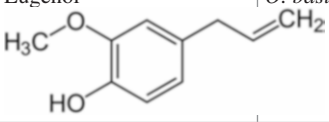
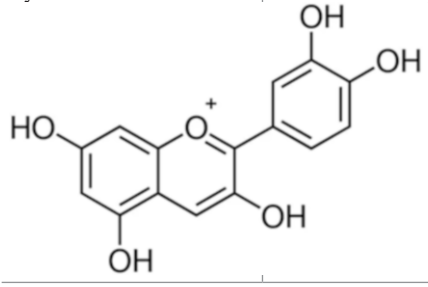
In vitro cultures, although were primarily developed for basic research on morphogenesis and differentiation of cells and tissues, today is considered one of the most prominent techniques of plant manipulation and sustainable plant biotechnology. Manipulation of plant explants in aseptic conditions, combined with growth

and differentiation under the defined temperature, humidity, and light intensity, allows maintaining of uniformity and the desired plant characteristics, while the production of biologically active secondary metabolites in the mentioned conditions enables the rational use of natural resources and conservation (Jakovljević and Stanković 2020; Jakovljević et al. 2022). Different varieties of basil, when considering their multiple importance, have been insufficiently studied under in vitro conditions. The most of investigations are related to propagation through different explants and on different substrates. Still, investigations from the last decade indicate increased production of secondary metabolites in basil plants under in vitro conditions, particularly through the callus, cell suspension, and hairy root culture. The most commonly identified compounds and their chemical structure are presented in Table 1. With the selection of an adequate type of tissue culture, the composition and concentration of the medium, and the application of exogenous elicitors, the synthesis of metabolites of interest can be induced in significantly higher concentrations than when growing the same basil genotype using standard cultivation methods (Jakovljević 2018; Jakovljević et al. 2022).

Through the callus culture, the undifferentiated plant cells can be maintained for a long period on a solid medium with a unique growth pattern of callus tissue established after callus induction and further cell division. The changes in the callus structure, the size of cells and tissues, and in cell metabolism can be seen through the different developmental stages of callus culture, whereas the placement of the callus on media and conditions which cause the increased synthesis of metabolites of interest can be of great importance in the production of bioactive components originating from plant sources. The great practical importance can be seen in successfully established callus culture since the growing tissue can have different metabolism compared to the plant from which callus culture is established. When it comes to the callus culture of basil with the aim of producing secondary metabolites, previous reports demonstrated high content of total PC (including flavonoids and phenolic acids) in *O. basilicum* (Guirgis et al. 2007; Abdel Rahman et al. 2015; Pandey et al. 2015; Duran et al. 2019; Nadeem et al. 2019), *O. basilicum* var. *purpurascens* (Nazir et al. 2019, 2020a, b), *O. basilicum* cv. "Thai basil" (Nazir et al. 2020c, 2021a, b), *O. sanctum* (Hakkim et al. 2007; Pandey et al. 2015), and *O. kilimandsharicum* (Pandey et al. 2015).

Cell suspension culture consists of cells and cell aggregates in a mobile liquid medium. During the incubation of cells in a liquid medium and in the initial stages of cell suspension, the amount of plant material is constantly increasing. This increase is time-limited, given that the cell suspension reaches the maximum amount of plant material at one point, so the progressive growth of the cell suspension is followed by a stationary phase. If the cell suspension is subcultured, i.e., placed in the initial conditions with the same cell content and the same medium concentration, it can be expected that in the following period, the cell suspension will go through the same or a similar growth pattern and yield a similar amount of plant material. In this way, the cell suspension can be propagated by successive passages for a long period. For the secondary metabolite production in cell suspension culture, the high metabolic rate, rapid cell mass proliferation, and rapid response to

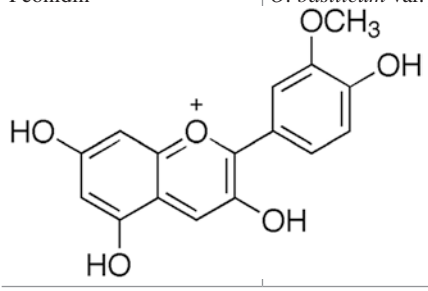
Table 1 The most commonly identified secondary metabolites in basil (*Ocimum* spp.) callus, cell suspension and hairy root cultures

Secondary metabolite	Species	References
Rosmarinic acid 	*O. americanum* *O. basilicum* *O. sanctum*	Rady and Nazif (2005) Nadeem et al. (2019) Nazir et al. (2020a, b, c) Pandey et al. (2015)
Betulinic acid 	*O. kilimandsharicum* *O. sanctum*	Pandey et al. (2015) Pandey et al. (2015)
Chicoric acid 	*O. basilicum*	Nadeem et al. (2019) Nazir et al. (2020a, b, c)
Caffeic acid 	*O. basilicum*	Nazir et al. (2019)
Eugenol 	*O. basilicum*	Nadeem et al. (2019)
Cyanidin 	*O. basilicum* var. *purpurascens*	Nazir et al. (2019)

(continued)

Table 1 (continued)

Secondary metabolite	Species	References
Peonidin	*O. basilicum* var. *purpurascens*	Nazir et al. (2019)



various stimulants are the most important advantages (Jakovljević et al. 2022). This type of tissue culture is used for the production of PC in *O. basilicum* (Kintzios et al. 2003, 2004; Pandey et al. 2019; Açıkgöz 2020, 2021), *O. basilicum* cv. 'dark opal' (Strazzer et al. 2011), and *O. sanctum* (Hakkim et al. 2011a, b).

Hairy root culture implies the cultures of genetically transformed plant roots caused by the infection with *Agrobacterium rhizogenes*. The hairy roots produced in this way possess a high growth rate and can be characterized by higher levels of secondary metabolites compared to untransformed (intact) plants (Giri and Narasu 2000). Due to fast and stable growth, growth on media free of hormones, genetic stability, and the production of secondary metabolites in concentrations higher than in untransformed roots, transgenic cultures of hairy roots represent an effective biotechnological approach for the secondary metabolites production (Bais et al. 2002; Sharan et al. 2019). Hairy root culture was established for *O. basilicum* to produce phenolic acids (Tada et al. 1996; Bais et al. 2002; Marzouk 2009; Srivastava et al. 2016; Kwon et al. 2021) and for *O. tenuiflorum* to produce various PC (Vyas and Mukhopadhyay 2014; Sharan et al. 2019).

4 Phytochemical Diversity of Basil Secondary Metabolites Produced In Vitro

Plant secondary metabolites from the group of PC are among the largest and most comprehensive products of plant secondary metabolism ubiquitously present in the plant kingdom but uncommon in algae, fungi, and bacteria. PC has received a lot of attention in the last few years since it is found that reduced risk of various disease development may be related to the intake of juices, brews, and vegetables with a high level of these aromatic compounds. Therefore, PC are applicable for the preparation of nutraceuticals, ingredients of functional food, dietary supplements, or cosmeceuticals (Lattanzio et al. 2008). Today the antioxidant ability of PC is well known. These natural antioxidants, mainly presented in medicinal and aromatic

herbs, make an increasing interest because of their ability to protect cell membranes from induced oxidative stress and free-radical-induced damage. These antioxidant properties of PC are a consequence of their ability to act as electron donors, as well as their ability to chelate metal ions (Filip et al. 2017; Kruk et al. 2022). Due to the carcinogenic, mutagenic, and overall toxic properties that synthetic antioxidants like BHA (butylated hydroxyanisole) and BHT (butylated hydroxytoluene) demonstrated in terms of human health, the usage of these compounds has been restricted in recent years (Cooper and Grover 2012). On the other hand, substances belonging to PC show flavor, color, and harshness – characteristics that are typically related to the organoleptic properties of foods. Additionally, the PC presented in a food matrix has significant effects on its availability, extractability, solubility, and biological activity, making them widely used in the industry of food (Karaś et al. 2017). Moreover, PC are linked with many functions like germination, pollination, resistance to predators and pests, seed development, reproduction, and sensorial properties. As it is mentioned, more than 8000 different PC synthesized by higher plants have been identified, and the amount is continually growing (Kabera et al. 2014).

Plant cells and tissues contain numerous compounds including flavonoids, proanthocyanidins, amides, esters, glycosides of hydroxycinnamic acids, and polymers with a phenolic structure such as pollen sporopollenin, suberin, and lignin. Several soluble PC, e.g., chlorogenic acid, are broadly widespread, while for diverse compounds, the distribution is restricted to specific species, genera, or families, which make them appropriate biomarkers in studies of plant taxonomic (da Silva et al. 2022). There are different ways of PC classification – from highly polymerized compounds to simple molecules. Still, there is no consensus regarding a classification system for phenolics in general. Santana-Gálvez and Jacobo-Velázquez (2017) divided PC from the most general to the most specific as follows: (1) flavonoids and non-flavonoids, (2) PC classified by the number of aromatic rings, (3) PC classified by carbon skeleton, and (4) PC classified by basic chemical composition. Kabera et al. (2014) presented a different approach to phenolic classification. Firstly, PC classification can be based on a number of hydroxyl groups. According to this classification, polyphenols are PC which contain more than one OH-group in the aromatic ring. Secondly, PC can be classified into mono-, di, oligo-, and polyphenols, and this classification is based on chemical structure. Thirdly, PC can be classified based on substitutes in the carbon skeleton, a number of carbon atoms, and aromatic rings in the side chain. Based on the third principle, PC can be further classified into four subgroups: (i) phenolics with one or (ii) two aromatic rings, (iii) quinones, and (iv) polymers. Numerous compounds possess one aromatic ring, including simple phenols (C6); phenols with one aromatic ring and one carbon atom attached (C6-C1), e.g., gallic acid and salicylic acid; phenols with one aromatic ring and with two carbon atoms (C6-C2), e.g., phenylpropanoids; and phenols with three carbon atoms attached (C6-C3), e.g., hydroxycinnamic acid, ferulic acid, and sinapic acid. Benzoquinones and xanthones (C6-C1-C6) are included in the group of PC which contains two aromatic rings linked with two atoms of carbon. Flavonoids contain three carbon atoms (C6-C3-C6).

The first group of polyphenolics, the largest among PC, belongs to the flavonoids. These pigments are water-soluble and widely distributed in nature. In plant cells, flavonoids are located in the vacuoles. More than 5000 flavonoids have been identified among plants, consistently present along with carotenoids and chlorophylls (Stalikas 2007). The definition of flavonoid is commonly used to describe phenolic with a chemical structure consisting of a C6-C3-C6 carbon skeleton, more specifically, an aromatic ring attached to a benzopyran moiety. In this structure, A ring is called an aromatic in the benzopyran group; the B ring is also aromatic but attached to the benzopyran group, whereas the C ring is with the O-heterocyclic. Flavonoids can be divided into three groups based on the position of the B ring: isoflavonoids (3-phenylbenzopyrans), neoflavonoids (4-phenylbenzopyrans), and flavonoids (2-phenylbenzopyrans) (Marais et al. 2007). Additionally, flavonoids can be classified into the following groups: flavonols, flavones, and anthocyanins. The most common forms of flavonoids occurring in the vacuolar juice of plant cells are either aglycons or glycosides. One flavone C-glycoside, six flavone O-glycosides, and seven flavonol O-glycosides were identified in species of *Ocimum* by Grayer et al. (2002). In higher plants, flavonoids are included in numerous functions including symbiotic nitrogen fixation, ultraviolet filtration, and flowers coloration related to the pollinator animal attractions. Additionally, flavonoids are cell cycle inhibitors, chemical messengers, and physiological regulators or may possess inhibitory activities against plant pathogens, e.g., *Fusarium oxysporum*. In recent years there is an increasing interest in flavonoids originating from plants, mainly due to their benefits related to human health, including antioxidant, anti-allergic, antiviral, anti-inflammatory, and anticancer activities, whereas sinusitis, asthma, eczema, and high fever can be relieved by flavonoid quercetin.

The other group of PC are tannins, commonly classified into condensed tannins and hydrolyzable tannins. Because of the various possibilities to form oxidative linkage, tannins show considerable structure diversity (Hassanpour et al. 2011). A large number of oligomeric compounds with molecular weights between 2000 and 5000 Daltons are produced via intermolecular oxidation processes. Flavan-3-oligomers or polymers known as condensed tannins are connected by an interflavan carbon bond. They are also known as proanthocyanidins because, when heated in acidic alcohol solutions, they undergo an oxidation reaction that is catalyzed by acid, converting them to anthocyanidins.

The primary PC in basil plants are flavonol-glycosides and phenolic acids (Kividompolo and Hyotylainen 2007; Castronuovo et al. 2019). Gallic, coumaric, caffeic, and ferulic acids are examples of acids that fall under the two categories of benzoic and cinnamic acid derivatives, respectively. Additionally, phenolic acids can be found in food plants as glycosides or esters that are conjugated to other organic substances such as glucosides, flavonoids, hydroxy fatty acids, sterols, and alcohols. The most common esterification of caffeic acid with quinic acid results in chlorogenic acid, which is the main PC in coffee. Caffeic acid is the most frequent phenolic acid in numerous fruits and vegetables. Ferulic acid (esterified to hemicelluloses in the cell wall) is another phenolic acid commonly found in cereals (D'Archivio et al. 2007). One of the most significant caffeic acid esters found in

Ocimum spp. is rosmarinic acid. This phenolic acid provides therapeutic and pharmacological benefits for the food industries or cosmetic sectors. These benefits include antibacterial, antiviral, antioxidant, and anti-inflammatory activities (Mastaneh et al. 2014; Nazir et al. 2020b).

Diverse types of chemicals, including essential oils, simple phenolics, coumarins, flavonoids, anthocyanins, terpenoids, fixed oils, and steroids, have been found in various *Ocimum* species which make them a fragrant herb used both in cooking and in medicinal (Kasem 2017; Zahran et al. 2020). Among these chemicals, rosmarinic acid, caffeic acid, chlorogenic and p-hydroxybenzoic acids, quercetin, rutin, and apigenin, as well as essential oil components methyl chavicol, linalool, eugenol, α-pinene, ocimene, β-pinene, 1,8-cineole, geraniol, borneol, B-caryophyllene, and n-cinnamate, are basil's most significant components regarding the antioxidant activity (Teofilović et al. 2017; Shahrajabian et al. 2020; Avetisyan et al. 2017). Gang et al. (2001) emphasize that on the surface of basil leaves, both capitate and peltate glandular trichomes can be found. Still, single or two distinct phenylpropene molecules are present in the essential oils they generate. Though, because of biochemical and genetic heterogeneity within the Lamiaceae family, and following individual variability, there is a significant challenge in the use of Lamiaceae species for medicinal reasons. Therefore, using in vitro techniques of micropropagation is an effective means for quick and extensive propagation of aromatic and medicinal plants in order to achieve high homogeneity of progeny (Ardelean et al. 2018).

One of the first studies devoted to secondary metabolites in basil carried out by Kintzios et al. (2003) reported rosmarinic acid accumulation in various liquid culture and cell suspensions immobilized in calcium alginate. Rosmarinic acid generally represented around 90% of the total phenolic content in every type of culture (callus, cell suspension, and immobilized cells). Rosmarinic acid accumulation in cell suspensions varied considerably with time and maximum values were observed during the second and fourth weeks of culture. In addition, cell immobilization in 1.5% calcium alginate completely inhibited rosmarinic acid production. Additionally, the accumulation of monoterpenes, sesquiterpenes, phenolic acids, anthocyanins, phenylpropanoids, and other PC with antioxidant potential in callus culture of sweet basil was shown in the study of Makri and Kintzios (2008).

The antioxidant activities and phenolic content of many *Ocimum basilicum* subspecies are still unknown, despite the fact that basil is widely used in a range of foods and medicines. Consequently, Bajomo et al. (2022) examined 22 commercial basil cultivars and divided them into distinct chemotypes using similarities in characteristics of phenolic acids. Furthermore, total phenolic content, oxygen radical absorbance capacity (ORAC), and cupric ion-reducing antioxidant capacity (CUPRAC) were all significantly influenced by the cultivar type. The findings demonstrated that a chemotype of caffeic acid-rich basil cultivars was defined as having the highest total phenolic content and strongest antioxidant capabilities. Also, significant variations in the content of phenolics among basil morphotypes were observed, e.g., high content of phenolics and better antioxidant activity was demonstrated for green Genovese-type basil in contrast to lettuce-leaf basil (Bajomo et al. 2022). When compounds with biological activities in in vitro cultures of *O.*

basilicum were detailly examined, it can be seen that researchers mostly focused on an effective methods of biologically active metabolites production. For this purpose, they mainly used hairy roots, callus, or suspension cultures and applicate as elicitors 6-benzylaminopurine (BAP), 2,4-dichlorophenoxyacetic acid (2,4-D), indole-3-acetic acid (IAA), 1-naphthylacetic acid (NAA), thidiazuron (TDZ), kinetin (KIN), methyl jasmonate (MeJ), melatonin, salicylic acid, chitosan, cadmium chloride, silver nitrate, yeast, and different light intensities (reviewed by Jakovljević et al. 2022).

According to some authors, optimization of callus culture conditions could be used as a low-cost and suitable basis of important phenolic acids: rosmarinic and cichoric acid, with substantial antioxidant activity (Rady and Nazif 2005; Abdel Rahman et al. 2015; Nazir et al. 2019). The supplementation of MS medium (Murashige and Skoog 1962) with 1 mg L^{-1} BA and 0.25 mg L^{-1} IAA led to the accumulation of high concentrations of rosmarinic acid in callus culture of *O. americanum* (Rady and Nazif 2005) and *O. basilicum* (Abdel Rahman et al. 2015). Nazir et al. (2020c) described the procedure for callus culture of sweet basil cv. 'Thai basil', according to which, MS medium with 1 mg L^{-1} NAA and 5 mg L^{-1} BAP was the best choice for the accumulation of fresh and dry biomass, and for the increased production of phenolics. Similarly, Nazir et al. (2019) cultured callus through the leaf explants on MS medium supplemented with various plant growth regulators (PGRs), such as α-naphthalene acetic acid (NAA), thidiazuron (TDZ), and 6-benzylaminopurine (BAP). According to this study, the highest mass growth (23.2 g L^{-1} DW) was achieved with 2.5 mg L^{-1} NAA, together with high total phenolic content (210.7 mg L^{-1}) and flavonoid (196.4 mg L^{-1}) production. What is more, the varying phenolic acid accumulation was proved through the HPLC (high-performance liquid chromatography) analysis: caffeic (44.67 mg g^{-1} DW), rosmarinic (52.22 mg g^{-1} DW), and chicoric acid (43.89 mg g^{-1} DW) as well as anthocyanins: peonidin (10.77 mg g^{-1} DW) and cyanidin (16.39 mg g^{-1} DW) as an effect of the PGRs treatment. Wongsen et al. (2015) demonstrated that *O. basilicum* callus had the highest fresh weight and the highest levels of flavonoids, phenolics, ascorbic acid, and β-carotene (7.38 mg g^{-1} FW, 6.54 mg g^{-1} FW, 0.64 mg g^{-1} FW, and 0.08 mg g^{-1} FW, respectively) after 7 days on the medium with 0.5 mg L^{-1} 2,4-D. Comparable results were obtained by Hakkim et al. (2011a), where the highest callus biomass production of *O. sanctum* grown on media with 0.1 mg L^{-1} KIN and 1 mg L^{-1} 2,4-D correlated with a high rosmarinic acid concentration. Bhuvaneshwari et al. (2016) grew *O. basilicum* and *O. tenuiflorum* from nodal explants in vitro on MS media with various concentrations of BAP, either alone or with KIN (0.25–2.0 mg L^{-1}) and IAA (0.25–2.0 mg L^{-1}). The maximum shoot induction was achieved for both species when 1 mg mL^{-1} BAP and 0.5 mg mL^{-1} KIN were combined (99% and 98%, respectively), and the leaves thus obtained had several times higher content of eugenol and total phenolic compared to leaves from field-grown plants. The highest concentration of total PC (185 mg g^{-1} DW) was obtained for *O. tenuiflorum*, whereas both *O. basilicum* and *O. tenuiflorum* contained comparable amounts of eugenol (approximately 85 μg g^{-1}). Additionally, the association and importance between total phenolics concentration and eugenol in all in vitro grown plant parts of *O. basilicum* and *O. tenuiflorum* were established. The holy basil (*O. sanctum*) chemical

composition and antioxidant properties were examined in the study by Hakkim et al. (2007). Parts of plants (inflorescence, stems, and leaves) grown under natural conditions in field were compared with corresponding callus cultures produced from each explant in vitro. For the first time, the distribution of PC, i.e., rosmarinic acid, ursolic acid, sinapic acid, carnosic acid, isothymusin, and eugenol, in each organ was examined using HPLC. Rosmarinic acid was found to be the most abundant phenolic acid in all callus extracts when compared to plants grown in the field. In comparison to the plants cultivated in the field, holy basil callus accumulated rosmarinic acid 10.8-fold more. The prior study by Kintzios et al. (2003), which discovered a significant increase in the concentration of rosmarinic acid in callus of *O. basilicum*, gives support to these findings. Additionally, 2,4-D and KIN used as PGRs for callus culture can generate the highest mass production with great secondary metabolite content, according to Kintzios et al. (2003). What is more, in all callus culture testing systems, the antioxidant activity increased, and the extracts obtained from callus had greater antioxidant activity than the extracts derived from field-grown plants at the same concentration of growth regulators. The information from the Kintzios et al. (2003) study revealed that in vitro callus cultures rather than field-grown plant parts of holy basil might be used to isolate rosmarinic acid with a high level.

Among the most successful method for enhancing a plant's production of beneficial secondary metabolites is elicitation. Elicitors, both biotic and abiotic, are widely used to improve plant metabolite production in cell cultures by speeding up the process and resulting in higher culture volumes and product concentrations (Mulabagal and Tsay 2004; Yue et al. 2016). One of the most significant benefits of cell cultures for the synthesis of secondary metabolites is the rapid multiplication of cells biomass through organogenesis or somatic embryogenesis, which typically results in a rise in the metabolic rates of developed cells (Jakovljević et al. 2022). Melatonin's effect on *O. basilicum* (sweet basil) callus initiation and PC production were examined by Duran et al. in 2019. According to the phytochemical evaluation, calluses grew in media with 100 and 200 μM of melatonin had the highest total phenolic acid concentrations (784.6 μg g^{-1} and 335.2 μg g^{-1}, respectively), as opposed to calluses produced with MS alone (192.0 μg g^{-1}). The findings showed that PC such as rosmarinic acid, caffeic acid, vanillin, and p-coumaric acid accumulated differently in response to melatonin. The amount of rosmarinic acid (754.2 μg g^{-1}) significantly increased in the callus grown on 100 μM melatonin medium by about fivefold, compared to the callus in the control group. In addition, the presence of the following aromatic compounds was found: 1,8-cineole, DL-limonene, methyl eugenol, 3-methylbutanal, 2-methylbutanal, hexanal, furan-2-carboxaldehyde, benzaldehyde, and bergamotene. Other results reported by Bahcesular et al. (2020) showed that melatonin and salt stress applications decreased total phenolics and total flavonoids; rosmarinic acid was not detected under salinity, while the concentration of caffeic acid and cichoric acid was decreased. Açıkgöz (2020) investigated biotic and abiotic elicitors that activate the defense mechanisms of plants in order to obtain an increasing concentration of biologically active metabolites in *O. basilicum* cell suspension cultures. The silver nitrate ($AgNO_3$), cadmium chloride ($CdCl_2$),

and yeast extract were applied at various concentrations, and their effects were studied on the cell growth, cell viability, total phenolic and flavonoid contents, and pharmaceutical active ingredients. Among the investigated elicitors, the treatment with 200 mg L^{-1} of yeast extract gave the greatest total phenolic and flavonoid concentrations. The HPLC analysis revealed that the yeast extract treatment caused the highest accumulation of rosmarinic acid (21.28 mg g^{-1} DW of rosmarinic acid in 200 mg L^{-1} of yeast extract treatment) and chicoric acid (6.45 mg g^{-1} DW of chicoric acid in 50 mg L^{-1} of yeast extract treatment) which were 0.92 and 1.25 times higher than in the control, respectively. Compared to the control culture, the application of 50 mg L^{-1} of yeast extract resulted in an increase of rutin for 1.91 times (6.54 mg g^{-1} DW) and isoquercetin for 1.86 times (3.72 mg g^{-1} DW), respectively. The $AgNO_3$ treatment (25 μM) resulted in the highest levels of linalool and estragole compared to the control culture (4.37 g g^{-1} DW and 3.30 g g^{-1} DW, respectively). Açıkgöz (2021) used sorbitol as an elicitor and showed that 50 mg L^{-1} of it was the most effective in terms of total phenolics, while 200 mg L^{-1} treatment was most effective for total flavonoid content. Based on HPLC analysis, the highest concentrations of rosmarinic acid and chicoric acid (12.32 mg g^{-1} DW and 4.52 mg g^{-1} DW, respectively) were obtained in 25 mg L^{-1} of sorbitol and 100 mg L^{-1} treatment, whereas the 50 mg L^{-1} of sorbitol affected the optimum biosynthesis of rutin (6.78 mg g^{-1} DW), isoquercetin (4.12 mg g^{-1} DW), linalool (4.58 μg g^{-1} DW), and methyl chavicol (4.40 μg g^{-1} DW) compared to the control culture. According to these findings, adding $AgNO_3$, $CdCl_2$, yeast extract, and sorbitol to cell suspension cultures of *O. basilicum* may be a suitable way to increase medicinal active components, particularly terpenoids and phenolics.

In the sweet basil hairy root cultures, Marzouk (2009) identified six triterpene acids including ursolic, oleanolic, betulinic, alphitolic, euscaphic, and 3-epimaslinic. Using *A. rhizogenes* strain LBA 9402 (with a kanamycin-resistant gene as a selectable marker), hairy roots were induced on stem and leaf pieces of *O. basilicum*. The extracts obtained from hairy roots showed a significant hepatoprotective potential against CCl_4-induced oxidative stress in female albino rats and demonstrated inhibition two- to threefold that of silymarin used as the positive control. Received results showed that hairy root culture had the same metabolic profile as the non-transformed roots but showed a tendency to accumulate secondary metabolites at higher amounts. The study of Srivastava et al. (2016) reported *A. rhizogenes*-mediated transformation of basil cultivars (‘holy green’, ‘red rubin’, and ‘Subja’) for hairy root establishing and selection for the production of PC. Hairy root growth was explant and virulence dependent. Differences between cultivars in the content of total phenolics, rosmarinic acid, and caffeic acid were observed. They varied among cultivars, with the highest rosmarinic acid content recorded in 60 days of culture (76.41 mg g^{-1} DW). It can be concluded that rosmarinic acid synthesis in hairy roots of ‘holy green’, ‘red rubin’, and ‘Subja’ basil cultivars are age dependent. The caffeic acid (in concentration from 0.11 mg g^{-1} DW to 1.74 mg g^{-1} DW) was detected in all tested samples, and the trend of this compound’s content differed between the tested lines of hairy roots within the age. On this basis, three superior lines of hairy roots were chosen because they had higher biomass production, rosmarinic acid, and

antioxidant capacity compared to untransformed roots. Prior studies by Tada et al. (1996) also corroborated the same trend involving the five clones of hairy roots grown well in MS, B5 (Gamborg et al. 1968), and woody plant (McCown and Lloyd 1981) liquid media. A high content of rosmarinic acid (14.1% DW) was recorded in the MS medium together with low content of other phenolics, including lithospermic acid (1.70% DW) and lithospermic acid B (0.17% DW). In addition to the commonly occurring rosmarinic acid, Pandey et al. (2019) recorded the presence of pentacyclic triterpenes in the suspension culture of *O. basilicum* for the first time: betulinic, oleanolic, and ursolic acids. The highest amount of rosmarinic acid (15.73 mg g^{-1} DW) was produced, followed by betulinic acid (14.63 mg g^{-1} DW), ursolic acid (4.71 mg g^{-1} DW), and oleanolic acid (0.91 mg g^{-1} DW). The cell suspension culture produced several times higher content of these pentacyclic triterpenes than the in vivo control leaves. Moreover, the promising influence of MeJ was ascertained on the overall productivities of sweet basil suspension culture relating to three investigated metabolites: rosmarinic, betulinic, and ursolic acids, compared to control culture. Though MeJ in the concentrations of 200 and 300 μM were successful in increasing the production of ursolic acid, only the lower MeJ concentration (200 μM) was successful in increasing the amount of betulinic acid, supporting the prior discoveries by Pandey et al. (2015). This conclusion is consistent with several past observations where MeJ induced the production of rosmarinic acid in several *Ocimum* species (Mathew and Sankar 2012). Misra et al. (2014) examined the transcriptional responses in sweet basil following MeJ treatment, which is thought to be an elicitor of secondary metabolites, and they found 388 potential MeJ-responsive individual transcripts. Transcript research reveals that MeJ upregulates transcripts of the numerous secondary metabolic pathways, including terpenoids and flavonoids, in addition to directing its production and stress responses.

Koca and Karaman (2015) examined the effects of MeJ, epibrassinolide, spermine, and l-phenylalanine on basil to quantify the production of PC and the activity of PAL. The concentration of total PC, including flavonoids (6.72 mg g^{-1} and 0.92 mg g^{-1}, respectively), was highest after the 1.0 mM spermine + MeJ application. In this way, the content of rosmarinic and caffeic acids can be significantly enhanced. Still, no alterations were seen in the contents of chicoric acid or PAL activity. The predominant phenolic acid in all samples was rosmarinic, with content ranged from 1.04 to 2.70 mg g^{-1} FW. Because of this, spermin + MeJ, as well as epibrassinolide + MeJ, can be regarded as efficient elicitors of secondary metabolites production, and these interactions can be crucial for the formation of phytochemicals in plants. *O. basilicum* var. *thyrsiflorum* cv. 'Thai basil' was the subject of research by Nazir et al. (2021a) which investigated the effect of elicitation with salicylic acid and varied light regimes on the formation of secondary metabolites. The researchers demonstrated that the combination of salicylic acid (10 μM) and constant light significantly improved the antioxidant capacity and content of secondary metabolites including the content of total phenolics (18.7 mg g^{-1} DW), total flavonoid (7.2 mg g^{-1} DW), rosmarinic acid (54.35 mg g^{-1} DW), chicoric acid (64.46 mg g^{-1} DW), eugenol (0.56 mg g^{-1} DW), peonidin (0.32 mg g^{-1} DW), and cyanidin (0.42 mg g^{-1} DW). The highest concentration of caffeic acid (0.54 mg g^{-1} DW) was obtained after the photoperiod and 25 μM of salicylic acid.

The study of Hammock (2018) and Sipos et al. (2021) demonstrates that supplemental narrow-wavelength light treatments from light-emitting diodes (LED) sources can be used to manipulate plant development and significantly influence the yield and accumulation of biologically active compounds. According to Shoji et al. (2009) and Lobiuc et al. (2017), especially red and blue LEDs can tailor the induction of improved growth and content of PC in green and red basil microgreens. In the experiment done by Ardelean et al. (2018), the effect of LED and fluorescent lamps was evaluated on in vitro growth and content of PC of basil cultivar 'Aromat de Buzau'. Basil seedlings were cultivated under red, yellow, green, and blue LEDs with peak wavelengths of 660, 525, 500, and 470 nm, respectively. This study showed that the growth of basil plants was not affected by the LEDs since the greatest fresh biomass and shoot height were recorded after 60 days under control light treatment. However, compared to the other treatments, blue LED light considerably increased the total phenolic and flavonoid content of basil plants. Nazir et al. (2020b) described metabolic variations in the purple basil *O. basilicum* var. *purpurascens* callus culture successfully induced by exposure to UV and different monochromatic lights. In comparison to the control, blue light caused the greatest accumulation of callus biomass, total phenolic, and flavonoid contents and the highest antioxidant activity. Nazir et al. (2020b) also demonstrated that dark conditions resulted in higher content of peonidin (0.13 mg g^{-1} DW), cyanidin (0.15 mg g^{-1} DW), and rosmarinic acid (87.62 mg g^{-1}), whereas the production of chicoric acid (14.65 mg g^{-1} DW) was enchased by red light. In the subsequent study, Nazir et al. (2020a) described how melatonin, UV-C, and their combinations can improve phenylpropanoid metabolite production in callus cultures of basil. The highest total phenolic and total flavonoid content (18.4 and 13.4 mg g^{-1} DW, respectively) was measured in the callus exposed for 50 min to UV-C. HPLC quantification of phenylpropanoid metabolites showed that UV-C (10 min) led to an increased concentration of rosmarinic acid (134.5 mg g^{-1} DW), whereas UV-C (50 min) led to an increased concentration of cichoric acid, cyanidin, and peonidin (51.52, 0.50, and 0.30 mg g^{-1} DW, respectively). Therefore, according to the most recent research, the use of elicitors like UV-C and narrow-wavelength LED light can appropriately affect secondary metabolites in purple basil, which suggests that this method may be an alternative to modifications in metabolism in other species to obtain bioactive secondary metabolites. Moreover, according to Hashim et al. (2021), among the several abiotic elicitors, light has drawn interest in boosting secondary metabolite production due to its versatility in terms of wavelengths, affordability, and durability.

5 Biological Activity

When it comes to the human organism, the balance between the pros and cons of free radicals is of vital importance for the normal functioning of the organism, given that oxidative stress triggered by an increased concentration of free radicals in the human organism can lead to various stress-related diseases, including

cardiovascular disease, cancer, and diabetes (Shoham et al. 2008; Benhammou et al. 2009). Although the human body has an antioxidant defense system that acts against free radicals, this system that prevents oxidative damage is very often not a sufficient component of cellular antioxidant protection (Rechner et al. 2002). The antioxidants introduced exogenously into the human organism can neutralize or mitigate the negative consequence of free radicals. For this reason, synthetic antioxidants (such as BHT and BHA) have been widely used in different aspects (Stanković et al. 2019). However, despite the confirmed antioxidant abilities and the increased interest in this group of synthetic compounds, some data indicate the toxic and carcinogenic effects of synthetic antioxidants (Jennings and Akoh 2009; Sindhi et al. 2013). Because of this, it is essential to replace synthetic antioxidants with antioxidants derived from natural sources. Secondary metabolites from plant samples are the most significant natural antioxidants (Stanković et al. 2019). They are mainly PC that can remove reactive oxygen species and free radicals and consequently prevent the occurrence of oxidative stress or reduce its harmful consequences. The high reactivity of PC as hydrogen or electron donors, the ability of the aromatic phenolic radical to localize the unpaired electron, the ability to react with transition metal ions, and the removal of superoxide anion radicals, hydroxyl radicals, and hydrogen peroxide are all factors that contribute to their antioxidant capabilities (Falleh et al. 2011; Stanković et al. 2019). Therefore, it is considered that plant polyphenolics have bioactive qualities that may be significant in the treatment of a variety of damages (Sousa et al. 2015; Zengin et al. 2015). As a result, recently, the use of plant antioxidants as additives, functional food, and many types of medical and pharmaceutical materials has increased (Stanković et al. 2019).

Plants of the genus *Ocimum* are known in many countries around the world based on their pharmaceutical active components, including alkaloids, phenols and flavonoids, terpenoids, and others. That is why basil extracts are of great practical importance in the pharmaceutical industries, food, and cosmetics. When it comes to the pharmaceutical industry, the most valued are essential oil components like linalool and methyl-chavicol (Piras et al. 2018; Talebi et al. 2018; Alkuwayti et al. 2019) and PC like rutin, isoquercetin, rosmarinic acid, and caffeic acid (Kwee and Niemeyer 2011; Jakovljević et al. 2019). The content of these metabolites varies, and it is determined by both genetic and environmental agents such as season, climate, and sampling period. Also, the growth and development processes, the plant parts, and the extraction procedures and further processing of the material additionally affect the quantity and quality of the active components of basil (Alkuwayti et al. 2019; Jakovljević et al. 2019, 2021). The production of biologically active components of basil, due to all the mentioned factors, may be constrained or expensive. The use of conventional planting can be expensive for in-depth basil research or screening of bioactive compounds in numerous genotypes that exist within the genus *Ocimum*. That is why it is necessary, with smaller financial investments, to establish an easily-managed system for smaller areas (Srivastava 2014). Also, the

use of biologically active substances in medicine and pharmacy requires homogeneity and a high degree of purity of the substances (Rao and Ravishankar 2002).

Because of its abundant phenolic acid and flavonoid content, *O. basilicum* is exploited in cosmetic and pharmaceutical treatments. It can act in the prevention of heart illnesses, inflammation reduction, and the occurrence of malignancies and diabetes because of its powerful antioxidant activity (Mastaneh et al. 2014). Antioxidants originating from plants can be extracted using a variety of techniques and conditions (process time, lightning) and with different solvents. A significant number of phenolic acids and flavonoids, which are holders of antioxidant activity, are produced during the maceration with organic solvents (Vidović et al. 2012). Teofilović et al. (2017) reported that the total phenolic content of ground parts of sweet basil ranged from 5.17 mg g^{-1} DW to 65.25 mg g^{-1} DW of extract and the content of the total flavonoids from 0.11 mg g^{-1} DW to 40.63 mg g^{-1} DW of extract. With longer extraction times, more polar solvents, and more plant fragmentation, the overall extraction yield increased. The extract produced by extraction with 96% ethanol for 30 min had the highest total phenolic content, while the extract produced by chloroform for 30 min had the highest flavonoid level. Properties of leaves from 'Genovese', 'dark opal', and 'sweet Thai' basil to phenolics accumulation were determined in the presence of potassium ions. The 5.0-mM potassium rate increased the concentration of total PC, with higher rosmarinic and chicoric acid concentrations compared to a lower (1.0 mM) potassium treatment level. The phenolic composition and antioxidant properties of basil were also affected by the type of cultivar. The cultivar 'sweet Thai' had lower total phenolics content and ferric-reducing antioxidant power (FRAP) compared to 'Genovese' and 'dark opal'. The concentrations of anthocyanin, unaffected by the amount of potassium ions, differed remarkably among the cultivars. Purple 'dark opal' basil showed higher anthocyanin levels than 'Genovese' and 'sweet Thai'.

Shafique et al. (2011) investigated the antimicrobial activities of various extracts obtained from in vitro and in vivo grown plants of *O. basilicum* and showed that extracts from basil grown in vitro demonstrated better antimicrobial activity against Gram-positive bacteria compared to extracts obtained from in vivo grown plants. Significant antioxidant activity was demonstrated for seedlings of various basil genotypes grown in vitro under conditions of nutrient deprivation (Jakovljević et al. 2019) with values similar to standardized extracts of ginkgo or green tea. Considering the purity of substances in standardized extracts and the mixture of substances in basil samples, it can be concluded that basil grown in vitro can be an important source of natural antioxidants. Guirgis et al. (2007) and Homhuan et al. (2008) showed that the application of gamma rays to basil callus culture can increase its antioxidant capacity. Wongsen et al. (2015) examined callus culture as a source of antioxidative compounds. It has been shown that the callus, originating from the leaf explants of *O. basilicum*, is a significant source of β-carotene, ascorbic acid, phenolics, and flavonoids but also of total antioxidant activity and that these biologically active substances can be obtained in high concentrations for 7 days after

callus induction. Extracts originating from the callus tissue of purple basil show a protective effect in stress conditions caused by radiation, indicating the great practical potential that the in vitro callus culture of this basil variety possess (Nazir et al. 2019). The influence of different abiotic and biotic stimuli on the secondary metabolites production in the cell suspension of *O. basilicum* was studied by Açıkgöz (2020). It was shown that the antioxidant activity, the content of total phenolics, and the concentration of total flavonoids are the highest with the addition of yeast extract (200 mg L^{-1}). Srivastava et al. (2016) determined that by the genetic transformation of three different varieties of basil ('holy green' 'red rubin', and 'Genovese') with *A. rhizogenes* significantly higher antioxidant activity can be achieved compared to non-transformed roots. The culture of genetically transformed hairy roots of *O. basilicum* shows a tendency to accumulate rosmarinic acid in a concentration three times higher compared to control (non-transformed) roots, whereby the synthesis of this phenolic acid can be further improved by the application of biotic stimulants. Rosmarinic acid synthesized in genetically transformed roots shows significant antimicrobial activity and has a significant effect on *Pseudomonas aeruginosa* (Bais et al. 2002). According to the study of Darrag et al. (2022), the volatile secondary metabolites obtained from the cell suspension of *O. basilicum* may be useful as a bio-insecticide against *Rhynchophorus ferrugineus*.

6 Concluding Remarks

In recent years, there can be seen an improvement in the implementation of in vitro tissue culture with the goal of producing compounds of interest originating from basil plants. This is mainly due to the fact that in vitro production of bioactive substances is stable, predictable and reliable, independent of geographic position, seasonal fluctuations, and environmental factors, and it allows content modification to achieve the production of bioactive compounds with the proper quantitative and qualitative composition. Callus culture, cell suspension, and genetically transformed roots provide the opportunities of obtaining biologically active compounds at higher concentrations compared to the standard cultivation methods. Through the hairy root culture, callus, or cell suspension culture, it is possible to obtain a significant amount of phenolic acids, particularly rosmarinic acid, caffeic acid, chicoric acid, betulinic acid, and flavonoids cyanidin and peonidin. These secondary metabolites possess various biological activities which can contribute to human health, and it is proved that extracts originating from basil grown in vitro act as antioxidant and antimicrobial agents.

Acknowledgments This work was supported by the Ministry of Science, Technological Development, and Innovation of the Republic of Serbia (Agreements No. 451-03-47/2023-01/200122).

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In Vitro Callus Induction from *Gymnema sylvestre* (Madhunashini) to Enhance the Production of Gymnemic Acid Using PGRs

Abhishek R. Vyas, Kaushik H. Nakum, Vipul B. Vaja, and Kalpeshkumar B. Ishnava

Abstract Human population has largely depended upon medicinally plant-based crude drugs for treatment of variety of illness and life-threatening diseases for centuries. Medicinal plants which are used in Ayurveda provide biologically active molecules which have better activity. Diabetes is a chronic disease. Globally, the number of diabetics has more than doubled during the last few decades. There are more patients today who prefer to treat themselves with natural medications. Therefore, medicinal plants for treating diabetes might give people more choice to consume medicinal herb. Plants synthesize secondary metabolite compounds, which are significantly used in therapeutic treatments for human health. Gymnemic acid is an active component synthesized in leaves of *Gymnema sylvestre* which is responsible for the antidiabetic activity. In vitro callus induction is reliable and highly beneficial to enhance the production of gymnemic acid in *Gymnema sylvestre*. The combination of IBA (2 mg/l) + 2,4-D (2 mg/l) + BAP (0.5 mg/l) + kinetin (1 mg/l) is give better response in the callus production and also higher amount of gymnemic acids. In vitro growth-regulator hormone studies have shown promise in increasing gymnemic acid in *Gymnema sylvestre.*

Keywords Gymnemic acid · *Agrobacterium* · Diabetes

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N. Kumar, R. S. Singh (eds.), *Biosynthesis of Bioactive Compounds in Medicinal and Aromatic Plants*, Food Bioactive Ingredients,
https://doi.org/10.1007/978-3-031-35221-8_17

1 Introduction

The source of life is plants. Plants have an indirect or direct impact on every living creature. As autotrophs, plants produce their own sustenance. All the other living organisms are depending on them. They provide us shelter, clothes, fuel, paper, etc. But more than this, they also have medicinal importance. The numerous ailments affecting humans are treated with them. The benefit of using plants as medicines is that they contain natural ingredients, meaning that there are less or no negative effects. They are nontoxic to animals. Native inhabitants in India from ancient times have used plant and plant-based products traditionally. India continues to have a leading position in the usage of medications derived from plants, and it is one of the few nations to have developed and practiced an indigenous system of medicine that is well known (Sivranjan and Balachandran 1993; Mohan et al. 2006; Kalpesh et al. 2011).

Medicinal plants have played an important role in Indian culture since the Rigveda (5600 BC) when about 67 medicinal plants are recorded. It is estimated that 80% of his approximately 4 billion inhabitants are dependent on traditional medicine (Krishna et al. 2012; Kalpesh et al. 2012). Medicinal plants have been used for thousands of years to treat ailments and conditions. They have gained economic importance through their use in the pharmaceutical, cosmetic, perfumery, and food industries. Interest in herbal systems is increasing day by day because nature can cure many ailments (Mahesh et al. 2014). Medicinal plants are used in the treatment of various diseases. *Asparagus racemosus*, *Withania somnifera*, *Glycerrhiza glabra*, etc. are used in the treatment of anemia. *Piper longum*, *Adhatoda vasica*, *Zingiber officinale*, etc. are used in bronchial asthma. *Phyllanthus emblica*, *Ricinus communis*, etc. are used in arthritis. *Terminalia chebula*, *Phyllanthus* sp., etc. are used in obesity. *Tribulus terrestris*, *Zingiber* sp., etc. are used in the treatment of paralysis. *Piper longum*, *Curcuma longa*, *Ocimum sanctum*, etc. are used to improve blood circulation. *Azadirachta indica*, *Holarrhena antidysenterica*, *Tinospora cordifolia*, etc. are used in cancer therapy. Medicinal plants of commercial significance include amla, Shatavari, tulsi, brahmi, Isabgol, Senna, cinchona, belladonna, kalmegh, Safed musli, Ashoka, ashwagandha, Bael, etc. (Emery and Stone 2013; Hiral et al. 2014).

Plants which are used in Ayurveda also provide biological active molecule and lead structure for developing new modified derivatives which have better activity and reduced toxicity. This is especially so when he was struck with ailments, both physical and mental. The World Health Organization (WHO) has listed over 21,000 plant species (including synonyms) that have been reported for medicinal uses around the world. India is rich in biodiversity. More than 7000 species of plants have been used in various medical systems throughout the country since ancient times. There are about 8000 known medicinal plants in India, and about 1000 plants are used in Ayurvedic, Unani, and Siddha traditional medicine systems, while the tribesmen use his 7500 plants for medicinal purposes. Of the 250,000 higher plant species on Earth, over 80,000 are medicinal. India has over 45,000 different plant

species. However, only 7000–7500 species are used in traditional, folk, and herbal medicines (Ved et al. 1998; Ankita and Kalpesh 2015).

Gujarat is known to have 4320 plant species among which 2205 are angiosperms (Singh and Parabia 2003). About 1315 (trees: 248, shrubs: 165, herbs: 754, climbers: 148) medicinal plant species have been available. Out of the seven non-angiosperm medicinal species, five are pteridophytes and two are gymnosperms (Pandey et al. 2005). The secondary sources indicate the presence of 61 species of grasses that have medicinal value (Shah 1978). Eleven mangrove species occur in Gujarat among which six mangrove species occurring in Gujarat have medicinal values (Pandey et al. 2005).

Despite the term "secondary metabolites," these compounds provide selective benefits to plant species by providing protection from predators, pathogens, and abiotic stresses. Attract pollinators and benefit animals and microbes. It acts as a signal for interaction with other plants (Dudareva and Pichersky 2008). In addition, they act as growth regulators, regulators of gene expression, and signal transducers at the cellular level. This is because plants are estimated to produce over 2,00,000 metabolites (Fiehn 2002). In recent years, there has been an increase in research on new natural products that can be used in pharmaceuticals, pesticides, and agro-industrial pharmaceuticals, biopesticides, and food additives. Yields of these compounds are low (less than 1% of dry weight) and depend on the physiological and developmental stage of the plant. For plants, very few phytochemical plant species are tested. Due to the complexity and expense of this study, so far only a small proportion of these secondary plant materials have been investigated for their biological properties. Two-thirds of his medicinal plants are wild-harvested, whereas only about 10% of commercial plants are cultivated in Europe (Canter et al. 2005). Today, a quarter of all pharmaceutical manufacturing countries contain compounds derived directly or indirectly from plants. In addition, 11% of the 252 medicines considered essential and essential by the WHO are derived exclusively from flowering plants (Rates 2001). The changing consumer demand for herbal and natural products in international markets has led to extensive research into the development of pharmaceuticals and health products from medicinal plants. According to a report by EXIM Bank, the international trading market for medicinal plants is worth about US$60 billion annually. The Indian market is valued in rupees $550 million ($140 million) annually. Phytochemicals such as terpenes and steroids account for the most significant share with estimated annual sales of US$12.4 billion. India's share of the US$62 billion market growing at a rate of 7–12% annually is less than 1% (Fabio et al. 2014).

1.1 Diabetes

Diabetes is a pathological condition created in the body by metabolic disorder in which the blood glucose level increased due to less secretion of hormone insulin, genetic defect, auto immunity, etc. The International Diabetes Federation estimated

that 382 million peoples are suffering from diabetes in the world. Among them 65.1 million peoples are from India in the year 2013. And among them every one person out of ten has type 2 diabetes. India has the second highest number of patient with diabetes in the world (International Diabetes Federation 2013).

Type 2 diabetes accounts for over 85% of all diabetes cases. Far from being a disease of the wealthy, type 2 diabetes is devastating in developing countries and increasingly affecting the poor. The global epidemic of obesity and sedentary lifestyles has made type 2 diabetes one of the fastest-growing public health problems in both developed and developing countries (Fig. 1). For example, in the Indian city of Chennai, diabetes prevalence increased by more than 70% in just 14 years (WHO 2010). In a similar period, the prevalence of diabetes in China tripled. Recent data from China show that the country's current prevalence of diabetes is double to what was estimated based on studies conducted 10–15 years ago. Moreover, the prevalence of diabetes in rural China appears to be almost as high as in urban areas. This data from China raises the question of whether similar prevalence underestimations are very likely for other low- and middle-income countries for which there are no recent data. The global burden of diabetes is projected to increase by 50% over the next 20 years, even as the trend toward higher rates of diabetes prevails. This is mainly due to the increase in developing countries where the disease affects an increasingly younger age group (Fig. 1). There are three types of diabetes.

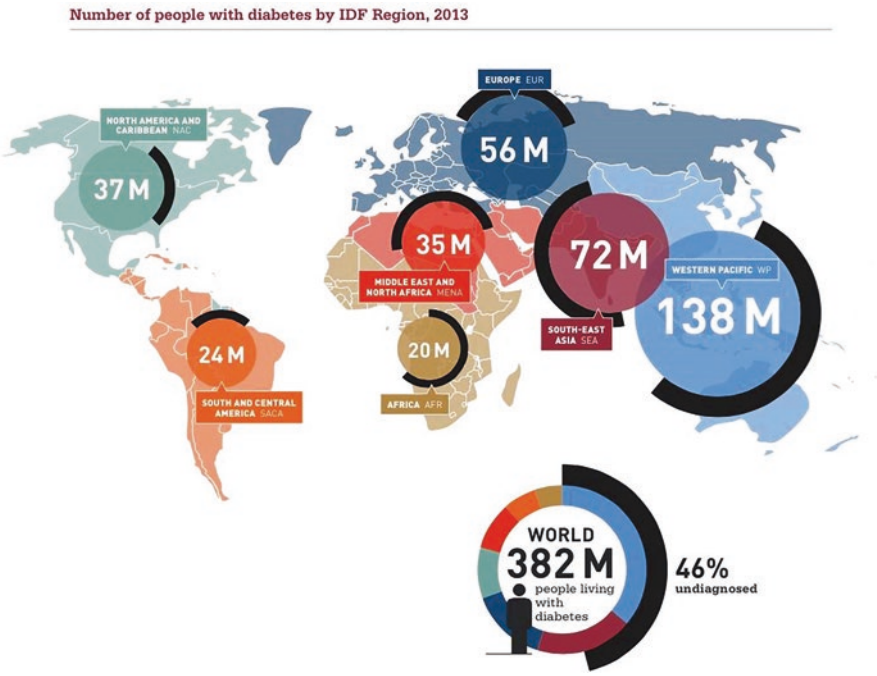

Fig. 1 Current scenario of diabetes and future possible scenario of diabetes

1.1.1 Type 1 Diabetes

In type 1 diabetes, the pancreas fails to produce insulin. Without insulin, the body's cells cannot use glucose. Type 1 diabetes is also known as insulin-dependent diabetes; it is an immune-mediated, recessive inheritance, exhibited by lymphocytic insulitis; type 1 DM occurs when the beta cells of the pancreas are damaged and does not produce the insulin. It is responsible for 5–10% of all cases of diabetes (Patidar and Dwivedi 2012).

1.1.2 Type 2 Diabetes

Also known as non-insulin-dependent diabetes, it is the most common type of diabetes. This occurs when the body can no longer use insulin effectively and gradually resists its effects. It is a slowly progressive disease that progresses through identifiable stages. In the early stages, both insulin and glucose levels are elevated (conditions known as hyperinsulinemia and hyperglycemia, respectively) (Fig. 2). About 90% of diabetes is type 2 (Olokoba et al. 2012).

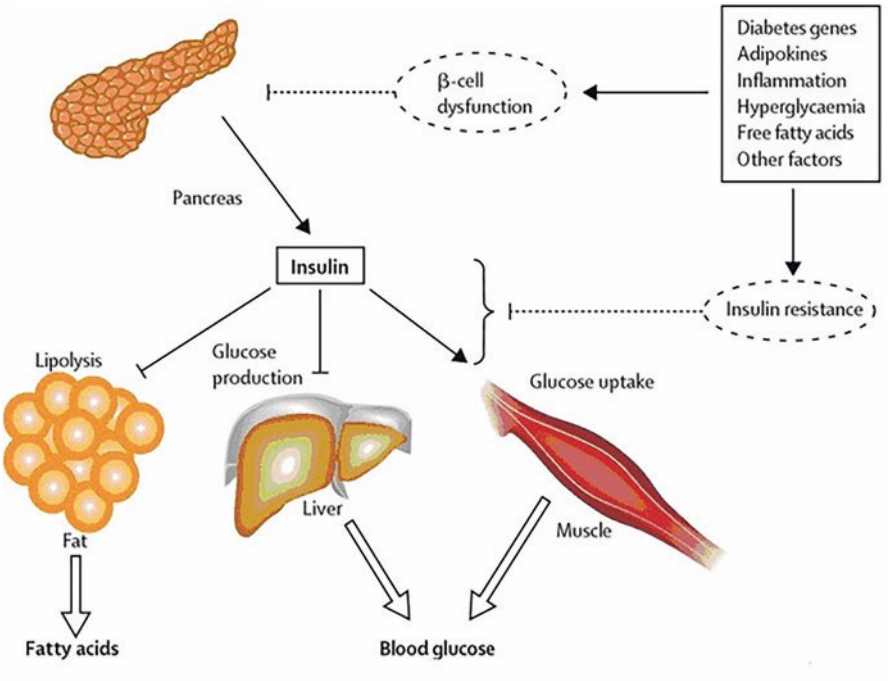

Fig. 2 Insulin resistance pathways affect the action of insulin in each of the major target tissues

1.2 Gestational Diabetes

Gestational diabetes occurs in pregnant women. It has been found that a transient state of diabetes is often formed in pregnant women. But neglecting personal care can lead to permanent diabetes. Gestational diabetes refers to the onset or early detection of impaired glucose tolerance during pregnancy, usually occurring between the sixth and nineth weeks. This occurs in approximately 4% of all pregnancies. Patients with GD have a 30–50% chance of developing diabetes, usually type 2 diabetes (Bastaki 2005).

2 Methods for Secondary Metabolite Production

Plants synthesize different metabolites for compound production. Some are known as primary metabolites, and some are known as secondary metabolites. Primary metabolites have significance in their metabolic activity. While secondary metabolites does not have well-recognized role in the plant, they play very important role for surviving in the environment (Fabio et al. 2014). These compounds give advantages to plant by suppressing the growth of weeds; providing protection from predators, pathogens, and abiotic stress; attracting pollinators; etc. Over 66% of antimicrobial (including antibacterial, antifungal, and antiviral compounds) and 60% of cancer drugs compounds on the market today are natural products made from the plant secondary metabolites (Fabio et al. 2014)

2.1 In Vitro Plant Tissue Culture

It is a technique of growing plant cells, tissues, and organs in an artificially prepared nutrient medium under aseptic condition (Kumar 2003; Mehul and Kalpesh 2015). Plant tissue culture media contain the entire essential nutrient components which are required for its growth. There are two types of nutrients micro and macro. Micronutrients are those which are require in small or micro concentration, while macronutrients are require in large or macro concentration (Murashige 1974).

Plant cell and tissue cultures can be routinely established under sterile conditions from explants such as plant leaves, stems, roots, and meristems for the growth and extraction of secondary metabolites (Karuppusamy 2009). Strain improvement, methods of selecting high-producing cell lines, and optimization of media can lead to increased production of secondary metabolites. The ability of plant cell, tissue, and organ cultures to produce and accumulate many of the same valuable compounds as their natural parent plants has been recognized almost since the dawn of in vitro technology. Today's growing market demand for natural, renewable products has drawn renewed attention to in vitro plant material as a potential factory for natural biofactories secondary metabolites, producing in vitro secondary products. Has paved the way

for new studies exploring the expression of secondary metabolites (Karuppsamy 2009). Producing valuable secondary products in plant cell cultures rather than in vivo in whole crops has many distinct advantages. These include the following:

- Production becomes more reliable, simpler, and more predictable.
- Phytochemical isolation is faster and more efficient than extraction from complex whole plants.
- Compounds synthesized in vitro can directly align compounds throughout the plant.
- Interfering compounds that occur in field plants can be avoided in cell culture.
- Tissue and cell cultures can provide large amounts of defined, standard phytochemical sources.
- Tissue and cell culture are potential models for testing induction.
- Cell cultures can be radiolabeled so that the resulting secondary products can be metabolically traced when fed to experimental animals.

While previous studies have succeeded in producing a wide range of valuable secondary metabolites in unorganized callus or suspension cultures, in other cases production requires more sophisticated microplants or require organ culture (Hussain et al. 2012).

2.2 Biotic Elicitation

Elicitors are microbial molecules that can increase the production of secondary metabolites in cultured cells. Recent developments in elevation have opened new avenues for the production of secondary metabolites. Synthesis and accumulation of secondary metabolites in cell culture can be triggered by applying elicitors to the culture medium (Devi 2011). Plants have many defined mechanisms against attack by physical, chemical, or biological agents. Defense signaling pathways are then triggered, leading to an increased production of secondary metabolites by low-molecular-weight phytoalexins induced in stressful situations.

2.3 Role of Agrobacterium rhizogenes *in Secondary Metabolite Production*

Agrobacterium spp. are soil-borne plant pathogens responsible for various neoplastic diseases, such as H. Crown gall (*Agrobacterium tumefaciens* and *Agrobacterium vitis*), hairy root (*Agrobacterium rhizogenes*), and tube gall (*Agrobacterium rubi*). A cell-free extract of *Agrobacterium rhizogenes* was used to recover gymnemic acids from suspension cultures of *G. sylvestre*, showing a maximum accumulation of 66.12 ± 1.76 mg/g at 24 h, 7.5 times higher than control culture. Because *Agrobacterium* is a Gram-negative bacterium, it serves as a source of lipopolysaccharide, peptidoglycan, and various other cell wall components that aid in the

induction process. Among all the bacterial triggers, the extract of *Agrobacterium rhizogenes* showed the greatest increase in gymnic acid production at a trigger dose of 1% v/v after 24 h (Chodisetti et al. 2013).

3 Gymnemic Acid

Gymnemic acid is the active ingredient in the medicinal plant *Gymnema sylvestre*, responsible for its antidiabetic effects. Gymnemic acids are pentacyclic triterpenoids. *Gymnema sylvestre* leaves contain 20 types of gymnemic acids (Fig. 3).

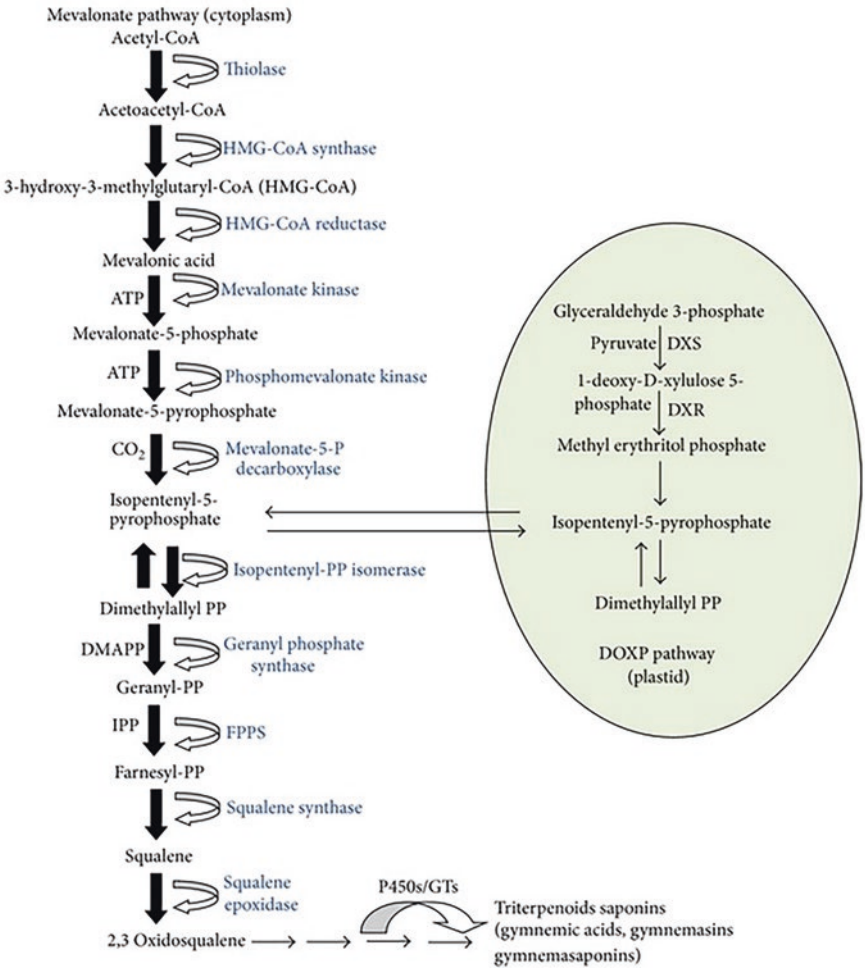

Fig. 3 Biosynthesis pathway of gymnemic acid

Below this, type 1 gymnemic acids exhibit the greatest anti-sweetening and antidiabetic effects. It can also inhibit the sweetness of the sweet proteins aspartame and thaumatin (Kurihara 1992).

Gymnemic acids include several acylated (tigrolyl, methylbutyryl, etc.) derivatives of deacyl gymnemic acid (DAGA). Individual gymnemic acids (saponins) include gymnemic acids I through VII, gymnemosides A through F, and gymnema saponins. Four new triterpenoid saponins, gymnemasins A, B, C, and D, isolated from *G. sylvestre* leaves were identified as 3-O-[β-D-glucopyranosyl(1 > 3)β-D-glucopyranosyl]. Gymnemanol, 3-O-[β-D-glucopyranosyl (1 > 3)-β-D-glucuronopyranosyl]-gymnemannol, 3-O-β-D-glucuronopyranosyl-22-O-tyroylgymnemannol, 3-O-β-D-glucopyranosyl-gymmethanol (Saneja et al. 2010).

3.1 Gymnemic Acid Mode of Action

The atomic arrangement of gymnemic acid molecules resembles that of glucose molecules. These molecules fill the receptor sites of the taste buds, thereby preventing their activation by sugar molecules present in food, thereby suppressing sugar cravings. It fills the outer layer of receptor sites and prevents absorption of sugar molecules through the intestine, causing hypoglycemia (Krishna et al., 2012).

Taxonomical Classification of Plant Gymnema sylvestre

Kingdom	Plantae
Subkingdom	Tracheobionta
Super division	Spermatophyta
Division	Magnoliophyta
Class	Magnoliopsida
Subclass	Asteridae
Order	Gentianales
Family	Asclepiadaceae
Genus	*Gymnema*
Species	*G. sylvestre*
Common name	Madhunashini, gurmar, and Rams horn

3.2 Geographical Distribution

It is found in tropical forest of India: Andhra Pradesh, Bihar, Chhattisgarh, Tamil Nadu, Uttar Pradesh, and West Bengal. It is also found in Banda, Konkan, Western Ghats, and northern India. It is found in Malaysia, Sri Lanka, Australia, Indonesia, Japan, Vietnam, and China.

3.3 Viability

Gymnema is propagated naturally by seed germination. Natural reproduction takes place by seeds which are pollinated in the hot summer, and germination takes place in the rainy season. The efficiency of germination is very low. Because when it is released, the environment has dry condition with low moisture contain and has less endosperm. Due to that although the mother plant produces a large number of seed, the germination efficiency of *Gymnema* is low.

3.4 Plant Description

About 119 species occur in genera *Gymnema* in which *Gymnema sylvestre* R.Br. is one of them. About 25 species are found in tropical or subtropical Asia, South Africa, and Oceania.

A large-branched shrub that reaches the tops of tall trees. Stem: The uppermost juvenile part is pubescent and often dense, i.e., columnar. Leaf: Leaf is 2.3–4.5 × 1–2.6 cm, caudate and male, opposite, ovate, ovate to elliptical or ovate, elongated, hairy, pointed or short apical, more or less hairy Yes, densely hairy below, especially on nerves, base rounded or heart-shaped, sometimes wedge-shaped, abruptly acute at apex. Petiole: 0.6–1.25 cm long, hairy (Fig. 4a). Inflorescence: In lateral umbels, stems are 1–1.5 cm long, densely hairy, shorter than petiole, may form continuous umbels or whorls of flowers. The flower is small, yellowish or pinkish-greenish, entire, radial, hermaphroditic, quintuple, hypogynymous, and ringed. The peduncle is 0.3–1.25 cm long and pubescent. Bracts are fine, ovate oblong, hairy, and ciliate (Fig. 4b). Calyx: There are five sepals, multi-sepals; hairy; and basally divided or mostly divided. Segments are 0.1–0.2 cm long, oblong, obtuse, ciliate, scaly, and glabrous. Flower crown: There are five petals, 0.4–0.5 cm wide, yellow, copetal,

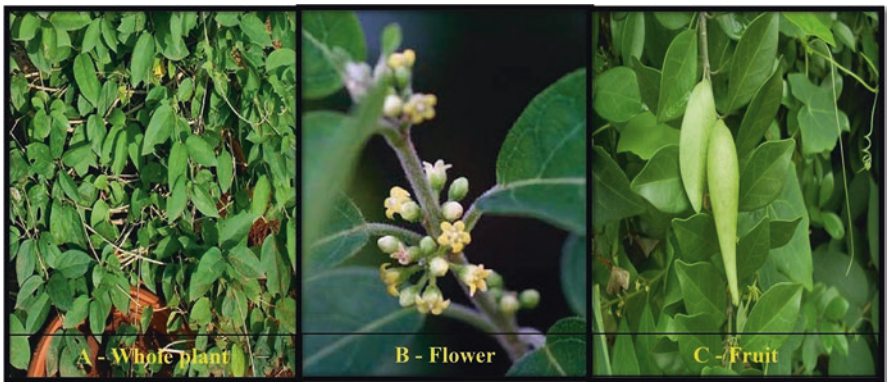

Fig. 4 Morphological characters of *Gymnema sylvestre*

tubular, medium, or more divided. Tubes are ±0.12 cm long, bell-shaped, and almost identical to the leaves. Lobe is thick, ovate deltoid, broad, reflective, and glabrous. Five-pronged coronal processes, inserted into the corolla canal, alternating with lobes, free at the short subacute apex of the deltoid, projecting beyond the sinuses, inferiorly branched, tubular, strongly ciliated rim, mouth of the crown that projects beyond the coronal crest. Androecium: A stamen extends from the base of the crown. The pollination bag is ±0.02 cm; anthers are short and erect, with short membranous appendages. The pollinia stands solitary in each anther cell. Gynoecium: The ovary is ±0.07 cm and the style ±0.05 cm long, thick at tip, hemispherical, extending beyond anther, and pearly white. Fruits: Follicle is 6.25–7.5 × 0.8 cm, paired, cylindrical, rigid, lanceolate, beak weak, and glabrous; one follicle is often suppressed. Seed: Body length is ±1.25 cm, many, narrow ovate-oblong, flattened broad wings with thin margins, brown, and glabrous (Fig. 4c).

3.5 Traditional Uses

Traditional healers from diverse parts of India use this plant in various ailments. This plant is traditionally used for antidiabetic, antibacterial, antiviral, anti-inflammatory, anti-sweeting agent, for weight loss, antipyretic, uterine tonic, anti-asthma, and cardio tonic.

The leaves are given in gastric troubles in Rajasthan. Traditional healers of Maharashtra prescribe it in urinary problems, whereas in Madhya Pradesh, it is used as stomachache. Tribal's and local healers apply the leaf extract in cornea opacity and other eye disease. In Andhra Pradesh, it is used in glycosuria (Fruit et al. 2012; Thakur et al. 2012).

4 Materials and Method

4.1 Collection of Plant Material

The plant *Gymnema sylvestre* was collected during July, 2014, to August, 2014, from the Medicinal Plant Garden, Anand Agriculture University (AAU), Anand, Gujarat, and plant leaves are collected from the National Research Centre of Medicinal and Aromatic Plant (NRC-MAP), Boriyavi, Anand, Gujarat, India (Fig. 4a–c). The plant was identified by Dr. Kalpesh Ishnava (plant taxonomist) at Ashok and Rita Patel Institute of Integrated Study and Research in Biotechnology and Allied Sciences (ARIBAS), New Vallabh Vidyanagar, Gujarat, India.

4.2 Glassware

The glassware used for the plant tissue culture is sugar tubes and test tubes (Borosil): 100-ml, 250-ml, 500-ml, and 1-l flask. Sterile pipettes of 1 ml, 5 ml, and 10 ml. The measuring cylinders are 100 ml, 250 ml, 500 ml, and 1 l. All the glassware used for the plant tissue culture must be sterile by autoclave to minimize the chances of contamination. Before use all the glassware was washed thoroughly by detergent and then washed with water, and put it inverted on the blotting paper to dry it. The plugs for the sugar tubes are made from a nonabsorbent cotton. The sugar tubes and bottles which are used for the inoculation and storage of the stock solution must be autoclave at 12 °C and 15-lbs pressure for 15 min.

4.3 Plant Tissue Culture

4.3.1 Culture Medium Preparation

The basal medium use for the plant tissue culture is Murashige and Skoog medium (MS media, 1962), with 3% sucrose and 100 mg/l myo-inositol. The MS media contain all the micro- and macronutrients essential for the growth of the plant like phosphate, sulphate, chloride, nitrate, carbohydrates, vitamins, and plant growth regulators in the optimum concentration. The micronutrients are required in micro quantity, and the macronutrients are required in more quantity than micronutrients. This constitute is dissolved in distilled water in appropriate amount. Likewise all the stock solution of the MS media are prepared. After making all stock solution, it will be stored in the refrigerator at 20 °C. To make the MS media, all the stock solutions are added in the 1-liter conical flask as shown in Table 1. (All constitutes are used is from Hi-Media). After making the MS media, 10-mg/l myo-inositol and 3% sucrose are added into it. The total volume makes up 800 ml by adding distilled water. The appropriate amount of plant growth regulators are added, and adjust the final pH 5.8 by adding 0.1 N HCL or 0.1 N NaOH as per requirement. After adjust the pH, the final volume makes up 1 liter after distilled water is added. After then add the 0.8% agar-agar powder in the media, and keep in the oven to dissolve it completely. After dissolving it, transfer the media to the sugar tubes. These sugar tubes are closed tightly by using cotton plug. Wrap these sugar tubes, and autoclave it at 15-psi pressure at 121 °C temperature for 15–20 min. As the sugar tubes are being autoclaved, put it into slant position to make slants for inoculation of the leaf, and allow it to solidify overnight.

4.3.2 Selection of Explant

The leaf is selected as the explants used for the inoculation. We collect the plant leaf from AAU is use for the inoculation. Both mature and young leaves are taken as explants from it.

Table 1 Stock solution and preparation of MS media

Name of stock	Constituents	Amount (g/l)	Amount to draw (ml/l)	Actual composition (mg/l)
A	NH_4NO_3	82.5	20	1650
B	KNO_3	95	20	1900
C	KH_2PO_4	34	5	170
	H_3BO_3	1.24		6.2
	$NA2MoO_4.2H_2O$	0.05		0.25
	KI	0.166		0.83
	$CoCl_2.6H_2O$	0.005		0.025
D	$CaCl_2.2H_2O$	88	5	440
E	$MgSO_4.4H_2O$	74	5	37
	$MnSO_4.4H_2O$	4.46		22.3
	$ZnSO_4.7H_2O$	1.72		8.6
	$CuSO_4.5H_2O$	0.005		0.025
F	$FeSO_4.7H_2O$	5.56	5	27.85
	Na2EDTA	7.40		37.3
Vitamins	Inositol			100
	Nicotinic acid	20 mg		0.5
	Pyridoxine	20 mg	5	0.5
	Thiamine	4 mg		0.1
	Glycine	8 mg		2

4.3.3 Explant Sterilization

Leaves were taken from the cultivated plant *Gymnema sylvestre*. Place these leaves in a beaker, cover with a mesh, and soak in running tap water for 30 min to remove all adhered dust and microbes from the surface; add 2–3 drops of liquid detergent to the water, and rinse again under running water 7–8 min until all detergent is removed from the water. After this, transfer to distilled water, and place in laminar flow to inoculate the medium.

4.3.4 Surface Sterilization of Explants

Surface sterilization is done to remove the microbes present on the explants surface. It is done by using 0.1% $HgCl_2$ (add 0.1 gm of $HgCl_2$ into 100 ml of distilled water). Put the explants into it for 1 min and/or 30 s for surface sterilization. After that wash with sterile distilled water three to four times.

4.3.5 Inoculation of Explant

The entire experiment should be performed under a laminar flow unit to maintain strict aseptic conditions. The surface of LAFU is disinfected with 70% alcohol through cotton. Forceps, Petri dishes, and surgical blades used in experiments

should be autoclaved. All instruments used in the experiment were placed in the LAFU under UV light for 20 min before performing the experiment. All worke space area and other accessories such as instruments (spatulas, tweezers, scalpels, blades, etc.), gas burners, lighters, tubes of absolute alcohol, etc. There was also alcohol disinfection. Hands and arms are also rubbed with 70% alcohol before vaccination. All instruments (forceps, scalpels, spatulas, etc.) were sterilized by immersing in 70% alcohol and flaming several times. Before using these instruments, they must be cooled. Wash the surface sterile explants 4–5 times with sterile distilled water, and store in sterile distilled water. Transfer these explants to a sterile Petri dish. Leaf midribs and leaf margins are removed using an autoclaved surgical blade. Leaves are cut into 1 cm × 1 cm pieces using sterile forceps and a sterile surgical blade. Tweezers are sterilized by dipping them in 70% alcohol and setting them on fire. It was then left to cool for a while. One of the sugar tubes has the cotton plug removed near the flame, and the mouth of the sugar tube is flame-wrapped to avoid contamination. The cut leaf pieces are picked from sterile forceps and inoculated into sugar tubes with MS medium. Reheat the mouth of the sugar tube and close it with a cotton plug. Disinfect the forceps again with 70% alcohol. This procedure is used for inoculating sugar tubes with all leaf explants and for inoculating bottles. These inoculated sugar tubes are placed in a sugar tube rack. The racks and flasks are now transferred to the growth chamber of the plant tissue culture laboratory where culture conditions are maintained.

4.3.6 Culture Condition

In the culture room all the inoculated explant culture providing the artificial environmental conditions are maintained. The temperature is maintained at 24–26 °C. The humidity is maintained at 80–85%. The light is given 2000–3000 lux. The light duration is kept at 16 h of light period and 8 h of dark period.

4.4 Establishment of Culture Media for In Vitro Callus Induction

In vitro callus induction is done for production of the callus from the plant leaf. For that different combination of the plant growth regulators (PGRs) is taken to find in which PGRs combination the maximum callus production occurs within a short time period. IBA (indole butyric acid), 2,4-D, kinetin (KIN), and BAP are taken as PGRs. In them IBA and 2,4-D are the auxin, while KIN and BAP are cytokinin (all the hormones used are from Hi-Media). The different combinations of them are shown in the Table 2. The stock of the hormones is made by dissolving the 10 mg of hormone into the 10 ml of distilled water. Thus the final solution is of 10 mg/10 ml, and by this the stock of the hormones will be 1 mg/1 ml. Preparation of the MS-media with different hormones concentration for inoculation is shown in below Table 3.

Table 2 Different combinations of PGRs

Sample	IAA (mg/l)	2,4-D (mg/l)	KIN (mg/l)	BAP (mg/l)
A	2	1	–	–
B	2	2	–	–
C	2	3	–	–
D	2	4	–	–
E	3	5	–	–
F	1	1	–	0.5
G	2	2	1	0.5

Table 3 Preparation of media for inoculation

Sample	MS media (ml)	Sucrose	Myo-inositol (mg)	IBA (ml)	2,4-D (ml)	KIN (ml)	BAP (ml)
A	500	15	50	1	0.5	–	–
B	500	15	50	1	1	–	–
C	500	15	50	1	1.5	–	–
D	500	15	50	1	2	–	–
E	500	15	50	1.5	2.5	–	–
F	250	7.5	25	0.25	0.25	–	0.125
G	250	7.5	25	0.5	0.5	0.25	0.125

Note: Hormones taken in ml are from stock solutions

4.5 Isolation of Gymnemic Acid from Gymnema sylvestre *Leaves (Krishna et al. 2012)*

Isolation of gymnemic acid from *Gymnema sylvestre* leaves involves four major steps. The steps used for the isolation of gymnemic acid are as follows:

Step: 1 Making Dry Powder of Gymnema sylvestre Leaves

Leaves of *Gymnema sylvestre* are taken and washed under running tap water. These leaves are now allowed to dry in oven at 40–50 °C temperature or put in the normal condition until dry. These dry leaves are used to form powder by crushing it into the mixture. From this dry powder of *Gymnema sylvestre*, the gymnemic acid will be extracted.

Step: 2 Extraction with Petroleum Ether

50 g of dry leaf powder was packed into a clean soxhlet extraction unit. 500 ml of petroleum ether was added and extracted for 3–4 h until all the components were solubilized in petroleum ether. The temperature is maintained at 60–70 °C. Petroleum ether extract is then collected. This extract is now poured into the plate, and put it overnight to evaporate the petroleum ether. After evaporation of the petroleum ether, we get a sticky paste of the extract collated.

Step: 3 Extraction with 90% Methanol

Extraction with methanol is carried out by the distillation unit. The above extract is dissolved in the 90% methanol. Then the distillation process is done. The methanol and extract is collected after 1 h of distillation process. The temperature is maintained at 70–75 °C as the boiling point of the methanol is 65 °C. After the extraction by the 90% methanol, the extract is transferred into the plate, and put it overnight so that excess methanol will evaporate from it. After 1–2 days when methanol is evaporated, we get the sticky extract collected.

Step: 4 Isolation of Gymnemic Acid from Methanol Extract

The paste of methanol-soluble extract was dissolved in 1% aqueous KOH solution, and put it on magnetic stirrer for continuous stirring for 45 min–1 h. Then diluted HCl was added slowly under constant stirring, during which the gymnemic acids were precipitated. The solution is then filtered through filter paper. The precipitates are then dried into the vacuum oven. This dried precipitates contain gymnemic acid.

4.6 Identification of Isolated Gymnemic Acid by TLC (Thin-Layer Chromatography)

The TLC was performed on pre-coated 20 × 20-cm and 0.25-mm-thick silica plates. Before running the solvent, the apparatus must be saturated with the same solvent system for 30 min by adding solvent in it, and then close the system. In the TLC there are two phases.

1. Stationary phase.
2. Mobile phase.

The percolated silica acts as a stationary phase, while the solvent system we choose acts as a mobile phase. A small spot of the solution is applied on the silica plate. During the development of the plate, the spot will diffuse with the mobile phase, and give a good separation. As the spot is larger, it is difficult to observe the separate bands and identify it. Small spots can be obtained by using fine capillary tubes as applicators. The different solvent systems are made to carry out separation: The following systems are used for separation of compound.

Solvent system 1: chloroform:methanol (3:2)
Solvent system 2: chloroform:methanol (3:2.5)
Solvent system 3: chloroform:methanol (2:3)
Solvent system 4: chloroform:methanol (2.5:3)
Solvent system 5: chloroform:methanol (2:3.5)
Solvent system 6: isopropyl alcohol:chloroform:methanol:acetic acid (5:3:1:0.5)

The sheets were run in the above solvent systems and dried at room temperature. Then the separated bands are observed by developing plate by spraying it with

vanillin sulfuric acid spray, and put it into the oven at 125 °C for 5 min. Then the Rf value will be measured.

$$\text{Rf value} = \frac{\text{Distance travels by solute}}{\text{Distance travels by solution}}$$

Rf value is specific for the specific component. Thus, if only one intense separate band is observed, it shows that your solution contains a higher concentration of one substance, and by comparing the Rf value, we can find which component.

4.7 Quantification of Gymnemic Acid by HPLC (Bhuvaneswari et al. 2013)

One gram of dried root powder was extracted three times with 10 ml of methanol. Extracts were evaporated to dryness and reconstituted in HPLC grade methanol according to the standard curve. HPLC analysis was performed on his Perkin Elmer (200 series) system in the USA. The system consisted of a quaternary gradient system pump, RI detector (range 1.00–1.75 RIV), autosampler, and DGU 20A3 degasser and was run in class VP software. Separation was performed on columns (250 × 4.6 mm, C 18 ODS with 5-μm particle size) C-18, RP-18, and PI gels with 5-μm particle size and acetonitrile and water (80: 20) at a flow rate of 1 ml/min. Gymnemic acids are detected with a UV detector at 210 nm, with a retention time of 2.8–3 min for gymnemic acids. Gymnemic acids are detected with a UV detector at 210 nm, with a retention time of 2.8–3 min for gymnemic acids. For analysis and comparative studies, run two samples with and without PGR treatment. Each analysis was repeated twice.

5 Result and Discussion

Gymnema sylvestre is a slow-growing climber. It belongs to the Asclepiadaceae family. It is occasionally cultivated for its high demand in indigenous medicine (Jayaweera and Senaratne 1980). Gymnema sylvestre is a slow-growing climber. It belongs to the Asclepiadaceae family. It is occasionally cultivated for its high demand in indigenous medicine (Jayaweera and Senaratne 1980. *Gymnema sylvestre* contains acidic glycosides and anthraquinones with antidiabetic, sweetening, and anti-inflammatory effects. The most medically important compound of this kind is gymnastic acid (Lee et al. 2006). This medicinal plant is also used to treat rheumatism, cough, ulcers, and eye pain. It is also effective against inflammation, indigestion, constipation, and jaundice. The roots of this plant have been described as a remedy for snake bites (Nadkarni 1993).

Gymnema sylvestre leaf extract (ethanol and water) contains triterpenes, and saponins, belonging to the classes of oleananes and dammaranes. In addition, flavones, anthraquinenes, hentriacontanes, alpha and beta chlorophylls, phytin, resins, inositol, D-quersite, alkaloids, tartaric acid, formic acid, butyric acid, lupeol, amylin, and stigmasterol have been isolated and characterized. (Kapoor 1990).

Natural populations of *Gymnema sylvestre* are rapidly disappearing and endangered due to discriminatory collection, commercial purposes, and abuse of natural resources to meet pharmaceutical needs. Vegetative propagation is the only method of cultivation, but it is a very slow growth process in different climatic conditions. In nature, the low germination rate of seeds with low viability causes population decline. It is poorly propagated coupled with indiscriminate collection from natural sources for its diverse medicinal uses. *Gymnema sylvestre* is in great demand in the herbal medicine industry and is an active ingredient commonly used for type 2 diabetes using herbal medicines or natural medicines, with demand for rapid disappearance and threat. Therefore, there is an urgent need to preserve plants through biotechnological approaches such as tissue culture.

Gymnema sylvestre's various important uses increase its commercial value every day. It is one of the important antidiabetic medicinal plants. The leaves of these plants are in high demand in the pharmaceutical or industrial world. To meet commercial demands and save them from extinction, we use in vitro culture methods to propagate them. Callus induction is generated in vitro in a short period of time to be a better alternative to the secondary metabolites of gymnemic acids using various growth regulators (PGRs). Therefore, in this study, we select plants for in vitro callus induction and use plant tissue culture methods to enhance the production of secondary metabolites.

5.1 Standardization of the Surface Sterilization Protocol for Gymnema sylvestre

Microbial contamination poses significant challenges to the initiation and maintenance of viable in vitro cultures. Sterility must be maintained in plant tissue culture and maintained by providing a chemical treatment. The first explant was washed under running tap water to remove dust particles. Clean with liquid detergent after removal. A few drops of liquid detergent were added to the water, and the explants were washed under running tap water for 10–15 min. After washing with detergent, wash again under running tap water to remove excess detergent. If microorganisms are present on the surface of the explant, inoculation into the appropriate medium will eliminate contamination. Therefore, it is necessary to remove these microorganisms present on the explant surface. This is called surface sterilization. Surface sterilization is performed by His $HgCl_2$ treatment of explants. However, this requires a suitable concentration of $HgCl_2$. Otherwise, adverse effects such as browning of the explants will occur. Therefore 0.1% $HgCl_2$ is used for this. Despite the best

timing and selection efforts, it is almost impossible to eliminate contamination from in-vitro-grown plants. In fact, according to Leifert et al. (1989), losses due to in vitro contamination in each subculture averaged 3–15% in most commercial and scientific plant tissue culture laboratories, mostly fungal, yeast, and bacterial contamination. The cumulative result is a huge waste of time, effort, and materials that can have serious economic consequences if not mitigated.

To establish the protocol for surface sterilization, treatment of $HgCl_2$ is given to explants for various time periods as shown in below Table 1. As the above table, we can show which type of the effect occurs of the different time period of washing on the leaf. In first case when we gave the treatment of 0.1% of $HgCl_2$ for only 1 min, the contamination% is high. It shows that the surface sterilization treatment time is very less or not appropriate for the remove microorganisms; there are still some microbes that are present or survived, but there is no effect observed on the appearance of the leaf.

I In the second case, where the concentration of $HgCl_2$ is maintained but the time period of washing is increased up to 2 min, it shows that less contamination occurred, but it causes adverse effects on the leaf (Table 4). The browning of the leaf occurred. The browning of the leaf occurred.

In the third case, again the concentration of the $HgCl_2$ is maintained, but the time period of washing is 1 min and 30 s. It shows less contamination without any adverse effect on appearance of the leaf and also observed proper growth and development of the leaf.

It is shows that to maintain aseptic culture condition and remove microbes present on the leaf, the 0.1% $HgCl_2$ treatment is given to explants for 1 min and 30 s which is more suitable compare to other treatment.

5.2 *Effect of Different Growth Regulator for Establishment Protocol for Callus Induction in* Gymnema sylvestre

5.2.1 Effect of Auxin on Callus Induction

Auxin is commonly used in plant cell culture at concentrations ranging from 0.01 to 10.0 mg/L. When added at appropriate concentrations, it can modulate cell elongation, tissue swelling, cell division, adventitious root formation, inhibition of adventitious and axillary bud formation, callus initiation and growth, and induction of embryogenesis. Auxins are involved in the maintenance of plant cell and tissue

Table 4 Standardization of surface sterilization in leaf explants in *G. sylvestre*

Sr. no.	$HgCl_2$ (%)	Washing time	Effect on leaf	Contamination %
1	0.1	1 min	Leaves are green colored	50
2	0.1	2 min	Browning of leaf occurred	20
3	0.1	1 min 30 s	Leaves are green colored	10

Table 5 Effects of different concentration of auxin and cytokinin for selections of leaf on callus induction

Sample	IBA (mg/l)	2,4-D (mg/l)	Leaf type	Callus response after the fourth week
A	2	1	Mature	No callus induction
B	2	2	Mature	No callus induction
C	2	3	Mature	No callus induction
D	2	4	Mature	No callus induction
E	3	5	Mature	No callus induction
F	2	1	Young	No callus induction
G	2	2	Young	White colored callus induce
H	2	3	Young	No callus induction
I	2	4	Young	No callus induction
J	3	5	Young	No callus induction

Table 6 Rate of contamination and callus induction

Sample	Contamination%	Callus induction%
A	5	0
B	5	50
C	10	0
D	5	0
E	10	0

culture systems and are involved in promoting growth, callus proliferation, root formation, and morphological diversity (Kim et al. 1999).

As shown in Table 5, we take mature and young leaf as explants and inoculate in different combinations of the auxin. After inoculation we put all the inoculated sugar tubes in the suitable environment. In Table 5, the time period is given in which the type of response generated is given. As per our observation, no single response is generated in the inoculated tubes in which mature leaf is inoculated. It may happened due to the cells of mature leaf that lost their ability to dedifferentiation or redifferentiation process. In the case of one batch observed, there is whitening of the leaf. The possible reason of it may be the lack of nutrition or death of the cells.

The sugar tubes in which young leaf is inoculated shows response. The auxin combination of IBA (2 mg/l) and 2,4-D (2 mg/l) shows response after the first week, but callus induction occurred after 21–25 days (Table 6). Other combinations of IBA and 2,4-D do not show any response or callus induction (Fig. 5a–c).

It was shown that the alone auxin combination has callus induction ability, but the rate of callus induction and its generated response is very low. The growth of callus occurred after the fifth week, while after transferring this callus to new MS media with the same combination of hormones, the callus production increased after the seventh week (Fig. 6a).

A combination of IBA (2 mg/l) and 2,4-D (2 mg/l) auxin showed a response in the experiment as the selected explants were very young. A similar type of result was observed with the same plant by his. Primary callus from *G. sylvestre* node and

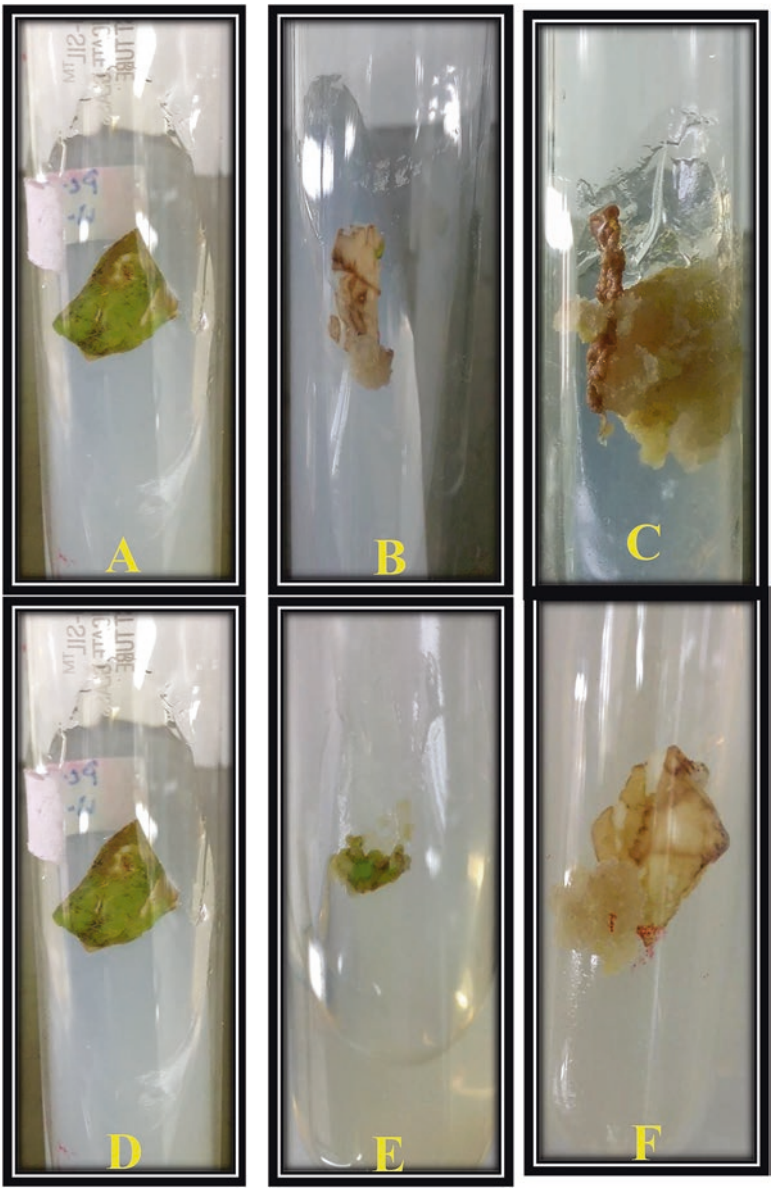

Fig. 5 Effect of plant growth regulators (PGR) on callus induction in *Gymnema sylvestre*. (**a**) Inoculation of leaf (MS media supplemented with 2,4-D (2 mg/l) + IBA (2 mg/l). (**b**) Response after the third week (MS media supplemented with 2,4-D (2 mg/l) + IBA(2 mg/l). (**c**) Response after the fifth week (MS media supplemented with 2,4-D (2 mg/l) + IBA(2 mg/l). (**d**) Inoculation of leaf (MS media supplemented with 2,4-D (2 mg/l) + IBA (2 mg/l) + BAP (0.5 mg/l). (**e**) Response after the third week (MS media supplemented with 2,4-D (2 mg/l) + IBA (2 mg/l) + BAP (0.5 mg/l). (**f**) Response after the fourth week (MS media supplemented with 2,4-D (2 mg/l) + IBA (2 mg/l) + BAP (0.5 mg/l)

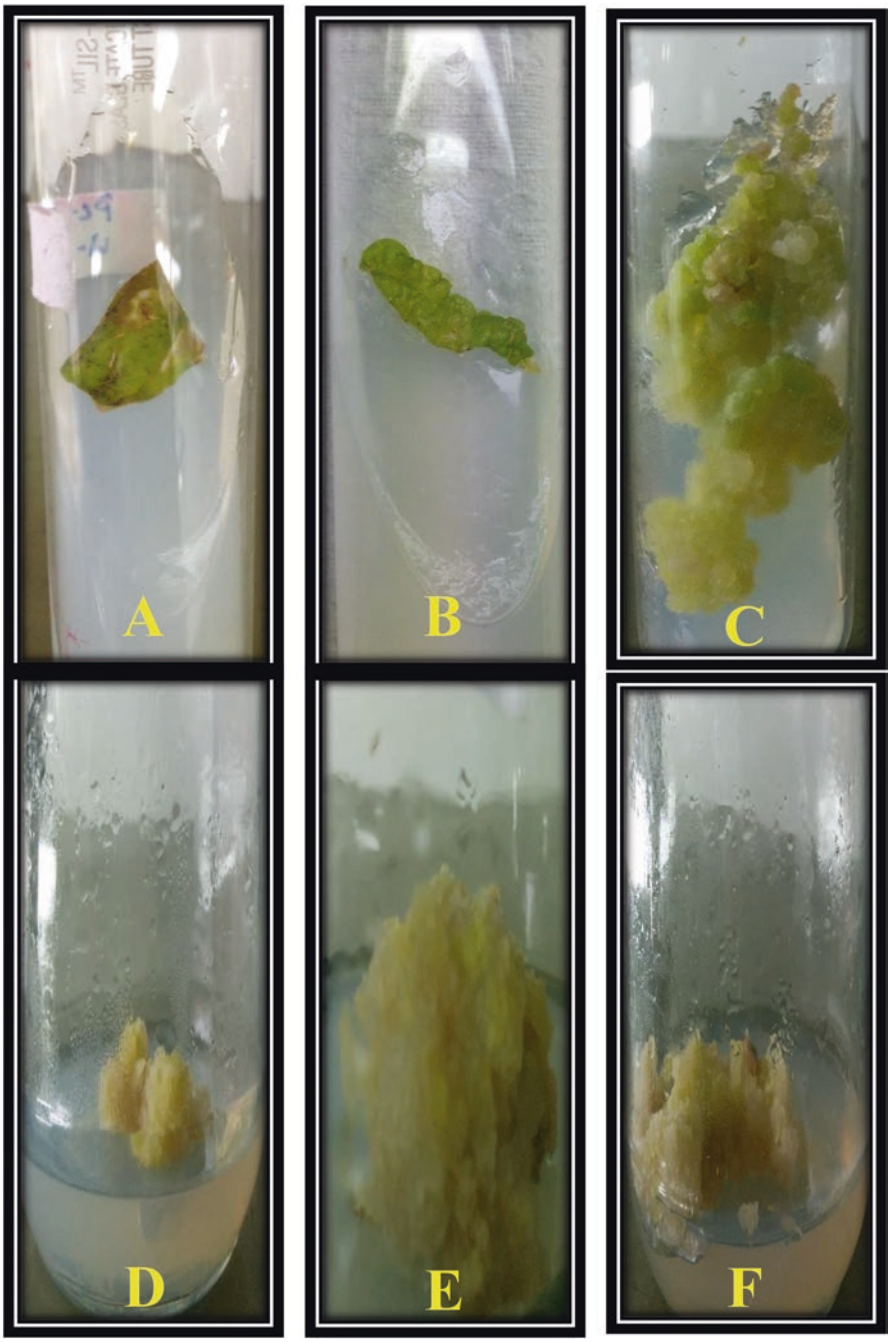

Fig. 6 Effect of plant growth regulators (PGR) on callus induction in *Gymnema sylvestre*. (**a**) Inoculation of leaf (MS media supplemented with 2,4-D (2 mg/l) + IBA (2 mg/l) + BAP

leaf explants were subjected to basal MS spiked with various concentrations (0.10, 0.25, 0.5, 1.0, 2, 5, and 5.0 mg/l) of 2,4-D in medium. The growth efficiency of callus from node explants was significantly higher than that from leaf explants at 4–5 weeks of culture incubation. Significant callus induction was obtained with MS medium containing 0.5 mg/L 2,4-D. The nature of the callus was pale yellow and delicate. Less time of callus induction was observed in our case. Auxin 2.0 mg/L 2,4-D alone induced very weak callus (56%) with an onset time of 18 days. Explants cultured in MS medium supplemented with 1.0 mg/l 2,4-D turned pale, but developed fluorescent green, well-developed, albino-like, spongy, and loosely arranged callus within 40 days. The time at which fluorescent green turns white Increasing the concentration of 2,4-D to 3.0 mg/L and 5.0 mg/L resulted in friable and fluorescent green callus within 38 days (Khatak et al. 2014).

5.2.2 Effect of Auxin and Cytokinin Combinations on Callus Induction

Cytokinins are generally used in plant cell culture at a concentration range of 0.1–10.0 mg/l. Two major properties of cytokinin are that it helps in the regulation of cell division and release lateral bud dormancy (Krikorian et al. 1995). Cell division is regulated by both auxin and cytokinin. Auxin affects DNA replication, while cytokinin has some controls on mitosis (Vesely et al. 1994).

Combinations of auxins and cytokinins are taken, as shown in Table 7. The combination of IBA (2 mg/l) + 2,4-D (2 mg/l) + BAP (0.5 mg/l) showed a response in the first week and callus induction started after 25 days (illustration: 5(D, E, F). After callus induction occurred, the callus production rate was high. Increased biomass produces callus, and auxin combination alone produces callus. According to a study by Buckledeen, BA (0.5 mg/l) (DW-113 mg/l), 2,4-D (1.5 mg/l), and KN (0.5 mg/l) (DW-144 mg/l) increased 35–45 days later. However, the combination of 2,4-D + BA and 2,4-D + KN (<3.0–5.0 mg/L) dramatically reduced callus biomass (Ahmed et al. 2012). Maximal callus induction (98.4%) was observed on MS medium supplemented with 2,4-D (0.5 mg/L) or NAA (1.0 mg/L) and 10% coconut water (Ahmed et al. 2009a, b). In our experiments, exchanging the growth regulators IBA, BAP, and 2,4-D improved the response to callus induction and resulted in high biomass production at day 25. This study shows that the combination of BA, KIN, and 2,4-D is superior to the combination of IBA, BAP, and 2,4-D.

Fig. 6 (continued) (0.5 mg/l) + kinetin (1 mg/l)). (**b**) Response after the third day of inoculation (MS media supplemented with 2,4-D (2 mg/l) + IBA (2 mg/l) + BAP (0.5 mg/l) + kinetin (1 mg/l)). (**c**) Callus induction after the eighth day (MS media supplemented with 2,4-D (2 mg/l) + IBA (2 mg/l) + BAP (0.5 mg/l) + kinetin (1 mg/l)). (**d**) Callus produce in MS media supplemented with 2,4-D (2 mg/l) + IBA (2 mg/l) after the seventh week. (**e**) Callus produce in MS media supplemented with 2,4-D (2 mg/l) + IBA (2 mg/l) + BAP (0.5 mg/l) after 21 weeks. (**f**) Callus produce in MS media supplemented with 2,4-D (2 mg/l) + IBA (2 mg/l) + BAP (0.5 mg/l) + kinetin (1 mg/l) after the third week

Table 7 Combinations of auxins and cytokinins

Sample	IBA (mg/l)	2,4-D (mg/l)	Kinetin (mg/l)	BAP (mg/l)	Leaf type
F	1	1	–	0.5	Young
G	2	2	1	0.5	Young

Table 8 Response of callus induction

Sample	Leaf	Week 1	Week 2	Week 3	Week 4
F	Young	No callus induction	No callus induction	No callus induction	Callus induce with brownish color
G	Young	Response is seen on the third day; callus formation started at on the seventh day	Green colored callus induce	Callus was grown	Rapid growth of callus occurred when it transfer to the bottle contain same media

Table 9 Rate of contamination and callus induction

Sample	Contamination %	Callus induction %
F	20	80
G	0	100

The combination of IBA (2 mg/l) + 2,4-D (2 mg/l) + BAP (0.5 mg/l) + kinetin (1 mg/l) shows curling, and callus induction response is generated at the fourth day (Table 8) (Fig. 6a–c). At the eighth day, green-colored callus is induced. When it is transferred to the new MS media with same hormones, the combination rate of callus production is enhanced, and at the fourth week, the rapid growth and more biomass of callus is produced (Fig. 6c). group reported the combination of the phytohormones, viz., NAA (2 mg/l) + 2,4-D (2 mg/l) + BAP (1 mg/l) + kinetin (1 mg/l) good ignition of callus induction. As a result, replacing NAA with IBA has a better response in combination with short-term callus production and high-yield biomass production within the 25th day. It is a better combination for the secondary metabolite production in vitro condition. The rate of contamination in callus induction in sample 20% and in sample G was not observed (Table 9).

The fact that callus was not induced by 2,4-D alone indicates that gurmari plants are not auxin specific. In contrast, MS medium supplemented with three different concentrations of 2,4-D (1.0 mg/L, 3.0 mg/L, and 5.0 mg/L) and a fixed concentration of kinetin (1.0 mg/L), a green fluorescence, develops into a non-hard, compact callus in 28–30 days (Khatak et al. 2014). *G. sylvestre* leaves were grown in MS medium supplemented with 2,4-D (1.5 mg/L) and KN (0.5 mg/L) (Ahmed et al. 2009a, b), raised, and elevated. MS medium supplemented with 2,4-D (1 mg/L) and kinetin (0.1 g/L) shows callus induction in leaf explants (Ahmed et al. 2009c).

The above results indicate that auxin alone is not sufficient to achieve maximum callus production, although callus is formed in it, but callus production is low. Although combinations of auxins and cytokinins have the ability to induce callus

formation in a short period of time and are also effective in increasing callus production. Thus, auxin and cytokinin regulate cell proliferation and differentiation and re-differentiation in callus production.

5.3 *TLC of* Gymnema sylvestre *Leaf and Callus Extracts*

The Rf value of the calla extracts indicates that the gymnemic acid present in the samples is in a detectable concentration. The Rf value of 0.71 compared to the standard oleic acid compound compared to the Rf value of the callus extract is in the range (Table 10). Thus, we can confirm that gymnemic acid is a synthesis in the callus formed by plant tissue culture. TLC examines standard gymnemic acid with an Rf of 0.71. The solvent system chloroform:methanol (6:5) gave better results compared to other solvent systems for a better separation of compounds in the methanol extract of *G. sylvestre* (Krishna et al. 2012). Krishna et al. (2012) reported that thin-layer chromatography studies were carried out using different solvent systems, viz., chloroform:acetone, chloroform:methanol, toluene:ethyl acetate: diethylamine, and ethyl acetate:petroleum ether. Nine spots with different Rf values were obtained from all gymnemic acid samples. Solvent system chloroform:methanol (6:5) gave better results compared to other solvent systems. TLC studies showed that the profiles are similar compared to conventional gymnemic acid with an Rf of 0.71. Based on the reported value and our sample result, it seems that callus kernel extract contains gymnemic acid. The blue color is obtained not only by the Rf value but also by the form of the vanillin-sulfuric acid reagent with the spray reagent.

5.4 *HPLC of Gymnemic Acid*

For the HPLC method of estimation, the basic frame work of gymnemic acids, deacyl gymnemic acid, is used as marker. The calibration curve for deacyl gymnemic acid was found to be linear over the range of 10–30 mcg/ml (Singh and Dixit 2008).

The above results show that maximum gymnemic acid production occurred in sample G (IBA (2 mg/l), 2,4-D (2 mg/l), BAP (0.5 mg/l), kinetin (1 mg/l), (6.25 mg

Table 10 TLC of callus extracts

Sample	Rf value
Leaf extract (crude)	0.73
B	0.78
F	0.74
G	0.72

Table 11 HPLC of gymnemic acid

Sample	Callus incubation period	Area	Height	Concentration (mg/ml) in 1 gm
Leaf extract	–	1,537,818.49	35,870.18	30.27 mg/ml
B	8-week-old callus	7,461,069.36	261,042.65	45.62 mg/ml
G	4-week-old callus	14,033,963.89	263,699.48	46.25 mg/ml
F	22-week-old callus	21,340,461.81	259,999.89	14.26 mg/ml

of the hormone combination). on a week-old callus (Table 11) (Photo: 7B). The combination of sample B (IBA (2 mg/l) 2, -D (2 mg/l) BAP (0.5 mg/l)) shows 5.62-mg/l gymnemic acid synthesis in 8-week-old callus (Fig. 7c). If the content of the leaves was gymnemic acid, it was 30.27 mg/ml (Photo: 7a). Thus, gymnemic acid production is higher in the callus products of in vitro leaf explants using the plant tissue culture technique. Subathra Devi et al. (2012) reported that leaves grown in Murashige and Skoog salts supplemented with IAA at 1.5 mg/L and BA at 0.5 mg/L produced maximum callus percentage compared to other treatments evaluated. Growth frequency and gymnemic acid accumulation in callus suspension culture were determined. *Xanthomonas* sp. was used as an inducer in the production of gymnemic acid. Compared to unstimulated cultures, a double yield of gynoic acids was observed in stimulated cultures. Gymnemic acid quantification was performed by HPLC. The total content of ginemagenin after the 21st day of incubation was 30.2389 mg/100 ml. This method can be used economically in pilot studies.

In vitro, salt stress also induces *Gymnema sylvestre* R.Br. production of gymnemic acid. Gymnemic acid content increased with increasing 2,4-D concentration along with NaCl (Kumar et al. 2010). 2,4-D (2.0 mg/l) and kinetin (0.1 mg/l) and 3% w/v sucrose were found to accumulate the best biomass and seed content (9.95 mg/g dry weight) (Nagella et al. 2011). Trivedia and Pundarikakshudu (2008) reported an HPTLC method for the indirect determination of gymnemic acids such as gymnemagenin in *Gymnema sylvestre*. Because the deconjugate lacks gymnemagenin, the common aglycone of gymnema acids and UV absorption is very poor. Thus, a post-derivatization method was used to quantify gymnemagenin. Linearity was observed between 180 and 10 ng/point. Thus, the method was found to be more sensitive when gymnemagenin was quantified at the nanogram level. The method was validated according to ICH guidelines and was successfully applied to quantify gymnemagenin from plant leaf powder, extract, and several herbal preparations. The percentage was found to be 98 ± 1.0%.

Xanthomonas sp. is used as a comparison in vitro tissue culture technique using a plant growth regulator (PGR) that gives a better response to the active phytochemical form; the callus produced by in vitro leaf explants has a higher level of gymnemic acid.

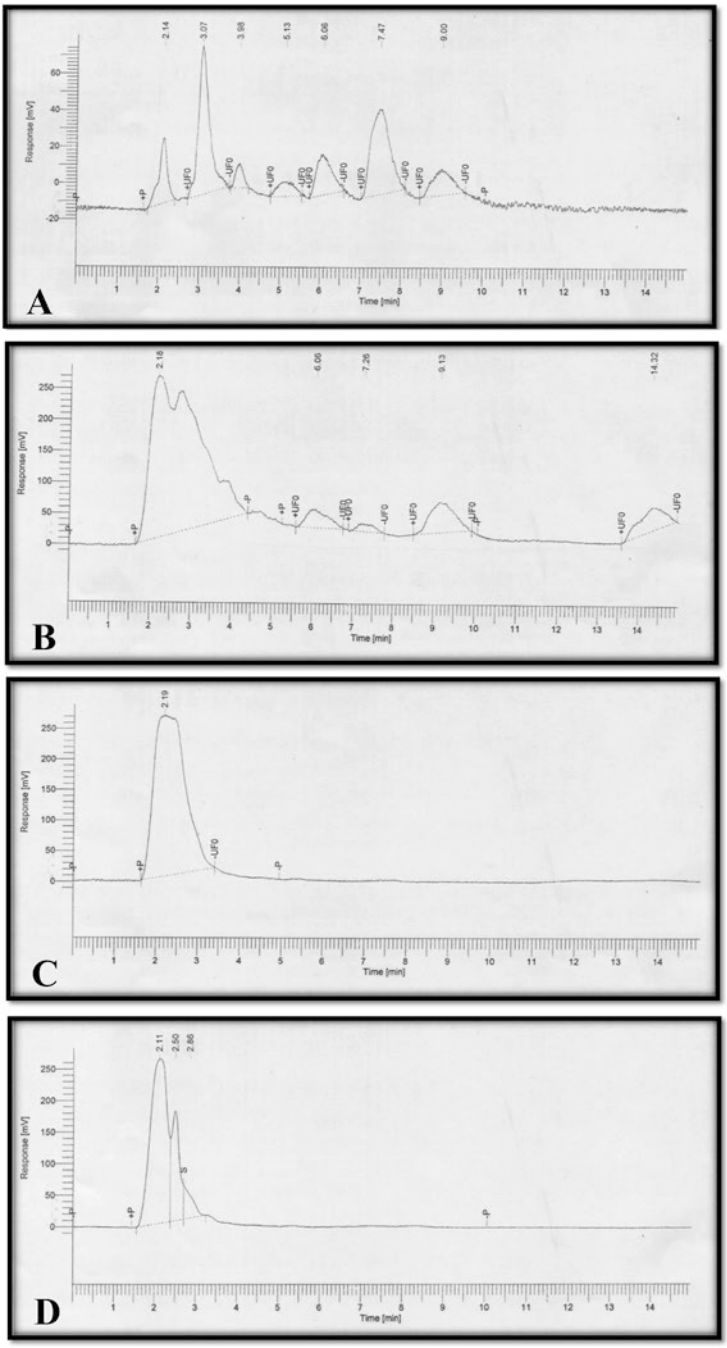

Fig. 7 HPLC of callus methanolic extract of *Gymnema sylvestre*. (**a**) HPLC of gymnemic acid from leaf extract of *Gymnema sylvestre.* (**b**) HPLC of sample G (MS media supplemented with 2,4-D (2 mg/l) + IBA (2 mg/l) + BAP (0.5 mg/l) + kinetin (1 mg/l)). (**c**) HPLC of sample B (MS media supplemented with 2,4-D (2 mg/l) + IBA (2 mg/l)). (**d**) HPLC of sample F (MS media supplemented with 2,4-D (2 mg/l) + IBA (2 mg/l) + BAP (0.5 mg/l))

6 Conclusion

In vitro callus induction of *Gymnema sylvestre* from leaf explants to improve the principle of phytochemical structure and plant growth regulator (PGR) in plant tissue culture techniques using selective metabolite production methods proved to be very useful for the commercial production of gymnemic acid. The combination of IBA (2 mg/l), 2,4-D (2 mg/l), BAP (0.5 mg/l), and kinetin (1 mg/l) gives a better response in callus production, more gym acids on the 25th day of production, and a tall increase in yield biomass with plant tissue culture techniques. Plant growth hormones can be used as enhancers of plant secondary metabolite synthesis and play an important role in biosynthetic pathways to increase the production of commercially important compounds. The increased production of secondary metabolites from plant tissue culture techniques through PGR (auxin and cytokinin) has opened a new field of research that can have significant economic benefits for the bioindustry. Research on growth hormones has shown promise for increasing yields and reducing production costs.

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