Biotechnological Approaches for Medicinal and Aromatic Plant-Based Products

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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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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

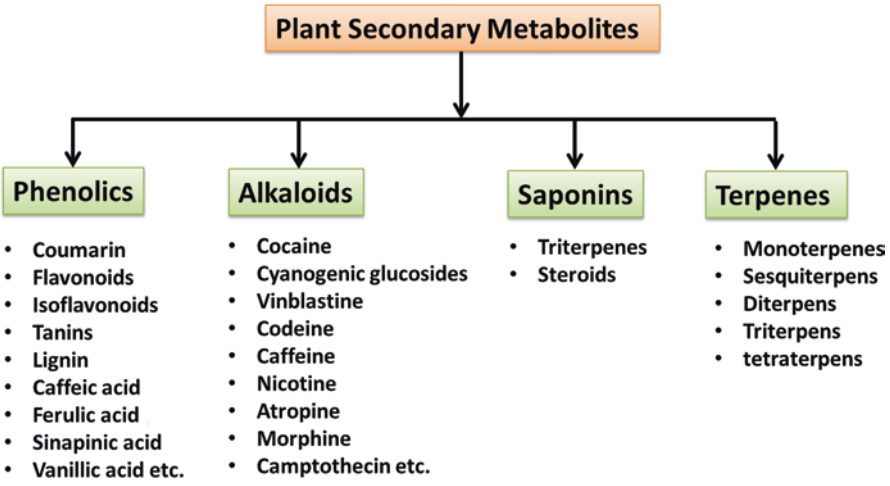

Fig. 1 Broad classification of secondary metabolites

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

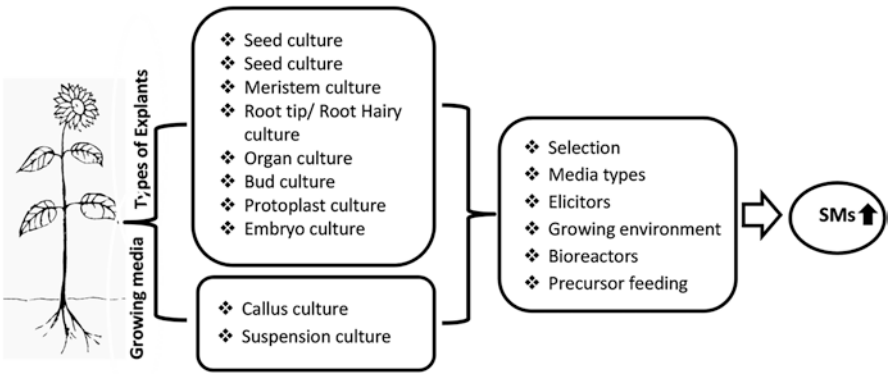

Fig. 2 Systematic representation of SM production under *in vitro* conditions

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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