Nitish Kumar *Editor*

Biotechnological Approaches for Medicinal and Aromatic Plants

Conservation, Genetic Improvement and Utilization

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Preface

Medicinal and aromatic plants are the most important source of life-saving drugs for the majority of the world's population. The biotechnological tools are important to select, multiply and conserve the critical genotypes of medicinal plants. In vitro regeneration holds tremendous potential for the production of high-quality plant-based medicine. Plant tissue culture and traditional methods provide an opportunity for conservation of endangered medicinal and aromatic plants. In vitro production of secondary metabolites in plant cell suspension cultures has been reported from various medicinal plants. Genetic transformation may be a powerful tool for enhancing the productivity of novel secondary metabolites, especially by *Agrobacterium rhizogenes*-induced hairy roots.

Biotechnological Approaches for Medicinal and Aromatic Plants-Conservation, Genetic Improvement and Utilization discusses the applications of plant biotechnology for enhancement of secondary metabolite production in vitro from medicinal and aromatic plants. This book contains 29 chapters divided into 3 parts.

Part I: In vitro production of secondary metabolite
Part II: In vitro propagation, genetic transformation and germplasm conservation
Part III: Conventional and molecular approach

Gaya, Bihar, India — Nitish Kumar

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About the Editor

Nitish Kumar has over 8 years of teaching and research experience in Plant Biotechnology and currently working as an Assistant Professor at Department of Biotechnology, Central University of South Bihar, Gaya, Bihar, India. He received his PhD degree in Botany from Council for Scientific and Industrial Research (CSIR)-Central Salt & Marine Chemicals Research Institute (CSMCRI)/Bhavnagar University, Bhavnagar, Gujarat, India. He has been the recipient of the Outstanding Faculty in Biological Sciences Award in the year of 2017 by the Venus International Foundation, Chennai, Tamil Nadu, India. He has received many awards/fellowships during his academic career from different organizations like CSIR, Department of Biotechnology (DBT), Indian Council of Agricultural Research (ICAR), and Department of Science & Technology (DST). He also got the Fast Track Young Scientist Award from DST, Government of India, in 2013. Dr. Kumar has published more than 40 research papers in peer-reviewed journals of national and international repute. He holds membership of several academic bodies/societies. He is also an Associate Editor of the journal *Gene*.

Part I
In Vitro Production of Secondary Metabolite

Chapter 1
Production of Plant Secondary Metabolites: Current Status and Future Prospects

P. Silpa, K. Roopa, and T. Dennis Thomas

Abstract Plants are the prime life-supporting system on earth. Despite its use as food, it is also utilized as a source of life-saving drugs for majority of the population in the world. Many plants yield phytochemicals known as secondary metabolites, which are pharmaceutically important and are extracted directly from the plants collected from natural habitat. Regardless of conventional methods, biotechnological approaches especially plant tissue culture techniques play a unique role in producing and extracting secondary metabolites at industrial level. This book chapter discusses the various strategies adopted for secondary metabolite production in plants.

Keywords Secondary metabolites · Tissue culture · Metabolic engineering · Abiotic stress

1.1 Introduction

Plants have been the go-to resource for most of man's needs from aboriginal times. Recently, there is an increased interest in medicinal plants due to the rise in the use of herbal medicine and its therapeutic effects. Plants being a great source of bioactive secondary metabolites play a vital role in the field of drug development (Jose and Thomas 2014). Thus, the alternation of these active components through tissue culture and other biotechnical methods acts as a pillar in drug research. However, the requirement of high quantity of raw material is the main hurdle in using plant material as a key resource in drug development. Modifying the genetic makeup through metabolic engineering and high biomass production through tissue culture of these plants would lead to quality and quantity efficient production of bioactive compounds required for drug research.

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1.1.1 Plant Secondary Metabolites

Plants synthesize a variety of organic compounds, mainly classified as primary and secondary metabolites. Primary metabolites are required for essential biochemical processes such as growth, development, photosynthesis and respiration. Secondary metabolites are mainly involved in defence and protect against environmental stresses and also give specific colour, odour and tastes to the plants. Bioactive compounds are also used in agriculture field to protect crops from pests and also in signal transduction to attract seed dispersal and pollination (Wink 2003). Plant secondary metabolites have no key role in maintenance of the life processes in plants. It forms a significant source for pharmaceuticals, insecticides, flavouring agents, drugs (morphine, codeine, cocaine, quinine, etc.) and many other important biochemicals. In addition to this, certain plant secondary metabolites like phenolics, terpenoids, flavonoids and sulphur and nitrogen derivatives play a critical role to prevent many human diseases (Leicach and Chludil 2014). Biotic and abiotic stresses have a pivotal role in accumulation of secondary metabolites in various plant species (Pavarini et al. 2012). Plant secondary metabolites have been divided into three classes, namely, terpenes, phenolic and nitrogen-containing compounds (Taiz and Zeiger 2004).

1.1.2 Terpenes

Terpenes are one of the largest classes of secondary metabolites, and it is built up from isoprene units. Terpene is derived from the word “turpentine”. Terpenes are further divided into monoterpenes (e.g. carvone, perillyl alcohol, geraniol, limonene), sesquiterpene, diterpene (retinoic acid and retinol), triterpene (lupeol, betulinic acid, olealonic acid) and tetraterpene (lycopene, β-carotene, α-carotene, lutein) based on the number of isoprene units (Thoppil and Bishayee 2011). In higher plants, terpene biosynthesis occurs through two pathways, namely, mevalonic acid pathway and MEP pathway. Minor changes in the enzyme terpene synthases can trigger new catalytic properties easily and thereby induce terpene production (Keeling et al. 2008). Terpenes provide a number of esteemed functions, to attract pollinators, signalling compounds in metabolic pathway, plant-pathogen interactions, environmental stresses and other plant defences (Chen et al. 2011). Certain reports have shown that an endophytic fungus *Hypericum perforatum* helps in the production of terpenes in some plants (Zwenger 2008).

1.1.3 Phenolic Compounds

Tannins, flavonoids, stilbenes, lignans, quinines, phenolic acids, coumarins, etc. are the major phenolic compounds from medicinal herbs. Phenolic compounds form an aromatic ring and contain one or more hydroxyl groups. It possesses various bioactivities like anti-inflammatory, antioxidant, anti-carcinogenic and anti-mutagenic effects (Huang et al. 2010). Phenolics are responsible for the colour of red fruits, wines, and juices and also act as flavouring agents (Cheynier 2012). It can be divided into simple and complex phenolic compounds. These biologically active compounds mainly involved in plant defences during stressed conditions. Flavones and flavanols, isoflavonoids, tannins, anthocyanins, and lignin are included in the complex phenolic compounds. These compounds have significant role in many physiological events like flower and root differentiation, characterization of developmental stages, gene activity determination and growth vigour (Akillioglu 1994).

1.1.4 Nitrogen-Containing Compounds

Alkaloids are heterogenous group of secondary metabolites, and in an estimate, about 12,000 alkaloids are isolated from various plants (Ziegler and Facchini 2008). The three major alkaloids, cyanogenic glycosides and glucosinolates and nonprotein amino acids, are the three groups of nitrogen-containing compounds. They mainly act as growth regulators, provide protection from predators and also maintain ionic balance. Cyanogenic glycosides and glucosinolates send out volatile poisons and also play a key role in defence mechanisms (Taiz and Zeiger 2004).

1.2 Production of Plant Secondary Metabolites

Plant secondary metabolites are antibiotic, antifungal or antiviral agents that have the primary function of protecting the plants from disease-causing organisms or pathogens. Plants are mainly exploited for pharmaceuticals, food colours, flavours, fragrances and sweeteners. In this high-tech era, man mainly depends on plant bioactive components for the development of drugs and plant-derived drugs, and intermediates constitute about 25% of the total prescription drugs. Biotechnological approaches such as plant tissue culture techniques have great potential as an alternative for production of useful medicinal compounds like alkaloids, terpenoids, steroids, saponins, phenolics, flavonoids and amino acids. Some commercially available secondary metabolites which are available in the market include shikonin and taxol. In natural conditions, uniform availability of secondary metabolites is not possible due to several reasons. However, cultivation of plant cells or tissues in

aseptic conditions in a bioreactor often leads to consistent production of secondary metabolites with improved quality and yield (Fowler 1985).

For improving the production of secondary metabolites, a number of strategies like screening and selection of high-yielding cell lines; culture of cells from various organs such as shoots, roots, leaves, callus, etc.; suspension culture; induction by elicitors; metabolic engineering; and optimization of media and plant growth regulators were adopted (Anand 2010).

Plant cell and tissue culture holds great potential for controlled production of numerous secondary metabolites which could be useful for various purposes. Secondary metabolite production under controlled condition is devoid of any environmental fluctuations and is cost-effective (Rao and Ravishankar 2002). Plant cell and tissue culture helps to produce important bioactive compounds, and advances in this area may enhance the production of these compounds. The secondary metabolites produced by plant tissue and organ culture are similar to secondary metabolites produced by intact plants. Commercially produced shikonin and taxol are now available in markets. About 20 recombinant proteins like enzymes, antibodies, growth factors and edible vaccines have been produced from tissue culture techniques. Large-scale production of secondary metabolites can be achieved through cell suspension culture by transferring friable callus to suitable medium. The advantage of using cell suspension culture for secondary metabolite production over field-grown plant is that it is devoid of production interfering compounds (Filova 2014).

Production of secondary metabolites in higher plants can be achieved through different explants under sterile condition. Plant cell and tissue culture technique is routinely employed to extract secondary metabolites from various plant species (Table 1.1). Many secondary metabolites are synthesized from primary metabolites in higher plants. About 100,000 secondary metabolites have been isolated from higher plants (Jeong and Park 2006). Its production is usually in lesser quantities, and it is determined by developmental stage and physiology of plants. Medium optimization is required to enhance the production. In many cases, suspension culture, organ culture, embryo culture and callus culture have been successful in increasing the yield of secondary metabolites. The interested metabolites may sometimes synthesize in plant from specialized tissue or organs. For example, saponin is produced from the root of *Panax ginseng*, and hence for large scale, in vitro production of saponin from *P. ginseng* requires root culture. At present several medicinal plants have been utilized to establish various culture systems like callus culture, organ culture, hairy root culture and suspension culture. There are many biotechnological approaches to produce bioactive components and are briefly described below.

Table 1.1 Recent studies on secondary metabolites production by using plant cell/tissue cultures

Plant	Compound/s	References
Anoectochilus roxburghii	Kinsenoside	Jin et al. (2017)
Withania somnifera	Withanolides	Ahlawat and Abdin (2017)
Stevia rebaudiana	Antioxidants	Ahmad et al. (2016)
Vitis species	Monoterpene and sesquiterpene	Alonso et al. (2015)
Dysoxylum binectariferum	Rohitukine	Mahajan et al. (2015)
Prunella vulgaris	Antioxidants	Fazal et al. (2014)
Withania somnifera	Withanolide	Sabir et al. (2013)
Vitis species	Nerolidol	Escoriaza et al. (2013)
Solanum tuberosum	Chlorogenic acid (phenolics)	Navarre et al. (2013)
Humulus lupulus	Prenylflavonoid	Matousek et al. (2012)
Ruta graveolens	Umbelliferone	Vialart et al. (2012)
Nicotiana tabacum	Anthocyanins	Huang et al. (2012)
Senecio species	Pyrrolizidine alkaloid	Karam et al. (2011)
Medicago sativa	Saponins	Szakiel et al. (2011)
Brachiaria species	Protodioscin	Barbosa-Ferreria et al. (2011)
Lavandula officinalis	Tetrahydrofurate (THF) acetate derivative	Patel et al. (2011)
Malus species	Anthocyanins	Lin-Wang et al. (2011)
Eugenia uniflora	Tannins and flavonoids	Santos et al. (2011)
Crotalaria retusa	Monocrotaline and pyrrolizidine	Anjos et al. (2010)
Echium plantagineum	Pyrrolizidine (alkaloid)	Lucena et al. (2010)
Vernonia species	Lactones and flavonoids	Keles et al. (2010)
Lychnophora species	Lactones and flavonoids	Gobbo-Neto et al. (2010)
Angelica gigas	Decursin, decursinol angelate	Rhee et al. (2010)
Lavandula officinalis	Deoxyartemisinin	Patel et al. (2010)
Lavandula pedunculata	Essential oils	Zuzarte et al. (2010)
Lavandula vera	Volatiles	Georgiev et al. (2010)
Lithospermum erythrorhizon	Shikonin	Zhang et al. (2010)
Argemone mexicana	Sanguinarine	Trujillo – Villanueva et al. (2010)
Picrorhiza kurroa	Picoside –I	Sood and Chauhan (2010)
Thevetia peruviana	Peruvoside	Zabala et al. (2010)
Abrus precatorius	Glycyrrhizin	Karwasara et al. (2010)
Silybum marianum	Silymarin	Khalili et al. (2010)
Artemisia species	Artemisin	Brown (2010)
Lavandula pedunculata	Camphor and 1,8- cineole	Zuzarte et al. (2010)

1.2.1 Callus Culture

Callus is the mass of undifferentiated cells containing meristematic loci (Bhojwani and Dantu 2013). For induction of calli from explants, 2, 4-D is the most preferred auxin. However, a combination of auxin and cytokinin or high concentration of auxin alone may be used by various workers for callus induction (Filova 2014). For secondary metabolite production, usually non-embryogenic calli were selected which have homogenous mass of dedifferentiated cells. For callus growth and multiplication, auxin is generally preferred, whereas it is omitted for secondary metabolite production. Screening and selection of high-yielding cell lines and standardization of media for optimum secondary metabolite production are some important strategies to improve secondary metabolite production. Suspension culture is an alternative strategy to obtain high level of secondary metabolite production. Small clumps of calli are transferred to liquid medium in flasks, and this will be followed by continuous agitation on an orbital shaker. Agitation often exerts a pressure on the cell clumps resulting in the breaking of larger clumps into smaller aggregates. Agitation also helps in the uniform distribution of cells and better aeration of cells inside medium. High rate of cell division can be achieved in suspension culture than normal callus culture.

1.2.2 Hairy Root Culture

Plant hairy root culture is the most promising technique among root culture for the production of secondary metabolites. Fast hormone-independent growth, genetic stability, lateral branching and lack of geotropism are the major characteristics of hairy roots. Inoculation of *Agrobacterium rhizogenes* helps to synthesize secondary metabolites in hairy roots (Karuppusamy 2009; Palazon et al. 1997).

A. rhizogenes have root-inducing plasmid (Ri plasmid) which contain a T-DNA; during the time of infection, T-DNA is further divided into TL and TR region in some strains (strain A4) of *A. rhizogenes* in induction processes. Two sets of PRi genes, *aux genes* (in TR region) and *rol genes* (in TL region) are involved (Jouanin 1984). Elicitation of hairy root promotes secondary metabolite production and also arrests feedback inhibition, preventing degradation of metabolites in the culture medium (Chandra and Chandra 2011).

In many plants, hairy root become green by culturing in continuous exposure of light. It generates photo-oxidative stress in hairy roots (Mukherjee et al. 2014). It produces excess H_2O_2 in the root (Behnke et al. 2010). Phenolics to volatile terpenoids shift occur in green hairy roots of carrot, due to the redirection of primary metabolites towards synthesis of volatile isoprenoid synthesis (Mukherjee et al. 2016).

1.2.3 Organ Culture

Organ culture techniques help the rapid propagation of plants. It also gets a high quantity of bioactive compounds and higher growth compared to plants grown in natural habitats. In *Fritillaria unibracteata*, rapid propagation is achieved through small bulb, and the content of alkaloids is higher in this bulb culture (Gao et al. 1999). Tropane alkaloids hyoscyamine and scopolamine were produced in high quantity in root culture (Fazilatun et al. 2004). Many valuable medicinal compounds were obtained from root culture (Pence 2011; Li et al. 2002). Secondary metabolites produced in plant aerial parts are produced from root culture (Bourgaud et al. 2001; Nogueira and Romano 2002; Smith et al. 2002; Kaimoyo et al. 2008). Organ culture exhibits less sensitivity of shear stress, but in biomass production, they show a high degree of spatial heterogeneity. Ginseng roots are the only example for commercially production of secondary metabolites by organ culture (Hibino and Ushiyama 1999).

1.2.4 Elicitation

Elicitors are substances which produce signals against pathogenic attack resulting in the accumulation of secondary metabolites in plants. Further it can improve the biosynthesis of specific compounds when introduced into a living cell system (Radman et al. 2003). Elicitation is the process in which the living cells will be treated with biotic or abiotic elicitors to obtain enhanced rate of secondary metabolites (Rao and Ravishankar 2002). The most commonly employed biotic elicitors include polysaccharides, glycoproteins, yeast extract and some fungi like *Fusarium oxysporum*, *Aspergillus niger* and *Rhizopus oryzae* (Dornenburg and Knorr 1995). The abiotic elicitors are non-biological origin and are mostly inorganic salts, jasmonic acid, salicylic acid, high pH and environmental stress conditions such as heavy metals, UV radiation, osmotic shock, etc. (Naik and Al-Khayri 2015). High stilbenes accumulation in root cultures of *Cayratia trifolia* was observed by Arora et al. (2009). The addition of alar (N-dimethylamino succinamic acid) along with the elicitor salicylic acid enhanced the stilbenes content up to 12-folds (Arora et al. 2009). In *Centella asiatica*, the presence of triterpenes in callus suspension culture derived from leaves showed an increase after incorporating amino acids (Kim et al. 2004). Adding the amino acid isoleucine at 2 mM in the medium enhanced the production of hyperforin in *Hypericum perforatum* (Karppinen et al. 2007). Hence elicitation is considered as one of the most effective methods for enhancing the secondary metabolite production in cultures (Oksman-Caldentey and Inze 2004). Heavy metals and increasing temperature affect the production of secondary metabolites. In *Robinia pseudoacacia* seedlings exposed to elevated carbon dioxide, high temperature and heavy metals (Pb-Cd) enhanced the production of secondary metabolites (Zhao et al. 2016).

1.2.5 Endophytes

Microbes, such as bacteria or fungus which lives inside the plant without making any indication of disease, are called endophytes. Many useful compounds were obtained from the plants with endosymbionts. For example, *taxol*, an anticancer agent, is obtained from *Taxus brevifolia* when infected with a fungi *Taxomyces andreanae* (Strobel et al. 1993). Accumulation of several valuable metabolites in plants is due to the synergistic effect of both plants and endophytes (Engels et al. 2008). The endophytic fungi of *Pinus sylvestris* and *Rhododendron tomentosum* produce useful secondary metabolites having various antibacterial and antioxidant activities (Kajula et al. 2010). It is reported that some endophytic fungi produce a number of beneficial phytochemicals in Leguminosae family (Wink 2013). In some plants, due to the presence of endophytic fungi, an alkaloid called indolizidine is produced (Ralphs et al. 2008). The endophytic fungus *Phoma medicaginis* produces a compound, hydroxy-6-methylbenzoic acid, which shows a noticeable antimicrobial activity (Yang et al. 1994). Phenylpropanoids, lignins, phenol and phenolic compounds, alkaloids, steroids, etc. are isolated from many mycoendophytes (Herre et al. 2007). Novel metabolites from the endophytes have a vital role in the treatment of many infectious diseases (Rai et al. 2012). It is reported that several genes and protein present in plants were seen in fungi and bacteria. This suggests that horizontal gene transfer may take place from endosymbiotic bacteria and fungi (Wink and Schimmer 2010).

Inoculation of arbuscular mycorrhizal fungi (AMF) increases the production of secondary metabolites in economically important plants (Maier et al. 1995). The presence of AMF can considerably enhance the growth and biomass of a plant (Silva et al. 2004). It can also improve the capability to absorb the micronutrients and macronutrients (Chu et al. 2001; Matsubara et al. 2009). Recently, there is an increase in research about the efficiency of AMF in improving the secondary metabolite production (Table 1.2). There is a qualitative and quantitative increase in secondary metabolite production in many plants due to the presence of AMF (Ponce et al. 2004; Ceccarelli et al. 2010). In addition to these, there is an increasing edible vegetable quality triggered by AMF (Baslam et al. 2013).

1.2.6 Nitric Oxide

Nitric oxide plays a crucial role in the production of some important phytochemicals. By the induction of some stresses, accumulation of nitric oxide takes place. Several studies showed that nitric oxide plays a significant role in the development, growth and defence responses of the plant (Flores et al. 2008; Hong et al. 2008). Pharmaceutically important secondary metabolites can be produced by elicitor-induced nitric oxide response (Xu and Dong 2008). Therefore, the significance of nitric oxide can be applied in various biotechnological processes resulting in the production of target secondary metabolites.

Table 1.2 Recent report on influence of inoculation of arbuscular mycorrhizal fungi (AMF) on secondary metabolite production

Host plant	Plant organ	Evaluated phytochemicals	AMF	Effects	References
Moringa oleifera	Leaves	Glucosinolates	*Rhizophagus intraradices*	+ ve	Cosme et al. (2014)
			Funneliformis mosseae	+ ve	
Helianthus annuus	Seeds	Fixed oil	*Funneliformis mosseae*	+ve	Heidari and Karami (2014)
Passiflora alata	Leaves	Total phenols and total flavonoids	*Gigaspora albida*	+ ve	Oliveira et al. (2015)
Stevia rebaudiana	Leaves	Stevioside and rebaudioside A	*Rhizophagus fasciculatus*	+ ve	Mandal et al. (2013)
Anadenanthera colubrina	Leaves	Total phenols, total flavonoids and total tannins	*Acaulospora longula* + *Gigaspora albida*	+ ve	Pedone-Bonfim et al. (2013)
Cucumis sativus	Leaves	Phenols, flavonoids and lignin	*Funneliformis mosseae*	+ ve	Chen et al. (2013)

1.2.7 Abiotic Stress

Abiotic stresses are important for the production of secondary metabolites. Water stress is one of the prominent abiotic stresses which can influence secondary metabolite production. Light has a crucial role in the induction of both primary and secondary metabolites. Light-grown suspension culture displays an increase in phenolic production, in antioxidant activity and also in the total plant metabolite production (Ali and Abbasi 2014). It is reported that there is a link between antioxidant activity and total phenolic content in the suspension culture of *Artemisia absinthium* (Ali and Abbasi 2014). High blue light ratio increases all phenolic acids and flavonoids in some plants (Ouzounis et al. 2014).

1.2.8 Bioreactor

Bioreactors were designed for the commercial production of secondary metabolites. Bubble column bioreactor (Huang and McDonald 2009) and stirred tanks (Su 2006) are the widely used bioreactors for the culture of plant cell. Plants like *Eurycoma longifolia* grow well in bioreactor, and it has a rapid growth compared to that of flask cultures (Lulu et al. 2015). Exposure to UV light brings out secondary metabolite production in bioreactor and also in flask cultures. In *Lavandula vera* cells, rosmarinic acid production showed a 32-fold increase in bioreactors as compared to normal shake flask cultures (Pavlov et al. 2005). Production of secondary metabolites from cells of *Digitalis lantana*, *Catharanthus roseus*, *Hypericum perforatum*,

Panax ginseng, *Sophora flavescens*, etc. has been cultured in various bioreactors (Filova 2014). Sharma et al. (2011) studied the puerarin accumulation in *Pueraria tuberosa* during shoot cultures in static and liquid medium with or without aeration. Shoots were grown in growtek bioreactor with different aeration, and the maximum puerarin content was 1484 μg/g dry weight, which was about 2.3-fold higher than puerarin content recorded in control cultures (Sharma et al. 2011). The genes involving in withanolides production in *Withania somnifera* were upregulated in bioreactor. About 1.5-folds increase in the production of withanolides was found in bioreactor as compared to shake flask (Ahlawat and Abdin 2017). Different bioreactors like continuous immersion bioreactor with net (CIB-N), continuous immersion bioreactor (CIB), temporary immersion bioreactor (TIB) and temporary immersion bioreactor with net (TIB-N) were used for the production of bioactive compounds (Jang et al. 2016). Of these, CIB system was found to be the most efficient bioreactor for the large-scale production of metabolites (Jang et al. 2016). Bioreactor culture of *Anoectochilus roxburghii* accumulated highest level of kinsenoside and other polysaccharides (Jin et al. 2017).

1.2.9 Metabolic Engineering

Metabolic engineering has proven to be a valuable tool for large-scale production of several biomedically as well as industrially relevant secondary metabolites from plants. Current studies employing transgenic and/or recombinant technologies have opened up opportunities for metabolic engineering/metabolomics, leading to the manufacturing of high-value secondary metabolites, even at the commercial level. Metabolic engineering mainly aims at the increase in the production of desired products, brings down the production of unwanted compounds and scales up the yield of novel compounds (Ludwing-Muller et al. 2014). Many of the medicinally and economically important terpenoids are produced through metabolic engineering (Elgar 2017). Patel et al. (2016) reported that the incorporation of squalene synthase gene in isoprenoid pathway of *Withania somnifera* increased the production of bioactive compounds.

1.2.10 Immobilization

Immobilization enhances the secondary metabolite production by entrapping plant cells in suitable matrix, which can protect cells from liquid shear forces and allow better cell-to-cell contacts. The viability of immobilized cells extended over a prolonged period of time (Brodelius 1985). The benefit of immobilized plant cell is that it can extend its production time, making the cells catalyse the same reaction almost indefinitely. The immobilized cells can overcome the task of isolating the compounds from the biomass; rather the products will be delivered in the medium itself.

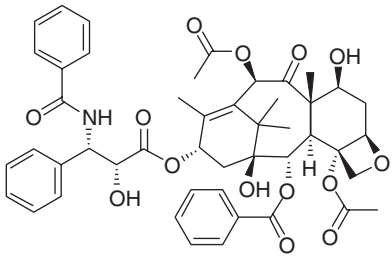

Fig. 1.1 Chemical structure of taxol

It can also perform multienzyme operations, and by using high-yielding cell lines, the productivity can be enhanced significantly (Smetanska 2008).

1.3 Production of Valuable Pharmaceutical Compounds Through In Vitro Culture Techniques

Most of the new therapeutics was evolved from secondary metabolites from plants. Advancement in the plant cell and tissue culture enhanced the production of several pharmaceutically active compounds, and some of such key compounds are described below.

1.3.1 Taxol

Taxol (Fig. 1.1) is a compound extracted from the bark of the Pacific yew tree (*Taxus brevifolia*) which possesses anticancer properties (Oksman-Caldentey and Inze 2004). In polymerized form of microtubules, taxol stabilizes it and thereby causes the death of cells. Due to the huge commercial use of taxol, *Taxus* species have been massively explored. In some studies, it was found that addition of certain amino acids like phenylalanine in the medium yielded maximum taxol in *T. cuspidata* (Long and Croteau 2005). The effect of both biotic and abiotic elicitors positively influenced the yield and accumulation of taxol in some species (Pavarini et al. 2012).

1.3.2 Morphine and Codeine

Morphine and codeine (Fig. 1.2a, b) are the pain-relieving drugs obtained from the members of the family Papaveraceae. Both these compounds occur naturally in Poppy plant (*Papaver somniferum*). Both morphine and codeine were commercially produced in cultures using callus and suspension culture (Yoshikawa and Furuya 1985). The optimum quantity of codeine and morphine was observed in cultures devoid of exogenous hormone (Furuya et al. 1972).

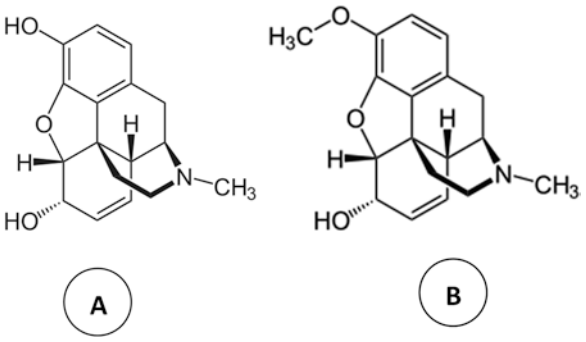

Fig. 1.2 Chemical structure of morphine (**a**) and codeine (**b**)

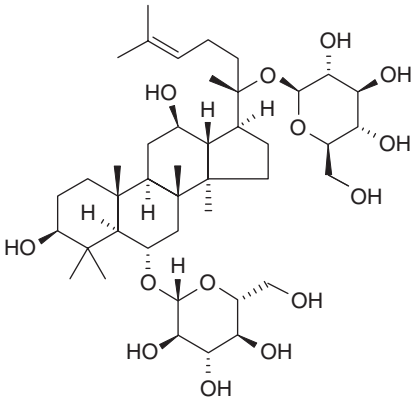

Fig. 1.3 Chemical structure of ginsenoside

1.3.3 Ginsenosides

The active component of the plant *Panax ginseng* is referred to as ginsenosides which are essential for various physiological activities. Chemically ginsenosides are a group of triterpene saponins (Fig. 1.3). The addition of spermidine in the medium enhanced the production of ginsenosides in cultures (Marsik et al. 2014). Further, an elicitor, casein hydrolyzate enhanced ginsenosides production without suppressing the biomass (Marsik et al. 2014). In root culture of *Panax ginseng*, jasmonic acid improves the ginsenosides production (Lambert et al. 2011).

1.3.4 L-DOPA

A nonprotein amino acid, L-DOPA (L-3, 4-dihydroxy phenylalanine; Fig. 1.4), is a potent drug obtained from the plant (*Mucuna hassjoo*) and is mainly used to cure Parkinson's disease (Brain and Lockwood 1976). It is the precursor of many secondary metabolites like alkaloids, melanin and betalain (Daxenbichler et al. 1971). The requirement of large quantities of L- DOPA led to the development of cell

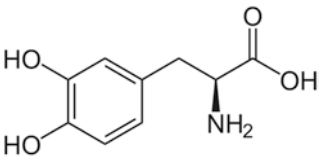

Fig. 1.4 Chemical structure of L-DOPA

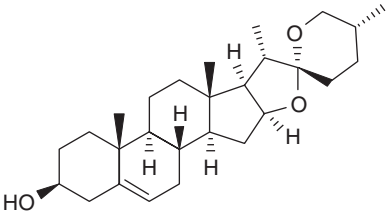

Fig. 1.5 Chemical structure of diosgenin

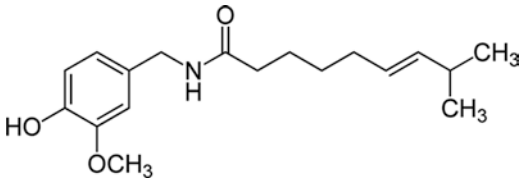

Fig. 1.6 Chemical structure of capsaicin

culture techniques to obtain optimum production of this compound. *Mucuna hassjoo* cells were cultured in MS medium supplemented with kinetin for obtaining optimum quantity of L-DOPA (Vanisree et al. 2004).

1.3.5 Diosgenin

Diosgenin (Fig. 1.5) is a biologically active metabolite, intermediate to various steroid drugs (Tal et al. 1984). Due to its high demand in the market, the production of diosgenin was enhanced by the application of in vitro techniques, and thereby it is beneficial to modern system of medicine. The optimum accumulation of diosgenin in culture was greatly influenced by the carbon and nitrogen level in the medium (Tal et al. 1984).

1.3.6 Capsaicin

The alkaloid, capsaicin (Fig. 1.6), is obtained from *Capsicum* species and mainly serves as a food additive (Ravishankar et al. 2003). The quantity of capsaicin differs significantly in suspension culture and immobilization technique. The amount of capsaicin was comparatively low in suspension culture, whereas it was increased substantially to about 100-fold in immobilization technique (Lindsey 1995). Further, the addition of isocaproic acid in the medium enhanced the production of capsaicin (Lindsey 1995).

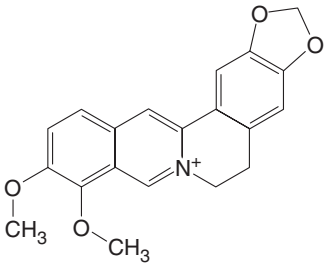

Fig. 1.7 Chemical structure of berberine

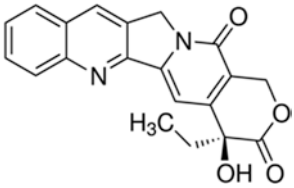

Fig. 1.8 Chemical structure of camptothecin

1.3.7 Berberine

The isoquinoline alkaloid berberine (Fig. 1.7) is obtained from the cell culture of *Coptis japonica* (Vanisree et al. 2004), *Thalictrum* species (Nakagawa et al. 1986) and *Berberis* species (Morimoto et al. 1988). In order to increase the berberine yield in cultures, several elicitors were employed by Funk et al. (1987). Further, Nakagawa et al. (1984) and Morimoto et al. (1988) standardized the nutrient medium for optimum berberine production.

1.3.8 Camptothecin

Camptothecin (Fig. 1.8), a potent antitumor alkaloid, is isolated from the plant *Camptotheca acuminate* (Padmanabha et al. 2006). The production of camptothecin from *C. acuminata* cells in cultures was optimum on MS medium supplemented with 4.0 mg/l NAA (Thengane et al. 2003).

1.3.9 Vincristine and Vinblastine

The plant *Catharanthus roseus* (also known as *Vinca rosea*) contains the vinblastine and vincristine (Fig. 1.9a, b) which are used in chemotherapy (Noble 1990). Due to its irreplaceable medicinal properties, application of biotechnological tools

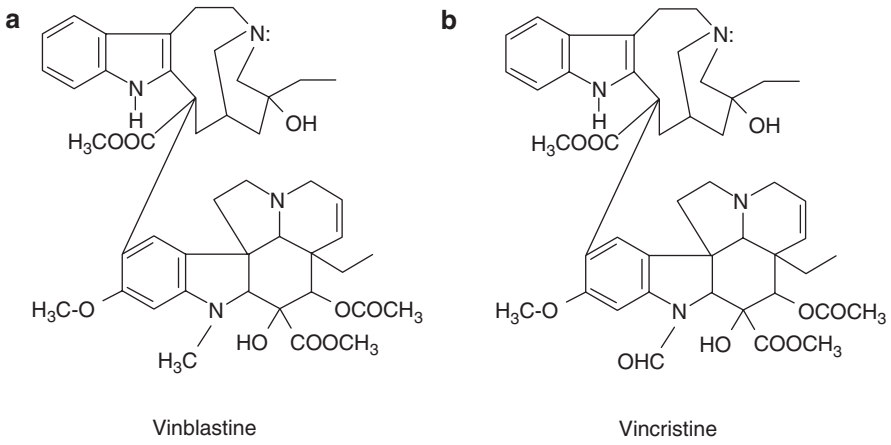

Fig. 1.9 Chemical structure of vinblastine (**a**) and vincristine (**b**)

especially plant tissue culture techniques was employed to produce large quantity of these compounds (Oksman-Caldentey and Inze 2004). Several chemicals such as oxalate, maleate, ferric chloride and sodium borohydride were added in the medium to increase the production of vinblastine (Verma et al. 2007). The other factors which influenced the production of these alkaloids include various stresses such as salinity, drought, heavy metals (Pandey 2017), UV stress (Binder et al. 2009) and presence of elicitors and addition of bioregulators (Zhao et al. 2001).

1.4 Conclusions

Plants are potent source of various useful phytochemicals. The secondary metabolites can be extracted from various parts of the plant. In recent years, there is an increased use of biotechnological tools to obtain continuous and reliable source of secondary metabolites. Among the various techniques, plant cell and tissue culture technology plays a crucial role in secondary metabolite production. The major advantage of using this technology is that it can provide bioactive secondary metabolites in controlled conditions irrespective of season and soil conditions. However, many challenges like difficulties in scaling-up, sustainability of culture and phytochemical recovery, etc. still remain to be challenging factors in this area. Continuous refinements of these techniques are necessary to overcome these limitations.

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Chapter 2
The Effects of *rol* Genes of *Agrobacterium rhizogenes* on Morphogenesis and Secondary Metabolite Accumulation in Medicinal Plants

Sayantika Sarkar, Ipshita Ghosh, Dipasree Roychowdhury, and Sumita Jha

Abstract Induction of hairy roots by *Agrobacterium rhizogenes* and regeneration of Ri-transformed plants from such transgenic roots are reported in a large number of taxonomically diverse plant species. Ri-transformed cultures (roots/calli/plants) have altered characteristics of their own compared to non-transformed ones. Four *rol* genes (*rolA*, *rolB*, *rolC*, *rolD*) of T-DNA of Ri-plasmid are known to be responsible for these phenomena. However, few attempts have been made to elucidate the role of individual *rol* genes on morphogenic ability. In addition, the effect of wild-type *A. rhizogenes* on the production of secondary metabolites is well studied in wide number of plant species. The popularity of this research has never declined through time which explains its immense value and provides a hope for a promising future. Based on such studies, several reviews have been written from time to time, explaining the '*rol* effect' on secondary metabolite accumulation in medicinal plants and to discuss the advances in this field of research. However, investigations dealing with the effect of individual *rol* genes are comparatively less and need further attention. Therefore, in this chapter, we have discussed in detail the effects of each of the four *rol* genes individually or in combination on in vitro morphogenesis and secondary metabolite accumulation in medicinal plants.

Keywords *Agrobacterium rhizogenes* · Medicinal plants · Morphogenesis · *rol* genes · Secondary metabolites

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2.1 Introduction

Plants are known for their ability to regenerate new tissues and organs to whole plants (morphogenesis) from damaged cells for survival due to their high cellular totipotency (de Almeida et al. 2015; Ikeuchi et al. 2016). It is well known that morphogenesis in vitro is affected by a variety of endogenous and exogenous factors with cumulative effects in order to acquire organogenic competence and organ initiation followed by its development (Hicks 1994; Das et al. 1996; Kumar and Reddy 2011; Sarkar and Jha 2017). It is clear from the pioneering work of Skoog and Miller (1957) that the type and concentration of auxin and cytokinin in the culture medium have a great influence on morphogenesis as they determine the developmental fate of regenerating organs: high ratios of auxin to cytokinin generally led to root regeneration, and high ratios of cytokinin to auxin tended to promote shoot regeneration.

Agrobacterium rhizogenes, a soil-borne gram-negative bacterium, is well known to have a unique capability to induce 'hairy root' formation at the site of infection in higher plants. Different strains of *A. rhizogenes* are known to induce such roots from the host plant cells by transferring its T-DNA (transfer DNA) from root-inducing (Ri) plasmid to the host genome (Tepfer 2017). Several studies have revealed that only four open reading frames (ORFs) of the T-DNA are critical for induction, growth and morphology of hairy roots in infected plants. These loci were thus called *rol* (root-inducing locus) oncogenes and named as *rolA* (ORF 10), *rolB* (ORF 11), *rolC* (ORF 12) and *rolD* (ORF 15) (White et al. 1985, Slightom et al. 1986). Such transgenic roots can be excised from the wound site and cultured indefinitely on hormone-free medium. The hairy roots exhibit fast, plagiotropic growth characterized by profuse lateral branching and rapid root tip elongation in growth regulator-free medium in contrast to non-transformed roots (Tepfer and Tempé 1981; Chilton et al. 1982; Tepfer 1984).

While in some plant species the Ri-transformed roots exhibit this typical 'Hairy root syndrome' (Tepfer and Tempé 1981; Chilton et al. 1982; Tepfer 1984), in other plants, the Ri- roots lack the presence of extensive root hairs (Chaudhuri et al. 2005; Roychowdhury et al. 2015a; Halder and Jha 2016). In addition to the variation of the hairy roots between the species, variation in growth and morphology was also noted among the different rhizoclones of a single species (Batra et al. 2004; Chaudhuri et al. 2005; Alpizar et al. 2008; Roychowdhury et al. 2015a; Bandhyopadhyay et al. 2007; Majumdar et al. 2011; Ray et al. 2014; Basu and Jha 2014; Halder and Jha 2016; Basu et al. 2015). The variation in hairy root morphology includes variation in thickness of primary root, lateral density of the roots (i.e. number of laterals per cm), presence of rooty callus, etc. These variations among the transformed rhizoclones were attributed due to the variation in nature, site and number of T-DNA integration into the host genome (Jouanin et al. 1987; Amselem and Tepfer 1992; Batra et al. 2004; Alpizar et al. 2008). Molecular variation among the rhizoclones in terms of T-DNA insertion and effect of T_L-DNA and T_R-DNA on root morphology has been investigated by different groups (Batra et al. 2004; Bandhyopadhyay et al.

Fig. 2.1 Differences in in vitro responses of five different strains of *Agrobacterium rhizogenes* in axenic shoots of *Tylophora indica* after 6 weeks of infection. Infection was done at two different sites, i.e., nodal wound site (NWS) and internodal wound site (INWS). (**a**, **b**) Control NWS (bar = 0.2 cm) and INWS (bar = 0.13 cm) showing no response; (**c**, **d**, **e**) root induction from NWS of explants infected with *A. rhizogenes* strain LBA 9402 (bar = 0.28 cm), A4 (bar = 0.25 cm) and HRI (bar = 0.2 cm); (**f**) wound callus induction in 100% of NWS and INWS of explants infected with *A. rhizogenes* strain 15,834 (bar = 0.4 cm); (**g**) wound sites showing swelling and necrosis in explants infected with *A. rhizogenes* strain R1000 (bar = 0.4 cm)

2007; Alpizar et al. 2008; Taneja et al. 2010; Roychowdhury et al. 2015a). While the morphology of hairy roots has been well characterized in many species, their anatomy has not been fully explored. The anatomy of *A. rhizogenes*-transformed roots is more or less similar to wild-type roots with some notable exceptions (Kim and Soh 1996; Odegaard et al. 1997; Park and Facchini 2000; Peres et al. 2001; Halder and Jha 2016). In addition to the above-mentioned variations, morphogenetic ability of *A. rhizogenes* varies with different strains within a single species (Vanhala et al. 1995; Kim et al. 2008; Ionkova et al. 2009; Thwe et al. 2016) (Fig. 2.1).

The morphogenic capability of the hairy roots is widely reported in a number of plant species. Regeneration of Ri-transformed plants from the hairy root cultures have been reviewed thoroughly from time to time (Christey 1997, 2001; Roychowdhury et al. 2013b). Ri-transformed roots have been observed to show spontaneous as well as induced, direct and/ or indirect, organogenesis and/or somatic embryogenesis forming complete transgenic plants as reviewed earlier in details (Fig. 2.2) (Roychowdhury et al. 2013b). Such plants showed altered phenotypes when compared with wild-type plants, some of which are wrinkled leaf, shortened internodes, decreased apical dominance, altered flower morphology, increase in the number of branches, reduced pollen and seed production and abundant

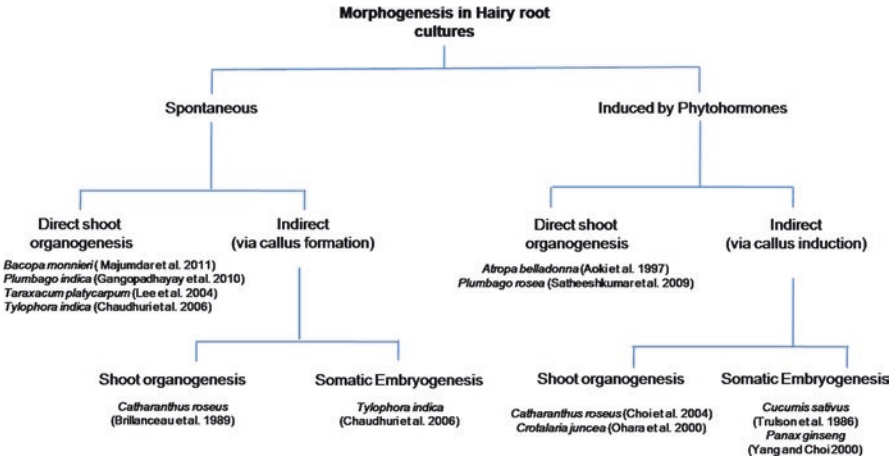

Fig. 2.2 Regeneration in hairy root cultures of different plant species

production of highly branched plagiotropic roots (Tepfer 1984). It has been shown that these abnormal morphological traits or 'hairy root syndrome' is due to the combined actions of *rolA*, *rolB*, *rolC* and *rolD* genes since each of the *rol* genes are associated with specific phenotypic alterations (Nilsson and Olsson 1997). Majority of the hairy roots and Ri-transformed plants are known to be stable in long-term culture, although some instability was also noted (Roychowdhury et al. 2013b, 2015a, b, 2017). Additionally, it has been reported that these Ri-transformed plants were able to transmit the traits to their offspring in a Mendelian manner (Tepfer 1984).

In general, it has been amply demonstrated that the morphogenic response in hairy roots transformed with wild-type *A. rhizogenes* is due to the presence of *rol* genes (Fig. 2.3). However, the role of individual *rol* genes on morphogenesis is not yet well documented. Therefore, in this chapter, we have summarized the effects of each of the four *rol* genes individually or in combination on in vitro morphogenesis of medicinal plants.

2.2 Effect of *rolA* Gene on Morphogenesis

Different explants infected with *Agrobacterium tumefaciens* strains harbouring *rolA* gene showed induction of shoot buds from the wound sites on hormone-supplemented media, either directly (Zhu et al. 2001a; Zia et al. 2010; Amanullah et al. 2016; Bettini et al. 2016b) or indirectly through callus formation (van Altvorst et al. 1992; Holefors et al. 1998). In all the reports, such hormone-supplemented media could trigger shoot bud formation in non-transformed explants as well. The developing shoots from the shoot buds were rooted in hormone-free (Bettini et al. 2016b) or

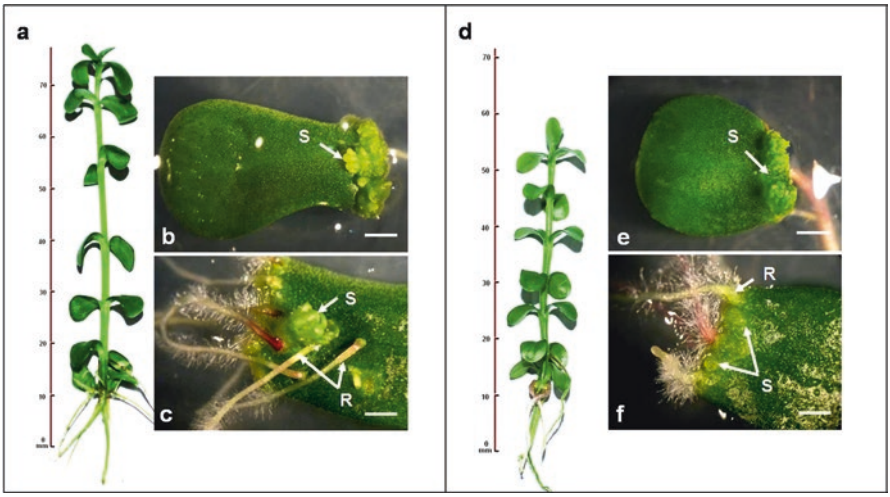

Fig. 2.3 Morphogenic potential of leaf explants excised from Ri-transformed plants of *Bacopa monnieri*. (**a**) Ri-transformed plant obtained following transformation with *A. rhizogenes* strain 9402, (**b**, **c**) morphogenesis in excised leaf explants excised from Ar 9402 Ri-transformed plants harbouring *rol* genes of TL-T-DNA on basal medium (R-root organogenesis, S-shoot organogenesis) (bar = 1.5 mm and 1.0 mm). (**d**) Ri-transformed plant obtained following transformation with *A. rhizogenes* strain A4 (**e**, **f**) morphogenesis in excised leaf explants excised from Ar A4-Ri-transformed plants harbouring *rol* genes of TL-T-DNA on basal medium (R root organogenesis, S shoot organogenesis) (bar = 1.0 mm and 0.9 mm)

hormone-supplemented media (van Altvorst et al. 1992; Holefors et al. 1998; Zhu et al. 2001a; Zia et al. 2010; Amanullah et al. 2016) to generate complete transgenic plants.

Alteration of phenotype of *rolA*-transgenic plants such as wrinkled leaves, lower percentage of rooting, early flowering, reduced flower bud length, hyperstyly, small fruits often lacking seeds and decreased pollen viability have been reported (Schmülling et al. 1988; Sinkar et al. 1988; van Altvorst et al. 1992; Carneiro and Vilaine 1993; Dehio et al. 1993; Holefors et al. 1998; Zhu et al. 2001a; Zia et al. 2010; Amanullah et al. 2016; Bettini et al. 2016b). However, these phenotypic alterations were found to be dependent on the level of transgene expression. When expressed under its own promoter, this gene caused dwarfism, severe wrinkling of leaves, shortened internodes, small leaves and condensed inflorescence (Schmülling et al. 1988; Sinkar et al. 1988). These phenotypes were even more exaggerated in 35S::*rolA*-transformed plants which showed stunted growth with small, dark-green, severely wrinkled leaves and were late-flowering, with a reduced number of flowers (Dehio et al. 1993). On the other hand, soybean *rolA* transformants showed enhanced rooting in presence of auxin and early flowering as compared to the control plants (Zia et al. 2010), while lower rooting percentage in *rolA*-transformed apple rootstock A2 in auxin-supplemented medium and delayed flowering in *rolA*-transformed *Artemisia dubia* plants are reported (Zhu et al. 2001a; Amanullah et al. 2016).

Therefore, the effect of *rolA* gene was also found to be species specific. However, phenotypic variations were also observed among different *rolA*-transformed clones regenerated from the same plant species. Several authors have suggested that this may be due to the copy number of transgene (Bettini et al. 2016b) or the position effect where transgene is integrated into the host genome (Holefors et al. 1998).

2.3 Effect of *rolB* Gene on Morphogenesis

White et al. (1985) first identified that *rolB* gene plays a critical role in the formation of adventitious roots from different explants after infection. Later on, several authors agreed and showed that plant vector constructions carrying only *rolB* gene were capable, to different extents, of triggering root differentiation on different plant tissues (Cardarelli et al. 1987; Spena et al. 1987; Altamura et al. 1994; Schmülling et al. 1988). However, the frequency of root formation differed when *rolB* gene was allowed to express under different promoters (Spena et al. 1987). More intense and earlier root formation was observed with pPCV002-B1100 (where *rolB* is under the control of its own 5′ flanking sequences) than with pPCV002-B300 (containing only 300 bp of *rolB* 5′ flanking sequences). It is interesting to note that the CaMVBT chimeric gene (where *rolB* is under the control of the cauliflower mosaic virus 35S promoter) showed a weaker response than pPCV002-B1100 probably due to overexpression of *rolB* transcript which is not conducive to root induction. On the contrary, this gene was found incapable of inducing roots on carrot discs when inoculated alone (Cardarelli et al. 1987). But hairy root symptoms almost comparable to those induced by wild-type *A. rhizogenes* were elicited when the discs were inoculated with *rolB* gene with 1200-bp-long 5′ upstream region along with the T_R-DNA (Capone et al. 1989). The roots induced by *rolB* were fast growing, highly branched and plagiotropic (Capone et al. 1989). For several years, *rolB* has then been regarded as a 'root-inducing gene' which is capable of turning on a specific morphogenetic programme in higher plants. Apart from direct root formation, indirect root formation was also reported in tobacco protoplast (Spena et al. 1987) and leaf explants of *Rubia cordifolia* (Bulgakov et al. 2002). Unlike the control calli which did not produce roots, pPCV002-B300-transformed calli showed spontaneous root formation on media containing either low or high auxin concentrations (Spena et al. 1987; Bulgakov et al. 2002).

The morphology of *rolB*-transformed roots was very similar to hairy roots transformed with wild-type *A. rhizogenes*. Compared to non-transformed roots, *rolB*-induced roots were fast growing, highly branched and plagiotropic as a result of increased sensitivity of auxin in plant cells transformed by this oncogene (Spena et al. 1987; Schmülling et al. 1988; Capone et al. 1989).

Shoot organogenesis and establishment of *rolB*-transformed plants from infected explants are reported on hormone-supplemented media, either directly (Koltunow et al. 2001; Carmi et al. 2003; Zhu et al. 2003; Zia et al. 2010; Arshad et al. 2014; Dilshad et al. 2015a; Bettini et al. 2016a, Kodahl et al. 2016) or indirectly via callus

induction (van Altvorst et al. 1992; Welander et al. 1998; Sedira et al. 2001; Zhu et al. 2001b). In all the reports, such hormone-supplemented media could induce shoot bud formation in non-transformed explants as well. However, when compared with the non-transformed plants, the *rolB*-transgenic plants showed numerous altered phenotypes such as profuse rooting with altered root morphology; reduced stem length, node number and apical dominance; shortened internodes; smaller, wider leaves with altered shape; increased trichome density; early necrosis of rosette leaves; altered floral morphology; more inflorescence; early flowering; high flower production; infertile flowers; decreased pollen viability; abnormal ovary and ovule development; early maturing of fruits; parthenocarpic fruits; and small size and less number of fruits (Schmülling et al. 1988; van Altvorst et al. 1992; Welander et al. 1998; Sedira et al. 2001; Koltunow et al. 2001; Zhu et al. 2001b; Carmi et al. 2003; Zhu et al. 2003; Zia et al. 2010; Arshad et al. 2014; Dilshad et al. 2015a; Bettini et al. 2016a; Kodahl et al. 2016).

rolB-transformed shoots showed enhanced rooting percentage and number of roots per shoot in absence of auxin suggesting that the endogenous auxin level in *rolB* transformants is sufficient for rooting (Welander et al. 1998; Sedira et al. 2001; Zhu et al. 2001a, 2003). In presence of auxin in the medium, transformed shoots produced profuse callus at the base of the stem and reduced both rooting percentage and number of roots significantly which was probably due to increased auxin sensitivity (Welander et al. 1998; Sedira et al. 2001). The morphology of the roots was even altered where the roots became shorter and thicker in auxin-supplemented medium (Sedira et al. 2001). However, multiple copies of *rolB* gene insertion into the plant genome also imposed a negative impact on rooting (Sedira et al. 2001; Zhu et al. 2003). Zhu et al. (2001b) suggested that this reduction was more associated with the position of the transgene on the plant genome.

rolB gene has been shown to significantly affect the phenotype of transformed calli as compared to non-transformed calli in different species. The non-transformed or empty vector transformed callus culture of *Maackia amurensis* was friable, aqueous and vigorously growing with light yellow or brown colour (Grishchenko et al. 2016). But the *rolB*-transformed calli displayed morphological variation and could be correlated with the level of *rolB* expression. The calli with low level of transcription were friable, globular and yellow-whitish to light-brown colour. In contrast, compact, non-watery, yellow-brown to brown callus with active growth was obtained in high level of *rolB* gene-expressing callus lines (Grishchenko et al. 2016). Similarly, inoculation of *Vitis amurensis* callus culture with *rolB* gene also produced *rolB*-transformed calli lines of friable and compact type (Kiselev et al. 2007). The colour of leaf-derived-*rolB*-transformed calli of *R. cordifolia* was reported to depend on the level of *rolB* gene expression – yellow in low-expressing callus, orange in moderately expressing callus and orange-red in highly expressing callus due to maximum accumulation of anthraquinones (Bulgakov et al. 2002; Shkryl et al. 2007). Some of the *R. cordifolia* calli transformed with *rolB* gene spontaneously formed small roots (Bulgakov et al. 2002).

The level of *rolB* gene expression was also found to affect greatly the growth of transformed callus (Kiselev et al. 2007; Shkryl et al. 2007). Compared to the

non-transformed callus culture which grew vigorously, the growth of *rolB*-transformed calli was reported to depend on the level of its expression. *rolB* gene when expressed at a low level supported the growth of callus (fast growing), but excessive expression of *rolB* gene inhibited callus growth and was associated with necrosis in callus tissues (Kiselev et al. 2007; Shkryl et al. 2007). This negative effect of *rolB* gene on the growth was found to be completely abolished when *rolB*-transformed callus was treated with a tyrosine phosphatase inhibitor (Kiselev et al. 2007; Shkryl et al. 2007). This result indicated that the growth of *rolB*-transformed cells is mediated by tyrosine dephosphorylation.

2.4 Effect of *rolC* Gene on Morphogenesis

The *rolC* gene was able to induce root formation directly from leaf explants in tobacco (Spena et al. 1987; Schmülling et al. 1988; Palazón et al. 1998) under 35S CaMV promoter and in *Atropa belladonna* under its own promoter (Bonhomme et al. 2000). However, in *Kalanchoe* leaves, this gene could not stimulate root formation when driven by its own or 35S CaMV promoter but induced roots when expressed along with *rolB* gene (Spena et al. 1987; Schmülling et al. 1988). On the other hand, hairy root induction has been reported from *rolC*-transgenic calli in absence or presence of auxin when expressed under strong CMV35S promoter in *Panax ginseng* and *R. cordifolia* (Bulgakov et al. 1998, 2002).

Apart from root induction, *rolC* gene also affected the growth and morphology of transgenic roots that were induced directly or indirectly from the explants (White et al. 1985; Schmülling et al. 1988). The morphology of *rolC*-transgenic roots also varied depending on the plant species as well as type of media used (Bulgakov et al. 1998, 2005; Palazón et al. 1998; Bonhomme et al. 2000). By analysing insertional mutants of *rolC* locus in the A4 Ri-plasmid, White et al. (1985) reported that the growth of roots induced from *Kalanchoe* leaves was attenuated. When combined with *rolB* gene, *rolBC* transgenic roots grew straight from these explants (Schmülling et al. 1988). In hormone-free medium, *rolC*-induced transgenic roots of *A. belladonna* and tobacco showed fast and plagiotropic growth and were highly branched (Schmülling et al. 1988; Palazón et al. 1998; Bonhomme et al. 2000). In contrast, Bulgakov et al. (1998) reported that *P. ginseng* transgenic roots derived from *rolC*-transformed calli were slow growing with reduced lateral branching in absence of hormone. These transgenic roots grew better in the medium supplemented with auxins (Bulgakov et al. 1998, 2005).

In *P. ginseng*, non-transformed callus obtained from the stem did not show any morphogenesis even after long-term culture in different combinations of hormone-supplemented media (Gorpenchenko et al. 2006). However, introduction of *rolC* gene into this callus resulted in morphological differentiation to form shoot buds in absence of hormones. The majority of the shoots regenerated displayed fasciated shoot apical meristems and fused leaf primordia.

Formation of *rolC*-transformed adventitious shoot buds has been reported from different explants on hormone-supplemented media either directly (Fladung 1990; Kurioka et al. 1992; Oono et al. 1993; Bell et al. 1999; Kaneyoshi and Kobayashi 1999; Zuker et al. 2001; Koshita et al. 2002; Kubo et al. 2006; Bettini et al. 2010; Zia et al. 2010; Dilshad et al. 2015a; Ismail et al. 2016) or indirectly via callus induction (Palazón et al. 1998; Zhang et al. 2006). *rolC* gene is known to cause substantial morphological and biochemical alterations in transgenic plants which were related to the degree of its expression (Schmülling et al. 1988; Kurioka et al. 1992; Kaneyoshi and Kobayashi 1999). Transgenic plants expressing *rolC* gene from its endogenous promoter had reduced apical dominance, plant height, internodal distance, node number and leaf area, enhanced branching, altered leaf morphology, small flowers, small fruits, more number of fruits and reduced seed production compared to wild-type plants (Schmülling et al. 1988; Bell et al. 1999; Kaneyoshi and Kobayashi 1999; Bettini et al. 2010; Kubo et al. 2006; Landi et al. 2009). However, *rolC*-transformed *A. belladonna* plants when expressed under native promoter did not exhibit any morphological alteration and resembled with wild-type plants (Kurioka et al. 1992).

When *rolC* is expressed under strong 35S CaMV promoter, these characteristics were exaggerated, with drastically reduced apical dominance and internodal length; highly dwarf, very small leaves with altered shape; more lateral branching; higher rooting capacity; increased axillary budbreak; dramatic promotion of flowering; reduced inflorescence; smaller flowers; and male sterile flowers (Schmülling et al. 1988; Fladung 1990; Kurioka et al. 1992; Oono et al. 1993; Palazón et al. 1998; Kaneyoshi and Kobayashi 1999; Zuker et al. 2001; Koshita et al. 2002; Dilshad et al. 2015a; Ismail et al. 2016). In *A. belladonna*, although majority of *rolC*-transformed plants showed typical altered phenotypes, only two unusual regenerants showed unexpected morphology of leaves where leaf periphery was severely wrinkled and darker than central region (Oono et al. 1993). Under the control of 70S CaMV promoter, *rolC*-transformed soybean plants were dwarf and altered leaf morphology, early flowering and lower number of flowers (Zia et al. 2010). Phenotypic alteration such as shortened internodes and increased branching suggested that the expression of *rolC* gene might be linked to an increase in cytokinin activity (Schmülling et al. 1988).

It has been hypothesized that the extreme dwarf phenotype and early flowering in *rolC*-transformed plants were due to the reduction in gibberellic acid. Bettini et al. (2010) reported that higher ratio of abscisic acid to indole-3-acetic acid (ABA/IAA) may be responsible for the stunted aspect of these plants. Furthermore, *rolC* leads to better rooting ability in transformed fruit trees (Kaneyoshi and Kobayashi 1999; Koshita et al. 2002), soybean (Zia et al. 2010) and carnation plants (Zuker et al. 2001) which indicates that the expression of this gene could exert auxin-like activity.

When callus culture of *P. ginseng* was transformed with *rolC* gene under strong promoter (35S CaMV), induction of somatic embryogenesis was observed (Gorpenchenko et al. 2006). Non-transformed calli of *P. ginseng* did not show any morphogenesis; however, introduction of *rolC* gene into the non-transformed calli

resulted in morphological differentiation to form proembryos and somatic embryos even in absence of hormone which indicated that *rolC* gene is also able to induce somatic embryogenesis (Gorpenchenko et al. 2006). But the proembryos and somatic embryos that were formed had enlarged and fasciated meristems and terminated at different stages of their development to form secondary adventitious meristems. According to them, overexpression or ectopic expression of WUSCHEL (WUS) gene and reduced CLAVATA (CLV) activities caused similar such developmental abnormalities. Whether *rolC* gene affects embryogenesis in *P. ginseng* callus through WUS/CLV signalling pathway is not clearly understood.

Establishment of *rolC*-transformed callus cultures has been reported in some species using *A. tumefaciens* harbouring 35S *rolC* gene in hormone-supplemented media (Bulgakov et al. 1998, 2002; Grishchenko et al. 2013). It was found that integration of *rolC* gene resulted in significant alteration of phenotype, growth and biomass accumulation when compared with non-transformed callus culture. The empty vector transformed callus of *M. amurensis* and *R. cordifolia* was friable and watery, while *rolC*-transformed callus was compact and non-watery (Bulgakov et al. 2002; Grishchenko et al. 2013). But in *P. ginseng*, the same *rolC* construct produced friable and almost watery-type callus in auxin-containing medium (Bulgakov et al. 1998). *rolC* gene also affects the growth and biomass accumulation of transformed callus cultures depending upon the plant species used. Both empty vector and *rolC*-transformed callus cultures of *M. amurensis* demonstrated active growth, but the latter accumulated twice the amount of dry biomass compared to the former (Grishchenko et al. 2013). The growth of *rolC*-transformed *P. ginseng* callus was also rapid in auxin-containing medium (Bulgakov et al. 1998). However, Bulgakov et al. (2002) reported that the growth of one of the *rolC*-transformed *R. cordifolia* callus lines was reduced (almost twofold) compared to the control culture.

2.5 Effect of *rolD* Gene on Morphogenesis

To date, unlike other *rol* genes, *rolD* gene has not been thoroughly investigated although it has been identified as a root locus (White et al. 1985). The mutants of *rolD* gene in *Kalanchoe* leaves produced roots of attenuated growth along with increased amount of callus (White et al. 1985). However, when *rolD* gene was introduced in tobacco stems under the control of long version of promoter, root formation was achieved on hormone-free medium (Mauro et al. 1996). But no difference was observed in adventitious root production, root morphology and its growth pattern between *rolD* and mock-infected plants.

rol-transformed plants (*rolD* gene under the control of 578 bp of its 5′ upstream non-coding region) have been established in tobacco (Mauro et al. 1996), tomato (Bettini et al. 2003) and *Arabidopsis* (Falasca et al. 2010). In tobacco, control as well as *rolD*-transformed leaf explants was able to show shoot induction in hormone-free MS medium (Mauro et al. 1996). In tomato, *rolD*-transformed plants were established in hormone-supplemented medium from cotyledons after infection;

however, non-transformed plants were also obtained when explants were cultured on non-selective medium (Bettini et al. 2003). Falasca et al. (2010) established seed derived non-transformed and *rolD*-transformed *Arabidopsis* plants in hormone-free medium.

In transgenic plants, the *rolD* gene does not seem to induce significant morphological modifications during vegetative growth except early bolting of the stem, smaller leaves with characteristically curved pointed tips in tobacco (Mauro et al. 1996), increased branching in tomato (Bettini et al. 2003) and an increased production of axillary buds and adventitious root meristems along with frequent occurrence of wrinkled leaves in *Arabidopsis* (Falasca et al. 2010). The most conspicuous alteration of *rolD* transgenic plants was precocity in floral transition leading to early flowering and increased number of inflorescences (Mauro et al. 1996; Bettini et al. 2003; Falasca et al. 2010). Since *rolD* gene product is assumed to catalyse the conversion of ornithine to proline, effect of *rolD* on flowering may be therefore due to the accumulation of proline or depletion of ornithine (Trovato et al. 2001). However, Falasca et al. (2010) suggested that proliferation of axillary meristems in *rolD* plants could be due to modification in cytokinin/auxin ratio in *rolD*-transformed plants.

2.6 Effect of *rolABC* Gene on Morphogenesis

When explants were transformed with *A. tumefaciens* harbouring *rolABC* together (expressed under its own promoter), root induction occurred in absence of exogenous hormone directly (Spena et al. 1987; Palazón et al. 1998; Bonhomme et al. 2000) or indirectly through callus induction (Spena et al. 1987; Rugini et al. 1991). Nearly half of *rolABC*-transformed calli obtained from tobacco leaf protoplasts were found to develop roots in the absence of exogenous auxin, whereas no root induction occurred in calli transformed with the binary vector (pPCV002) (Spena et al. 1987). The same construct when used to transform leaf explants of tobacco and *Kalanchoe* was also able to induce roots in hormone-free medium (Spena et al. 1987; Palazón et al. 1998; Bonhomme et al. 2000). However, in *Kalanchoe*, co-inoculation with *A. tumefaciens* strain pGV3297 harbouring a Ti-plasmid with auxin-producing genes was needed for root formation although *A. tumefaciens* strain pGV3297 itself did not form roots (Spena et al. 1987). In kiwi, the emergence of some roots from leaf-derived *rolABC*-transformed callus was noticed when cultured in callus induction medium containing auxin (Rugini et al. 1991). On the other hand, high concentration of auxin in this medium prohibited root formation from control calli which indicates that root morphogenesis was directed by *rol* genes in transformed cells.

Compared to non-transformed roots, *rolABC*-transformed roots could grow well in hormone-unsupplemented medium and showed the typical hairy root phenotype as observed in roots transformed with wild-type *A. rhizogenes* (Palazón et al. 1998; Bonhomme et al. 2000). Non-transformed roots obtained from in vitro grown plants grew slowly with no lateral branching when cultured on MS basal medium (Palazón

et al. 1998). In contrast, transformed roots expressing *rolABC* together grew more vigorously, were highly branched with a plagiotropic growth and were thick (more than 3 mm diameter) in the medium without phytohormone (Palazón et al. 1998; Bonhomme et al. 2000). Schmülling et al. (1988) reported that *rol ABC*-transformed tobacco roots grew better in hormone-free medium than non-transformed roots. The growth rate of transformed root lines was also significantly higher than non-transformed roots (Palazón et al. 1998; Bonhomme et al. 2000).

The morphology of *rolABC*-transformed plants was more or less similar to that of Ri-transformed plants and showed typical hairy root syndrome. Tobacco plants transgenic for *rolABC* exhibited high growth rate of plagiotropic roots, reduced apical dominance in roots and stems, wrinkled and epinastic leaves with altered morphology, shorter internodal length, small flowers and reduced seed production (Schmülling et al. 1988; Palazón et al. 1998). van Altvorst et al. (1992) reported that *rolABC*-transformed tomato plants showed similar morphology with control plants with respect to leaf shape, leaf wrinkling, apical dominance and pollen production. However, these transformed plants produced small, thin roots, low pollen viability and reduced flower bud length compared to control plants (van Altvorst et al. 1992). Rugini et al. (1991) reported that in vitro grown *rolABC*-transformed kiwi plants had shorter internodes, dark-green wrinkled leaves and high rooting ability. In general, three T-DNA genetic loci indicated as *rolA*, *rolB and rolC* act synergistically in the induction and morphology of hairy roots as well as hairy root phenotype of regenerated plants.

2.7 The '*rol* Effect' on Secondary Metabolites in Plants

The application of plant tissue culture and plant genetic transformation for successful production of highly valuable secondary metabolites is not a new trend and can be traced back to the early works of Flores and Filner (1985), Kamada et al. (1986), Mano et al. (1989) and Robins et al. (1991). It has been found that hairy roots, Ri-transformed plants and Ri-transformed callus cultures showed activation of secondary metabolites in more than a hundred, taxonomically diverse medicinal plant species (Ray et al. 1996, 2014, Ray and Jha 1999; Christey 1997, 2001; Chaudhuri et al. 2005, 2006; Bulgakov 2008; Bulgakov et al. 2005, 2011; Majumdar et al. 2011; Roychowdhury et al. 2013a, b, 2015a, b; Basu and Jha 2014; Basu et al. 2015; Paul et al. 2015; Halder and Jha 2016). In some cases decreased content of some of the target metabolites has also been observed (Bulgakov et al. 2005). These changes could be attributed to the variation in the pattern of T-DNA integration within the genome of the host plant which caused the differential expression of key regulators of biosynthetic pathways (Jouanin et al. 1987; Amselem and Tepfer 1992; Bulgakov 2008). The preference for 'Ri-transformed plant or hairy roots or calli' system for production of secondary metabolites was due to their high growth rates acting as a factory for continued production of high amounts of important compounds and the

stability in metabolite accumulation in long-term cultures (Häkkinen et al. 2016, Roychowdhury et al. 2017). Though there are various other factors affecting the accumulation of secondary metabolites, which include media composition and pH, effect of hormones, bacterial strain used for inoculation, temperature, light and effect of elicitors (Chaudhuri et al. 2009; Majumdar et al. 2011; Simic et al. 2014; Khalili et al. 2015; Paul et al. 2015; Sivanandhan et al. 2016; Basu et al. 2017); in this review, we intended to confine our attention to the influence of *rol* genes on the increase or decrease of secondary metabolites produced in medicinal plants. The effect of wild-type *A. rhizogenes* on the production of secondary metabolites is well studied in plants. Based on such studies, several reviews have been written from time to time, explaining the '*rol* effect' on secondary metabolite accumulation in medicinal plants and to discuss the advances in this field of research (Hamill et al. 1987; Tepfer 1990; Toivonen 1993; Constantino et al. 1994; Bourgaud et al. 2001; Rao and Ravishankar 2002; Verpoorte et al. 2002; Guillon et al. 2006; Srivastava and Srivastava 2007; Bulgakov 2008; Bulgakov et al. 2013; Karuppusamy 2009; Pistelli et al. 2010; Chandra 2012; Sharma et al. 2013; Roychowdhury et al. 2013a, 2017; Matveeva et al. 2015; Parr 2017; Mitra et al. 2017). The popularity of this research has never declined through time which explains its immense value and provides a hope for a promising future.

However, the knowledge regarding the respective roles of *rolA*, *rolB*, *rolC* and *rolD* genes or when integrated in combinations is still insufficient. Very few attempts have been reported, some of the pioneers being Palazón et al. (1997) and Bulgakov et al. (1998). Years later, for the first time, Bulgakov (2008) took an initiative to review how the individual *rol* genes impacted secondary metabolism. In the following section of this chapter, the effects of individual and combined *rol* genes on secondary metabolites of medicinal plants have been discussed in details based on updated list of reports. During the compilation it was observed that *rolC* gene has been the most popular choice for this study followed by *rolB* and then *rolA* (Fig. 2.4). Surprisingly, the role of *rolD* in the accumulation of secondary metabolites has remained unexplored.

2.8 Effect of *rolA* Gene on Plant Secondary Metabolite Accumulation

There are very few reports on the effect of *rolA* gene on secondary metabolite production (Palazón et al. 1997; Shkryl et al. 2007; Amanullah et al. 2016). The earliest report where *rolA* was seen to stimulate nicotine production in transformed root lines of *Nicotiana tabacum* was that of Palazón et al. (1997). Later, Shkryl et al. (2007) transformed *R. cordifolia* plant with *A. tumefaciens* (strain GV3101) harbouring *rolA* construct pPCV002-A controlled by its own native promoter. In this study, the effect of *rolA* gene on anthraquinone accumulation was investigated. There was a 2.8-fold increase in the content of anthraquinones in the

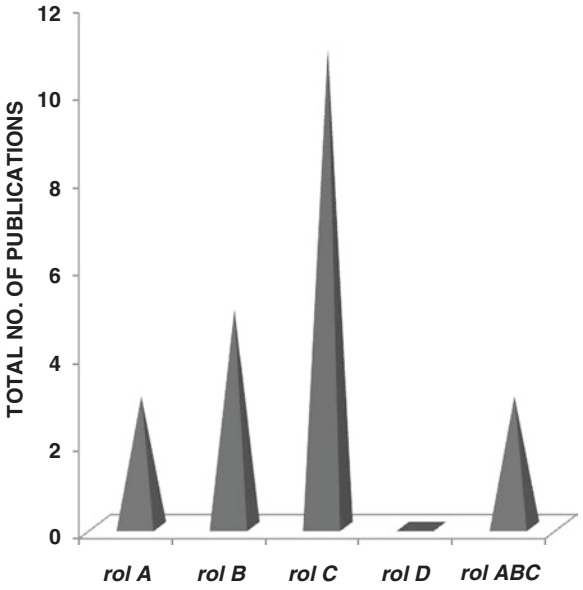

Fig. 2.4 Graphical representation of relative number of publications available on the effect of individual *rol* genes on secondary metabolism in medicinal plants

rolA-transformed calli compared to wild-type non-transformed ones. In *rolA*-transformed cultures of *A. dubia* plants, artemisinin and its derived compounds were found to be comparable to that of the non-transformed plant (Amanullah et al. 2016). Therefore, on one hand, *rolA* showed enhancement of secondary metabolites in transformed *N. tabacum* plants and *R. cordifolia* calli (Palazón et al. 1997; Shkryl et al. 2007) and, on the other hand, maintained the levels of secondary metabolite production comparable to non-transformed *A. dubia* plants.

2.9 Effect of *rolB* Gene on Plant Secondary Metabolite Accumulation

rolB gene has been a master regulator in secondary metabolite accumulation in majority of the studies (Shkryl et al. 2007; Kiselev et al. 2007; Arshad et al. 2014; Dilshad et al. 2016; Grishchenko et al. 2016). Presence of *rolB* gene has been co-related to the enhanced secondary metabolite content in transformed plants with respect to the non-transformed plants and plants transformed with *rolA*, *rolC* and *rolABC* genes (Shkryl et al. 2007).

Transformed callus culture of *R. cordifolia* showed a 15-fold increase in anthraquinone levels when compared to the non-transformed callus culture (Shkryl et al. 2007). In this study, stimulation of isochorismate synthase gene (ICS) was positively correlated with the enhancement in anthraquinone content since ICS is a key gene involved in biosynthesis of anthraquinones. Transformed callus lines express-

ing low levels of *rolB* produced twofold higher anthraquinones, whereas transformants expressing medium and higher levels of *rolB* gene produced 2.8-fold and 4.3-fold anthraquinones, respectively. The effect of *rolB* on production of another target metabolite, resveratrol, was studied by Kiselev et al. (2007) in transformed calli lines of *V. amurensis* Rupr. Compared to non-transformed callus, a striking 100-fold increase was seen in the *rolB*-transformed calli. It was further shown that tyrosine phosphatase inhibitors played antagonistic role with the stimulatory effects of *rolB* gene, suggesting the involvement of tyrosine phosphorylation in plant secondary metabolism. Grishchenko et al. (2016) established *rolB*-transformed callus cultures of *M. amurensis* Rupr., and the yield of isoflavonoids was studied in the transformed calli. Isoflavonoid accumulation in *rolB*-transformed calli ranged from 1.4 to 2.1% DW (dry weight) compared to 1.22% DW in empty vector control.

A. carvifolia Buch. plants transformed by *rolB* gene showed an increase in flavonoid levels (Dilshad et al. 2016). Caffeic acid, quercetin, isoquercetin, rutin, catechin, apigenin, gallic acid and kaempferol were some of the flavonoids which were compared among the transformed and non-transformed plants. Of these, apigenin and catechin were absent in wild-type plants but present (75 mg/g DW) in the transformed shoots. The transgenics showed an increased content of quercetin (sixfold), rutin (2.4-fold) and isoquercetin (1.9-fold) in the transformed plants. *Solanum lycopersicum* L. transformed with *A. tumefaciens* harbouring *rolB* gene of *A. rhizogenes* (Arshad et al. 2014) showed up to 62% increase in the lycopene content in *rolB*-expressing tomato fruit lines compared to the control non-transformed fruits.

Hence, *rolB* gene can be considered having a positive effect on enhanced secondary metabolite production in medicinal plants because transformation with *rolB* gene has resulted into higher metabolite content in most cases (Kiselev et al. 2007; Shkryl et al. 2007; Dilshad et al. 2016).

2.10 Effect of *rolC* Gene on Plant Secondary Metabolite Accumulation

Several reports are available which explained the role of *rolC* as a modulator of secondary metabolite production among diverse group of medicinal plants (Bulgakov et al. 1998, 2005; Palazón et al. 1998; Shkryl et al. 2007; Dubrovina et al. 2010; Grishchenko et al. 2013; Vereschagina et al. 2014; Dilshad et al. 2015a, b, 2016; Ismail et al. 2016).

Palazón et al. (1998) examined levels of nicotine production in *rolC*-transformed plants of *N. tabacum*. In comparison with the non-transformed control, the roots of *rolC*-transformed plants accumulated twice the amount of nicotine, and the transformed leaves showed a threefold increase. Similarly, transformation of *P. ginseng* with *rolC* oncogene resulted into production of threefold higher levels of ginsenoside (Bulgakov et al. 1998). *rolC*-transformed callus cultures of

R. cordifolia showed a 4.3-fold higher levels of anthraquinone compared to the control calli (Shkryl et al. 2007). Dubrovina et al. (2010) in their study with *V. amurensis* showed that *rolC*-transformed callus lines produced 3.7- to 11.9-fold increase in resveratrol content compared to non-transformed calli. A stable two- to fourfold increase (stable over a period of 2 years) in the polyphenol levels was recorded in *rolC*-transformed calli of *Cynara cardunculus* var. *altilis* (Vereshchagina et al. 2014).

Artemisinin content in *rolC*-transgenic plant of *A. annua* showed a 4- to 4.6-fold increment (Dilshad et al. 2015b). In the same study, artesunate and dihydroartemisin also increased up to 9.1-fold and 2-fold, respectively. A similar investigation was conducted with another species of *Artemisia*, *A. carvifolia* (Dilshad et al. 2015a), where the artemisinin content recorded in transgenic plants was up to six-fold higher than determined in non-transformed plants. The increase in contents of artesunate, dihydroartemisinin and artemether was measured to be up to 8.9-, 3.2- and 5-fold, respectively. Dilshad et al. (2016) reported twofold increase of caffeic acid in *rolC*-transformed plants of *A. carvifolia* compared to non-transformed controls. In addition, such *rolC*-transformed plants showed increased levels of quercetin (fourfold), isoquercetin (1.6-fold) and rutin (1.6-fold) compared to control. *rolC*-transformed plants of *Lactuca sativa* showed enhancement of flavonoid content in the range of 7.5–8.2 μg/ml in contrast to 5.1 μg/ml in control (Ismail et al. 2016).

The stimulatory effect of *rolC* on secondary metabolite accumulation was quite evident in all of the above examples; however, it was interesting to note that *rolC* gene has shown a reverse effect on production of certain metabolites in transformed cultures of *Eritrichium sericeum* and *Lithospermum erythrorhizon* (Bulgakov et al. 2005). *rolC*-transformed cultures of *E. sericeum* (root and calli) and *L. erythrorhizon* (calli) showed reduced rhabdosin and rosmarinic acid content than the respective controls. Grischenko et al. (2013) reported *rolC*-transformed callus cultures of *M. amurensis* with slightly higher isoflavonoid productivity compared to control. Interestingly, on one hand, in the *rolC* callus cultures, increased contents of six isoflavonoids were obtained; on the other hand, genistin production decreased compared to control. This effect of *rolC* on isoflavonoid production was stable for 4 years.

Therefore, *rolC* gene showed both stimulatory effect (Bulgakov et al. 1998; Palazón et al. 1998; Shkryl et al. 2007; Dubrovina et al. 2010; Vereshchagina et al. 2014; Dilshad et al. 2015a, b, 2016; Ismail et al. 2016) and inhibitory effect (Bulgakov et al. 2005) on the accumulated levels of target secondary metabolites. *rolC* gene might be considered responsible for differential regulation of different secondary metabolites within the same transformed plant causing increase in level of one compound and reduction in level of others simultaneously (Grishchenko et al. 2013).

2.11 Synergistic Effect of *rol ABC* Gene on Plant Secondary Metabolite Accumulation

The *rol* genes have individually shown to exert a neutral or a stimulatory as well as negative effect on secondary metabolites in various medicinal plants (Bulgakov et al. 2005; Kiselev et al. 2007; Shkryl et al. 2007; Dilshad et al. 2015a,b; Amanullah et al. 2016; Grishchenko et al. 2016). To study the combinatorial effect of *rol* genes (*rolA*, *rolB*, *rolC*), transformed cultures have been reported to be established with *A. tumefaciens* harbouring *rolABC* genes (Palazón et al. 1998; Bonhomme et al. 2000; Shkryl et al. 2007).

Palazón et al. (1998) established *rolABC* transgenic root lines of *N. tabacum* $_{CV}$. Xanthi, where the mean nicotine level showed a drastic enhancement (86 mg) in comparison to that of the non-transformed root lines (0.8 mg). Shkryl et al. (2007) performed an experiment with *rolABC* transformed callus cultures of *R. cordifolia* to monitor the effect on anthraquinone accumulation. Total anthraquinone content in *rolABC* transformed calli was measured to be almost 2 times the anthraquinone levels recorded in non-transformed callus line and 1.4 times the anthraquinone levels in wild-type (A4) transformed calli. Bonhomme et al. (2000) reported similar increase in accumulation of total alkaloid contents in Ri-transformed and *rolABC* transformed root lines of *A. belladonna* compared to non-transformed roots, suggesting *rolABC* genes to be enough for increasing the tropane alkaloid content in this plant.

Hence, the *rolABC* showed a considerably stronger effect on the enhancement of secondary metabolite productivity of transformed plants of *Nicotiana* (Palazón et al. 1998). However, the stimulatory effect of *rolABC* gene on transformed *R. cordifolia* calli (Shkryl et al. 2007) was relatively weaker than on *Nicotiana* as measured in terms of fold increment of their respective target metabolites. Differential regulation of two different compounds within the transformed hairy root lines of the same plant is yet another remarkable aspect of *rolABC* gene effect (Bonhomme et al. 2000).

2.12 Conclusion

Transformation of plants with wild-type *Agrobacterium rhizogenes* has been subject of many studies. However the functions of individual oncogenes of the Ri-plasmid are not well known. Rhizogenic property of *A. rhizogenes* is a well-known phenomenon in higher plants, as well as morphogenesis from such hairy roots. Four *rol* genes (*rolA*, *rolB*, *rolC*, *rolD*) are known to be responsible for such ability for a long time. However, few attempts have been made to elucidate the morphogenic ability of individual *rol* genes. Transformation with individual *rol* genes results in transformed cultures (roots/calli/plants) having altered characteristics, extent of which varies with plant species, choice of promoter and number of

transcripts of the respective *rol* gene. In contrast, direct evidence of rhizogenic ability (direct and indirect) has been found in case of *rolABC* genes together and the morphology of such transformed cultures is comparable to typical Ri-transformed ones.

While numerous reports are available on production of secondary metabolites from Ri-transformed plants or callus or hairy root cultures, investigations dealing with the effect of individual *rol* genes on secondary metabolite accumulation are comparatively less and need further attention. Majority of the reports have highlighted the role of *rolC* on secondary metabolite accumulation in medicinal plants. We observed that phenylpropanoids were the most common group of target secondary metabolites studied for the effects of individual *rol* genes followed by terpenoids, alkaloids, quinones and steroids, respectively. While *rolA* caused a stimulatory effect in the accumulation of secondary metabolites, the *rolB* and *rolC* genes were found to play dynamic roles leading to a differential regulation of the target metabolites even in the same species. It was interesting to note that the *rolABC* genes exerted a greater effect on secondary metabolite synthesis than individual *rol* genes. From the overall study, the lack of reports for effect of individual *rol* genes suggests that there is ample scope of research in this field in spite of being in the business for more than three decades.

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Chapter 3
Conventional and Biotechnological Approaches to Enhance Steviol Glycosides (SGs) in *Stevia rebaudiana* Bertoni

Arpan Modi and Nitish Kumar

Abstract *Stevia rebaudiana* Bertoni (Asteraceae) is a perennial herb with many secondary metabolites present mainly in the leaf and other plant parts. Major secondary metabolites, for which the plant is consumed, are steviol glycosides (SGs) containing diterpene steviol, attached to which are one to four molecules of glucose by glycosidic bond(s). They impart very less calorie in consumer's diet, thus widely used as a sweetener in food and beverage industries. The amount of SGs in the plant varies from 8 to 10%, enhancement of which is always in demand. Both conventional and biotechnological approaches are being made till date to increase the level of SGs in the plant. In the present chapter, we discussed various ways to enhance the level of these sweeteners with the prime focus on conventional and biotechnological approaches.

Keywords Micropropagation · Physical factors · *Stevia rebaudiana* · Steviol glycosides

3.1 Introduction

Steviol glycosides (SGs) are present mainly in the leaf of Bertoni (2n = 22), a medicinal herb, native to Paraguay (Megeji et al. 2005). Steviol glycosides are found predominantly from *Stevia rebaudiana*, *Stevia phlebophylla*, and *Rubus sauvissimus* (a Chinese sweet tea) (Richman et al. 1999). They are low caloric sweeteners. Major steviol glycosides are stevioside, rebaudioside, steviolmonoside, steviolbioside, and dulcosides. These steviol glycosides, along with other secondary metabolites like alkaloids, phenols, and flavonoids in *Stevia rebaudiana*, make the plant suitable for medicinal use and food additives (Tadhani et al. 2007). Steviol

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glycosides share the common pathway with gibberellic acid (GA_3) as they are terpenoids, and both are derived from 2-C-methyl-d-erythritol 4-phosphate (MEP) pathway operated within plastids, and due to that, these glycosides are synthesized exclusively in the leaf and not in any other organ of the plant. All terpenoids are derived from five-carbon precursor iso-pentenyl diphosphate (IPP) and its isomer dimethyl allyl diphosphate (DMAPP). The most abundant plant pigment after chlorophyll is carotenoid which is a tetraterpene. In the 1950s, mevalonic acid pathway for the production of isoprenoids in yeast and animals was reported, but few years later, MVA-independent pathway, called MEP pathway, was detected in bacteria and plants. Efforts were made to upregulate the genes of MEP pathway in plants so that respective metabolites can be enhanced (Walter et al. 2000 and Veau et al. 2000). Methylerythritol phosphate pathway (MEP pathway) was proven to produce the wide variety of terpenoids in plants. From the precursor to end product, the pathway involves plastid, cytosol, endoplasmic reticulum, and vacuole in *Stevia rebaudiana* B. Apart from biochemical characterization of enzymes of this pathway, high-throughput expressed sequence tags have provided very useful database from which isolation and characterization of genes of the pathway were made possible (Brandle and Telmer 2007). The same group of researchers have also reported that in *Stevia rebaudiana* only six genes were found putative and others were characterized by several researchers. The concentrations of steviol glycosides are 10,000 times higher than gibberellic acid which shows the dedication of this plant toward SGs in view of MEP pathway. Biosynthesis of steviol glycoside pathway (MEP pathway) highlights many secondary metabolites, some of which are commercially and biologically important to the plants. Pickens et al. (2011) explained how microbes can be utilized in the enhancement and production of natural compounds. Strategies to achieve this goal include increasing the precursor supply, overexpressing or increasing the efficiency of respective enzymes, altering the regulation of gene expression, reducing flux toward unwanted by-products or competing pathways, and reconstituting the entire pathway in a heterologous host.

Due to their extensive use as a sweetener, they are paid more and more attention by pharmaceutical industries since the last two decades. On the other hand, various studies have been conducted to increase the level of SGs inside the leaf of the plant. For this purpose, two prerequisite studies were conducted, viz., transcriptome sequencing of stevia leaves (Brandle et al. 2002; Mandhan et al. 2012; Chen et al. 2014; Kaur et al. 2015) and monitoring the regulation of genes involved in steviol glycoside biosynthesis under various experimental conditions (Modi et al. 2014a, b; Hajihashemi and Geuns 2016). The former studies elucidate the information of all the co-expressed genes, EST-SSR, transcriptional factors, and micro-RNA (as regulatory factors), and later studies highlight the performance of key regulatory genes involved in biosynthetic pathway again under various experimental conditions. SGs' enhancing experiments involve both conventional and biotechnological ways. The present chapter describes how both the ways were utilized to accumulate these important compounds of *Stevia rebaudiana* Bertoni. In the present chapter, micropropagation techniques are considered under biotechnological approaches.

3.2 Conventional Approaches

The most reliable way, to enhance any desired character in plant, is a conventional breeding strategy. Through selection and intercrossing, several plant types have been patented. Several lines were also developed in which important characters like total glycosides, rebaudioside A, and rebaudioside A/stevioside ratios were enhanced. Other yield contributing characters were also targeted for the improvement. Fixing these characters with improvisation through breeding methods may give permanent solution toward enhanced SG content (Yadav et al. 2010).

Ever since 1970s various attempts have been made to enhance the production of steviol glycosides through tissue culture techniques (Yamazaki and Flores 1991). Several synthetic plant hormones have shown positive effect to enhance the stevioside production (Striedner et al. 2004). The concentration of the SGs inside the leaf is highly controlled by environmental conditions (Evans 2014; Pal et al. 2015), leaf position on the plant (Kumar et al. 2012), various physical factors (Ceunen et al. 2012; Pandey and Chikara 2015; Khalil et al. 2014), and plant growth regulators (Modi et al. 2011). Traditional ways were employed by several researchers by giving light treatment, stress conditions, and even gamma rays as mutagenic agents. As we are discussing about the enhancement of secondary metabolites, their concentration tends to increase when plant faces these kinds of stressful conditions. Mostly, these approaches are carried out on field or greenhouse conditions, and thus, chances of a better yield of compounds are more than in vitro experiments, but at the same time, the risk of less uniformity is also associated with it. Here, mainly physical factors are discussed.

3.2.1 Enhancement by Physical Factors

The concentration of these glycosides is modulated by the physical treatment given to the plant. These include daylight condition, spectra of light (Ceunen et al. 2012), abiotic stresses (Pandey and Chikara 2015), and mutation created by gamma rays (Khalil et al. 2014). The quantity and quality of light have a significant role in the accumulation of steviol glycoside content as determined by Ceunen et al. (2012). In order to enhance steviol glycoside levels, which were believed to present in higher amount during short-day conditions, an interruptive treatment of far-red LED light in long night condition grown plants was given both in field and phytotron. They observed almost twofold higher steviol accumulation in young stevia seedling (under short-day condition) treated with red LED light. They also found that the enhanced levels of steviol glycosides were independent of cultivar. Growth stages of stevia seedlings were found to be phytochrome mediated and arrested for some time in vegetative period in which steviol glycosides got accumulated. Moreover, general phenomenon of decreased steviol glycoside content was also not seen in the plants treated with far-red LED light as observed in control plants. Interruption of plants'

light/dark cycle by red LED light caused delay in flowering and thus, SGs were accumulated. In other study conducted by Pandey and Chikara (2015), two abiotic stresses, salinity and drought, as induced by 0 (control), 25, 50, 75, and 100 mM treatment of NaCl and mannitol, respectively, were employed to in vitro-raised plantlets. Under salinity stress, there were accumulation of stevioside content as well as upregulation of genes encoding stevioside biosynthesis (UGT74G1); however, in drought stress only the upregulation of the respective gene was observed, but stevioside content was not increased significantly. Gamma rays are potent physical factor which induce mutation in an organism. Stevia seedlings and callus mutated with gamma rays exhibited at par and significantly higher content of stevioside, respectively. On the other hand, contents of rebaudioside A were found at par in both callus and seedling with control. However, there was a significant reduction in dry biomass and germination percentage in calli and seeds, respectively, treated with gamma rays. Even 2.5 Gy gamma-irradiated seeds of stevia showed more than one-third decreased germination percentage (from 42% to less than 14%) when they were sown in soil; thus, this technique may not be suitable for the enhancement of SGs under in vivo as well as in vitro conditions (Khalil et al. 2014).

3.2.2 Enhancement by Chemical Factors

Various plant growth regulators can be applied in vivo to enhance the steviol glycoside levels. In an experiment conducted by Modi et al. (2011), in vivo-grown plants of stevia were treated with different levels of gibberellic acid (15, 30, and 60 μM) which enhanced the level of stevioside up to more than twofold. In another study, where plants were treated with elicitor, methyl jasmonate, with four different concentrations, viz., 50, 100, 150, and 200 μM, it was observed that the levels of stevioside were increased from 8.14% (control) to 10.33% (200 μM). However, the gene responsible for the stevioside biosynthesis behaved differentially during these treatments (Modi 2013).

3.3 Biotechnological Interventions

The abovementioned examples are approaches to enhance SGs with simple methods. These methods involved mostly in vivo applications of physical or chemical agent for the enhanced production of steviol glycosides. As a broad subject, biotechnology includes cell, tissue, and organ culture-based technology along with metabolic engineering to enhance secondary metabolites in plants. Tissue culture methods produce uniform plantlets with less variation in commercially important traits like concentrations of secondary metabolite. Here, micropropagation methods are not just limited to plant production, but they are also utilized to enhance the biomass and secondary chemicals (Karuppusamy 2009).

3.3.1 Micropropagation

Although in vitro techniques result in lesser content of secondary metabolites than the plants' respective in vivo counterparts, they are still feasible because of utilization of less space and less variations in the final product as compared to in vivo plants. Tissue culture techniques give an advantage to experimenter to try with many different treatments starting from media composition, plant growth regulators, and physical conditions. Numerous experiments were conducted till date to enhance SGs at in vitro level. Table 3.1 describes various experiments conducted with micropropagation techniques to enhance these glycosides by several researchers.

Results of various experiments conducted by Bayraktar et al. (2016) could be used as a platform for the production of steviol glycosides at large scale through bioreactor systems where physical and chemical conditions for the growth of the plant can be precisely controlled and that will contribute toward the accumulation of SGs. Considering the time, as an important factor for the large-scale production of plantlets, Yucesan et al. (2016) optimized an efficient micropropagation protocol for the rapid multiplication of *S. rebaudiana* plants through which more than half million of plants can be produced from the single node within 6 months. This study could be employed to enhance the production of steviol glycosides per year.

Micropropagation techniques surely contribute to increase the secondary metabolites like steviol glycosides with the limitations of 2–3% of steviol glycoside production in dry plant material as compared to field-grown material with 8–10%. Apart from the micropropagation techniques mentioned in Table 3.1 to enhance SGs in the plant, several researchers also tried to enhance the content with callus culture (Gupta et al. 2010, 2016).

3.3.2 Enhancement by Biological Factors

Although *Agrobacterium*-mediated transformation governed engineering of plant secondary metabolites is a common way to enhance secondary chemicals in plant cells, some of the biological agents help with the effect of symbiosis (non-transformation based) and can perform the same role. Metagenome of stevia was found to be vital for the accumulation of rebaudioside in the leaf. Four "housekeeping" endophytes, viz., *Proteobacteria*, *Actinobacteria*, *Bacteroidetes*, and *Firmicutes*, were observed throughout the life cycle of the plant. A total 12 phyla with 22 families were found in different growth stages of the plant. From the data of metagenomic analysis, two major endophytes *Sphingomonas* and *Salinibacterium* were positively correlated with stevioside content, and *Methylobacterium* and *Acinetobacter* were positively correlated with UGT74G1 and UGT76G1 gene expression, respectively. Application of growth substances like fulvic acid enhanced the community of such endophytes and thereby increased the growth parameters as

Table 3.1 Tissue culture experiments for enhanced production of stevioside and/or rebaudioside in *Stevia rebaudiana*

Sr. no	Experiment	Treatment details	Observations	References
1	Effect of elicitors on in vitro plantlets	WPM + elicitor + 3% sucrose + 0.65% agar (pH 5.8)	Control plants showed 1.69 mg of stevioside per gram of dry weight (DW) sample	Bayraktar et al. (2016)
		Elicitor:		
		1. Alginate (0.05%)	1. 14.69 mg/g of DW	
		2. Casein hydrolysate (0.05%)	2. 13.05 mg/g of DW	
		3. Pectin (0.1%)	3. 10.74 mg/g of DW	
		4. Yeast extract (0.2%)	4. 14.54 mg/g of DW	
		5. Chitin (100 µM)	5. 7.02 mg/g of DW	
		6. Methyl jasmonate (50 µM)	6. 12.53 mg/g of DW	
		7. Salicylic acid (50 µM)	7. 13.84 mg/g of DW	
2	Combined effect of cytokinins with agar	MS + 3% Sucrose + cytokinins + agar (pH 5.6–5.8)	In control plants, concentration of dulcoside A (DA), stevioside (ST), and rebaudioside A (RA) were recorded as 50.81, 70 and 7.39 µg/g of DW, respectively	Aman et al. (2013)
		1. 3 ppm BA + 3 ppm Kn + 0.35% agar		
		2. 3 ppm BA + 3 ppm Kn + 0.7% agar		
		3. 1 ppm BA + 0.7% agar		
			1. 71.8 µg/g of DW (DA)	
			2. 82.48 µg/g of DW (ST)	
			3. 12.35 µg/g of DW (RA)	
3	Tissue cultured plantlets vs seedlings	MS + 1 ppm Kn + 3% sucrose + 0.8% agar (pH 5.7) for shooting and MS + 0.25 ppm IAA + 3% sucrose + 0.8% agar (pH 5.7) for rooting	No significant differences found between 12-week-old micropropagated plantlets transferred to field (6.7% ST) and 12-week-old seed-raised population (6.9% ST)	Yucesan et al. (2016)
4	Effect of culture vessel	For traditional culture vessel, the media composition was MS + 3% sucrose + 0.8% agar (pH 5.7)	SG production was increased up to 1.5–2.0-fold higher in shoots cultivated in roller bottle bioreactor than in shoots cultivated in traditional 80 ml culture vessel	Bondarev et al. (2002)
		For roller bottle bioreactor, the media was same as mentioned above without the addition of agar with four revolutions per minute		

well as steviol glycoside content in the plant (Yu et al. 2015). Arbuscular mycorrhizal fungi are also known to enhance secondary metabolites like essential oils (Copetta et al. 2006), rosmarinic acid, caffeic acid (Toussaint et al. 2007), hypericin, and pseudohypericin (Zubek et al. 2012) in plants. Successful efforts were also made by Mandal et al. (2013) to increase stevioside and rebaudioside level, and

these glycoside levels along with chlorophylls and glandular trichome density were significantly increased as compared to control, phosphorous application, and mycorrhizal + phosphorous application. Due to enhanced rate of photosynthesis and carbohydrate production, synthesis of precursor molecules like IPP and DMAPP increased which results in the end products like stevioside and rebaudioside along with other terpenoids. This mycorrhization mainly targeted two regulatory enzymes, viz., copalyl diphosphate synthase (CPPS) and kaurenoic acid hydroxylase (KAH), which could be further confirmed with upregulation in transcript accumulation of these enzymes. Expressions of genes involved in glycosylation of steviol (synthesized by upregulated enzyme KAH) were found to be more than sevenfold in mycorrhizal plants as compared to non-mycorrhizal plants (Mandal et al. 2015).

3.3.3 Hairy Root Culture and Metabolic Engineering

Like other medicinal plants, stevia was also targeted for hairy root culture by several researchers. The work was started before two decades by Yamazaki and Flores (1991) who observed no stevioside in the root generated after the infection of *Agrobacterium rhizogenes*. Moreover, they also observed stevioside in shoot cultures treated with cytokinins. They proposed the involvement of plastids in the formation of stevioside, and thus, it could not be synthesized in the root. However, several researchers also tried to optimize the hairy root culture protocol to enhance the level of stevioside. Michalec-Warzecha et al. (2016) optimized the efficient transformation protocol of hairy root culture from leaves and internodes infected with two strains LBA 9402 and ATCC 15384 and different inoculum density in light and dark. They established up to 50% of transformation efficiency. However, they did not determine the level of stevioside in any of the sample. On the other hand, Iiaei et al. (2016) produced two kinds of roots, viz., yellow-white and green after incubation of *A. rhizogenes* (strains ATCC15834, R1000, GM, and C58C1)-infected material in dark and light, respectively. Dark-grown roots produced no stevioside, but light-grown roots had 18.67 mg of stevioside per gram of dried sample. Hairy root culture might not help in the formation of stevioside as the site of synthesis of the compounds is primarily a leaf and not roots, thus giving importance to other secondary metabolites; Fu et al. (2015) enhanced the level of chlorogenic acid and its derivatives (105.58 mg/g of dry weight) in the hairy roots of stevia. After successfully establishing direct organogenesis protocol from leaf by Sreedhar et al. (2008) as well as indirect organogenesis by other researchers, genetic transformation in stevia to alter the expression of desired character became more feasible. To check the molecular basis of stevioside formation, an experiment was conducted by Guleria and Yadav (2013) in which they employed *Agrobacterium*-mediated transformation with RNA interference (RNAi) system to block the synthesis of four important enzymes, viz., KA13H and UGT85C2, UGT74G1, and UGT76G1. Out these four, the first two (KA13H and UGT85C2) were found to be key regulatory enzymes which were very region-specific and had region-selective activities. Other

two enzymes were found to be involved in the main as well as alternative pathway leading to the formation of rubusoside and 10-O-β-glucopyranosyl steviol. Corresponding genes of these enzymes can be targeted to overexpress in future transgenic approaches.

3.4 Conclusion

Stevia rebaudiana Bertoni is a continuously demanding plant for its sweet diterpene glycosides. These glycosides are highly modulated by external and internal factor. Many efforts have been carried out using various growth regulators, elicitors, biological agents, and physical conditions to enhance the steviol glycosides. Conventional approaches, except breeding strategies, may be considered as temporary solution for the increased SGs. The content of steviol glycosides could be increased for the time being in either in vivo or in vitro plants. For permanent accumulation of steviol glycosides in *Stevia rebaudiana*, biotechnological approaches are good alternative. Enhancing the expression of genes involved in glycosylation especially UGT74G1 and UGT76G1 in the plant's constitution should be the strategy of interest. So far, *Agrobacterium*-mediated transformation protocols are well optimized, but the overexpression of target enzymes is not yet done. Pathway for the steviol glycoside synthesis is also well characterized. Increasing demand of these low caloric sweeteners requires efforts to be carried out with transgenic approach.

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Chapter 4
Effect of Chemical Elicitors on Pentacyclic Triterpenoid Production in In Vitro Cultures of *Achyranthes aspera* L.

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Abstract Bioprocess technology for the production of phytochemicals from plant cell cultures mainly depends upon elicitation for enhancing the yields. The application has been successfully demonstrated in various plant species for a number of metabolites of interest. *Achyranthes aspera* L. is a highly traded medicinal plant known for a wide array of pharmacological properties. In this study, the effect of different concentrations of salicylic acid (SA), methyl jasmonate (MeJA), jasmonic acid (JA), and chitosan (CH) on growth and accumulation of betulinic acid (BA), oleanolic acid (OA), and ursolic acid (UA) in cultures of *A. aspera* was investigated using RP-UFLC technique. Results for in vitro cultures grown on various concentrations of selected elicitors (50, 100, and 200 μM) were collected and studied at 7-, 15-, and 30-day intervals. Two separate experiments for elicitors with and without plant growth regulators (PGRs), i.e., 6-benzylaminopurine 3.0 mg/L and thidiazuron 0.5 mg/L, were studied. Higher fresh and dry weights were observed in all the treated tubes as compared to control. Optimum cell growth along with higher BA content was observed in MeJA-treated cultures with and without PGRs. Increased OA content was evident in 30-day cultures growing on 100 and 200 μM MeJA supplemented with PGRs. Chitosan both in media supplemented with and without PGRs produced higher content of UA at 30th day. Furthermore, it becomes important to understand the biochemical conversions in light of the metabolic pathways so that we can use this data in maneuvering production of metabolites in *A. aspera*.

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4.1 Introduction

Achyranthes aspera Linn. is a highly traded medicinal plant from the wastelands of India belonging to the family Amaranthaceae (Prain 1963; Ved and Goraya 2007). It is used in Ayurveda, which is practiced widely by traditional practitioners in India, and is reported to treat a wide range of ailments (Anonymous 2007, 2017; Tripathy et al. 2017). In many parts of Southern India, *A. aspera* individually or in combination with other plants/plant extracts is widely used in traditional medicine (Harsha et al. 2004; Hebbar et al. 2004; Nadakarni 2009; Upadhya et al. 2009; Upadhya 2015).

4.1.1 Taxonomy of the Plant

Family Amaranthaceae contains ~850 species assigned to 71 genera distributed worldwide. India is endowed with 20 genera with 60 species (Mishra and Singh 2001; Singh et al. 2000). Genus *Achyranthes* contains ~15 species distributed in the tropics and subtropics (Shu 2003). There are three species of *Achyranthes* described from the Indian subcontinent, and they are *Achyranthes aspera*, *A. bidentata*, and *A. coynei* (Punekar and Lakshminarshiman 2011).

Achyranthes aspera is a widespread cosmopolitan distributed species in different regions, viz., Tropical Asia, Africa, Australia, and America (Prain 1963; deLange et al. 2004; Upadhya et al. 2015). In India, this plant can be found in many places grown as a troublesome weed of roadsides and waste places. The taxonomical identity of these varieties has to be confirmed beyond doubts by means of molecular techniques (Upadhya 2015).

Achyranthes aspera is a stiff herb (Fig. 4.1a), branching from the base; leaves of this plant show variability in shape; however, usually the leaves are orbicular to obovate. Young leaves show presence of hair on both upper and lower surface, and the older leaves were glabrous. *A. aspera* bear swollen node on the stem and presence of opposite or whorled leaves in nodal region. The plant develops many terminal flower spike inflorescences. Flowers are greenish pink in nature with spinescent bract and bracteoles. Basal portion of the bracteole is winged in nature, and flowers are deflexed on the axis after anthesis. Perianth of the flowers has free five segments herbaceous to coriaceous and one nerved. Stamens are five alternating with pseudostaminodes, and the filaments of anthers unite to form a cup shape at the base. Staminodes are truncate and fibrate. The ovary is one ovuled with a short style and capitate stigma. Fruit of *A. aspera* is indehiscent, shed with the persistent pungent perianth.

Fig. 4.1 (**a**) *Achyranthes aspera* L. Habit; (**b**) Plants grown inside the laboratory on a Petri plate containing coco peat; (**c**) Induction of multiple shoots using MS media supplemented with BAP 3 mg/l + TDZ 0.1 mg/l; (**d**) Multiplication of subculture used in elicitation experiment

Flowering and fruiting can be observed from September to April. Fruits or seeds get dispersed when they are caught on the skin of the animals and clothes that brushes against them and also through wind and water (Cook 1901–1908; Sing et al. 1996; Yadav and Sardesai 2002; Punekar and Lakshminarshiman 2011).

4.1.2 Ethnobotanical Significance

Achyranthes aspera is also known as Prickly Chaff flower in English language. It is well-known as Apamarg (Sanskrit name). The plant is reported to treat a variety of diseases in codified and non-codified traditional systems of medicine in India and elsewhere (Nadakarni 2009; Tondon 2011; Upadhya et al. 2015; Anonymous 2017; Tripathyet al. 2017), including kidney infections and problems, fever, cold, piles, asthma, cough, boils, eruptions of skin, pneumonia, astringent in bowel complaints, ophthalmia and opacities of the cornea, ulcers, warts, bites of poisonous snakes and

reptiles, night blindness, cutaneous diseases, and gastric disorders and is also used in pregnancy. It is also useful for the treatment of haemorrhoids, emetic, hydrophobia, carminative, swelling, digestive problems, phlegm, strained back, bite of wasps, liver complaints, rheumatism, scabies and a number of other skin diseases. The root of this plant is used as toothbrushes. In Ayurveda it is described as pungent, antiphlegmatic, antiperiodic, diuretic, purgative, and laxative properties (Sing et al. 1996; Anonymous 2007; Khare 2007; Nadakarni 2009; Tondan 2011; Anonymous 2017). The plant is also reported to be used in veterinary medicine (Battaraj 1992).

4.1.3 Pharmacology

Pharmacological investigations in the genus *Achyranthes* mainly focus on its biological effects on disorders pertaining to immunology, nervous, bone metabolism, reproduction, hypoglycemic, blood-activating, cancer chemopreventive (Chakraborty et al. 2002; Subbarayana et al. 2010), cardiovascular (Neogi et al. 1970; Ram et al. 1971; Gupta et al. 1972), hepatoprotective (Bafna and Mishra 2004), anti-inflammatory, and antiarthritic activities (Gokhale et al. 2002; Vetrichelvan and Jegadeesan 2003; Kumar et al. 2009), and it is also recorded for several other diseases (Varuna et al. 2010; Srivastav et al. 2011). Review on *A. aspera* by Sharma and Chaudhary (2015) reported other pharmacological activities like anthelmintic, antiviral, antimicrobial, larvicidal, nephroprotective, thermolytic, and wound healing.

4.1.4 Chemical Properties

He et al. (2017) report 133 compounds from the genus until date, the major group being triterpenoid saponins, ketosteroids polysaccharides, and polypeptide. Thirty-one compounds have been reported from *A. bidentata*, *A. aspera*, and *A. fauriei* (Misra et al. 1991, 1993, 1996; Ali 1993; Sharma et al. 2009; Tang et al. 2013; Rameswar 2007; Dong 2010; Fujii et al. 2010) Additionally, compounds such as eugenol, hydroquinones, asarone, α-ionone, spathulenol, allantoin, dibutyl phthalate, N-butyl-β-D-fructopyranoside, and many others are also reported from *Achyranthes* plants (Chao et al. 1999; Wei et al. 1997; Meng et al. 2002; Meng 2004; Rameswar 2007).

Oleanolic acid (OA: 3β-hydroxyolean-12-en-28-oic acid) is a pentacyclic triterpenoid responsible for a wide display of biological activities in plants, including *A. aspera* (Tokuda et al. 1986; Hsu et al. 1997; Liu 1997; Jeong 1999; Aparecida et al. 2006; Ovesna et al. 2006; Aeri et al. 2010; Tondon 2011). Similar activities are also attributed to betulinic acid (BA: 3-hydroxylup-20(29)-en-(28)-oic acid) and ursolic acid (Fontanay et al. 2008; Woźniak et al. 2015), another triterpenoid widely studied in many plant species. Reports suggest OA to be the most studied compounds in *A.*

aspera; however, both BA and UA were also reported from this plant. Pai et al. (2014, 2016) have studied and reported BA from the plant. All the three triterpenoids, BA, OA, and UA, share similar chemical formula ($C_{30}H_{48}O_3$) but differ in their structures (Fig. 4.2a–c).

Although *A. aspera* is considered among the 46 identified high volume trade species from India, the quality requirements of the species are the major concern (Ved and Goraya 2007). Previous studies suggested variations in content of the OA and other constituents of *A. aspera* growing in wild (Srivastav et al. 2011; Varuna et al. 2010; Tondon 2011). Studies have also proved physiological and environmental conditions to regulate the quality and quantity of secondary metabolites (Verpoorte et al. 2002). Biotechnological techniques facilitated to produce required quality and quantity of secondary metabolites. Thus, the experiment was undertaken to check the effect of chemical elicitors on the production of triterpenoids in *A. aspera.*

4.2 Methodology

4.2.1 Sampling and Collection

Sampling was done for the plant from Belagavi and nearby locations; GPS reading for the collection spots was marked and noted (GPS: N 15.88°; E 74.52°, 801 M above MSL). Young leaves and axillary buds of *A. aspera* were collected for the study.

4.2.2 Sample Authentication

Plant specimen was identified taxonomically by Dr. Harsha Hegde, Scientist C, Herbal Medicine Division, National Institute of Traditional Medicine (formerly RMRC), Indian Council of Medical Research (ICMR), Belagavi. Herbarium of authenticated specimen was deposited at Herbarium, NITM (formerly RMRC), Belagavi, Karnataka, India, for future reference (Vouch. No. RMRC-1250).

4.2.3 Sample Processing

The explants (apical and axillary nodes) were obtained from seeds germinated in lab using coco peat (Fig. 4.1b) washed with running tap water for 15 min. Pretreatments prior to disinfection per se included immersion in fungicide carbendazim 50% WP (Bensaan 50, India). Later explants were washed thoroughly in sterile water; standardization of surface sterilization using different concentrations of sodium

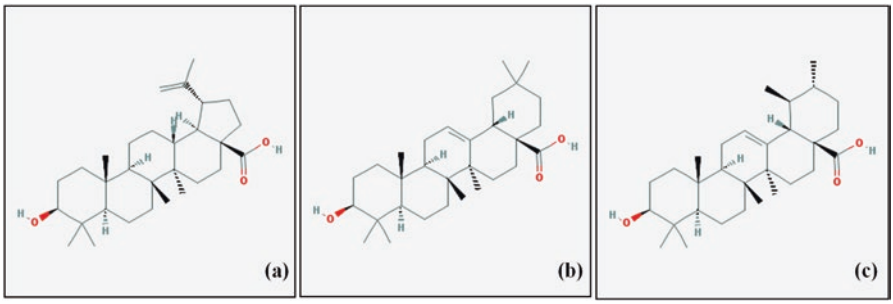

Fig. 4.2 Chemical structure of (**a**) 3-hydroxylup-20(29)-en-(28)-oic acid (BA, betulinic acid), (**b**) 3β-hydroxyolean-12-en-28-oic acid (OA, oleanolic acid), (**c**) 3-beta-3-hydroxy-urs-12-ene-28-oic-acid (UA, ursolic acid). (*Source: PubChem*)

hypochlorite (NaOCl: 1–4% v/v) and mercuric chloride ($HgCl_2$: 0.1–0.4% w/v) solutions with addition of Tween 20 was studied.

4.2.4 Effect of Plant Growth Regulators on Shoot Induction and Rate of Multiplication

Single axillary buds obtained from the field were pretreated and disinfected as mentioned above and were inoculated on different combination of plant growth regulators (PGRs) (data not shown). Multiplication rate (number of plantlets that originated from each initial explant) and the number of shoots and roots produced per explant were used for evaluation. Percent response, shoots per explants, and average shoot length were determined after 8 weeks of culturing with one subculture after 4 weeks.

4.2.5 Effect of Elicitors with and Without PGRs for Production of Triterpenoids

Achyranthes aspera cultures were obtained from excised plant material (apical meristem) on MS basal medium fortified with BAP 3 mg/L, TDZ 0.5 mg/L. All flasks were maintained at 25 ± 2 °C with a dark/light regime of 18/6 h.

Elicitation: Salicylic acid (SA), methyl jasmonate (MeJA), jasmonic acid (JA), and chitosan (CH) were selected as chemical elicitors for the study. Different concentration, viz., 50, 100, 200 μM of each, was compared with control at regular intervals on 7, 15, and 30 days after inoculation.

Effect of elicitors with and without PGRs was observed in the production of selected triterpenoids.

Parameters: Fresh weight, dry weight, and content (%) of betulinic acid (BA), oleanolic acid (OA), and ursolic acid (UA).

4.2.6 Culturing Conditions

Shoot tips (apical meristem) from the fully grown individual were cultured separately on Linsmaier and Skoog (LS) culture medium with 3% (w/v) sucrose and 0.8% (w/v) agar. The pH was adjusted to 5.8 before autoclaving at 121 °C for 15 min. Culture conditions were 25 ± 2 °C temperature and a photoperiod of 16 h light/8 h dark (light intensity of 30 μ mol m^{-2} s^{-1}, Philips TL 34).

4.2.7 Extraction

Ultrasonic extraction was performed on ultrasonic bath (Sonics Vibracell, USA) by subjecting 1 g of fresh in vitro grown 8 weeks old plant material in 20 mL of 95% aqueous methanol in 100 mL Erlenmeyer flask. The flask was exposed to a working amplitude of 60 kHz for 15 min at ambient temperature. The sample was filtered through Whatman filter paper No. 1, re-volumized to 20 mL with 95% aqueous methanol to obtain a 5% extract. The filtrate was passed through 0.45 μ nylon filters before analysis to remove impurities and was stored at 4°C until use.

4.2.8 RP-UFLC Analysis of Selected Triterpenoids

The method described by Pai et al. (2014, 2016) was used for quantification of triterpenoids. The RP-UFLC analysis was performed on Shimadzu chromatographic system (Model no. LC-20 AD) consisting of a quaternary pump, manual injector, degasser (DGU-20A5), and dual λUV absorbance diode array detector SPD-M20A. The built-in LC Solution software system was used for data processing. Chromatographic separation was achieved on a Hibar 250–4.6 mm, 5 μ, Lichrospher 100, C18e column. A mobile phase consisting of methanol and water was used for separation with 90:10, and pH 5.0 was adjusted using glacial acetic acid in an isocratic mode. Injection volume of standard and sample was 20 μL. The flowrate was 1 mL min^{-1}, and the detection wavelength was set at210 nm. The analysis time was 15 min for both standard and sample.

The limit of detection (LOD) and limit of quantification (LOQ) were determined with the signal–noise method. Signal–noise ratios of 3.3 and 10 were applied for estimating the LOD and LOQ, respectively. The system suitability test was assessed by three replicate injections of the standard solutions at a particular concentration. The peak areas were used to evaluate the repeatability of the method, and their

peaks were analyzed for resolution. A validated method for detection and quantification of BA, OA, and UA within a concentration range of 0.05, 1, 10, 20, 40, and 80 ppm was used (Fig. 4.3a).

4.3 Results and Discussion

4.3.1 RP-UFLC Analysis of Selected Triterpenoids

Six different concentrations (0.05, 1, 10, 20, 40, and 80 ppm) of standard triterpenoids (BA, OA, and UA) were detected at 210 nm wavelength using RP-UFLC technique. With the above given RP-UFLC conditions, chromatogram profiles with retention time 11.592 ± 0.026 (BA), 12.962 ± 0.045 (OA), and 13.534 ± 0.035 (UA) min were obtained (Fig. 4.3b). The linearity curves for standards were obtained with R2 not more than 0.998 (Fig. 4.3b). LOD of BA, OA, and UA were 0.035, 0.042, 0.033 ppm, and LOQ were 0.107, 0.126, and 0.101 ppm, respectively.

The results of the study indicated good linearity and significant validity of the calibration values used. Similarly, three injections each of three different concentrations of the analytes (1, 10, 20 ppm) had significant inter- and intraday precision. Lower RSD (< 0.72%) value of retention time indicated acceptable reproducibility of the method. The theoretical plate number (N) was found to be 9111.151 (BA), 9400.486 (OA), and 9547.568 (UA) for the column used during the study (250 mm × 4.6 mm i.e., particle size 5 μm), demonstrating the acceptable column efficiency. All these results assure the competence of the current UFLC method for analysis of BA, OA, and UA. The results obtained using this UFLC method was in line with the studies of Pai et al. (2014, 2016) and Upadhya et al. (2014).

4.3.2 Use of Salicylic Acid (SA), Methyl Jasmonate (MeJA), Jasmonic Acid (JA), and Chitosan (CH) in Media Fortified with and Without PGRs for Production of Triterpenoids

Salicylic acid, MeJA, JA, and CH are seen as some of the common elicitors used in plant secondary metabolite elicitation. Salicylic acid is a secondary molecule produced in plants as resistance against fungal, bacterial, and/or viral pathogens. MeJA and JA induce production of various proteins for resistance against insects, and CH has showed antimicrobial action. Chitosan has been used to control disease spread, to chelate the minerals, and also to prevent attack of pathogens, thus triggering the secondary metabolite production.

Achyranthes aspera shoot tip cultures were derived from the apical and axillary meristems. The best growth was obtained on Murashige and Skoog medium con-

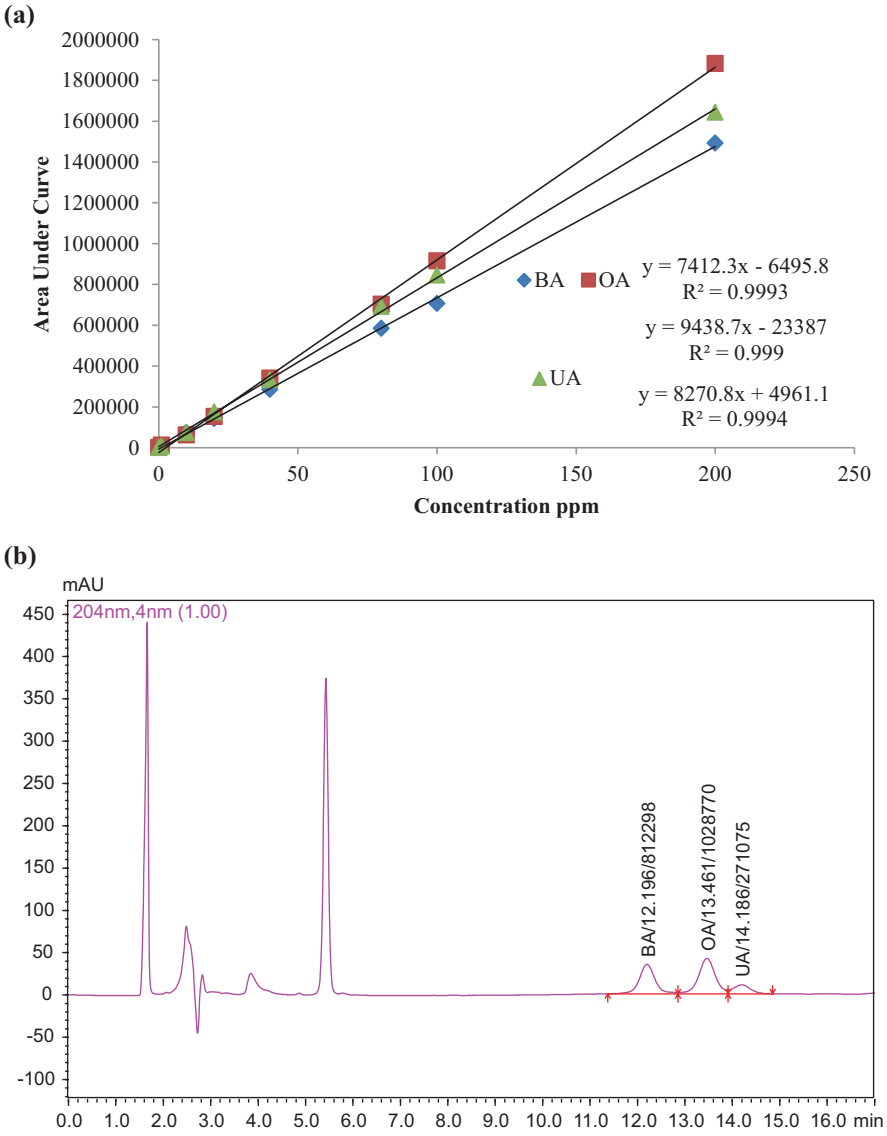

Fig. 4.3 (**a**) Standard graph of betulinic acid (BA), oleanolic acid (OA), and ursolic acid (UA); (**b**) RP-UFLC chromatogram of BA, OA, and UA 25 ppm

taining 3.0 mg/L BAP and 0.5 mg/L TDZ (Fig. 4.1c). The effects of elicitation with SA, MeJA, JA, and CH on production of triterpenoids were observed at concentrations of 50, 100, and 200 μM with 7-, 15-, and 30-day intervals. Two separate experiments for elicitors with and without PGRs were attempted (Fig. 4.1d).

4.3.3 *Quantification of BA, OA, and UA from In Vitro-Elicited Cultures Using RP-UFLC Analysis*

Six different concentrations (0.05, 1, 10, 20, 40, and 80 ppm) of standard triterpenoids (BA, OA, and UA) were detected at 210 nm wavelength using RP-UFLC technique. Earlier published chromatographic method for detection of betulinic acid, oleanolic acid, and ursolic acid has been used (Pai et al. 2016).

The results of the study are presented in Tables 4.1, 4.2, 4.3, 4.4, 4.5, 4.6 and Figs. 4.4, 4.5, 4.6. Higher fresh weight and dry weight were observed in all the treated tubes as compared to control. Out of the elicitors tested, MeJA showed promising results over the others. Chitosan failed to produce any elicitation. It was interesting to note that the addition of PGRs in the medium also helped in higher production of triterpenoids.

Betulinic acid: Earlier, elicitation was observed within 15 days of treatment. Optimum cell growth along with higher content was observed in MeJA-treated cultures with and without PGRs (Fig. 4.4a and b). MeJA with PGRs provided better results over that of MeJA without PGRs. 100 μM of MeJA within 7 days yielded higher BA. All the higher contents were higher to the control.

Oleanolic acid: Almost equal elicitation compared to control was observed in the case of only elicitors, whereas a difference was observed in the case of elicitors subjected with PGRs. An increase in OA content was evident in 30-day cultures of *A. aspera* growing on 100 and 200 μM MeJA supplemented with PGRs. However, the highest content was observed in the 15-day culture of *A. aspera* growing in 200 μM MeJA without PGRs (Fig.4.5a, b).

Ursolic acid: Unlike BA and OA, here CH played important role in elicitation of OA. Chitosan both in media supplemented with and without PGRs was responsible to produce higher content of UA at 30th day. Increasing concentration of chitosan in media with PGRs showed an optimum yield at 100 μM (Fig. 4.6a, b).

Altogether, the results showed that ursolic followed by betulinic and oleanolic acid had a higher content elicited. Although even results of elicitation were observed for OA, which is also the most commonly detected and studied compound from *A. aspera*.

Most pharmacologically active phytocompounds, pentacyclic triterpenoids, have complex structures, making their chemical synthesis a financially uncompetitive choice. Plant cell culture has been another option to elevate production of such compounds of pharmaceutical intrigue (Giri and Naraseu 2000; Gaines 2004; Naik and Al-Khayri 2016), with the advantages being ease of maneuvering and eliciting the production of these compounds experimentally in culture. Currently, the relationship between mechanisms controlling cell differentiation, tissue organization and biosynthesis of secondary metabolites is not very clear. Production of secondary metabolite requires association among leaves and roots wherein the precursors are supposed to be produced in roots and its bioconversion occurring in leaves (Giri and Naraseu 2000). Secondary metabolite biosynthesis is tissue specific (Aziz et al.

Table 4.1 Effect of in vitro elicitation on production of BA (%) in *A. aspera*

Elicitor	Concentration µM	Fresh weight (g/tube ±sd)		Dry weight (g/tube ±sd)		% of betulinic acid ±sd					
						7th day		15th day		30th day	
Ctrl	–	3.1200	±0.1560	0.2800	±0.0140	0.0058	±0.0003	0.0010	±0.0001	0.0010	±0.0001
JA	50	5.1000	±0.2550	0.3500	±0.0175	0.0016	±0.0001	0.0011	±0.0001	0.0010	±0.0001
	100	5.9300	±0.2965	0.4000	±0.0200	0.0013	±0.0001	0.0011	±0.0001	0.0054	±0.0003
	200	7.2300	±0.3615	0.5000	±0.0250	0.0012	±0.0001	0.0012	±0.0001	0.0029	±0.0001
MeJA	50	7.1000	±0.3550	0.4500	±0.0225	0.0010	±0.0001	0.0671	±0.0034	0.0000	±0.0000
	100	7.2000	±0.3600	0.4900	±0.0245	0.0012	±0.0001	0.0331	±0.0017	0.0010	±0.0001
	200	8.2500	±0.4125	0.4500	±0.0225	0.0381	±0.0019	0.0545	±0.0027	0.0013	±0.0001
SA	50	8.5100	±0.4255	0.5500	±0.0275	0.0000	±0.0000	0.0053	±0.0003	0.0015	±0.0001
	100	6.3800	±0.3190	0.5200	±0.0260	0.0010	±0.0001	0.0014	±0.0001	0.0010	±0.0001
	200	6.2700	±0.3135	0.5700	±0.0285	0.0023	±0.0001	0.0011	±0.0001	0.0172	±0.0009
CH	50	4.3600	±0.2180	0.3500	±0.0175	ND		ND		ND	
	100	4.4700	±0.2235	0.3900	±0.0195	ND		ND		ND	
	200	3.0500	±0.1525	0.2500	±0.0125	ND		ND		ND	

Table 4.2 Effect of in vitro elicitation with PGRs on production of BA (%) in *A. aspera*

Elicitor	Concentration μM	Fresh weight (g/tube ±sd)		Dry weight (g/tube ±sd)		% of betulinic acid ±sd					
						7th day		15th day		30th day	
Ctrl	–	4.0200	±0.2010	0.2900	±0.0145	0.0010	±0.0001	0.0193	±0.0010	0.0014	±0.0001
JA	50	9.7500	±0.4875	0.6000	±0.0300	0.0011	±0.0001	0.0010	±0.0001	0.0014	±0.0001
	100	11.8000	±0.5900	0.7700	±0.0385	0.0011	±0.0001	0.0014	±0.0001	0.0020	±0.0001
	200	8.8700	±0.4435	0.4900	±0.0245	0.0011	±0.0001	0.0011	±0.0001	0.0011	±0.0001
MeJA	50	9.6000	±0.4800	0.4700	±0.0235	0.0538	±0.0027	0.0265	±0.0013	0.0000	±0.0000
	100	8.8700	±0.4435	0.4700	±0.0235	0.0644	±0.0032	0.0391	±0.0020	0.0011	±0.0001
	200	10.7200	±0.5360	0.5800	±0.0290	0.0526	±0.0026	0.0429	±0.0021	0.0031	±0.0002
SA	50	8.5500	±0.4275	0.6500	±0.0325	0.0011	±0.0001	0.0014	±0.0001	0.0020	±0.0001
	100	7.4900	±0.3745	0.6000	±0.0300	0.0000	±0.0000	0.0011	±0.0001	0.0011	±0.0001
	200	3.7300	±0.1865	0.2000	±0.0100	0.0217	±0.0011	0.0056	±0.0003	0.0011	±0.0001
CH	50	6.2400	±0.3120	0.4500	±0.0225	ND		ND		ND	
	100	5.2200	±0.2610	0.4100	±0.0205	ND		ND		ND	
	200	4.5800	±0.2290	0.3500	±0.0175	ND		ND		ND	

Values in table are data obtained mean ± sd from three injections
JA jasmonic acid, *MeJA* methyl jasmonate, *SA* salicylic acid, *CH* chitosan, *ND* not detected

Table 4.3 Effect of in vitro elicitation on production of OA (%) in *A. aspera*

Elicitor	Concentration µM	Fresh weight (g/tube ±sd)		Dry weight (g/tube ±sd)		% of oleanolic acid ±sd					
						7th day		15th day		30th day	
Ctrl	–	3.1200	±0.1560	0.2800	±0.0140	0.0028	±0.0001	0.0031	±0.0002	0.0153	±0.0008
JA	50	5.1000	±0.2550	0.3500	±0.0175	0.0026	±0.0001	0.0028	±0.0001	0.0033	±0.0002
	100	5.9300	±0.2965	0.4000	±0.0200	0.0042	±0.0002	0.0029	±0.0001	0.0038	±0.0002
	200	7.2300	±0.3615	0.5000	±0.0250	0.0034	±0.0002	0.0026	±0.0001	0.0028	±0.0001
MeJA	50	7.1000	±0.3550	0.4500	±0.0225	0.0116	±0.0006	0.0027	±0.0001	0.0029	±0.0001
	100	7.2000	±0.3600	0.4900	±0.0245	0.0035	±0.0002	0.0027	±0.0001	0.0032	±0.0002
	200	8.2500	±0.4125	0.4500	±0.0225	0.0027	±0.0001	0.0332	±0.0017	0.0312	±0.0016
SA	50	8.5100	±0.4255	0.5500	±0.0275	ND		0.0027	±0.0001	0.0039	±0.0002
	100	6.3800	±0.3190	0.5200	±0.0260	0.0026	±0.0001	0.0030	±0.0001	0.0030	±0.0001
	200	6.2700	±0.3135	0.5700	±0.0285	0.0036	±0.0002	0.0037	±0.0002	0.0193	±0.0010
CH	50	4.3600	±0.2180	0.3500	±0.0175	ND		ND		ND	
	100	4.4700	±0.2235	0.3900	±0.0195	ND		ND		ND	
	200	3.0500	±0.1525	0.2500	±0.0125	ND		ND		ND	

Table 4.4 Effect of in vitro elicitation with PGRs on production of OA (%) in *A. aspera*

Elicitor	Concentration μM	Fresh weight (g/tube ±sd)		Dry weight (g/tube ±sd)		% of oleanolic acid ±sd					
						7th day		15th day		30th day	
Ctrl	–	4.0200	±0.2010	0.2900	±0.0145	0.0026	±0.0001	0.0063	±0.0003	0.0073	±0.0004
JA	50	9.7500	±0.4875	0.6000	±0.0300	0.0159	±0.0008	0.0026	±0.0001	0.0049	±0.0002
	100	11.8000	±0.5900	0.7700	±0.0385	0.0027	±0.0001	0.0031	±0.0002	0.0031	±0.0002
	200	8.8700	±0.4435	0.4900	±0.0245	0.0029	±0.0001	0.0028	±0.0001	0.0025	±0.0001
MeJA	50	9.6000	±0.4800	0.4700	±0.0235	0.0027	±0.0001	0.0027	±0.0001	0.0031	±0.0002
	100	8.8700	±0.4435	0.4700	±0.0235	0.0039	±0.0002	0.0028	±0.0001	0.0129	±0.0006
	200	10.7200	±0.5360	0.5800	±0.0290	0.0027	±0.0001	0.0028	±0.0001	0.0112	±0.0006
SA	50	8.5500	±0.4275	0.6500	±0.0325	0.0027	±0.0001	0.0032	±0.0002	0.0046	±0.0002
	100	7.4900	±0.3745	0.6000	±0.0300	0.0144	±0.0007	0.0052	±0.0003	0.0026	±0.0001
	200	3.7300	±0.1865	0.2000	±0.0100	0.0026	±0.0001	ND		0.0026	±0.0001
CH	50	6.2400	±0.3120	0.4500	±0.0225	ND		ND		ND	
	100	5.2200	±0.2610	0.4100	±0.0205	ND		ND		ND	
	200	4.5800	±0.2290	0.3500	±0.0175	ND		ND		ND	

Values in table are data obtained mean ± sd from three injections
JA jasmonic acid, *MeJA* methyl jasmonate, *SA* salicylic acid, *CH* chitosan, *ND* not detected

Table 4.5 Effect of in vitro elicitation on production of UA (%) in *A. aspera*

Elicitor	Concentration µM	Fresh weight (g/tube ±sd)		Dry weight (g/tube ±sd)		% of ursolic acid±sd					
						7th day		15th day		30th day	
Ctrl	–	3.1200	±0.1560	0.2800	±0.0140	0.0012	±0.0001	0.0021	±0.0001	0.1195	±0.0060
JA	50	5.1000	±0.2550	0.3500	±0.0175	0.0019	±0.0001	0.0070	±0.0003	0.0044	±0.0002
	100	5.9300	±0.2965	0.4000	±0.0200	0.0020	±0.0001	0.0023	±0.0001	0.0062	±0.0003
	200	7.2300	±0.3615	0.5000	±0.0250	0.0143	±0.0007	0.0024	±0.0001	0.0021	±0.0001
MeJA	50	7.1000	±0.3550	0.4500	±0.0225	0.0050	±0.0002	0.0020	±0.0001	0.0025	±0.0001
	100	7.2000	±0.3600	0.4900	±0.0245	0.0008	±0.0000	0.0012	±0.0001	0.0044	±0.0002
	200	8.2500	±0.4125	0.4500	±0.0225	0.0137	±0.0007	0.0177	±0.0009	0.0023	±0.0001
SA	50	8.5100	±0.4255	0.5500	±0.0275	ND		0.0029	±0.0001	0.0057	±0.0003
	100	6.3800	±0.3190	0.5200	±0.0260	0.0011	±0.0001	0.0009	±0.0000	0.0293	±0.0015
	200	6.2700	±0.3135	0.5700	±0.0285	0.0019	±0.0001	0.0030	±0.0002	0.0903	±0.0045
CH	50	4.3600	±0.2180	0.3500	±0.0175	ND		ND		1.8219	±0.0911
	100	4.4700	±0.2235	0.3900	±0.0195	ND		ND		0.3301	±0.0165
	200	3.0500	±0.1525	0.2500	±0.0125	0.5686	±0.0284	0.0638	±0.0032	0.8583	±0.0429

Table 4.6 Effect of in vitro elicitation with PGRs on production of UA (%) in *A. aspera*

Elicitor	Concentration μM	Fresh weight (g/tube ±sd)		Dry weight (g/tube ±sd)		% of ursolic acid ±sd					
						7th day		15th day		30th day	
Ctrl	–	4.0200	±0.2010	0.2900	±0.0145	0.0025	±0.0001	0.0046	±0.0002	0.0024	±0.0001
JA	50	9.7500	±0.4875	0.6000	±0.0300	0.0063	±0.0003	0.0042	±0.0002	0.0028	±0.0001
	100	11.8000	±0.5900	0.7700	±0.0385	0.0051	±0.0003	0.0017	±0.0001	0.0085	±0.0004
	200	8.8700	±0.4435	0.4900	±0.0245	0.0010	±0.0001	0.0015	±0.0001	0.0211	±0.0011
MeJA	50	9.6000	±0.4800	0.4700	±0.0235	0.0132	±0.0007	0.0008	±0.0000	0.0210	±0.0010
	100	8.8700	±0.4435	0.4700	±0.0235	0.0135	±0.0007	0.0043	±0.0002	0.0085	±0.0004
	200	10.7200	±0.5360	0.5800	±0.0290	0.0375	±0.0019	0.0432	±0.0022	0.0155	±0.0008
SA	50	8.5500	±0.4275	0.6500	±0.0325	0.0015	±0.0001	0.0015	±0.0001	0.0327	±0.0016
	100	7.4900	±0.3745	0.6000	±0.0300	ND		0.0029	±0.0001	0.0304	±0.0015
	200	3.7300	±0.1865	0.2000	±0.0100	0.0010	±0.0001	0.2243	±0.0112	0.0012	±0.0001
CH	50	6.2400	±0.3120	0.4500	±0.0225	0.3128	±0.0156	0.4414	±0.0221	0.4398	±0.0220
	100	5.2200	±0.2610	0.4100	±0.0205	ND		0.6448	±0.0322	1.7856	±0.0893
	200	4.5800	±0.2290	0.3500	±0.0175	ND		0.7966	±0.0398	0.3159	±0.0158

Values in table are data obtained mean ± sd from three injections
JA jasmonic acid, *MeJA* methyl jasmonate, *SA* salicylic acid, *CH* chitosan, *ND* not detected

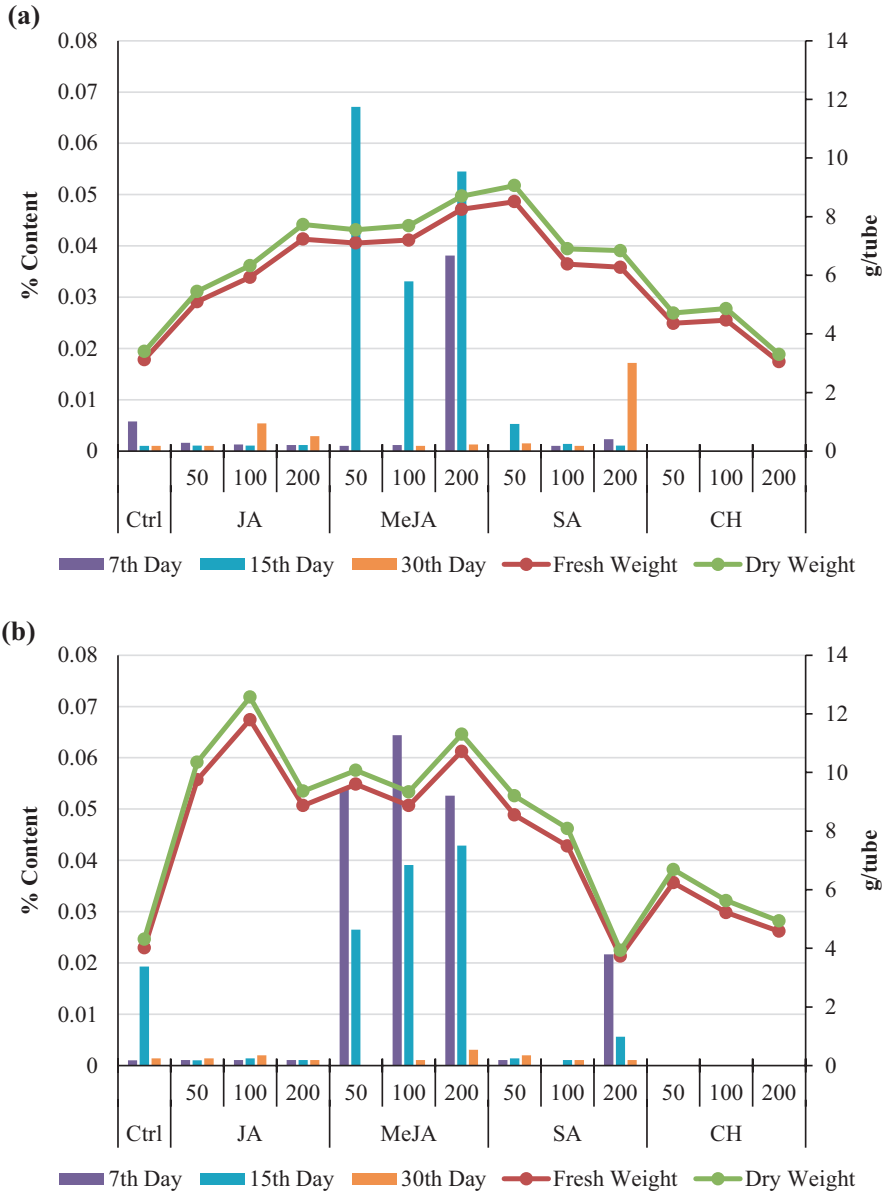

Fig. 4.4 Effect of in vitro elicitation on production of BA (%) in *A. aspera* cultures growing on (**a**) MS basal and (**b**) MS basal + PGRs

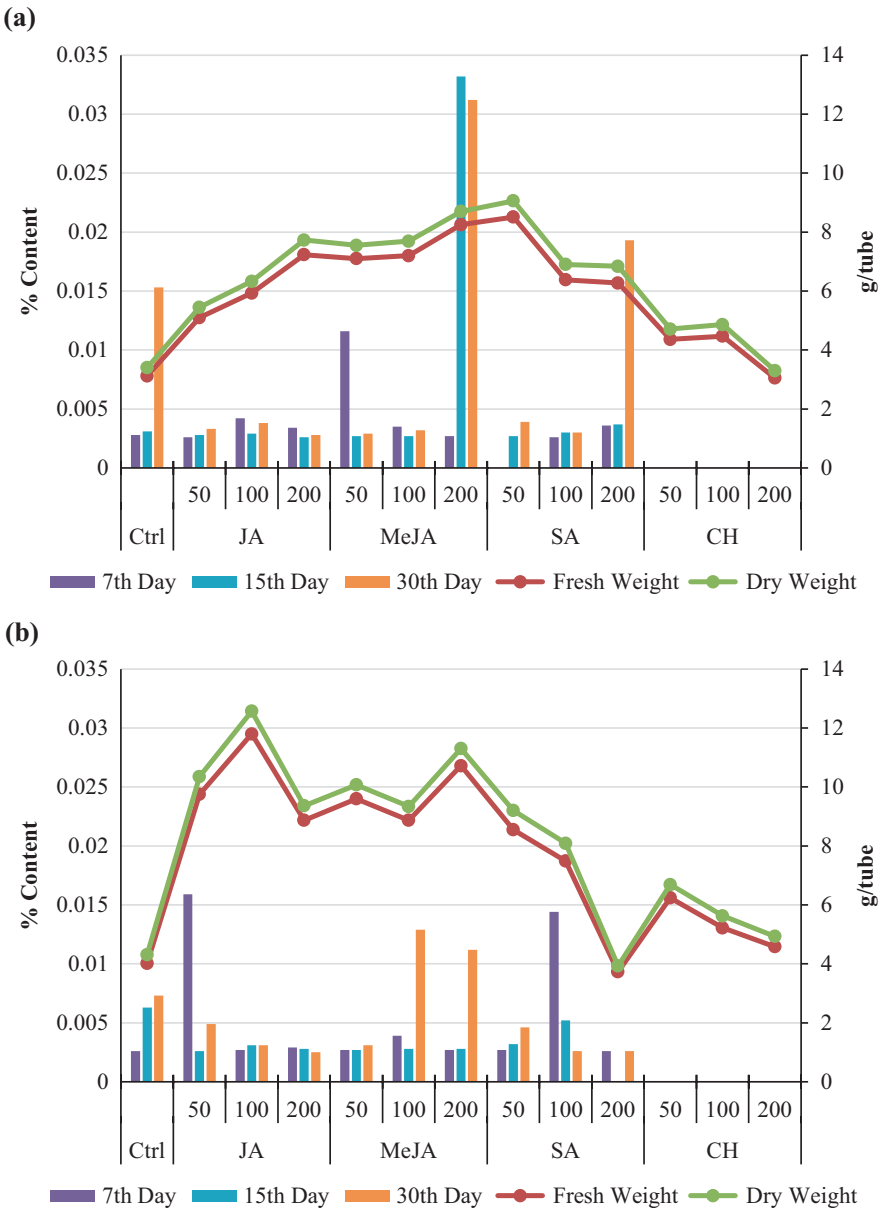

Fig. 4.5 Effect of in vitro elicitation on production of OA (%) in *A. aspera* cultures growing on (**a**) MS basal and (**b**) MS basal + PGRs

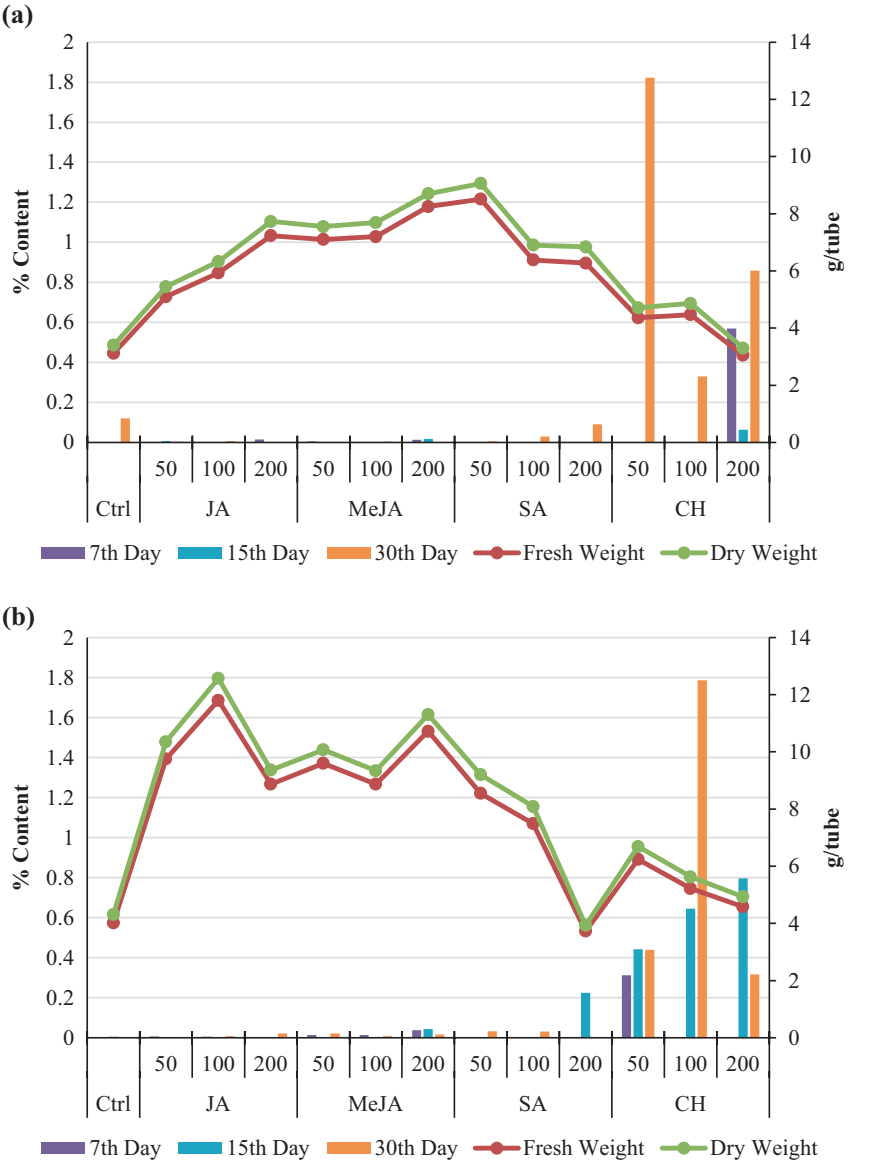

Fig. 4.6 Effect of in vitro elicitation on production of UA (%) in *A. aspera* cultures growing on (**a**) MS basal and (**b**) MS basal + PGRs

2007). Secondary metabolites from plants are generally synthesized by specific cells, at a particular development stage (Kim et al. 2002).

The approach advocated for regulation of metabolic pathways favoring production of particular secondary metabolites is by using precursors in medium (Bouhouche et al. 1998). Encouraging stress in cultures by biological and/or chemical elicitors has been the method used for enhanced production of biologically active secondary metabolites. Plant-particular flag entities, like methyl jasmonate (MeJa), control enzyme levels. It is realized that externally supplied MeJa can prompt biosynthesis of many secondary metabolites, including triterpenoid saponins (Hayashi et al. 2003). Comparable perceptions have also been recorded in the present study with references to triterpenoids and methylated jasmonic acid. Enzymes SQS (squalene synthase) and OSC (oxidosqualene cyclases) were reported to be upregulated by MeJa treatment in cultured *Glycyrrhiza glabra* cells (Hayashi et al. 2003). This upregulation was accompanied by more suitable concentrations of triterpenoid saponins. Hence, inhibition of branch factor enzyme cyclases seems to bring about elevation in flux through the triterpenoid pathway.

Other method for improving terpenoid levels is by increasing the flux of isopentenyl diphosphate (IPP) and dimethylallyl diphosphate (DMAPP) with the aid of overexpression in their respective genes (Roberts 2007). This probably allows for increased synthesis of all the triterpenes. Additionally, specific terpene synthases and OCSs can be modified or overexpressed to either alter or enhance specific terpenoids (Degenhardt et al. 2003).

4.4 Conclusion

Medicinal significance and proven pharmacological properties have generated great attention on *A. aspera*. There have been studies on its various pharmacological properties alongside studying their biosynthetic pathway. Production of selected triterpenoids in differentiated tissues using various chemical elicitors has been investigated. Furthermore, it becomes important to understand the biochemical conversions in light of the metabolic pathways so that we can use this data in maneuvering production of metabolites in *A. aspera*.

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Chapter 5
The Current Status and Future Applications of Hairy Root Cultures

Nisha Dhiman, Vanita Patial, and Amita Bhattacharya

Abstract Hairy roots are produced when the soil phytopathogen, *Agrobacterium rhizogenes*, infects a host plant. Just like normal roots, the hairy roots have the capacity to absorb target elements and produce valuable phytochemicals. Hairy roots have thus been exploited in applications like large-scale production of secondary metabolites and recombinant proteins, upscaling in bioreactors, phytomining and phytoremediation. The hairy roots have industrial applications and are used as important research tool for elucidation of secondary metabolite biosynthetic pathways and also expression and function of key genes and regulatory elements. The status of research conducted till date on hairy roots of medicinally important plants with respect to secondary metabolites production, elicitation, recombinant proteins, genetic manipulation, phytoremediation and phytomining is reviewed in the present chapter.

Keywords *Agrobacterium rhizogenes* · Bioreactor · Elicitation · Heavy metals · Phytoremediation · Recombinant proteins · Secondary metabolites

Abbreviations

ABA	Abscisic acid
ASA	Acetylsalicylic acid
AS	Acetosyringone
BA	Benzyladenine
BAP	6-Benzylamino purine
B5	Gamborg's B5 medium (Gamborg et al. 1968)

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bp	Base pair
Cd	Cadmium
2, 4-D	2, 4-Dichlorophenoxy acetic acid
2, 4-DCP	2, 4-Dichlorophenol
4′-DM6MPTOX	4′-Demethyl-6-methoxy podophyllotoxin
DDT	Dichlorodiphenyltrichloroethane
H_2O_2	Hydrogen peroxide
IAA	Indole-3-acetic acid
IBA	Indole-3-butyric acid
JA	Jasmonic acid
Kn	Kinetin
kb	Kilobase
L-DOPA	L-3, 4-dihydroxyphenylalanine
L	Litre
LS	Linsmaier and Skoog medium (Linsmaier and Skoog 1965)
MES	2-(N-morpholino)ethanesulfonic acid
MPTOX	6-Methoxy podophyllotoxin
MS	Murashige and Skoog medium (Murashige and Skoog 1962)
MSRT	MS + *RT vitamin complex (Khanna and Staba 1968)
NAA	α-Naphthalene acetic acid
Ni	Nickel
$NiSO_4$	Nickel sulphate
MeJa	Methyl jasmonate
mM	Millimole
nM	Nanomole
PCBs	Polychlorinated biphenyls
pM	Picomole
ppm	Parts per million
rpm	Revolutions per minute
SA	Salicylic acid
SH	Schenk and Hildebrandt medium (Schenk and Hildebrandt 1972)
TCE	Trichloroethylene
TDZ	Thidiazuron
TNT	2, 4, 6-Trinitrotoluene
µM	Micromole
UM	Uchimiya and Murashige medium (Uchimiya and Murashige 1974)
U	Uranium
WPM	Woody Plant Medium (Lloyd and McCown 1981)
YE	Yeast extract
YPS	Yeast polysaccharide

5.1 Introduction

Plants are a rich repository of a diverse array of secondary metabolites ranging from indole alkaloids, terpenoids, steroidal compounds, phenolics, saponins, etc. These secondary metabolites range from highly priced essential oils to pigments and compounds of pharmaceutical value. Many among these, have a huge market potential and have been the subject of intense research with respect to their function, biological properties and applicability. Studies have revealed a significantly variable distribution pattern of secondary metabolites among families, genera, species, plant parts and habitats (Sampaio et al. 2016; Zlatić and Stanković 2017). Depending on the mechanism(s) required by the plant for adaptation and defence against biotic and abiotic stresses, secondary metabolites are localized in specific plant parts or are distributed throughout the plant body (Fang et al. 2012; Talamond et al. 2015). While the leaves and flowers of some plants are the only source of novel pigments and essential oils, the underground parts of others are the major sources of secondary metabolites of high pharmaceutical value (Borkataky et al. 2014).

Plants inhabiting extreme climatic conditions including the ones in high altitude locales of different mountains survive various environmental extremes (Körner 2016). Such plants adapt to stressful environments by perennating via their underground parts and accumulate most of their secondary metabolites in the roots and other underground parts like rhizomes, tubers, corms, bulbs, etc. As a result, there have been indiscriminate uprooting of plants with secondary metabolites-rich underground parts. Till date, a large number of plants have become threatened and fall either in the rare, vulnerable, endangered or critically endangered category (Patial et al. 2012; Patel 2015). Therefore, different workers have employed various in situ and ex situ conservation strategies for the conservation of these plants (Chen et al. 2016). Among the different biotechnological methods employed for plant conservation till date, hairy root cultures have played an important role. Hairy root cultures, besides having a high proliferation rate, ensure a stable and continuous production of comparatively homogenous secondary metabolites (Mishra and Ranjan 2008; Pirian et al. 2012). Therefore, small- to large-scale production of important phytochemicals have been achieved in hairy root cultures. A classic example of this is the large-scale production of camptothecin and podophyllotoxin in hairy root cultures by the Swiss company, ROOTec in Witterswil, Switzerland.

5.2 Biology of Hairy Roots

Hairy roots are actually the result of a disease caused by the soil phytopathogen, *Agrobacterium rhizogenes*. The organism was earlier known as *Phytomonas rhizogenes* (Riker et al. 1930). The term ‘hairy root’ was coined in 1900 by Stewart et al. However, it was Ackermann, who first demonstrated its usage in plant transformation in 1973. Detailed biology of *A. rhizogenes* has revealed that the bacterium infects by transferring its ‘T-DNA’ or ‘transferred DNA’ into the nuclear genome of host plants

(Kayser and Quax 2007). The T-DNA is actually a 10–30 kb stretch of DNA present on the 200 kb Ri or root-inducing plasmid housed within the bacterial cell. The T-DNA contains four genetic loci, i.e. *rolA*, *rolB*, *rolC* and *rolD*, and is flanked by 25 bp borders of direct but imperfect repeats (Lee and Gelvin 2008). A complex machinery involving the Ri-plasmid and its (i) transferred DNA with its *rol* genes, (ii) the virulence or the *vir* region and its *vir* genes and (iii) the *chv* genes located on the bacterial chromosomal DNA facilitates the successful transfer of the T-DNA and subsequent infection of a host plant. The process of T-DNA transfer involves seven distinct steps and begins with the induction of *vir* genes by phenolic and sugar compounds secreted by the wounded tissues of plants and ends with the expression of *rol* genes and integration of the T-DNA into the host genome (Hwang et al. 2017). Studies have revealed that each of the *rol* genes, i.e. *rolA*, *rolB* and *rolC*, have distinct function and play important roles in the formation and growth of hairy roots (Pavlova et al. 2014). All the *rol* genes contribute towards the normal growth of hairy roots and are also responsible for the production and accumulation of bioactive compounds. Individually, however, *rolA* is responsible for the formation and growth of roots, *rolB* is involved in root initiation and callus formation, *rolC* is for root growth and *rolB* and *rolD* are for suppression of callus growth. After the integration of T-DNA, the genes encoding the synthesis of opines are activated, while the oncogenes on the T-DNA regulate the biosynthesis of auxins and cytokinins leading to the formation of abundant adventitious roots at the site of infection. Being hairy in appearance, the roots are named as the 'hairy roots' (Nilsson and Olsson 1997). The hairy roots are fast growing and have the ability to produce secondary metabolites that are either comparable to or higher than that of normal roots. Thus, the hairy roots are extensively used as alternative organs of secondary metabolites production.

Till date, hairy roots have been induced in over 100 medicinal plants (Siwach et al. 2013). A diagrammatic representation of the mechanism of hairy root development and their various applications are elucidated in Fig. 5.1.

5.3 Secondary Metabolite Production in Hairy Root Cultures

The hairy roots are generally stable and similar to the roots of wild type plants growing in nature. Hairy roots have thus, been extensively employed for secondary metabolite production. The production of optimal levels of secondary metabolites is, however, governed by several factors. Some of the important ones include culture medium and its composition like nitrogen source, sucrose concentration, plant growth hormones, culture growth conditions like light, temperature, relative humidity and type of culture vessel, use of elicitors and precursors, precursor feeding, cell permeabilization, plant species, and *A. rhizogenes* strain, etc. In addition to these, key biosynthetic pathway genes have been manipulated to increase the yield of secondary metabolites. All these have affected the growth and biomass of hairy roots and in turn the secondary metabolite production. These have been summarized in Tables 5.1 and 5.2.

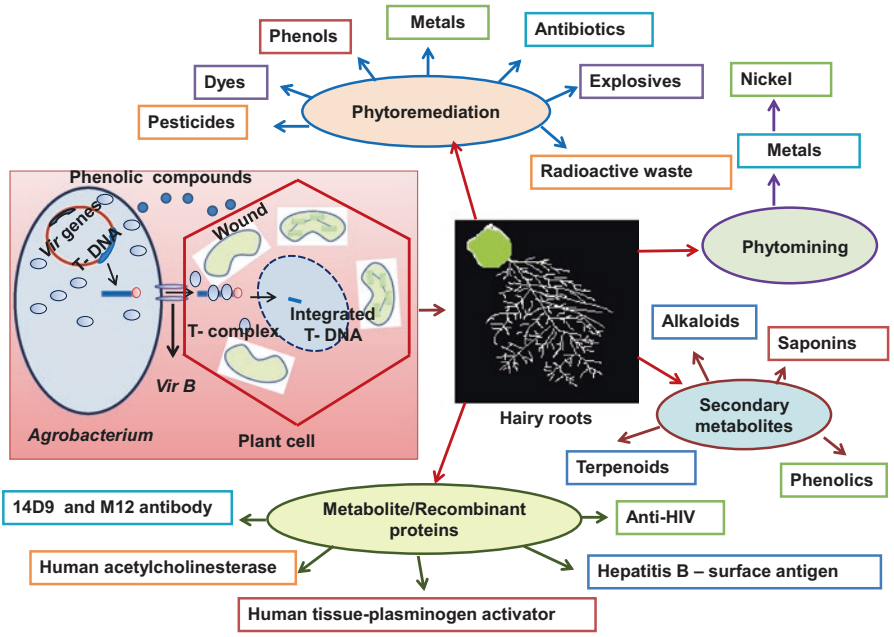

Fig. 5.1 Schematic representation of the mechanism of hairy root development and its applications

Culture medium has a strong influence on the hairy root growth and secondary metabolite production. This is evident from the various media that have been used for the induction and growth of hairy roots in different plant species. Even for the same species, the induction and growth of hairy roots and subsequent secondary metabolite production require different types of media. For example, different workers have reported the development of hairy root cultures of *A. annua* for artemisinin production (Weathers et al. 2005; Mannan et al. 2008; Patra et al. 2013). However, the difference in the process employed by each worker has been significant. Weathers et al. (2005) employed B5 medium supplemented with 3% sucrose for highest yield, whereas MS medium was preferred by Patra et al. (2013) and Mannan et al. (2008). While the former developed hairy root cultures on MS medium for artemisinin production, the latter improved the production by fortifying the MS medium with casein acid hydrolysate precursors. Growing of hairy root cultures of the well-known medicinal herb, *Atropa belladonna*, for the production of atropine, hyoscyamine and scopolamine (widely used in the treatment of rheumatism, sciatica and neuralgia) is another example.

As stated above, the strain of *A. rhizogenes* proved to be a crucial factor governing the induction and growth of hairy roots in a number of plant species (Ondrej and Protiva 1987; Jung and Tepfer 1987; Sharp and Doran 1990; Chashmi et al. 2010; Yang et al. 2011). Besides the *A. rhizogenes* strain C58C1, liquid MS medium, the

Table 5.1 Summary of plants transformed with different strains of *Agrobacterium rhizogenes* for the development of hairy roots

Plant species	Explant	Secondary metabolite	Strain	Method	Culture conditions	Media + PGR + sucrose			Remarks	Reference
						Induction	Maintenance	Regeneration		
Actinidia deliciosa	Hypocotyl	Mikimopine	NIAES 1724	Shaking	Cocultivation for 2 days	½ MS (Murashige and Skoog 1962) in dark	½ MS in dark	½ MS in light	Shorter internodes, darker-wrinkled leaves and active roots of regenerated plants	Yazawa et al. (1995)
Agastache rugosa	Leaves and stem	Rosmarinic acid	R1000	Dipping in the bacterial culture	Cocultivation for 2 days	MS + 200 mg/l timetin +3% sucrose	MS + 3% sucrose at 25 °C and 100 rpm in dark	–	116 mg/g dry wt. rosmarinic acid	Lee et al. (2008)
Ajuga multiflora	Petiole	20-hydroxyecdysone	A4	Infection of cut surface with bacteria	Cocultivation for 1 day	½ MS + 3% sucrose +500 mg/l cefotaxime in dark	½ MS + 3% sucrose at 25 °C in dark	–	Ten times higher 20-hydroxyecdysone content	Kim et al. (2005)
Ajuga reptans var. *atropurpurea*	Leaf disc	Phytoecdysteroids, 20-hydroxyecdysone, norcyasterone B, cyasterone and isocyasterone	MAFF 03-01724	Soaking for 5 min in 10 X 10^8/ml bacterial culture for 5 min	Cocultivation for 3 days	MS + 0.2% gellan gum +500 µg/ml carbenicillin +500 µg/ml vancomycin	Liquid MS at 25 °C and 120 rpm in dark	-	Biomass of AR-4 hairy root line increased by 230 times in airlift reactor The 20-hydroxyecdysone content was four times higher (0.12% on dry wt. basis)	Matsumoto and Tanaka (1991)
A. reptans var. *atropurpurea*	Plantlet	Phytoecdysone	Do	Do	Do	Do	Liquid MS at 25 °C in dark and 120 rpm	–	Phytoecdysone was incorporated with acetate and cholesterol	Nagakari et al. (1994)
Ambrosia maritima	Plantlet	Thiarubrine A and its epoxide, thiarubrine A diol and precursor pentayneene	ATCC 15834	Infection of cut surface with bacteria	–	MS; MS + 0.5 g/l claforan	MS	–	Maximum yield of thiarubrine A diol and 9.6 times higher yield of pentayneene	Zid and Orihara (2005)

Ammi majus	Stem and leaves	Umbelliferone	A4, LBA9402 and ATCC 15834	Inoculation with bacterial culture		MS in dark	MS + 3% sucrose at 22 °C and 110 rpm	–	Hairy roots with umbelliferone content equivalent to seeds and higher than cell suspension	Królicka et al. (2001)
Arnebia hispidissima	Shoot tip, leaf, nodal and internodal segments	Shikonin	A4	Immersion in bacterial culture with shaking	Cocultivation for 5 days	MS + 2.0 mg/l IBA + 250 mg/l cefotaxime at 25 °C in dark	MS	–	Hairy roots with shikonin content (0.85 mg/g fresh wt.) of tissues after 50 days of culture	Chaudhury and Pal (2010)
Armoracia lapathifolia	Leaf disc.	–	A4	Immersion for 10 min in 10^9 cells per ml of bacterial culture with shaking	Cocultivation for 3 days	MS + 3% sucrose +500 mg/l carbenicillin +200 mg/l vancomycin at 25 °C and light	MS + 3% agar at 25 °C in dark	MS + 3% sucrose at 25 °C and light	Hairy root cultures and plant regeneration	Noda et al. (1987)
Artemisia annua	Leaf blade and petiole	Artemisinin and stigmasterol	1601	–	–	–	Liquid MS +100 µg/ml kanamycin +3% sucrose	Liquid MS + 100 µg/ml kanamycin at 25 °C and 110 rpm in light	Artemisinin production (0.54% on dry wt. basis) and 201 times higher stigmasterol (108.3% on dry wt. basis)	Xie et al. (2000)
A. annua	Stem	Artemisinin	LBA 9402, 8196, A4 1601	Wounding, direct infection or injection	–	White's + 3% sucrose at 25 °C in dark	B5 + 3% at 25 °C and 100 rpm	–	Higher artemisinin production in 2-ip supplemented media than control	Weathers et al. (2005)
A. annua	Apical meristem	Artemisinin	LBA 301	Cut ends dipped in bacterial inoculum for 20 minutes	2 days of cocultivation in dark	MS + 500 mg/l cefotaxime +50 µg/l casein acid hydrolysate precursors +500 µg/l sodium acetate +40 µg/l MeJa	MS	–	Maximum artemisinin content (3.45 mg/g) after 15 days	Patra et al. (2013)

(continued)

Table 5.1 (continued)

Plant species	Explant	Secondary metabolite	Strain	Method	Culture conditions	Media + PGR + sucrose			Remarks	Reference
						Induction	Maintenance	Regeneration		
A. dubia and *A. indica*	Stem portions of seedlings	Artemisinin	LBA 9402 and 8196	Dipping for 20 min in 0.8 OD at 600 nm bacterial culture containing 100 µM acetosyringone	Cocultivation for 3 days on MS + 100 µM acetosyringone	MS + 500 µg/ml cefotaxime for 10 days	½ MS	–	Highest root biomass (3.9 g on fresh wt. basis) and artemisinin content (0.042%)	Mannan et al. (2008)
A. pallens	Leaf and stem	Artesunate	NCIM 5140	Immersion for 30 min and shaking at 90 rpm and 30 °C	Cocultivation for 3 days on basal MS	Basal MS + 400 mg/l cefotaxime	½ MS + 0.5 mg/l BA or Kn at 26 °C in dark	–	Hairy roots with twofold higher artesunate content as compared to aerial parts	Pala et al. (2016)
A. acuminata	Young leaves	Atropine and scopolamine	LBA 9402	Pricking and immersion in bacterial culture of O.D. 0.9–1.0 at 600 nm	Cocultivation for 3 days on MS at 25 °C in dark	MS + 3% sucrose +1.0 g/l cephalexin	Liquid ½ MS + 3% sucrose +1.0 g/l cephalexin at 80 rpm	–	First use of DART technique for chemical profiling of hairy roots and successful structural confirmation of two alkaloids	Banerjee et al. (2008)
Atropa belladonna	Seed hypocotyls	Alkaloids	8196 and C58C1 harbouring pRiA4b	Injection of hypocotyl with bacterial culture	Cocultivation for 4 weeks on MS + 2% sucrose	MS + 2% sucrose +0.5 mg/l ticarpen	MS + 2% sucrose + BAP in dark	–	Alkaloid synthesis in hairy roots transformed with A4 strain	Ondrej and Protiva (1987)
A. belladonna and *Calystegia sepium*	Stems	Cuscohygrine, atropine, hyoscyamine and scopolamine	A4 and 8196	Cut surface of stems touched with bacterial culture	–	MS + 0.5 g/l carbenicillin	MS + 0.5 g/l carbenicillin at 100 rpm	–	Increased biomass and tropane alkaloids synthesis	Jung and Tepfer (1987)

A. belladonna	Seedlings	Atropine	A4 and TR105	Wounding with syringe	Cocultivation at 25 °C	MS + 3% sucrose +200 mg/l cefotaxime	MS + 3% sucrose at 25 °C and 90 rpm in 16 h light	–	Atropine from substrate (1.4 mg/g in shake flasks and 0.46 mg/g in airlift reactor) Atropine levels higher in reactor grown roots (0.37% dry wt.) as compared to roots from shake flasks (0.25% dry wt.)	Sharp and Doran (1990)
A. belladonna	Leaves	Scopolamine and hyoscyamine	AR15834	Direct infection	Incubation at 28 °C	MS + 200 mg/l cefotaxime	MS + nitrate (0, 15, 35 and 95 mM) in dark at 27 °C and 110 rpm	–	Decreased hairy root growth at increased nitrate concentration but 3–20 times more alkaloid at 35 mM KNO_3	Chashmi et al. (2010)
A. belladonna	Leaf discs	Hyoscyamine and scopolamine	*A. tumefaciens* C58C1 carrying *pmt* encoding N-methyltransferase and *h6 h* encoding hyoscyamine 6-hydroxylase in pRiA4 pXI and A4	Inoculation with bacterial culture	Incubation at 25 °C	½ MS + 3% sucrose +250 mg/l carbenicillin +50 mg/l kanamycin at 25 °C in dark	Liquid MS + 3% sucrose at 25 °C and 100 rpm in dark	–	Overexpression of key genes encoding putrescine biosynthesis	Yang et al. (2011)
Azadirachta indica	Leaves, stem and callus	Azadirachtin, nimbin, salannin, 3-acetyl-1-tigloylazadirachtinin, 3-tigloylazadirachtol	LBA9402	Scratching with sterile needle dipped in bacterial culture	Cocultivation for 3 days at 25 °C	MS + 200 mg/l ampicillin; Liquid MS + 100 mg/l ampicillin	Liquid MS	–	100-fold increased biomass; Azadirachtin, nimbin, salannin, 3-acetyl-1-tiglolazadirachtinin and 3-tigloylazadirachtol detected in hairy roots	Allan et al. (2002)

(continued)

Table 5.1 (continued)

Plant species	Explant	Secondary metabolite	Strain	Method	Culture conditions	Media + PGR + sucrose			Remarks	Reference
						Induction	Maintenance	Regeneration		
Beta vulgaris and *Nicotiana rustica*	6–8 weeks old plants	Nicotine, β-cyanins and β-xanthins	LBA940	Wounding with hypodermic needle dipped in bacterial culture	–	B5 (Gamborg et al. 1968) + 3% sucrose +0.5 g/l ampicillin	Liquid B5 + 3% sucrose at 90 rpm	–	Betalains and nicotine alkaloids in hairy roots and medium, respectively	Hamill et al. (1986)
B. vulgaris and *Tagetes patula*	Hypocotyl	Betalaine and thiophene	LMG 150 and 63	Infection with bacterial culture and	Dark	MS + 3% sucrose in dark	Liquid MS + 3% sucrose at 25 °C and 90 rpm in dark. Treatment of hairy roots with	–	Increased thiophene content in hairy roots treated with *Haematococcus pluvialis* and *Spirulina platensis* on day 20 but betalain content on day 15 and 25 with *H. pluvialis* and *S. platensis*, respectively	Rao et al. (2001)
B. vulgaris	Leaves	Betacyanin and betaxanthin	ATCC 15834	Immersion in bacterial inoculum	Cocultivation in dark for 3 days at 26 °C and 11 rad/s	MS+ 3% sucrose +5.5 g/l agar +0.25 g/l claforan	MS+ 3% sucrose +5.5 g/l agar at 26 °C and dark	–	Total pigment production (13.27 mg/g dry wt.)	Pavlov et al. (2002)
B. vulgaris	do	do	do	do	do	do	do	–	Temporary immersion suitable for cultivation of hairy roots	Pavlov and Bley (2006)
Brugmansia candida	Seedling	Hyoscyamine and scopolamine	LBA 9402	Direct infection	–	MS + *RT vitamin complex (Khanna and Staba 1968) + 3% sucrose +1.0 g/l cefotaxime at 24 °C and 16 h light	Liquid MSRT +3% sucrose at 24 °C, 100 rpm and 16 h light	–	Alkaloid production biased towards scopolamine	Pitta-alvarez and Giulietti (1995)

B. candida	do	do	do	do	–	do	½ strength B5 + 15 g/l sucrose at 24 °C, 100 rpm and 16 h light	–	Twofold increase in scopolamine and fourfold increase in hyoscyamine	Pitta-Alvarez and Giulietti (1998)
B. candida	do	do	do	do	–	do	½ strength B5 + 15 g/l sucrose + JA or 25 and 250 mM $AlCl_3$ at 24 °C and 100 rpm in 16 h light	–	Increased accumulation of hyoscyamine (1200%) and scopolamine (30%). Increased accumulation of scopolamine and hyoscyamine (43–83%) by $AlCl_3$. Increased release of scopolamine (150%) in the medium	Spollansky et al. (2000)
B. candida	Leaves	Uncommon polyamines like cadaverine, putrescine, spermidine, spermine	LBA 9402	do	–	do	do	–	Detection of cadaverine in the hairy roots for the first time	Carrizo et al. (2001)
Catharanthus roseus	Seedling	Ajmalicine, serpentine, catharanthine and vindoline	ATCC 15834	Wounding and inoculation stem tips	Cocultivation for 12–24 h	½ B5 + 3% sucrose +6 g/l agar +0.25 g/l cefotaxime at 26 °C in dark	Liquid half-strength B5 + 3% sucrose at 26 °C and 110 rpm in dark	–	Ajmalicine, serpentine, catharanthine and vindoline levels in hairy roots equivalent to that in cell suspension	Bhadra et al. (1993)
C. roseus	Hypocotyl	Catharanthine	ATCC 15834	–	–	SH (Schenk and Hilderbrandt 1972) at 25 °C in dark	SH + 1.0 mg/l NAA + 0.1 mg/l Kn at 25 °C and 100 rpm in dark	–	Catharanthine (1.5 mg/g on dry wt. basis)	Jung et al. (1995)

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Table 5.1 (continued)

Plant species	Explant	Secondary metabolite	Strain	Method	Culture conditions	Media + PGR + sucrose			Remarks	Reference
						Induction	Maintenance	Regeneration		
C. roseus	Leaves	Alkaloids	R1000 and LBA 4404 carrying the plasmids pBI121/GUS and pBI121/DAT	Wounding and infection	Cocultivation for 2 days	MS + 400 ppm cefotaxime	½ strength B5 + 2% sucrose at 25 °C and 100 rpm in dark	–	Enhanced activity of deacetylvindoline-4-O-acetyltransferase	Magnotta et al. (2007)
Chaenactis douglasii	Petiole	Thiarubrine A and B	TR7	Cut surface infected with bacterial culture	–	SH + 0.2 mg/l vancomycin +0.5 mg/l carbenicillin +0.3 mg/l cefotaxime	Liquid SH at 25 °C and 100 rpm in dark	–	Enhanced levels of antifungal polyines and thiarubrines	Constabel and Towers (1988)
Cinchona ledgeriana	Shoot	Quinoline alkaloids	LBA9402 and R1000	Wounding with hypodermic needle and 5 to 10 µl of bacterial culture	–	Full or ½ B5 + 0.5 g/l ampicillin +3% sucrose	Full or ½ B5 + 3% sucrose	–	Maximum alkaloids (50 µg/g fresh wt.) after 45 days	Hamill et al. (1989)
Cinchona officinalis	Leaves	Tryptamine and strictosidine	LBA 9402	Dipping in bacterial inoculum	12 h light at 28 °C	1/4 strength B5 macrosalts, 50 mg/l L-cysteine +3% sucrose +100 µg/ ml augmentin	1/4 strength B5 at 25 °C	–	Tryptamine (1200 mg/g) and strictosidine (1950 mg/g)	Geerlings et al. (1999)
Cichorium intybus	Leaves	Sesquiterpene lactones and their glycosides	LBA 9402	–	–	Liquid MS + ½ macroelements +3% sucrose +500 mg/l cefotaxime at 25 °C and 110 rpm in 16 h light	Liquid MS + ½ macroelements +3% sucrose at 110 rpm and 16 h light at 25 °C	–	8-desoxylactucin yield reached 0.03 g/l at early stationary phase	Malarz et al. (2002)
Coleus forskohlii	Axenic plantlets	Forskolin	MAFF 03–01724	Wounding of newly cut surface with hypodermic needle	–	Woody plant medium (WPM) (Lloyd and McCown 1981) + 3% sucrose +0.5 g/l claforan	WP, MS and B5 + 3% sucrose at 25 °C in dark with 100 rpm	–	Highest forskolin yield (1.6 mg/100 ml flask) after 5 weeks on WPM medium	Sasaki et al. (1998)
C. *forskohlii*	Leaf and stem	Forskolin	A4, ATCC18534, MTCC533	Infection of cut/ wounded surface	Cocultivation for 2 days	MS + 250 mg/ml cefotaxime	Basal MS in fermentor at 21/18 °C under 16 h light	–	Confirmation of forskolin production in hairy roots	Maheswari et al. (2011)

Coluria geoides	Shoots	Polyprenols	LBA 9402	–	–	–	Liquid B5 + sucrose or different concentrations of glucose (0.25–1.5%) at 22 °C and 105 rpm in dark	–	Polyprenols yield (approx. 300 pg/g dry wt.)	Skorupińska-Tudek et al. (2000)
Cucumis melo	Cotyledons	Volatile compounds (*Z*)-3-hexenol, (*E*)-2-hexenal, 1-nonanol, and (*Z*)-6-nonenol	MAFF 03–01724 harbouring pRi1724	Dipping for 10 min in bacterial inoculum		Agar gelled MS + 0.5 g/l carbenicillin +0.1 g/l cefotaxime at 26 °C in continuous light	Liquid MS at 100 rpm	–	Higher volatiles production in hairy roots as compared to ripe melon fruits	Matsuda et al. (2000)
Daucus carota	Leaves	Peroxidase	15,834	Infection with hypodermic needle	–	MS + 3% sucrose +0.5 mg/ ml carbenicillin	MS + 3% sucrose	–	High peroxidase activity (19.2 UK-fresh cell wt.)	Kim and Yoo (1996)
Datura quercifolia	Stem	Hyoscyamine	LBA 9402	Inoculation with the bacterial culture	–	3/2 B5 + 0.25 g/l cefotaxime +1 g/l ampicillin	3/2 B5	–	Hyoscyamine (1.24% on dry wt. basis)	Dupraz et al. (1994)
Datura stramonium	Leaves	Hyoscyamine	LBA9402	Direct infection	–	B5 + 3% sucrose at 26 °C in 16 h light	B5 + 3% sucrose at 26 °C in 16 h light	–	Hyoscyamine (0.3% on dry wt. basis)	Payne et al. (1987)
D. stramonium and *Hyoscyamus niger*	Hypocotyl	Hyoscyamine and scopolamine	ATCC 15834	Wounding with sterile needle	25°c in continuous light	MS + 1.0 g/l carbenicillin	Liquid MS in dark	–	Increase in scopolamine content (0.56% on dry wt. basis)	Jaziri et al. (1988)
Duboisia leichhardtii	Leaf discs and stem segment	Scopolamine and hyoscyamine	ATCC 5834 and A4	Wounding with sharp scalpel and immersion in bacterial culture	–	–	Liquid Heller's medium (Heller 1953) at 25 °C in dark	–	Twofold more scopolamine in hairy roots than leaves	Mano et al. (1989)
D. myoporoides	In vitro shoots	Scopolamine and hyoscyamine	HRI	Infection using sterile tooth-pick	–	Linsmaier and Skoog medium (LS) (Linsmaier and Skoog 1965)	Liquid LS + 25 mg/l at 25 °C and 100 rpm in dark	–	Hyoscyamine content higher than control but lower scopolamine content	Deno et al. (1987)

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Table 5.1 (continued)

Plant species	Explant	Secondary metabolite	Strain	Method	Culture conditions	Media + PGR + sucrose			Remarks	Reference
						Induction	Maintenance	Regeneration		
Echinacea purpurea	Cotyledons	Polysaccharides and phenolic compounds	A4, R1601 and R1000	Immersion in bacterial culture	–	MS + 500 mg/l cefotaxime	Liquid MS at 28 °C and 16 h light (2000 lux)	–	Polysaccharides and phenolic compounds (236.0 and 18.9 mg/g dry wt., respectively)	Wang et al. (2006a)
Fagopyrum esculentum	Hypocotyl segments	(+)-Catechins, (−)- epicatechins, (−)-epicatechin-3-0-gallate, procyanidin B2 and procyanidin BZ3–0-gallate	15,834	–	–	Liquid B5	Agar gelled B5 + 0.3 g/l carbenicillin	–	Highest content of procyanidin B2-3′-O-gallate	Trotin et al. (1993)
F. esculentum	Leaves	Rutin	R1000	Dipping in bacterial culture of 1.0 O.D. at 600 nm	Cocultivation for 2 days	MS+ 3% sucrose +500 mg/l carbenicillin	MS + 3% sucrose	–	Highest biomass (378 mg dry wt. per 30 ml flask) and rutin yield (1.4 mg/g dry wt.) in clone H8	Lee et al. (2007)
Gentiana acaulis, *G. cruciata*, *G. lutea* and *G. purpurea*	In vitro shoots	–	ATCC 15834 and A4M70GUS	Dipping or smearing of cut surface with inoculum after wounding	YEB + 100 mg/l neomycin at 28 °C and 220 rpm	MS or WPM macronutrients + MS micronutrients + LS vitamins +3% sucrose +200 mg/L cefotaxime	MS or WPM macronutrients + MS micronutrients + LS vitamins +3% sucrose	WPM or BM + 0.1–4.0 mg/l Kn	Stable genetic transformation and plant regeneration	Momčilović´ et al. (1997)
G. scabra	Leaves	Loganic acid, swertiamarin and gentiopicroside	ATCC15834	Immersion	Cocultivation for 2 days	MS + 100 mg/l cefotaxime	WPM + 3% sucrose +0.3% gelrite at 25 °C in dark	–	Enhanced contents of loganic acid, swertiamarin and gentiopicroside	Huang et al. (2014)

Geranium thunbergii	Petioles	1,2,3,4,6-penta-O-galloyl-β-D-glucose, tannins, gallic acid, ellagic acid, (+)-catechin, β-glucogallin, 1,6-di-O-, 1,2,3,6-tetra-O-, 1,2,3,4,6-penta-O-galloyl- β -D-glucoses, corilagin and geraniin	A4	Smearing of cut surface with inoculum	–	½ MS + 2.0 g/l gelrite + 0.5 mg/l claforan at 25 °C in dark	½ MS + 3% sucrose and also B5 + 2% sucrose at 25 °C in dark at 100 rpm	–	Major yield of 1,2,3,4,6-penta-O-galloyl-β-D-glucose in MS and geraniin in liquid B5	Ishimaru and Shimomura (1991)
Gloriosa superba	Tubers and callus	Colchicine and colchicoside	MTCC 2364	Immersion in bacterial inoculum	Cocultivation for 2 days	MS + 1%mannitol +250 mg/l cefotaxime at 25 °C in dark	MS + 250 mg/l cefotaxime	–	Production of colchicines and colchicoside in the hairy roots	Bai and Agastian (2013)
Hyoscyamus albus	Leaf discs	Tropane alkaloids	A4	Coculture of leaf discs	–	MS + 0.5 g/l claforan	Liquid WPM + 3% sucrose at 25 °C and I00 rpm in dark	–	Enhanced growth and yield of hyoscyamine, 6 β-hydroxyl-hyoscyamine, scopolamine, 7 β-hydroxy hyoscyamine and littorine in B5 medium containing Cu^{2}+ ions	Christen et al. (1992)
H. muticus	Leaf segment	Hyoscyamine	A4, LBA 9402 and ATCC15834	Infection by wounding with a needle	–	B5, LSO or LSA + 50 µM AS	Liquid LS, B5 and W63 + 100 mg/l ampicillin +500 mg/l claforan	–	Maximum hyoscyamine accumulation in hairy roots transformed with LBA 9402	Oksman-Caldentey et al. (1989)

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Table 5.1 (continued)

Plant species	Explant	Secondary metabolite	Strain	Method	Culture conditions	Media + PGR + sucrose			Remarks	Reference
						Induction	Maintenance	Regeneration		
H. muticus	Leaf of seedlings	Tropane alkaloids	ATCC 15834	Direct infection	–	½ MS + 3% sucrose +1.0 g/l claforan	Liquid ½ MS + 3% sucrose at 25 °C and 100 rpm in dark	–	6β- and 7 β-hydroxy hyoscyamine, scopolamine, hyoscyamine and littorine	Jaziri et al. (1994)
H. niger	Leaves	Scopolamine	A4 and LBA 9402	Direct infection	–	½ B5 + 3% sucrose +100 µg/ml kanamycin +500 µg/ml cefotaxime	Liquid ½ B5 + 3% sucrose at 26 °C and 100 rpm in dark	–	Significantly higher levels of scopolamine in either of *pmt* and *h6 h* expressing hairy root lines	Zhang et al. (2004a, b)
Isatis tinctoria	Petioles	Flavonoids	LBA 9402	Immersion in bacterial culture	Cocultivation on ½ MS + 1 mM arginine +125 µM AS for 2 days at 25 °C in dark	½ MS + 300 mg/l cefotaxim at 25 °C in dark	½ MS + 3% sucrose at 25 °C in dark	–	Highest total flavonoid accumulation (438.10 µg/g dry wt.) in 24 days old hairy roots	Gai et al. (2015)
Lactuca virosa	Leaves	Sesquiterpene lactones	LBA 9402	–	½ MS + 3% sucrose	Liquid MS with ½ macronutrients +3% sucrose at 110 rpm in dark	–	NA	Eight sesquiterpene lactones, six glycoside derivatives, stigmasterol triterpenes and their acetates	Kisiel et al. (1995)
L. virosa	Leaves	Crepidiaside B and lactuside A (sesquiterpene lactones)	LBA 9402	–	–	–	MS with ½ macroelements +3% sucrose at 110 rpm in dark	–	60% higher crepidiaside B in hairy roots and 27% higher lactuside A after 48 h	Malarz and Kisiel (1999)

Lippia dulcis	Stem	Terpenoids	A4	Direct infection	–	MS + 0.5 mg/l claforan	Liquid MS + 2% sucrose at 25 °C in dark or 16 h light and 100 rpm; auxins or 0.2–10.0 mg/l chitosan	–	Accumulation of hernandulcin and 20 other monoterpenes in green hairy roots under light Fivefold enhanced production of hernandulcin by chitosan	Sauerwein et al. (1991)
Lithospermum erythrorhizon	Shoot	Shikonin	ATCC 15834	Direct infection	–	MS + 1.0 g/l carbenicillin	Root culture (RC) (EMBO course 1982) + 3% sucrose at 25 °C and 100 rpm in dark in airlift fermenter equipped with XAD-2 column	–	Threefold enhanced and continuous production of shikonin (5 mg/day) for more than 220 days after addition of adsorbents	Shimomura et al. (1991)
Lobelia inflata	Stems	Lobetyolin, lohetyol	ATCC 15834	Smearing of inoculum on cut surface	–	MS at 25 ° C and 16 h light	Liquid MS at 25 °C in dark and 80 rpm	NA	Lobeline content in hairy roots equivalent to that in normal roots	Yonemitsu et al. (1990)
L. inflata	Plants	Lobeline	R1601	Microinjection	–	MS or B5 salts and vitamins +2% sucrose or + 250 mg/l cefotaxime +1.0 g/l ampicillin	B5 + 0.2–20.0 mg/l IAA/ NAA + 0.2–5.0 mg/l Kn at 24 °C in dark	–	Maximum amount of lobeline at 0.2 mg/l IAA	Bálványos et al. (2001)
Medicago sativa	Stem	–	NCPPB 1855	Direct injection with hypodermic syringe/ wounding	–	Liquid MS + 1.0 mg/ml carbenicillin	UM (Uchimiya and Murashige 1974) + 2.0–5.0 mg/l 2,4-D + 0.25 mg/1 Kn	MS + 2% sucrose	Hairy roots with altered phenotype and plantlet regeneration from transformed calli	Spano et al. (1987)
Mitragyna speciosa	Stem and leaves	Mitragynine	ATCC 15834	Wounding +30 min immersion in bacterial suspension	Cocultivation for 3 days	WPM + 500 mg/l cefotaxime disodium at 25 °C in dark	Liquid WPM at 25 °C in dark with 80 rpm	WPM + 0.5 mg/l NAA at 80 rpm	High contents of mitragynine in regenerated plants	Phongprueksapattana et al. (2008)

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Table 5.1 (continued)

Plant species	Explant	Secondary metabolite	Strain	Method	Culture conditions	Media + PGR + sucrose			Remarks	Reference
						Induction	Maintenance	Regeneration		
Nicotiana cavicola, *N. velutinu*, *N. hesperis*, *N. africana* and *N. umbrarica*	Plantlets	Alkaloids	LBA9402	Wounding with hypodermic needle and 5–10 µl of bacterial culture	–	B5 + 3% sucrose and 0.5 g/l ampicillin	B5 + 3% sucrose at 90 rpm and continuous dim light	–	Alkaloids synthesis in the hairy roots and release in the media	Parr and Hamill (1987)
N. hesperis	Plantlets	Anabasine	LBA9402	do	do	B5 + 80 mM sucrose +0.25 g/l ampicillin	B5 + 80 mM sucrose at 25 °C and 90 rpm in dim light	–	Stimulation of anabasine production	Walton and Belshaw (1988)
N. rustica	do	Alkaloid	LBA9402	Puncturing and infecting with bacterial culture	–	MS + 2% sucrose	B5 + 80 mM sucrose +50 mM MES	–	Increased nicotinic acid altered the accumulation pattern of alkaloids	Robins et al. (1987)
Nicotiana rustica	do	Anabasine	do	do	do	do	B5 + 80 mM sucrose +0.67 mM ampicillin +50 mM MES at 25 °C and 90 rpm in 300 lux light	–	1–10 mM cadaverine stimulated the production of anabasine in hairy root cultures	Walton et al. (1988)
Ocimum basilicum	Leaf discs	Rosmarinic acid, lithospermic acid and lithospermic acid B	ATCC 15834 and MAFF 03-01724	Inoculation with the bacterium	Coculture for 2 days in dark at 100 rpm	1/2 strength MS + 0.5 mg /ml claforan	1/2 strength MS in dark	–	Production of rosmarinic acid (14.1% dry wt.), lithospermic acid (1.70% dry wt.) and lithospermic acid B (0.17% dry wt.) by J-1	Tada et al. (1996)
Ophiorrhiza pumila	Stem	Camptothecin	ATCC 15834	Scratching	–	B5 + 2% sucrose +200 mg/l cefotaxime	Liquid B5 + 2% sucrose +200 mg/l cefotaxime +2% sucrose at 25 °C and 60 rpm in light	–	Emergence of hairy roots after 80 days with camptothecin production (0.1% per dry wt.)	Saito et al. (2001)

O. pumila	Stem	Camptothecin (monoterpenoid indole alkaloid)	do	do	do	do	B5 + 2% sucrose at 25 °C and 60 rpm in dark	–	Coordinated regulation of two camptothecin biosynthesis genes (*OpSTR* and *OpTDC*) resulted in accumulation of monoterpenoid indole alkaloids in hairy roots	Yamazaki et al. (2003)
Panax ginseng	Root callus	Saponins	A4	Immersion bacterial culture for 15 h at 25 °C		MS + 250 mg/l vancomycin, 200 mg/l carbenicillin +200 mg/l tetracycline at 25 °C	Liquid MS at 25 °C and 140 rpm in dark	–	Up to 2.4 times higher accumulation of saponins and ginsenosides in hairy roots	Yoshikawa and Furuya (1987)
P. ginseng	Root callus	do	do	do	do	do	Liquid MS +2.0 mg/l IBA + 0.1 mg/l Kn at 145 rpm for 3 weeks	–	Biotransformed hairy roots with abilities for glycosylation and malonylation for 18β-glycyrrhetinic acid synthesis	Asada et al. (1993)
P. ginseng	Callus derived from stem	Ginsenosides	GV3101 strains harbouring *rol* genes and A4	Immersion in bacterial culture	Cocultivation for two days	MS + 4-chlorophenoxyacetic acid +500 mg/l cefotaxime	Liquid MS + 4-chlorophen-oxyacetic acid +250 mg/l cefotaxim +100 mg/l kanamycin sulphate at 25 °C and 100 rpm in dark	–	Increased ginsenoside contents in hairy roots expressing *rolC*	Bulgakov et al. (1998)
P. ginseng	Root segments	Ginsenosides	KCTC 2703	Infection of cut surfaces	Cocultivation for 2–3 days at 28 °C for 16 h	MS + 3% sucrose +300–500 mg/l claforan	Liquid MS + 3% sucrose at 25 °C and 100 rpm in dark	–	Improved production of total ginsenoside at 1.0 mg/l Ja but low production due to 300 mg/l peptone	Yu et al. (2000)

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Table 5.1 (continued)

Plant species	Explant	Secondary metabolite	Strain	Method	Culture conditions	Media + PGR + sucrose			Remarks	Reference
						Induction	Maintenance	Regeneration		
P. ginseng	Root discs	Ginsenosides	A4	Wounding and infection with bacterial culture	Cocultivation for 2 days	SH + 3% sucrose +500 mg/l cefotaxime	Liquid SH at 26 °C and 100 rpm in dark	–	Highest ginsenoside production in HRM root lines and variations in morphotypes	Mallol et al. (2001)
P. ginseng	Roots, stem and leaves	Ginsenosides	KCTC 2703	Cut and infected with bacteria	Overnight cocultivation	½ MS + 300 mg/l cefotaxime	MS + 2.0 mg/l NAA + 3% sucrose	–	Highest production of ginsenosides at 2.0 mg/l Ja	Yu et al. (2003)
P. japonicus	Root	Saponins	15,834	Infection of cut surfaces	Cocultivation for 2 days	MS + 200 mg/l cefotaxime	MS at 21 °C and 110 rpm in dark	–	Maximum saponin yield on 30th day of growth	Zhou et al. (1999)
P. vietnamensis	Shoots	Majonoside R2 (a dammarane saponin)	ATCC 15834, ICPB TR7, ICPB TR107	Wounding with a sterile needle	Cocultivation for 2 days	½ MS + 3% sucrose +250 mg/l cefotaxime	Liquid SH + 3% sucrose at 25 °C and 100 rpm in dark	–	Recovery of trace amounts of majonoside but large amounts of ocotillol saponins, pseudoginsenoside F11 and vinaginsenoside R1	Ha et al. (2016)
Perezia cuernavacana	Internode	Perezone (sesquiterpene quinone)	AR12	Infection of basal extremes of each internode	Cocultivation for 2 days	MS + 8.0 g/l agar +300 mg/l cefotaxime for 48 h in dark	Liquid MS + 3% sucrose +5 mM MES + 150 mg/l ascorbic acid	–	IR spectroscopy revealed the production of perezone	Arellano et al. (1996)
Picrorhiza kurroa	Leaf stem and root	Kutkoside and picroside I	LBA9402 and A4	Pricking	Cocultivation for 2 days	MS + 2.0 mg/l BAP + 0.1 mg/l NAA + 1.0 g/l cephalaxin	½ and full strength B5 + 3% sucrose	–	Relative transformation efficiency of leaf (66.7%) and root (8.76%) with LBA9402 strain	Verma et al. (2007)

P. kurroa	Leaf discs	Kutkoside and picroside I	LBA 9402	Pricking	Cocultivation for 2 days	MS + 2.0 mg/l BAP + 0.1 mg/l NAA + 1.0 g/l cephalexin	Liquid B5 + 3% sucrose at 90 rpm	–	1.1 and 1.3 times higher kutkoside and picroside I in ½ B5 as compared to MS medium	Verma et al. (2015)
P. kurroa	Shoot tip, Leaf and internode	Picrotin and picrotoxinin	A 4 and PAT 405	Wounding with sterile needles and dipping	Cocultivation for 2 days	MS + 250 mg/l carbenicillin	Liquid ½ MS + 3% sucrose at 25 °C with 90 rpm	–	Highest picrotin and picrotoxinin content (8.8 and 47.1 g/g dry wt., respectively, in 8 weeks old hairy roots	Mishra et al. (2011)
Pogostemon cablin	Leaves	-	ATCC15834 and C58C1	Dipping in inoculum	Cocultivation for 1,2 or 3 days	MS + 500 mg/l cefotaxime at 25 °C and 14 h light	MS at 25 °C and 14 h light	MS + 0.1 mg/l BA +0.1 mg/l NAA at 25 °C in 14 h light	Highest hairy root formation with ATCC15834 (83.3%) and C58C1 (80.5%)	Yan et al. (2016)
Psoralea corylifolia	Hypocotyl explants	Psoralen	A4 harbouring pRiA4 and ATCC 15834	Direct infection	Cocultivation for 1–3 days at 25 °C in dark	MS +0.25 g/l cefotaxime	½ liquid MS +1–5% sucrose	–	Higher psoralen content (3.0 mg/g dry wt.) in hairy roots	Baskaran and Jayabalan (2009)
Rauvolfia serpentina	Leaves	Vomilenine and reserpine	A4	Pricking with inoculum (O.D. 0.9 at 600 nm)	Cocultivation for 2 days	MS + 1.0 g/l cefotaxime in dark	½ and full MS + 0.0 g/l cefotaxime +3% sucrose at 25 °C in dark with 80 rpm	–	DART technique for the characterization of compounds	Madhusudanan et al. (2008)
Rhamnus fallax	Stem segments with internodes	Anthraquinones	A4M70GUS	Dipping in bacterial culture and stabbing with infected needle	Cocultivation for 2 days	MS + 300 mg/dm^{-3} cefotaxime	WPM +1/2 macronutrients +300 mg/ dm^{-3} cefotaxime at 25 °C and 16 h light/dark	–	Hairy roots with 50% increase in anthraquinone content provided the second node is stabbed	Rosić et al. (2006)
Rhinacanthus nasutus	Leaves, stems and cotyledons	Rhinacanthin	MTCC. 532	Dipping in bacterial culture	Cocultivation for 2 days on MS + 3% sucrose at 25 °C in dark	MS + 3% sucrose +300 mg/l cefotaxime	MS + 3% sucrose in dark	–	Highest frequency of induction (73%) of hairy roots from cotyledons	Cheruvathur et al. (2015)

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Table 5.1 (continued)

Plant species	Explant	Secondary metabolite	Strain	Method	Culture conditions	Media + PGR + sucrose			Remarks	Reference
						Induction	Maintenance	Regeneration		
Rhodiola sachalinensis	Leaves	Sachalinensis	A4	Dipping in shake cultures of bacterial suspension	Cocultivation for 3 days	MS + B5 vitamins +500 mg/l cefotaxime +250 mg/l carbenicillin	MS at 24 °C in dark	–	Enhanced biomass accumulation and salindroside production	Zhou et al. (2007a)
Rubia akane	Leaves	Anthraquinones (alizarin and purpurin)	R1000	Dipping in bacterial culture	Cocultivation for 2 days	MS salts +0.8% agar + vitamins +3% sucrose +200 mg/l timentin	Liquid MS at 25 °C and 100 rpm in 16 h light	–	Maximum biomass (10.4 g/l) with alizarin (3.9 mg/g dry wt.) and purpurin (4.6 mg/g dry wt.) contents within 20 days	Park et al. (2009)
R. tinctorum	Leaves	Anthraquinones	LBA 9402	Wounding with an infected scalpel	Cocultivation for 4 days at 24 °C in dark	B5 + 2% sucrose +1.0 g/l ampicillin +0.8% agar	Liquid ½ B5 or WPM + 2% sucrose at 25 °C and 100 rpm in 16 h light	–	Release of anthraquinones, ruberythric acid, lucidin, primeveroside, alizarin, rubiadin and lucidin ω-methyl ether (~10% of total) in culture medium	Perassolo et al. (2017)
Salvia miltiorrhiza	Plantlet	Lithospermic acid B and rosmarinic acid	ATCC 15834	Infection of cut ends of plantlets	–	½ MS + 0.5 g/l sodium cefotaxime	MS at 25 °C in dark	–	Root growth and production of phenolics	Chen et al. (1999)
S. miltiorrhiza	Plantlets	Diterpenoids, tanshinone-I, tanshinon-IIA and cryptotanshinone	ATCC 15834	do	do	do	MS without ammonium nitrate at 25 °C in dark	–	Increase in total tanshinone content by 6.6 fold upon renewal of medium and treatment with silver ions	Zhang et al. (2004b)
S. miltiorrhiza	Plantlets	Tanshinone	ATCC 15834	do	do	do	MS + 3% sucrose without ammonium nitrate at 25 °C in dark	–	Four- to fivefold increase in tanshinone content in hairy roots	Shi et al. (2007)

S. miltiorrhiza	Plantlets	Cryptotanshinone, tanshinone-I and tanshinone-IIA	ATCC 15834	Infection of cut ends of plantlets with a syringe	do	do	MS without ammonium nitrate +0.8% agar +3% sucrose +0.5 g/l casein hydrolysate at 25 °C in dark	–	About 12-fold enhanced production of tanshinone on root bacteria coculture	Wu et al. (2007)
S. miltiorrhiza	Plantlets	-	ATCC 15834	do	do	do	Liquid, MS without ammonium nitrate +3% sucrose at 25 °C in dark at 110–120 rpm	–	NO level increased at 10–100 mM ATP	Wu and Wu (2008)
S. miltiorrhiza	Leaves	Tanshinone I and II A, Cryptotanshinone	BCRC15010, O.D. = 0.4	Immersion in shake cultures for 30 minutes	Cocultivation for 2 days	MS + 200 mg/l cefotaxime	B5 + 200 mg/l cefotaxime at 25 °C and 100 rpm in dark	–	Tanshinone I and cryptotanshinone accumulation increased by 5- and 7.5-folds, respectively	Gupta et al. (2011)
S. sclarea	Shoots	Terpenoids	LBA 9402	Direct wounding with a needle	-	MS + 3% sucrose at 26 °C in dark	½ B5 + 3% sucrose at 26 °C and 100 rpm in dark	–	Diterpenoids, viz. ferruginol, salvipisone, aethiopinone and 1-oxoaethiopinone and ursenetype triterpenoids, viz. 2a,3a-dihydroxy-urs-12-en-28-oic acid and 2a,3a,24-trihydroxy-urs-12-en-28-oic acid	Kuzma et al. (2006)
Saussurea involucrata	Seedlings	Phenylpropanoids, syringin and hispidulin	R1601	Immersion	Cocultivation for 2 days	1/2 MS + 500 mg/l cefotaxime sodium +3% sucrose	MS + 3% sucrose at 25 °C, 90 rpm and 12 h light	1/2 MS ± 1.0 mg/L BA	Higher levels of syringin and hispidulin in hairy roots	Fu et al. (2006)

(continued)

Table 5.1 (continued)

Plant species	Explant	Secondary metabolite	Strain	Method	Culture conditions	Media + PGR + sucrose			Remarks	Reference
						Induction	Maintenance	Regeneration		
Solanum aviculare	Plantlets	Solasodine	A4, ATCC 11325, ATCC 15834, and ATCC 43057	Stabbed with sterile toothpicks	–	Liquid MS + 0.6 to 1.0 g/l ampicillin	Liquid MS	–	Hairy root formation with ATCC 15834 (90%), A4 (83%), ATCC 43057 (43%) and ATCC 11325 (20%)	Kittipongpatana et al. (1998)
Stevia rebaudiana	Leaves	Chlorogenic acid, 3,5-dicaffeoylquinic acid and 4,5-dicaffeoylquinic acid	C58C1	Immersion in bacterial culture	Cocultivation for 2 days	½ MS + 500 mg/l cefotaxime	Liquid ½ MS at 28 °C in dark at 115 rpm	–	Higher production of chlorogenic acid and its derivatives	Fu et al. (2015)
Swertia japonica	Aerial parts	Phenyl glucosides	15,834 harbouring pRi 15,834	Direct infection	–	–	Liquid RC	–	Isolation of two new phenyl glucosides	Ishimaru et al. (1990)
Symphytum officinale	Leaves	Invertase activity in hairy roots	15,834	Wounding with a hypodermic needle	–	MS + 3% sucrose	MS + 1/5 nitrogen +3% sucrose +500 mg/l ampicillin at 25 ° C in dark	–	Similar trends in invertase enzyme activity in hairy roots and cell cultures	Shimon-Kerner et al. (2000)
Tanacetum parthenium	Stem	Spiroketal enol ether type diacetylene	LBA 9402	–	–	–	MS + ½ macronutrients +3% sucrose at 110 rpm in 16 h light	–	Decrease in spiroketal enol ether type and diacetylenes	Stojakowska et al. (2008)
Withania somnifera	Leaves	Withaferin A	A4, LBA 9402 and LBA 9360	Infection of wounded explants	Cocultivation for 3 days	MS + 250 mg/l cefotaxime	1/2 MS + 3% sucrose at 22 °C and 80 rpm in dim light	–	Production of withaferin A in hairy roots as well culture medium	Banerjee et al. (1994)
W. somnifera	Seedling parts and nodal segments	Withanolide A	R1601	Immersion in bacterial culture	Cocultivation for 2 days	MS + 400 mg/l cefotaxime	Liquid MS at 100 rpm	–	2.7-fold higher withanolide A content in hairy roots	Murthy et al. (2008)

Table 5.2 Effect of elicitors on secondary metabolite production

Elicitor	Plant species	*A. rhizogenes* strain	Metabolite/target	Remarks	References
Methyl jasmonate (MeJa)	*Abrus precatorius*	MTCC 532 and MTCC 2364	Glycyrrhizin in hairy roots	Higher glycyrrhizin production (2.5 times)	Sajjalaguddam and Paladugu (2016)
	Centella asiatica	R1000	Asiaticoside	Production of asiaticoside (7.12 mg/g, dry wt.) after elicitation with MeJa	Kim et al. (2007)
	Glycyrrhiza inflata	ATCC 15834	Glycyrrhizin	Enhanced glycyrrhizin production (up to 109 µg/g dry wt.) on day 5 of elicitation with 100 µM MeJa	Wongwicha et al. (2011)
	Hyoscyamus niger	C58C1	Scopolamine	Increased levels of scopolamine after elicitation with 50 µM MeJa	Zhang et al. (2007)
	Panax ginseng	A4	Rg3 ginsenoside in hairy roots	Rg3 accumulation (0.42 mg/g on dry wt. basis) after 7 days of elicitation	Kim et al. (2013)
		A4	Triterpene saponins, protopanaxadiol (Rb group) and protopanaxatriol saponins	Increase in both types of saponins by 5.5–9.7 times and 1.85–3.82 times, respectively	Kim et al. (2009)
		KCTC 2703	Ginsenoside	Enhanced production of total ginsenoside after elicitation	Yu et al. (2000)
	Plumbago indica	A4M70GUS	Plumbagin	Increased yield of plumbagin to 5.0 and 3.8% on dry wt. basis after 48 h of elicitation with 50 µM MeJa and 100 µM acetylsalicylic acid, respectively	Martin et al. (2011)
Light, MeJa or cyclodextrin	*Scutellaria lateriflora*	ATCC 15834	Flavones like wogonin, baicalein, scutellarein and their respective glucuronides and also verbascoside	Significantly higher levels of aglycones, baicalein and wogonin but not scutellarein in cultures incubated under continuous light and elicited with 15 mM methyl-β-cyclodextrin for 24 h	Marsh et al. (2014)

(continued)

Table 5.2 (continued)

Elicitor	Plant species	*A. rhizogenes* strain	Metabolite/target	Remarks	References
MeJa/nitric oxide	*Catharanthus roseus*	A4	Catharanthine	Large increase in catharanthine and associated transcripts and pathway genes after elicitation with MeJa	Zhou et al. (2010)
MeJa and salicylic acid (SA)	*Azadirachta indica*	LBA 9402	Azadirachtin	Enhanced production of azadirachtin (i.e. ≃ six- and ≃ ninefold on dry wt. basis) after elicitation with 100mM each of MeJa and SA, respectively	Satdive et al. (2007)
	Glycine max (L.) Merrill	R1000	Isoflavones in 30 days old hairy root cultures	Enhanced production of total isoflavones by (i) 10.67-fold after 72 h of elicitation with 100 M MeJa and (ii) 5.78-fold after 96 h with 200 M SA	Theboral et al. (2014)
	Gossypium barbadense	ATCC 15834	Gossypol, 6-methoxygossypol and 6,60 –dimethoxygossypol	Enhanced levels of gossypol (eightfold) after elicitation with 100 μM MeJa	Frankfater et al. (2009)
	Withania somnifera	R1000	Withanolide A, withanone and withaferin A in 40 days old harvested hairy roots	Enhanced biomass production (1.23-fold higher), withanolide A (58-fold higher), withanone (46-fold higher) and withaferin A (42-fold higher) after 4 h of elicitation with 150 M SA	Sivanandhan et al. (2013)
SA and acetylsalicylic acid (ASA)	*Datura stramonium*	A4	Hyoscyamine	Significant changes in dry weights after 24 h of elicitation	Belabbassi et al. (2016)
SA and ethephon	*Prunella vulgaris*	ATCC15834	Rosmarinic acid	Maximum accumulation (1.66- and 1.48-folds) after 8 and 2 days of elicitation with SA, respectively	Ru et al. (2016)

Sodium acetate	*Arachis hypogaea*	ATCC 15834	Resveratrol	60-fold increase in the release of trans-resveratrol into the culture medium after elicitation	Medina-Bolivar et al. (2007)
Sodium acetate		ATCC 15834	Resveratrol and prenylated stilbenoids (arachidin-1 and arachidin-3)	Accumulation of 90% total resveratrol (arachidin-1 and arachidin-3) in culture medium	Condori et al. (2010)
Tannic acid, selenium, nickel sulphate and sodium chloride	*P. ginseng*	KCTC 2744	Saponins	Increased production by (i) 1.31 and 1.33 times after elicitation with 0.5 mM selenium, (ii) 1.20–1.23 times by 20 µM $NiSO_4$ and (iii) 1.15–1.13 times by sodium chloride	Jeong and Park (2006)
YE, *Pectobacterium carotovorum* lysate	*Scutellaria lateriflora*	A4 (ATCC 31798)	Acteoside, wogonoside	Increased production of acteoside and flavones by 1.4- and 1.7-folds, respectively, after 7 and 14 days of elicitation with 50 µg/ml YE. Wogonin accumulation after elicitation with *P. carotovorum* lysate in stationary phase	Wilczan'ska-Barska et al. (2012)
Sorbitol, YE, polysaccharide fraction of YE	*Salvia miltiorrhiza* Bunge	ATCC 15834	Tanshinone	Increase in tanshinone content by tenfolds and volumetric yield by ninefolds after elicitation with 50 g/l sorbitol and 100 mg/l YE	Shi et al. (2007)
Heptasaccharide and an octasaccharide from *Paris polyphylla* var. *yunnanensis*	*P. ginseng*	A4	Total saponins in hairy roots	Accumulation of saponins (5–30 mg/l) after elicitation	Zhou et al. (2007b)

(continued)

Table 5.2 (continued)

Elicitor	Plant species	*A. rhizogenes* strain	Metabolite/target	Remarks	References
Oligogalacturonides	*Artemisia annua*	R1601	Artemisinin	Maximum production of artemisinin (11.3 mg/l or 55.2% increase over control)	Zhang et al. (2010)
Oligosaccharides from *Colletotrichum gloeosporioides*			Artemisinin	Maximum production of artemisinin (up to 13.51 mg/l or 51.63% increase over control)	Wang et al. (2006b)
Oligosaccharide from *Fusarium oxysporum* mycelium		R1601	Artemisinin	Artemisinin accumulation	Zheng et al. (2010)
Polysaccharide fraction of YE and 150 mg/l chitosan		ATCC 15834	Artemisinin	Increased production of artemisinin (sixfolds in 6 days) after elicitation with 150 mg chitosan, 0.2 mM MeJa and 2.0 mg/ml YE	Putalun et al. (2007)
Polysaccharides from *Bacillus cereus*	*S. miltiorrhiza*	ATCC 15834	Tanshinone	Sevenfolds higher tanshinone accumulation	Zhao et al. (2010)
Exogenous yeast polysaccharide (YPS)	*Fagopyrum tataricum*	Ri1601	Flavonoids	Increased flavonoids yield (about 3.2-folds) after elicitation with 200 mg/l YPS and medium renewal	Zhao et al. (2014)
Chitosan (10 mg/l) and/or 50 M SA	*Hypericum pulchrum* and *H. annulatum*	A4 and ATCC 15834	Xanthones	Highest levels of total xanthone after 24 h of elicitation with SA followed by gradual decline	Zubrická et al. (2015)
Chitosan, vanadyl sulphate or MeJa	*P. ginseng*	A4	Ginsenosides	Increase in ginsenoside content (2.0, 1.8 and 4.0 times in C-M, HR-M and T-M root lines, respectively) after 28 days of elcitation	Palazón et al. (2003a)
PEG 8000 (2%), YE (0.1%)	*Glycyrrhiza uralensis*	A4	Flavonoids	Higher flavonoids content (17.12, 60.33 and 129.22%) as compared to control	Zhang et al. (2009)

CFYE, chitosan, manganese chloride, copper chloride and MeJa	*Plumbago indica*	ATCC 15834	Plumbagin	Maximum yield of total plumbagin (11.96 mg/g) after 3 days of elicitation with 200 mg/l chitosan and 80 μM MeJa	Gangopadhyay et al. (2011)
YE, chitosan, SA, putrescine and spermidine	*Psoralea corylifolia*	LBA 9402	Isoflavones	Enhanced daidzein and genistein production (1.3-fold) after elicitation with 2 mM phenylalanine, 2 mg/l chitosan	Shinde et al. (2009)
YE	*S. miltiorrhiza*	ATCC15834	Tanshinones	Twofold increase in total tanshinone from 0.46 to 1.37 mg/g dry wt.	Yan et al. (2005)
YE (200 mg/l) and silver ions (15 mM) as an abiotic elicitor		ATCC15834	Rosmarinic acid	Maximized yield of rosmarinic acid after elicitation with YE	Yan et al. (2006)
Silver ions and carbohydrate fraction of YE (CFYE)		ATCC15834	Tanshinone	Tanshinone accumulation	Ge and Wu (2005)
Silver ions (2 mM)	*Silybum marianum*	AR15834	Silymarin	Two fold higher production of silymarin	Khalili et al. (2010)
Phenylalanine, cysteine, SA, ASA, MeJa, β-aminobutyric acid, YE	*Tropaeolum majus*	LBA 9402	Glucotropaeolin	Enhanced yield of glucotropaeolin by two and fourfolds after elicitation with precursor amino acids or PAL inhibitor alone or in combination, respectively	Wielanek and Urbanek (2006)
Mycelial extracts from *Colletotrichum* sp. (0.4 mg total sugar/ml)	*A. annua*	R1601	Artemisinin	Artemisinin content increased from 0.8 mg/g dry wt. to 1.0 mg/g dry wt.	Wang et al. (2001)
Glycans of microbial origin (200–500 mg/l), extracts of whole microbial cultures (0.25–1.25%), culture filtrates (5–25%) and tenfolds higher metal ions than that in MS medium	*Beta vulgaris*	LMG 150	Betalain	Significantly higher productivity of betalain (158 mg/l) in *Penicillium notatum* DCP-treated cultures on the 7th day of elicitation	Savitha et al. (2006)

(continued)

Table 5.2 (continued)

Elicitor	Plant species	*A. rhizogenes* strain	Metabolite/target	Remarks	References
Pythium aphanidermatum and *Phytophthora parasitica* var. *nicotianae*	*Cichorium intybus* L.cv. Lucknow local	LMG 150	Esculin and esculetin	Maximum accumulation of endogenous spermine titers on the 28th day of elicitation with 1.0% media filtrate of *P. parasitica* (v/v)	Bais et al. (2000)
Culture filtrates of root endophytic fungus, *Piriformospora indica*	*Linum album*	LBA 9402	Podophyllotoxin and 6-methoxypodophyllotoxin	Maximum improvement in podophyllotoxin and 6-methoxypodophyllotoxin concentrations by 3.8- and 4.4-folds	Kumar et al. (2012)
Elicitor compounds released by *B. cereus*	*S. miltiorrihiza*	ATCC15834	Tanshinone in hairy roots	Enhanced production after elicitation	Wu et al. (2007)

supplementation of 3% sucrose and culturing under dark conditions were the other important variables governing the successful production of hairy roots in *A. belladonna.* The factors also regulated the overexpression of key genes encoding putrescine biosynthesis (Yang et al. 2011). Similarly, the success of hairy root cultures of *Beta vulgaris* depended upon the LMG 150 strain of *A. rhizogenes* along with culturing in liquid MS medium containing 3% sucrose (Hamill et al. 1986; Rao et al. 2001; Pavlov et al. 2002). Another worker, Rao et al. (2001), improved the production of thiophene in hairy roots of *B. vulgaris* by treating them with *Haematococcus pluvialis* and *Spirulina platensis.* In case of *Brugmansia candida* however, the LBA 9402 strain of *A. rhizogenes* and half-strength B5 medium were optimal for the induction and further growth of hairy root cultures (Pitta-Alvarez and Giulietti 1995; Spollansky et al. 2000). Moreover, a two- and fourfold increase in the contents of scopolamine and hyoscyamine, respectively, were recorded (Pitta-alvarez and Giulietti 1995).

Another effective method of enhancing the secondary metabolites yield in hairy root cultures is elicitation. Elicitors are of two types depending upon their origin: (1) abiotic and (2) biotic. Abiotic elicitors mainly include inorganic compounds and physical factors, while the biotic elicitors include the compounds of biological origin. Biotic elicitors include plant signalling molecules (methyl jasmonate, salicylic acid and ethephon), molecules derived from microorganisms (chitosan, polysaccharide fractions of yeast and bacterial extracts, mycelial extracts, glycans and culture filtrates of root endophytic fungus) and precursors of important metabolites like phenylalanine and cysteine.

Inorganic salts such as sodium acetate, sodium chloride, manganese chloride, copper chloride, vanadyl sulphate, nickel sulphate and metal ions like silver, selenium and Zn ions are some examples of abiotic elicitors. The various biotic and abiotic elicitors that were used for enhanced production of secondary metabolites in hairy root cultures are presented in Table 5.2.

The plant signalling molecule, methyl jasmonate, has been used by several researchers for improved production of secondary metabolites such as saponins, ginsenosides, terpenoids, flavonoids, alkaloids and phenylpropanoids (Yu et al. 2000; Zhou et al. 2010; Sivanandhan et al. 2013; Marsh et al. 2014; Sajjalaguddam and Paladugu 2016). In this regard, the level of protopanaxadiol was increased by 1.85–3.82 times and protopanaxatriol by 5.5–9.7 times after elicitation of *Panax ginseng* hairy root cultures with methyl jasmonate (Kim et al. 2009). Increased accumulation of hyoscyamine (1200%) and scopolamine (30%) after elicitation with jasmonic acid is another example. In another study, the yield of scopolamine and hyoscyamine increased to 43 and 83% after elicitation with jasmonic acid and 25 and 250 mM $AlCl_3$ (Spollansky et al. 2000). Salicylic acid is another elicitor that has been extensively used in hairy root cultures of plants like *Datura stramonium*, *Prunella vulgaris*, *Glycine max*, *Withania somnifera* and *Gossypium barbadense* (Satdive et al. 2007; Frankfater et al. 2009; Sivanandhan et al. 2013; Theboral et al. 2014; Belabbassi et al. 2016; Ru et al. 2016). Elicitors derived from microorganisms have also improved the yield of secondary metabolites in hairy root cultures of *Artemisia annua*, *P. ginseng*, *Psoralea corylifolia, Scutellaria lateriflora*, etc. (Zhou

et al. 2007b; Shinde et al. 2009; Zheng et al. 2010; Wilczańska-Barska et al. 2012). Sugars have also served as elicitors in enhancing the yield of secondary metabolites significantly. Thus, in hairy root cultures of *Salvia miltiorrhiza*, 50 g/l sorbitol and 100 mg/l polysaccharide fraction of yeast extract enhanced the tanshinone yield by tenfolds. On the other hand, the yields of tanshinone and silymarin were significantly enhanced upon elicitation with silver ions (Ge and Wu 2005; Khalili et al. 2010).

5.4 Metabolic Engineering

An important application of *A. rhizogenes*-mediated genetic transformation has been the expression and modulation of secondary metabolite pathway genes and regulatory elements. This has facilitated the elucidation and modulation of key intermediates and enzymes of secondary metabolite biosynthetic pathway(s). Another important application of the method has been the production of recombinant proteins in the hairy roots, particularly, when its production is difficult in either of bacterial, yeast or other expression systems. However, the method requires extensive optimization of parameters that govern the stability, structural integrity and activity of the recombinant proteins being produced in the hairy roots. Regulation of post-translational stability of recombinant proteins in a heterologous environment is also extremely crucial (Tokmakov et al. 2012). Therefore, one has to take into account the tissue and organ specificity of recombinant proteins with respect to their glycosylation profile and subcellular compartmentalization, as well as susceptibility to proteolytic degradation while optimizing their production in hairy root cultures (Streatfield 2007). The production of the recombinant protein, human acetyl cholinesterase (Woods et al. 2008), and the IgG1 type, 14D9 murine monoclonal antibodies, in the hairy roots derived from transgenic tobacco plants are important examples (Table 5.3). In one study, the production of the recombinant protein, human-secreted alkaline phosphatase, was increased by five- to sevenfolds in hydroponically grown hairy root cultures of transgenic tobacco (Gaume et al. 2003). In still another study, approximately, threefold higher accumulation of acetylcholinesterase was recorded in the hairy roots of transgenic *Nicotiana benthamiana* as compared to wild type plants (Woods et al. 2008). Hairy root cultures of *N. tabacum* were also employed by Moghadam et al. (2016) for the production of the recombinant anti-HIV and antitumour protein MAP 30 and by Lonoce et al. (2016) for mAb H10 – a monoclonal tumour-targeting antibody. In the following year, Gurusamy et al. (2017) reported the production of recombinant human erythropoietin in the hairy root cultures of *N. tabacum.* Clinical trials of recombinant proteins are also being conducted in countries like the USA and Canada. This is a clear indication of the upcoming bright future of hairy root-derived recombinant proteins.

Table 5.3 Production of recombinant proteins production in plants transformed with *Agrobacterium rhizogenes*

Plant species	Recombinant protein metabolite/target	*Agrobacterium* sp. and strain	Remarks	References
Althaea officinalis	Anti-HIV microbicide cyanovirin-N	LBA 9402 containing *cvn* gene in the plasmid pL32 (pL32:CV-N)	CV-N (2.4 µg/g fresh wt.) production in root tissue and secretion of 0.02 µg/ml/24 h in the medium	Drake et al. (2013)
Arabidopsis thaliana	Heterologous proteins	pRP49 containing His tag fused to *gfp* and coding a plant signal peptide from *At*1g69940	GFP production (130 mg/l) in the medium	Mai et al. (2016)
Beta vulgaris	Precursor of insecticidal proteinase inhibitor	p35SNaPI-15,834 carrying the *NaPI* gene from *Nicotiana alata*	Altered folding of recombinant protein and reduced post-translational processing efficiency of *NaPI* precursor	Smigocki et al. (2009)
Brassica oleracea var. *italica*	hGH1 isoform as a model therapeutic protein	LBA 9402 carrying the plasmid pRi1855	Production of 7.8 µg/g hGH1 protein (dry wt.) in 1.5 l airlift reactor with mesh	Lopez et al. (2014)
Brassica rapa	Heterologous protein-GFP	TR7 carrying the pEGFP	GFP production remained stable for more than 3 years	Huet et al. (2014)
Cucumis melo	The thrombolytic protein – human tissue plasminogen activator (t-PA)	K599 transformed with p221 containing t-PA gene under transcriptional control of single, dual, triple and quadruple *rolD* promoter fragments	Hairy roots with maximum t-PA content under the control of double *rolD* promoter fragments	Kang et al. (2011)
C. melo	Human tissue plasminogen activator – a thrombolytic protein	K599 transformed with p221 containing fragments encoding t-PA and its synthetic form	Production of enzymatically active t-PA (798 ng/mg) in hairy roots	Kim et al. (2012)
Cucumis sativus	Recombinant *Digitalis lanata* EHRH cardenolide 16-O-glucohydrolase	ATCC15834 harbouring pBI121*cgh*	Hairy roots with *cgh* 1 indicating biotransformation of natural compounds by recombinant enzymes	Shi and Lindemann (2006)

(continued)

Table 5.3 (continued)

Plant species	Recombinant protein metabolite/target	*Agrobacterium* sp. and strain	Remarks	References
Cucurbita moschata	*linA* gene from *Sphingobium japonicum* UT26 for degradation of γ-hexachlorocyclohexane (γ-HCH) and phytoremediation	*A. tumefaciens* MAFF03-01724 harbouring the expression vectors pRelinA and pAOs::relinA. Gene *linA* fused to endoplasmic reticulum targeting signal peptide for stable accumulation	90% degradation of 1.0 ppm γ-HCH	Nanasato et al. (2016)
Daucus carota	Recombinant human interferon-2b	*A. tumefaciens* GV3101 containing the leukocyte human interferon gene *alpha2b* fused with plant calreticulin apoplast targeting signal under 35S CaMV promoter or root specific MII sugar beet promoter	Antiviral activity in hairy roots (4.42 × 103 IU/mg TSP comparable to transgenic carrot leaf extracts)	Luchakivskaya et al. (2012)
Nicotiana benthamiana	Human acetylcholinesterase	*A. rhizogenes* (R 1000)-mediated transformation of transgenic plants expressing cDNA encoding human AChE-R fused to C-terminal SEKDEL	Threefold higher expression of acetylcholinesterase in hairy roots	Woods et al. (2008)
Nicotiana tabacum	Human secreted alkaline phosphatase (SEAP)	A4-mediated transformation of transgenic plants with human placental SEAP gene	Five to seven times higher secretion of SEAP in the hairy roots	Gaume et al. (2003)
N. tabacum	THCA synthase from *Cannabis sativa*	ATCC 15834 transformed with pBI121 vector having pUC119/THCAS cDNA	FAD-dependent THCA synthase reaction with binding of His-114 to FAD	Sirikantaramas et al. (2004)
N. tabacum	Secreted alkaline phosphatase (SEAP)	*A. rhizogenes* containing pRYG transformation vector with expression cassette for SEAP	SEAP yield of about 28 μg/g root dry wt./day	Komarnytsky et al. (2004)
N. tabacum	GFP recombinant protein	ATCC 15834	Almost 20% of the total secreted protein (i.e. more than 800 μg/l of GFP yield) after 21 days of incubation	Medina-Bolívar and Cramer (2004)

N. tabacum	14D9 antibody	LBA 9402-mediated transformation of transgenic plants expressing 14 V9 murine monoclonal antibody	Hairy roots with a yield of about 5.95 μg ml^{-1} 14D9 antibody	Martínez et al. (2005)
N. tabacum	Recombinant thaumatin	ATCC 15834 with N-terminal of *thaumatin* gene fused to calreticulin signal sequence (Z71395)	Recombinant thaumatin secretion in the medium (0.21 mg/l)	Pham et al. (2012)
N. tabacum	M12 – a monoclonal antibody	LBA9402 with M12 HC and LC coding sequences under the expression of 35S promoter	57% of antibody secretion in induction medium	Häkkinen et al. (2014)
N. tabacum	Recombinant anti-HIV and antitumour protein MAP30	ATCC AR15834 carrying the expression vector, pBI121-MAP30	Extracted total proteins with effective expression of rMAP30-KDEL	Moghadam et al. (2016)
N. tabacum	mAb H10 – a monoclonal tumour-targeting antibody	A4 carrying the plant expression vectors p35S-HC and p35S-LC	Stable hairy root cultures with mAb H10 having a human-compatible glycosylation profile	Lonoce et al. (2016)
N. tabacum	rhEPO – a recombinant human EPO	ATCC15834 carrying the EPO expression construct with or without the calreticulin apoplast targeting signal peptide	rhEPO (14.8 ng/g of total leaf protein) in first generation of transgenic plantlets	Gurusamy et al. (2017)

(continued)

Table 5.3 (continued)

Plant species	Recombinant protein metabolite/target	*Agrobacterium* sp. and strain	Remarks	References
Plumbago zeylanica	A single chain variable fragment antibody against plumbagin	Recombinant ATCC 15834 containing *PL-scFv* gene	Hairy roots with modulated PL biosynthesis pathway and 2.2 times higher yield of PL	Sakamoto et al. (2012)
Sesamum indicum	Recombinant fungal phytase	DC-AR2 containing the phytase expressing vector (pMOG413)	Efficient production of recombinant fungal phytase	Jin et al. (2005)
Solanum khasianum	Viable antibody for anti-solasodine glycoside single-chain fragment (scFv)	ATCC 15834 carrying the plant expression vector containing SUC2 fused to scFv-KDEL	Production of 2.3-fold higher solasodine glycosides	Putalun et al. (2003)
Solanum lycopersicum	Rabies glycoprotein and ricin toxin B chain (RGP-RTB) – a vaccine antigen	pCAMBIA1300 harbouring the chimeric fusion gene, *cal-rgp-gp-rtxB-SEKDL* gene under the control of CaMV35S double enhancer	Successful expression of (1.14%) RGP-RTB protein	Singh et al. (2015)
Solanum tuberosum	Hepatitis B – a surface antigen	*Agrobacterium rhizogenes* harbouring the pEFEHBS/pEFEHER with hepatitis B virus gene	HBsAg expression in hairy roots	Kumar et al. (2006)

5.5 Upscaling of Hairy Roots in Bioreactors

Hairy roots are the alternative but stable resources of pharmaceutically important compounds and require upscaling for applications in commercial ventures. Thus, researchers across the globe have scaled up the production of valuable secondary metabolites and/or hairy roots of medicinally important plants in various kinds of bioreactors like the conventional airlift, bubble column, stirred tank, airlift balloon and nutrient mist bioreactors (Table 5.4). These bioreactors being different from the ones used in plant cell cultures were chosen on the basis of metabolite localization characteristics and structural features of hairy roots. The bioreactors used by different researchers till date for hairy root cultures are either of gas phase, liquid phase or hybrid reactors with a combination of gas and liquid phases (Kim et al. 2002). The airlift and the bubble column bioreactor systems have proven to be the most successful for hairy root cultures and secondary metabolite production. On the other hand, the mist bioreactor or airlift mesh-draught bioreactor with wire helixes is reported to support high homogenous partitioning of biological materials while decreasing the volume of the culture medium. It also yields a concentrated form of secreted metabolites. This explains the extensive use of airlift mesh-draught type bioreactor by the Swiss company, 'ROOTec' for the production of camptothecin in the hairy root cultures of *Camptotheca acuminata*.

5.6 Regeneration of Whole Plants in Recalcitrant Species

Secondary metabolite biosynthesis is not always limited to roots and underground parts only. In many plants, the precursors of pharmaceutically important secondary metabolites are produced in the roots, but the final products are synthesized in the leaves and other aerial parts of the plants. In such cases, the regeneration of whole plants from hairy roots is particularly advantageous. The production of high amounts of vincristine and vinblastine in whole plants regenerated from hairy roots of *C. roseus* is a model example of this approach. Similarly, whole plants of mint- and rose-scented *Pelargonium* derived from hairy roots have yielded essential oil amounts equal to that of normal plants. On the other hand, the whole plants regenerated from hairy roots of *Centaurium erythraea* produced about eightfold higher secoiridoids and about 83% higher camptothecin in the *Ophiorrhiza pumila* plants derived from hairy roots. Significant increase in the hairy root biomass and 160–280% increase in tylophorine content were also recorded in *Tylophora indica* plants regenerated from hairy roots, whereas, the contents of solasodine, scopolamine and hyoscyamine were lower than the control in the *Solanum nigrum* and *Duboisia myoporoides* × *D. leichhardtii* plants regenerated from hairy roots, respectively (Celma et al. 2001). The *A. rhizogenes*-mediated transformation has been also used to initiate rhizogenesis in plants that are generally recalcitrant to rooting (Häggman and Aronen 2000).

Table 5.4 Biomass and secondary metabolite production in bioreactors

Bioreactor	Plant species	Strain	Metabolite/target	Response	References
Airlift principle based bioreactors					
Airlift column with polyurethane foam	*Armoracia rusticana*	*Agrobacterium rhizogenes*	Biomass growth	Linear relationship between biomass of hairy roots and decrease in medium conductivity (i.e. 11 kg dry cells m^3 in 31 days culture)	Taya et al. (1989)
Bubble column, modified bubble column and modified inner-loop airlift (2.5 l)	*Artemisia annua*	*A. rhizogenes*	Artemisinin	Artemisinin production reached to 368, 446 and 536 mg/l after 20 days, respectively	Liu et al. (1998a)
Modified inner-loop airlift (2.5 l)		*A. rhizogenes*	Artemisinin	Artemisinin production reached to 577.5 mg/l after 20 days	Liu et al. (1998b)
Modified stirred tank (3.0 l)		ATCC 15834	Artemisinin	Increased artemisinin production on addition of MeJa	Patra and Srivastava (2014)
Modified stirred tank		ATCC 15834	Artemisinin	Accumulation of 1.0 mg/g artemisinin after 16 days in fed-batch culture	Patra and Srivastava (2015)
Airlift (30.0 l)	*Astragalus membranaceus*	LBA 9402	Astragaloside IV and polysaccharides	Astragaloside IV production (about 11.5 g/l dry wt.)	Du et al. (2003)
Fermentor (2.0 and 30.0 l)	*Atropa belladonna* and *Calystegia sepium*	A4 and 8196	Cuscohygrine, atropine, hyoscyamine and scopolamine	Alkaloid production (2.3 mg/l per day)	Jung and Tepfer (1987)
Stirred with stainless steel net (30.0 l)	*A. belladonna*	MAFF 03-01724	Atropine	Production of approx. 1500 mg tropane alkaloids	Lee et al. (1999)
Self-made with impellers and air (1.5 l)		ATCC 15834	Scopolamine	Production of scopolamine (1.59 mg/g dry wt.)	Habibi et al. (2015)

Stirred tank	*Azadirachta indica*	LBA 920	Azadirachtin	Productivity of azadirachtin (3.89 mg/l per day)	Srivastava and Srivastava (2012)
Bubble column		LBA 920	Azadirachtin	Production of azadirachtin (3.2 mg/g per day)	Srivastava and Srivastava (2013)
Turbine blade	*Beta vulgaris*	*A. rhizogenes*	Betalains	Release of betalain pigment into culture medium on oxygen starvation	Kino-oka et al. (1992)
Single column		*A. rhizogenes*	Betalains	Reduced viability of growing points at hairy root tips on increasing pressure drop	Hitaka et al. (1997)
Bubble column		A4	Betalain	Release of betalains and its adsorption on XAD-16 resin at pH 2.0	Mukundan et al. (2001)
Airlift		A4	Betacyanin	Accumulation of betacyanin (27 mg/g dry wt.)	Shin et al. (2002)
Bubble column (3.0 l)		A4	Betalaine	Enhanced betalaine production (1.23- and 1.4-folds higher) after elicitation with spermidine, putrescine and MeJA	Suresh et al. (2004)
Bubble column		LMG-150, A 2/83, A4, A 20/83	Peroxidase	Production of peroxidase (8000–9000 U/g on fresh wt. basis and 1.18 x 106 U/l with a specific activity of 600 U/mg protein)	Rudrappa et al. (2005)
Bubble column		LMG-150	Betalain	Increase in betalain production by 47% with biotic and abiotic elicitors	Savitha et al. (2006)
Bubble column (3.0 l)		ATCC 15834	Betalain	11% higher betalain in batch cultures	Pavlov et al. (2007)
Bubble column		LMG-150	Betalain and peroxidase	High production of both betalain and peroxidase in fed-batch reactor	Neelwarne and Thimmaraju (2009)

(continued)

Table 5.4 (continued)

Bioreactor	Plant species	Strain	Metabolite/target	Response	References
Airlift with mesh (1.5 l)	*Brassica oleracea* var. *italica*	LBA 9402 (plasmid pRi1855)	Therapeutic protein, hGH1 isoform	Production of hGH1 (7.8 µg/g DW)	Lopez et al. (2014)
Stirred tank (1.5 l)	*Brugmansia candida*	LBA 9402	Scopolamine, anisodamine and hyoscyamine	Predominant production of anisodamine (approx. 10.05 mg/g dry wt.)	Cardillo et al. (2010)
Sparged and stir (3.5 l) reactor with medium circulation (1.5 l)	*Catharanthus roseus*	ATCC 15834, A4, A2-83, R1000 and TR7	Indole alkaloid	Sparged reactor supported maximum biomass growth and alkaloid production	Nuutila et al. (1994)
Bioreactor (14.0 l)		ATCC 15834, A41027, R100 and A4	Ajmalicine and catharanthine	Production in culture	Moreno-Valenzuela et al. (1999)
Two phase liquid		A4	Indole alkaloids	Tabersonine and lochnericine content increased by 100–400 and 14–200%	Tikhomiroff et al. (2002)
Airlift, sparged		A4, LBA 9402 and ATCC 11325	Catharanthine	Highest production of catharanthine in 5.0 l bioreactor	Verma et al. (2012)
Bubble column, rotating drum bioreactor, modified bubble column with polypropylene mesh support		*A. rhizogenes*	Ajmalicine	Higher levels of ajmalicine	Thakore et al. (2017)
Immersion	*Centaurium maritimum*	A40M70GUS	Secoiridoid glycosides	Eight times higher production	Mišić et al. (2013)
Isolated impeller (batch and continuous), modified stirred tank (14.0 l)	*Datura stramonium*	*A. rhizogenes*	Hyoscyamine	Release of sevenfold higher contents (3.6%) in the medium in continuous culture at 30/35 °C	Hilton and Rhodes (1990)

Turbine blade and immobilized rotating drum	*Daucus carota*	*A. rhizogenes*	Hairy roots biomass	10 g/l of hairy roots, irrespective of reactors	Kondo et al. (1989)
Amberlite XAD-2 column combined with turbine blade	*Duboisia leichhardtii*	*A. rhizogenes*	Scopolamine	Release of 1.3 g/l scopolamine in culture medium after 11 weeks	Muranaka et al. (1993)
Modified airlift	*Echinacea purpurea*	ATCC 43057	Cichoric acid	Enhanced production	Abbasi et al. (2009)
Stirred tank (5.0 l)	*Glycyrrhiza glabra*	K599	Biomass only	20 times increase in biomass	Mehrotra et al. (2008)
Bubble column (3.0 l)	*Harpagophytum procumbens*	ATCC 15834	Cinnamic acid derivatives, amino acids, sugars	Higher levels of ABA, salicylic acid, amino cyclopropane carboxylic acid, sugars and amino acids	Ludwig-Müller et al. (2008)
Bubble column, hybrid bubble column and spray	*Hyoscyamus niger*	LBA 1334	Tropane alkaloids, scopolamine, anisodamine, hyoscyamine and cuscohygrine	Higher productivity of scopolamine on elicitation with MeJa and 3.5-fold higher anisodamine productivity in hybrid bioreactor	Jaremicz et al. (2014)
Bubble	*Lobelia inflata*	E1601	Polyacetylenes	Detected in bioreactor	Bányai et al. (2003)
Airlift (2.0 l)	*Lithospermum erythrorhizon*	15,834	Shikonin	Threefolds higher release in culture medium	Shimomura et al. (1991)
Two phase bubble column (1.5 l)		A4	Shikonin	Three times higher production in shake flasks (572.6 mg/l after 54 days)	Sim and Chang (1993)

(continued)

Table 5.4 (continued)

Bioreactor	Plant species	Strain	Metabolite/target	Response	References
Column (1.5 l)	*N. tabacum*	*A. rhizogenes*	Nicotine	Release of fourfold increased nicotine in culture medium	Wilson et al. (1987)
Airlift		A4 and TR105	Murine IgG monoclonal antibody	1.7 times higher levels of extracellular antibody as compared to shake flasks	Wongsamuth and Doran (1997)
With stainless steel (3.0 l)	*Ophiorrhiza pumila*	*A. rhizogenes*	Camptothecin	Release of 17% higher camptothecin in the culture medium	Sudo et al. (2002)
Air bubble column or stir	*Panax ginseng*	*A. rhizogenes*	Hairy roots biomass	16-fold increase in growth rate of hairy roots	Jeong et al. (2002)
Bench top fermenter with spargers (5.0 l)	*Picrorhiza kurroa*	LBA 9402	Picrosides	27-fold increased growth rate	Verma et al. (2015)
Sparger type	*Plumbago indica*	ATCC 15834	Plumbagin	Production of 13.16 mg/g dry wt. of total plumbagin on pre-exposure to chitosan and MeJa for 3 days	Gangopadhyay et al. (2011)
Air sparged and stirred tank	*P. rosea*	A4 (ATCC 43057)	Plumbagin	Increased production of plumbagin (1.425%)	Pillai et al. (2015)
Airlift (2.5 l)	*Pueraria phaseoloides*	ATCC15834	Puerarin	Accumulation of 200 times more puerarin (5570 μg/g dry wt.) than shake flask	Kintzios et al. (2004)
A glass-draught internal loop (2 l). Basic design airlift reactor with a novel modified mesh-draught and wire helixes	*Solanum chrysotrichum*	*A. rhizogenes*	Biomass	Increased growth rate	Caspeta et al. (2005)
Airlift (3.0 l)	*Silybum marianum*	*A. rhizogenes*	Silymarin	Enhanced production of silymarin after elicitation with MeJa	Rahimi et al. (2012)
Two phase organic-aqueous liquid system (1.3 l)	*Tagetes patula*	*LBA 9402*	Thiophene	Increased production by up to 30–70%	Buitelaar et al. (1991)

Airlift with mesh	*Trigonella foenum-graecum*	LBA9402	Sotolone	Production of sotolone and 3-amino-4, 5-dimethyl-2(5H)-furanone (1.2 and 17% of volatile fraction, respectively)	Peraza-Luna et al. (2001)
Stirred tank (5.0 l)	*Vinca minor*	A4	Vincamine	Eightfold increase in biomass after 40 days	Verma et al. (2014)
Mist principle based bioreactors					
Bubble column and mist (liquid and gas phase)	*Artemisia annua*	ATCC 15834	Biomass	Highest biomass (15.3 g/l and 14.4 g/l) on dry wt. basis in bubble column and mist reactors, respectively	Kim et al. (2002)
Mist and bubble column		ATCC 15834	Terpenoids gene expression	Expression of genes of terpenoids biosynthesis equivalent to that in shake flask grown roots	Souret et al. (2003)
Mist 1.0 to 20.0 l	*Artemisia annua* and *Arachis hypogaea*	ATCC 15834	Biomass	Increased biomass as compared to shake flasks	Sivakumar et al. (2010)
Bubble column, nutrient sprinkle and acoustic mist	*Cichorium intybus*	LMG-150	Coumarins, esculin and esculetin	Increased levels of esculin (18.5 g/l) in acoustic mist	Bais et al. (2002)
Mist and airlift (disposable)	*N. tabacum*	ATCC 15834	mIL-12 protein	First successful and higher production of pharmaceutical protein in mist bioreactor	Liu et al. (2009)
Nutrient sprinkle	*P. ginseng*	ATCC 15834	Ginsenoside	Ginsenoside production of (6 mg/g dry wt.)	Kochan et al. (2012)
Nutrient sprinkle with high concentration of sucrose, nitrogen and phosphorus	*P. quinquefolium*	ATCC 15834	Ginsenoside	Production of 2.75 times higher levels of Rg group of saponins	Kochan et al. (2014, 2016)
Nutrient sprinkle	*P. quinquefolium*	ATCC 15834	Ginsenoside	Enhanced production of ginsenosides (1.57 times higher) by YE	Kochan et al. (2017)

(continued)

Table 5.4 (continued)

Bioreactor	Plant species	Strain	Metabolite/target	Response	References
Mist	*Platycodon grandiflorum*	ATCC 15834	Saponins	High yield (5.93 g/100 g on dry wt. basis)	Urbańska et al. (2014)
Sprinkle bioreactor (10.0 l)	*Salvia sclarea*	LBA 9402	Diterpenoids from hairy roots	Highest accumulation of ferruginol, salvipisone, aethiopinone and 1-oxoaethiopinone after pre-exposure to 125 µM MeJa for 7 days	Kuzma et al. (2009)
Nutrient sprinkle	*Salvia officinalis*	ATCC 15834	Rosmarinic acid, diterpenoids, carnosic acid and camosol	Increased biomass (18-folds) after 40 days but hyper-hydricity of leaves and stem	Grzegorczyk and Wysokinska (2010)
Mesh hindrance mist trickling	*Stizolobium hassjoo*	*A. rhizogenes*	L-DOPA	2.2-fold increase in production	Sung and Huang (2006)
Air sparged, droplet and mist	*Fragaria x ananassa Duch.*	A4	Biomass	Highest biomass in air-sparged bioreactor (up to 3.7 g dry wt./l)	Nuutila et al. (1997)
Single use bag (20.0 l)	*Nicotiana tabacum* L. cv. Petit Havana SR1)	pBIN2.4VoGES1in LBA 9402 for the expression of *VoGES*	Terpenoids and indole alkaloid	Geraniol production	Ritala et al. (2014)
Plastic sleeve	*N. tabacum*	ATCC 15834	GFP recombinant protein	About 20% of total secreted protein (> 800 µg GFP/l) after 21 days of incubation	Medina-Bolívar and Cramer (2004)
Wave or spray	*Panax ginseng*	A4	Ginsenoside from hairy roots	First successful and threefold higher production of ginsenoside (145.6 mg/l) after 14 days in wave bioreactor	Palazón et al. (2003b)

5.7 Phytoremediation

Apart from secondary metabolite production, hairy root cultures have been used in phytoremediation and also phytomining. In phytoremediation, the hairy roots of some plants have been made to absorb and accumulate contaminants/toxic organic molecules from polluted soil and water. These toxic molecules are then enzymatically converted into nontoxic forms. At times, the toxic elements are made to accumulate in vacuoles of plant cells. The large surface area and highly branched nature of the hairy roots in general bring about higher contact between the contaminants and plant tissues (Suza et al. 2008). Some hairy roots with an ability to detoxify or sequester harmful organic and inorganic contaminants have been used to exude huge quantities of enzymes and metal chelating compounds.

The potential of hairy roots in accumulating and biodegrading hazardous compounds like heavy metals, textile dyes, PCBs, TNT, pharmaceuticals, phenolics, radionuclides, antibiotics, etc. is now a well-demonstrated fact (Table 5.5). While some plants like *Alyssum bertolonii* and *Thlaspi caerulescens* are hyperaccumulators of some heavy metals from polluted soil or water (Boominathan and Doran 2003; Boominathan et al. 2004), others like Indian mustard and *Chenopodium amaranticolor* take only a short duration for phytoremediation of toxic/hazardous compounds (Eapen et al. 2003). Still other plants have specificity for a particular contaminant. For example, the hairy root cultures of black nightshade are capable of metabolizing and removing PCBs from solutions spiked with PCB congeners (Macková et al. 1997; Kucerova et al. 2000; Rezek et al. 2007), whereas, the hairy roots of *S. nigrum* and *S. aviculare* can take up only cadmium. The hairy roots of *B. napus*, *B. juncea* and *Helianthus annuus* can detoxify dichlorodiphenyltrichloroethane and 2,4-dichlorophenol (pesticides), phenol and also tetracycline and oxytetracycline (antibiotics) from industrial effluents (Agostini et al. 2003; Suresh et al. 2005; Gujarathi et al. 2005; Singh et al. 2006). Among all the tested plant species, *Solanum aviculare* was the most efficient in removing 98.6% phenol from liquid medium, but hairy roots of *D. carota* removed 83.0% chlorophenol.

Studies also showed that the level of toxic/hazardous compounds removed from a medium or substrate varied between the hairy roots of different plant species. Thus, the hairy roots of *B. napus* were capable of removing phenol 100–1000 mg/l 2,4-DCP in the presence of H_2O_2 (Singh et al. 2006; Agostini et al. 2003). Even the hairy roots of *Brassica juncea*, *Beta vulgaris*, *Raphanus sativus* and *Azadirachta indica* were shown to remove phenol from the culture medium (Singh et al. 2006), but the hairy roots of *B. juncea* were found to have the best phenol removing capacity (97%). In the same year, the hairy roots of *Lycopersicon esculentum* were shown to be effective in removing 100 mg/l phenol but in the presence of 5 mM H_2O_2 (González et al. 2006). In vitro studies on the phenol and chlorophenol removing potentials of *Daucus carota*, *Ipomoea batatas* and *Solanum aviculare* were also tested by Araujo et al. (2006).

The level of tolerating toxic/hazardous compounds by the hairy roots of different plant species was found to vary significantly. Thus, while the roots of *D. carota*

Table 5.5 Phytoremediation through hairy roots

Aim	Contaminant	Source plant	Explant	A. *rhizogenes* strain	Culture medium & Condition	Remarks	Reference
Developing model(s) for cadmium (Cd) accumulation	Cd	*Beta vulgaris*, *Nicotiana tabacum*, *Calystegia sepium*	Various plant parts	A4	Modified MS + 1/5 N salts	Model for evaluating Cd accumulation from sewage sludge	Metzger et al. (1992)
Optimization of pH, buffer type, temperature, exposure time for maximum Cd^{2+} uptake		*Solanum nigrum*	–	Ri C58ci	MS in dark at 27 °C	MES buffer and borate ions promoted the uptake of 0.2 to 2000 ppm Cd^{2+}	Macek et al. (1994)
Accumulation of Cd by hairy roots		*Thlaspi caerulescens*	Seedlings	15,834	B5 at 25 °C and 100 rpm in dark	Hairy roots (10.6 mg dry wt.) accumulated 100 ppm Cd	Nedelkoska and Doran (2000)
Phytoremediation	Nickel (Ni)	*Alyssum* sp.	Seedlings	15,834	B5 at 25 °C and 100 rpm in dark	Accumulation of 4000 ppm Ni (17,500 µg/g dry wt. hairy roots)	Nedelkoska and Doran (2001)
Phytoremediation		*Alyssum murale*	Shoots 1.5–2.0 cm	A4M70GUS, pRiA4	MS	Accumulation of up to 23,700 µg/g dry wt.	Vinterhalter et al. (2008)

Effect of phosphate ions on phytoremediation of uranium	Uranium (U)	*Armoracia rusticana*	Callus	A4	Liquid MS at 28 °C in dark	Accumulation of U by hairy roots (4.0 mg/g, however, uptake reduced to half in absence of phosphate ions (2.0 mg/g	Soudek et al. (2011)
Phytoremediation		*Chenopodium amaranticolor*, *Brassica juncea*	Seedling	A4	Liquid MS at 40–50 rpm	Removal of up to 20–23% U from aqueous solution (5000 mM) by *B. juncea* hairy roots	Eapen et al. (2003)
Determining the tolerance to chronic uranium exposure		*Daucus carota*	–	–	–	U accumulation (4.0 and 563.0 mg/kg fresh wt.) by hairy roots after 34 days of exposure to 2.5 and 20.0 mg/l uranium	Straczek et al. (2009)
Accumulation of zinc by hairy roots	Zn	*Solanum nigrum*	–	A4 and K1	Liquid ½ MS + 3% sucrose at room temperature and 100 rpm in dark	Up to 90 and 98% Zn accumulation by hairy roots obtained from K1 and A4 strains within 18 and 15 days, respectively	Subroto et al. (2007)
Evaluation of colonization of hairy roots by *Rhizophagus irregularis*	Aqueous solution of Cd, Co, Cu, Pb and Zn	*Nicotiana tabacum*	Leaves	8916 and 9402	Liquid MS + 1.0 g/l ampicillin at 25 °C and 100 rpm	Colonization of hairy roots by *R. irregularis* – A promising tool for phytoremediation	Neagoe et al. (2017)

(continued)

Table 5.5 (continued)

Aim	Contaminant	Source plant	Explant	A. *rhizogenes* strain	Culture medium & Condition	Remarks	Reference
Removal of phenol, a major pollutant in aqueous effluents	Phenol	*Brassica juncea*	Seedlings	9402	Liquid MS at 50 rpm	*B. juncea* hairy roots tolerant to 50, 100, 200, 500 and 1000 mg/l phenol	Singh et al. (2006)
Phytoremediation of 10–250 mg/l phenol		*B. napus*	Leaf	LBA 9402	Liquid MS enriched with vitamins at 100 rpm at 25 °C in darkness	Removal of 80–100% phenol from solutions by hairy roots	González et al. (2012)
Phytoremediation		*Lycopersicon esculentum*	Leaves and stalk segments	LBA 9402	Liquid MS enriched with vitamins at 25 °C and 100 rpm in dark	Removal of 85% phenol by transgenic clone eight. Clones with higher peroxidase activity removed almost 50% of remaining phenol	Wevar-Oller et al. (2005)
Phytoremediation of 100 mg/l phenol from waste water		*L. esculentum*	Leaf explants	LBA 9402	Liquid MS at 25 °C and 100 rpm in dark	Removal of phenol by hairy roots in the presence of 5 mM H_2O_2	González et al. (2006)
Phytoremediation		*Nicotiana tabacum* hairy roots expressing *tpx1* and *tpx2* genes from tomato	Leaf	LBA 9402	Liquid MSRT at 25 °C and 100 rpm in dark	Removal of phenol by *tpx1* overexpressing tobacco	Alderete et al. (2009)

Determining the uptake and metabolism of phenol and chloro derivatives	Phenol and chloro derivatives	*Daucus carota*	Discs (3.0–5.0 mm)	LBA 9402	B5 + 3% sucrose +500 mg/l ampicillin	Removal of more than 90% of phenolic compounds from culture medium within 120 h	De Araujo et al. (2002)
Phytoremediation	2, 4-dichlorophenol (2, 4-DCP)	*Brassica napus*	Leaf	LBA 9402	Liquid MS + vitamins at 25 °C and 100 rpm in dark	Removal of 97–98% 2, 4-DCP from aqueous solutions of 100–1000 mg/l 2,4-DCP and 5–10 mM H_2O_2	Agostini et al. (2003)
Phytoremediation of phenol and chlorophenol	Phenol, 2-chlorophenol and 2, 4-DCP	*Daucus carota*, *Ipomoea batatas* and *Solanum aviculare*	Carrot discs, plants	LBA 9402	B5 medium +30 g/l sucrose at 25 °C under 100 rpm in dark	Removal of 72.7, 90.7 and 98.6% phenol and 83.0, 57.7 and 73.1% 2, 4-DCP within 72 h by *D. carota*, *I. batatas* and *S. aviculare*, respectively	Araujo et al. (2006)
Investigating the role of peroxidases in the removal of 2, 4-DCP	2, 4-DCP	*Nicotiana tabacum* expressing *tpx1* and *tpx2*	Leaves	LBA 9402	Liquid MS at 25 °C and 100 rpm in dark	Total peroxidases extracts from hairy roots can remove 2, 4-DCP from waste waters	Angelini et al. (2014)
Studying the metabolism of *N*-acetyl-4-aminophenol	N-acetyl-4-aminophenol	*Armoracia rusticana*	Nodal segment	A4	MS + 1 mM acetaminophen	Hairy roots – a suitable model for studying the fate of acetaminophen in plant tissue	Huber et al. (2009)

(continued)

Table 5.5 (continued)

Aim	Contaminant	Source plant	Explant	A. *rhizogenes* strain	Culture medium & Condition	Remarks	Reference
Phytoremediation of tetracycline, oxytetracycline from aqueous media	Tetracycline and oxytetracycline	*Helianthus annuus*	Seedlings	ATCC 15834	B5 salt mixture + vitamin +3% sucrose at room temperature and 110 rpm in dark	Hairy roots – a potential system for phytoremediation	Gujarathi et al. (2005)
Role of *C. intybus* and *B. juncea* hairy roots in phytoremediation	Dichlorodiphenyltrichloroethane (DDT)	*Brassica juncea*, *Cichorium intybus*	Seedlings	15,834	Liquid MS at 80 rpm in dark	Residual 14C DDT in hairy roots decreased from 77 to 61% in 10 days	Suresh et al. (2005)
Metabolism of trichloroethylene (TCE)	TCE	*Atropa belladonna* expressing p450 2E1	Leaves	A4 with rabbit P450 2E1	½ MS salts + 4% sucrose	Increased levels of metabolites in hairy roots	Banerjee et al. (2002)
Degradation of polychlorinated biphenyls (PCBs)	PCBs	*Solanum nigrum*	NA	–	LS + 2% sucrose at 26 °C in dark	Only 40% of residual PCBs left after 30 days of incubation	Macková et al. (1997)
Phytoremediation	Reactive green 19A HE4BD	*Sesuvium portulacastrum*	Leaf and stem	NCIM 5140	MS in dark	Degradation of up to 98% reactive green 19A HE4BD within 5 days of incubation	Lokhande et al. (2015)

showed normal growth on medium supplemented with 1000 μM phenol, *I. batatas* and *S. aviculare* could not tolerate phenol beyond 500 μM. Again, the hairy roots of all the three species, i.e. *D. carota*, *I. batatas* and *S. aviculare*, could not tolerate chlorophenol beyond 50 μM.

Various naturally hyperaccumulating plants as well as the model plant *Arabidopsis* were used to identify genes involved in metal uptake, sequestration and translocation. These genes were also employed to develop transgenic hairy roots and/or whole plants with enhanced phytoremediation potential. For example, the hairy roots of transgenic *Atropa belladonna* expressing the heterologous protein, p450 2E1, were used to metabolize trichloroethylene without the exudation of the protein in the culture medium (Banerjee et al. 2002). In another study, the hairy roots of *tpx1* and *tpx2* expressing transgenic *N. tabacum* were used for effective removal of 2, 4-dichlorophenol from liquid medium (Angelini et al. 2014). The details of these studies are summarized in Table 5.5.

5.8 Phytomining

Plants are known to absorb valuable metals from low-grade mining ores and metal-polluted soils. Thus, their use in phytomining of valuable metals is fast becoming an attractive approach. However, such plants are generally required to have large biomass, high ability to absorb metals from soil and also a capacity to accumulate metals in cells and tissues. The metal hyperaccumulating plants include *Alyssum bertolonii*¸ *Berkheya coddii*, *Haumaniastrum* sp., *Atriplex confertifolia*, *Thlaspi* sp., *Astragalus pattersoni*, *Iberis intermedia*, *Macadamia neurophylla*, *Streptanthus polygaloides*, etc. (Brooks and Robinson 1998). Even non-hyperaccumulators like *Eleocharis acicularis* can be induced to absorb and accumulate metals (Ha et al. 2011). All of these plants have been found to absorb, collect and store valuable metals in their tissues and use them to deter the herbivores that feed on them. The metals that are absorbed and stored in plant tissues include gold, uranium, platinum, nickel, thallium, rhenium, cadmium, zinc and gold (Brooks and Robinson 1998; Sheoran et al. 2013; Novo et al. 2017). The extraction of these highly priced metals from soil is extremely laborious, difficult and expensive. Their extraction from a large biomass of plants/parts using phyto-based extraction technologies and smelting is also highly inexpensive and simple. Hairy roots of both hyper- and non-hyperaccumulating species have been effectively employed for phytomining of metals like Cd and Ni (Al-Shalabi and Doran 2013). Earlier, dried hairy root biomass of Ni hyperaccumulator, *A. bertolonii*, was shown to yield Ni-enriched bio-ore after treatment in laboratory-scale horizontal tube furnace (Boominathan et al. 2004). When the accumulated Ni in *A. bertolonii* was compared with that of whole plant, it was found to be about 15 times higher. On the other hand, considerably lower accumulation of Ni was recorded in the hairy root cultures of *A. tenium* (Boominathan et al. 2004; Nedelkoska and Doran 2001). In recent years, the hairy

roots of metal hyperaccumulators are fast emerging as efficient systems for the production of quantum dot nanocrystals and their use as potential tools in the manufacture of peptide-capped semiconductor quantum dots (Al-Shalabi and Doran 2013).

5.9 Conclusion

Hairy root cultures have emerged as an important biotechnological tool for various fundamental studies as well as industrial or environmental applications. These have ranged from conservation of high value threatened medicinal plants to production of valuable recombinant proteins and their upscaling. Phytoremediation and phytomining of valuable metals and synthesis of nanocrystals with applications in engineering are the other important uses of hairy roots.

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Chapter 6
In Vitro Culture and Production of Secondary Metabolites in *Centella asiatica*

Shweta Kumari, Shashikant, Nitish Kumar, and Maheshwar Prasad Trivedi

Abstract Plants are ever a valuable source of secondary metabolite which is used for curing various diseases whether it is mild or chronic. Due to huge medicinal importance of plants, studies on plants have been focused worldwide. *Centella asiatica* is an important medicinal plant, used as brain tonic all over the world. In a broad spectrum application of *Centella asiatica*, it has been listed in threatened species. The rapid depletion and high demands of their bioactive molecules feel the necessity for their conservation. In vitro culture and micropropagation are basic tools for conserving this medicinal plant and for production of secondary metabolites. In the present book chapter, we focused on conservation of *C. asiatica* through in vitro culture, production of secondary metabolites and strategies employed for the enhancement of secondary metabolites through manipulation in culture media, effect of growth regulators and elicitation.

Keywords *Centella asiatica* · Elicitation · Growth regulators · In vitro culture · Secondary metabolite

6.1 Introduction

Since many years plants with secondary metabolites have been used for curing diseases whether it be mild or chronic and thus have gained worldwide attention for their medicinal role. The increasing focus on research of plants have certified the effective curing property of its (Vaidya 1997; Dahanukar and Kulkarni 2000; Stafford et al. 2008). *Centella asiatica* is a tropical medicinal plant which belongs

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to family Apiaceae. This plant is found in South Asia such as India, Sri Lanka, China, Indonesia and Malaysia and also distributed in South Africa, South Pacific and Eastern Europe (Gohil et al. 2010; Orhan 2012). *C. asiatica* is a perennial herb, commonly known as mandukparni or Indian pennywort. This medicinal plant re-energizes brain cells and nerves thus also known as brain food of India (Gohil et al. 2010; Bhavna and Jyoti 2011; Seevaratnam et al. 2012; Rao et al. 2015). The major primary active constituent of *C. asiatica* is triterpenoids (Gohil et al. 2010; Gandi and Giri 2012; Rao et al. 2015). These active constituents or secondary metabolites are mainly responsible for these biological activities to curing diseases (James and Dubery 2009; CH et al. 2011; Mahapatra and Kumar 2012; Prasad et al. 2014). The active constituent comprises asiaticoside, asiatic acid, madecassic acid, asiaticoside, madecassoside, brahmic acid, brahminoside, thankiniside, isothankunisode, centelloside, madasiatic acid, centic acid and cenellicacid. Among all these active constituents, asiatic acid, madecassic acid, asiaticosside and madecassoside are the most active compounds (James and Dubery 2009; Gohil et al. 2010; CH et al. 2011; Orhan 2012; Seevaratnam et al. 2012; Govarthanan et al. 2015). Besides treating neurological disturbances, this medicine shows numerous activity such as wound healing activity (Shukla et al. 1999; Somboonwong et al. 2012), anti-inflammatory (Park et al. 2017), antioxidant (Sugunabai et al. 2015), antibacterial (Arumugam et al. 2011), antifungal (Singh and Maurya 2005), antidiabetic (Chauhan et al. 2010), antitumor (Bunpo et al. 2004), anxiolytic (Ramaswamy et al. 1970), antiviral (Yoosook et al. 2000) and antiproliferative activity (Mutua et al. 2013). According to reports due to diverse pharmacological properties, wild stock of this plant has been notably reduced, because of its unrestricted exploitation with limited cultivation. Hence it has been listed as threatened plant species by International Union for conservation of nature and natural resources (Naidu et al. 2010; Bhavna and jyoti 2011; Seevaratnam et al. 2012). The rapid depletion of medicinal plants and high demands of their bioactive molecules feel necessity for their conservation. Biotechnological tools such as micropropagation, in vitro culture, genetic transformation and development of DNA banks endeavour new methods for conservation of medicinal plants. The application of in vitro culture has the potential to enhance propagation and yield high value bioactive molecules of medicinal plants. In vitro plant cell shows physiological and morphological changes in the vicinity of microbial, physical or chemical agents known as elicitor's molecule. Elicitation is the process in which production of secondary metabolites enhances in plant cell culture. Induction of elicitor to enhancement of secondary metabolites production in *Centella asiatica* has reported by various researcher (Narula et al. 2004; Kim et al. 2004a, b; Mangas et al. 2006;Rai 2010; Prasad et al. 2013; Goyal et al. 2014; Rao et al. 2015). The current review emphasizes on conservation of *Centella asiatica* species through in vitro culture and enhancement of secondary metabolite production as well as future prospect of large-scale production of secondary metabolites through the bioreactor and how elicitor altered the secondary metabolite production in *C. asiatica* plant species.

6.2 In Vitro Culture and Secondary Metabolite Production in *Centella asiatica*

In vitro culture of medicinal plants is basic tool for conservation of threatened species as well as producing bioactive molecule in pharmaceutical industries. Micropropagation maintains consistent supply of valuable medicinal product and their consumption; thus development of a regeneration protocol has necessity to conserve medicinal plant (Dakah et al. 2014). In vitro regeneration has immense potential to develop explants into a whole plant. The plantlets produced by the tissue culture are pathogen free and offered enormous amount of secondary metabolites (Sharma and Dubey 2011). Elicitors as well as also growth regulators effects on asiaticoside production in *C. asiatica* species (Kim et al. 2004a, b).

6.2.1 Direct Shoot Regeneration Through Various Explants in Centella asiatica

Development of plants without involving callus stage is termed as direct regeneration. Direct regeneration may facilitate consistent genetic uniformity as compared to involving the callus stage. Several researches have been reported on direct regeneration in elite species of *C. asiatica* by the used of various explants. Axillary bud proliferation is obtained from nodal segment of CA. Synergistic combination of 22.2 μM BA and 2.68 μM NAA shows maximum response (91%) as well as four to five shoots per explants (Tiwari et al. 2000). An efficient multiple shoot regeneration protocol has been reported from shoot tip of *C. asiatica* species. The highest multiple shoot regeneration is obtained at MS having 4 mg/l BAP and 0.1 mg/l NAA at approximately 76.67% (Das et al. 2008). In *Centella asiatica* petiole showed better explants for induction of adventitious roots than leaf. Explants inoculated in MS augmented with IAA, IBA and NAA at 0, 1, 3, 5 and 7 mg/l. IBA showed best PGR for formation of adventitious roots as compared to NAA and IAA. Leaf explants showed maximum number of roots, as well as length of root at IBA 7 mg/l, while IBA 5 mg/l showed maximum roots for petiole explants (Ling et al. 2009). A regeneration protocol is established from a shoot tip of *C. asiatica*. A maximum number of shoots as well as leaf were obtained on MS supplemented with 4 mg/l BA and 0.1 mg/l NAA in CA species. Microshoots were further transferred into full strength MS comprising IBA (1–3 mg/l) and (0.5–2 mg/l) NAA. Profuse rooting (46.8 per shoot) was obtained on MS supplemented with 2 mg/l IBA with root length of 197.7 cm (Nath and Buragohain 2003). Multiple shoot regenerations are obtained from a shoot tip on MS augmented with 17.76 μM and 1.44 μM GA_3 in CA. A maximum number of roots (27.66) are obtained from MS comprising NAA at 10.74 μM (Sivakumar et al. 2006). In vitro culture of leaf explants showed maximum regeneration (81.6%) with maximum shoot length as well as shoot height as compared to petiole explants having callus formation at cutted base end of

C. asiatica. The highest multiple shoots are obtained from MS 3 mg dm^{-3} BAP and 0.05 mg dm^{-3} NAA. Regenerated shoot was further transferred into half-strength MS media augmented with 0.5 mg dm^{-3}. IBA shows maximum root regeneration (76.8%) with three to four roots per shoot in CA (Mohapatra et al. 2008). A reproducible multiple shoot regeneration protocol was developed from nodal explants in *C. asiatica*. Best regeneration of shoot is obtained from MS supplemented with 2 mg/l BAP and 0.5 mg/l KN (90.2% average, 16.3 shoots). The highest root regeneration was observed in MS comprising 1 mg/l NAA and 1 mg/l. IBA shows 92.2% average of 16.5 roots per shoot (Singh et al. 2014). Rapid multiplication of multiple shoots using leaf explants was established. The best multiple shoot regeneration was obtained on MS containing 2 mg/l 6-benzylaminopurine and 0.5 mg/l α-naphthalene acetic acid with 30 gm/l sucrose and 8 mg/l agarose. Further rooting regenerated shoots was transferred in MS comprising 1 mg/l 2,4-dichlorophenoxy acetic acid or 2, 4, 5-trichlorophenoxy acetic acid. MS medium supplemented with 1 mg/l of indole-3-butyric acid shows primary roots as well as secondary roots (Kumar 2017). Figure 6.1 shows direct regeneration of shoots from leaf explants.

6.2.2 Indirect Regeneration in Centella asiatica

Indirect regeneration depicts about development of complete plantlets with intervening callus stage. It has two different stages, dedifferentiation and redifferentiation. Dedifferentiation initiates when mature cells regress into meristematic cells and produce an unorganised mass of cells called callus. Redifferentiation initiates after callus formation in which differentiated callus forms a specialise group of primordial cells and regenerates into complete plantlets. A very few research have been reported in regeneration in *Centella asiatica* intervening callus stage which depends on combination as well as composition of plant growth regulators. In *C. asiatica* leaf explants showed the highest regeneration in comparison with stem explants (42.4 and 54.8 shoot/culture in stem and leaf). MS medium supplemented with 2 mg/l Kn and 4 mg/l NAA forms callus. The highest regeneration is observed on 4 mg/l benzyladenine, 2 mg/l kn and 0.25 mg/l NAA and 20 mg/l adenine sulphate. Regenerated shoots were transferred into half-strength MS augmented with 0.5 mg/l IAA with 2% sucrose for root induction (Patra et al. 1998). Regeneration through callus by using nodal explants was developed in *C. asiatica*. Different combinations and compositions of PGR with MS achieved well-developed regenerated plantlets. Maximum callus induction is observed on MS supplemented with 4 mg/l NAA and 2 mg/l 2,4-D (92%), while the highest regeneration is obtained on BAP 1.5 mg/l and Kn 1.5 mg/l (Naidu et al. 2010). Good quality callus was obtained on MS supplemented with 6-benzyladeanine alone or combination with NAA. Different combinations and compositions of plant growth regulators mostly produced

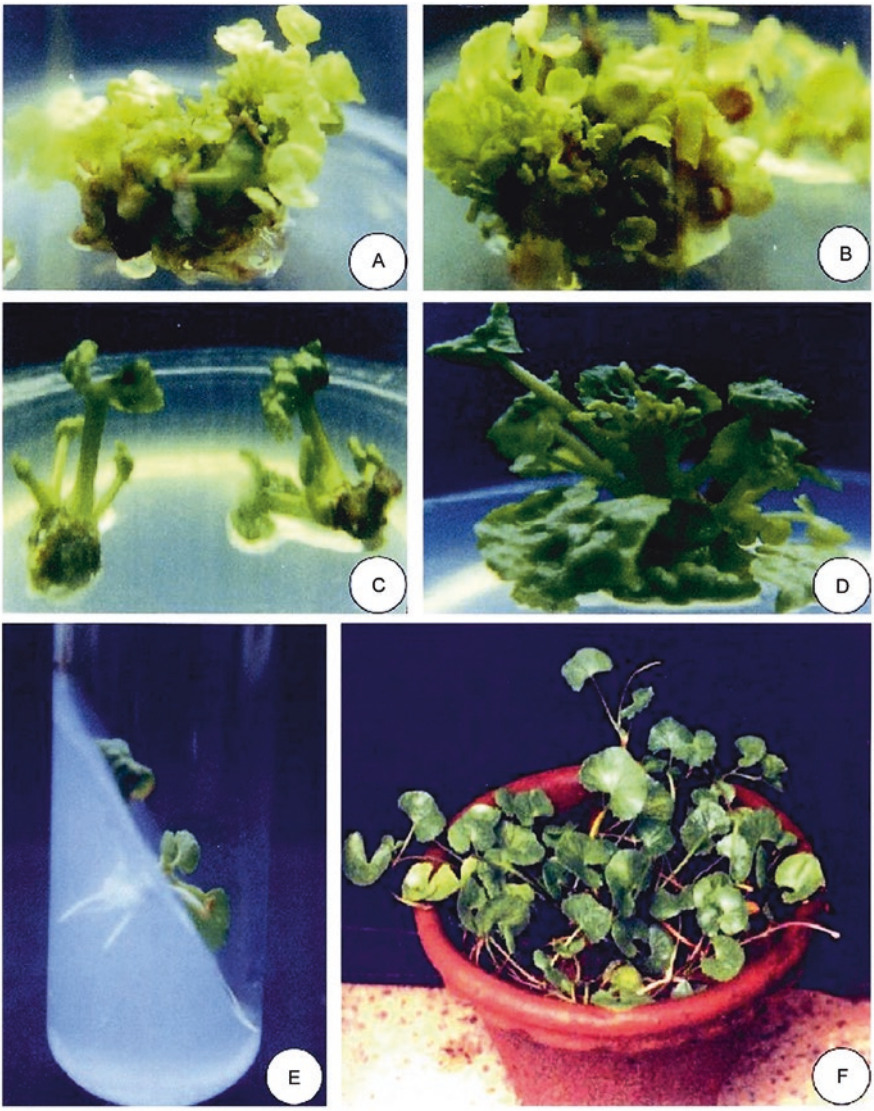

Fig. 6.1 (**a**) Multiple shoot initiation from leaf explant on MS + 3.0 mg dm-3 BAP + 0.05 mg dm-3 NAA at 12 d of culture, (**b**) elongation of in vitro shoots on the same medium at 27 d of culture, (**c**) multiple shoot initiation from nodal explant on MS + 3.0 mg dm-3 BAP + 0.05 mg dm-3 NAA with slight callus at the base after 15 d of culture, (**d**) elongation of in vitro shoots of on the same medium at 27 d of culture, (**e**) a rooted shoot on ½ MS + 0.5 mg dm-3 IBA after 12 d of culture, (**f**) an acclimated plant in garden soil. (Source Mohapatra et al. 2008 Licence No- 4178880162801)

embryogenic and green callus. Non-embryogenic callus can be converted into embryo or plantlets when cultured in suitable media. Maximum shoot regeneration was obtained on MS augmented with 4.42 μM BA and 5.37 μM NAA (ten shoots per callus). The best rooting is observed on MS comprising NAA at 5.37 μM and 10.47 μM, 20 roots per explants (Bibi et al. 2011). In *C. asiatica* species, stem explants showed best callusing as well as shooting via callus-mediated regeneration than leaf explants. The highest callusing was observed on MS comprising 0.5 mg/l BAP and 0.3 mg/l NAA (75% for leaf and 83.33% for stem explants, respectively). Leaf and stem explants show best callus-mediated regeneration on BAP at 0.5 mg/l and 0.75 mg/l, respectively. For rooting regenerated shoots were inoculated on MS augmented with IBA 0.5 mg/l (Joshi et al. 2013). The increasing concentration of 2,4-D leads to direct organogenesis which has been reported in *C. asiatica*. Morphology and texture of calluses also change by increasing the concentration of 2, 4-D. Maximum frequency of callus is obtained on 2 mg/l 2,4-D and 0.5 mg/l BAP. The highest shoot regeneration is obtained on BAP 2.5 mg/l and NAA 0.5 mg/l. Regenerated microshoots were further inoculated in rooting media, maximum rooting frequency as well as root length observed on MS augmented with 1 mg/l IBA (Panathula et al. 2014). The combination of NAA and BAP reduced multiple shoot regenerations, while combination of Kn and BAP enhanced maximum shoot regeneration on MS supplemented with 1 mg/l kn and 1 mg/l BAP. Callus was obtained from pale green leaf on MS comprising 1 mg/l 2,4-D, 0.5 mg/l kn and 4 mg/l TRY (Palengara 2017).

6.2.3 Somatic Embryogenesis in Centella asiatica

Somatic embryogenesis is the development of somatic cell into bipolar embryo structure and resembles zygotic embryo morphologically which bears typical embryo organ. Plant regeneration through the shoot bud has multicellular origin; this may result into a production of variable and chimeric plants, while somatic embryos assume to single cell origin either directly or indirectly and can lead to develop into genetically stable and nonchimeric plants. Paramageetham et al. (2004) first reported somatic embryogenesis in *Centella asiatica*. Maximum somatic embryos were obtained on MS which comprises 9.29 μM kinetin and 2.26 μM 2,4-D. Granular, white, shiny callus produced heart- and cotyledonary-shaped embryos on 9.29 μM kinetin and 2.26 μM 2,4-D. Further somatic embryos are inoculated into MS supplemented with 2.32 μM kn with 2.89 μM GA_3 for germination of somatic embryos (Paramageetham et al. 2004). A significant effect of plant growth regulators and type of explants on somatic embryogenesis as well as regeneration has been reported in *C. asiatica*. Best callus was obtained on Kn with NAA as compared to Kn with 2,4-D and showed earlier induction and maturation of embryo. Half-strength MS is fortified with NAA 2.69 μM and Kn 1.16 μM with induced mean of 204.3 somatic embryos per 100 gm of callus, while half-strength MS augmented with 0.45 μM 2,4-D and 1.16 μM Kn showed mean of 303.1 somatic embryos per

100 gm of callus. Combination of KN with 2,4-D developed high somatic embryos in comparison with Kn with NAA. Maximum percentage of somatic embryos is obtained from callus on MS supplemented with 4.52 μM 2,4-D and Kn 2.32 μM or NAA 5.37 μM and 2.32 μM Kn. Transfer of somatic embryo into half-strength semisolid MS media comprising 0.054 μM NAA either 0.044 μM BA or 0.046 μM Kn induced the development of somatic embryos into plantlets (Martin 2004). Effect of 2,4-D on somatic embryogenesis by using leaf and stolon tip has been demonstrated in *Centella asiatica* plant species. MS supplemented with 0.45 μM 2,4-D-induced somatic embryos exhibits various stages such as globular, heart and cotyledonary stages. Leaves produced calli on two different concentrations of 2,4-D (2.26 and 4.52 μM), while stolon tip produced calli on only one concentration of 9.04 μM 2,4-D. Further maturation and development of embryos into plantlets are induced by withdrawing treatments of 2,4-D (Joshee et al. 2007). Effective regeneration via somatic embryogenesis by the leaf explants has been reported. Compact, light green callus on MS fortified with 0.5 mg/l, 1 mg/l, 1.5 mg/l and 2 mg/l 2,4-D was obtained. Higher concentration of 2,4-D has induced embryoid formation. Maximum shoot regeneration is achieved on Kn and BA 1.5 mg/l and 2 mg/l with 0.2 mg/l NAA and IAA. MS augmented with 2 mg/l NAA and 2 mg/l IAA showed maximum root frequency (Biradar 2017).

6.2.4 Secondary Metabolite Production Through Tissue, Hairy Root Culture and Bioreactor

Secondary metabolites are not necessary to the survival of plants, but they play an important role to cope with environmental stresses for staying alive. These metabolites always protect plants from biotic and abiotic stresses. *Centella asiatica* contains triterpenoid saponins and bioactive molecules which have significant therapeutic properties. Several biotechnological approaches have been developed for the bioproduction of secondary metabolites such as in vitro cell, organ and hairy root culture of plant as well as scaling up from small scale to large scale through the bioreactor. Plant cell tissue and hairy root culture have immense potential to synthesize and store high value secondary metabolites along with conserve consistent flow of demand and supply of secondary metabolites (Rao and Ravishankar 2002; Karuppusamy 2009; Sree et al. 2010; Galleo et al. 2014; Pagare et al. 2015). Few researches have been reported in secondary metabolite production through the tissue culture. Terpenoid content of *C. asiatica* is tissue specific and varied between in vitro and in vivo of two phenotypes. Two different phenotypes such as fringed (F) and smooth (S) leaf were used to analyse terpenoid content of three explants such as leaf, root and petiole of those phenotypes in CA. Terpenoid content was highest in leaf in comparison with root and petiole. Fringed leaf contains (0.79 ± 0.03) asiaticoside and (0.97 ± 0.06) madecassoside, while smooth leaf contains (1.15 ± 0.10) asiaticoside and (1.65 ± 0.01) madecassoside % dry mass, respectively. F phenotype

comprises (0.12 ± 0.01%) dry mass asiaticoside in root, whereas S phenotypes contain 0.16 ± 0.01% of dry mass of asiaticoside and 0.18 ± 0.14% of dry mass of madecassoside in petiole explants (Aziz et al. 2007). Tan et al. (2010) first reported the establishment of suspension for flavonoid production by using different pant growth regulators in *C. asiatica*. Inoculated leaf explants comprises accession UPM01, UPM02, UPM03 and UPM04 on MS containing B_5 and augmented with different concentrations of (0.5–2.5 mg L − 1) 2,4-D, NAA, dicamba, picloram and IBA alone or combination with kinetin, BAP and TDZ (0.5–1.5 mg L − 1). Maximum callus achieved on 2 mg/l 2,4-D (86.67%) whereas highest biomass production (0.27 gm DW/culture) obtained from 2 mg/l 2,4-D along with 1 mg/l Kn for highest biomass production (0.27 gm DW/culture). HPLC analysis reveals that UPM03 accession contains the highest flavonoid content (10.75 mg/g DW) as well as biomass 0.41 g DW/culture (Tan et al. 2010). Suspension culture contains (45.35 mg/g DW) significantly higher asiaticoside such as 4.5-fold than in vitro culture (10.55 mg/g DW) which has been demonstrated in *Centella asiatica*. Petiole explants were inoculated on MS fortified with 20gm/l sucrose, 1 mg/l BAP and 1 mg/l NAA for callus induction. Further 2 gm of this callus is transferred into 50 ml of same medium without solidifying agent with agitation speed of 100 rpm. After 24 days of culture 9.03 g/50 ml biomass of callus produced with agitation speed 120 rpm contains 30 gm/l sucrose and 3 gm of inoculums size on the same MS medium (Loc and An 2010). Similarly, asiatic acid content was analysed and observed that callus contains higher concentration as compared to shoot bud in *Centella asiatica*. Regenerated shoot bud is having 1.02 ± 0.03 mg/g FW and 0.47 ± 0.08 mg/g FW asiatic acid content, respectively, while callus contains 1.46 ± 0.06 asiatic acid (Gandi and Giri 2013). Quantification of bioactive molecules by using various explants, viz. root, stem, leaf and leaf-derived callus, has been reported in CA. The maximum callus was obtained on NAA 1 mg/l and 0.5 mg/l BAP and quantified various secondary metabolites such as saponins, flavonoid and terpenoid by using standard protocol (Rao et al. 2015). A very few research has been reported regarding secondary metabolite production through infection of *Agrobacterium rhizogenes* in *Centella asiatica*. This bacterium infects the plant and causes hairy root disease. *Agrobacterium* transformed the root of CA and produced high growths in hormone-free media as well as secondary metabolite in comparison with untransformed root. Elicitation or manipulation in culture media may enhance growing capability of bacteria that produces significant amount of secondary metabolites (Bensaddek et al. 2008; Chandra and Chandra 2011). An efficient protocol of transformation through *Agrobacterium rhizogenes* has been reported in *Centella asiatica*, but no asiaticoside is detected in transformed root (Kim et al. 2007). In *Centella asiatica* asiaticoside production was enhanced by 166–172% after transformation through *A. rhizogenes* which may follow by the treatment of elicitor molecules (Ruslan et al. 2012). A suspension culture protocol in bioreactor and optimization of culture condition as well as inoculums size on effect production of asiaticoside was first reported by Loc and Nhat (2013). The cell growth and

production of asiaticoside peaked at 24 days of culture. Cell growth achieved maximum value of 302.45 g fresh weight ((31.45 g dry weight) and growth index of 3.03 with inoculum size 100 gm, aeration speed 150 r/min and aeration speed 2.5 l/min, although maximum asiaticoside (60.08 mg/g dry weight) is obtained from inoculum size of 50 gm in *Centella asiatica* (Loc and Nhat 2013).

6.3 Strategies for Enhancement and Analysis of Secondary Metabolite in *Centella asiatica*

Enhancement of secondary metabolite production is a prime requirement to overcome the problem of insignificant amount of secondary metabolite production. Since supply is limited

and increasing demand to consistent production faces constrains, thus researcher has developed an alternate technique to control supply of these bioactive molecules independent of availability of plants and season. Successful attempts to enhance huge quantity of these secondary metabolite productions offered a way to endure these constrains in CA (Kim et al. 2007; Monica et al. 2013).

6.3.1 Enhancements by Manipulation in Culture Media and Plant Growth Regulators

Plants faced stress condition in tissue culture medium, but genome of plant is able to protect them by secreting secondary metabolite. Some compound act as signal molecules such as sugar, PGR and elicitors which enhance secondary metabolite production in medicinal plants. Some studies have been reported on enhancement of secondary metabolite through the change of PGR and sugar concentration in *Centella asiatica*. Positive effect of TDZ on enhancement of secondary metabolite production has been identified in *C. asiatica*. Nodes and B_5 medium augmented with 0.01 mg/l 2,4-D reduce the asiaticoside production and growth in *C. asiatica* plants. Among the all used cytokinins (TDZ, BA, zeatin and kinetin), TDZ is the best for asiaticoside biosynthesis in *Centella asiatica* (Kim et al. 2004a). Effects of different combinations and concentrations of auxin on rhizogenesis as well as culture condition on enhancement of secondary metabolites on different root morphotypes produced by rhizogenesis in leaf explants of *Centella asiatica* have been studied. They also analysed the effect of culture condition such as pH, nature of carbon source (glucose, fructose, mannitol) as well as sucrose on induction of root morphotypes and enhancement of secondary metabolites production in *Centella asiatica* species. HPLC analysis revealed the enhancement of triterpenoid production and validated by expression of key gene coupled with their biosynthetic pathway in nontransformed root morphotypes of *Centella asiatica* (Singh et al. 2014).

Influence of $NH4^+$ -N: NO_3^--N or Cu^{2+} was confirmed on enhancement of asiaticoside production in *C. asiatica*. Maximum asiaticoside concentration (3.8 mg/g) and growth index (6.06 mg/g) are obtained on MS supplemented with 2.5 mg/l Kn in 35th day of culture. When concentration of nitrogen decreases from 60 mM to 50 mM or 40 Mm in culture media, the accumulation of asiaticoside enhances from 5.3 to 8.9, and dry weight is 8.7 mg/g in *C. asiatica* species. Effect of Cu^{2+} and sucrose on asiaticoside production has also been observed. Medium containing 0.10 lM Cu^{2+} produced 4.4 mg/g dry weight asiaticoside and growth index of 5.8, while medium without Cu^{2+} produces 7.05 mg/g asiaticoside and has growth index of 7.7.Thus Cu^2 reduces the asiaticoside production. Higher sucrose concentration enhances production of asiaticoside as well as biomass. Sucrose 5% or 7% contains growth index (17.1) and asiaticoside content (7.2) and sucrose (3%) having growth index (16.9) and asiaticoside (5.2 mg/g) dry weight, respectively (Prasad et al. 2012). An efficient protocol on effect of carbon source (sucrose, fructose) as well as elicitors (Malt extract, salicylic acid and jasmonic acid) on enhancement of secondary metabolite in different accessions of *Centella asiatica* has been explored. MS supplemented with 3 mg/l sucrose and 1.5 mg/l BAP shows better for enhancement and biomass production (Kundu et al. 2016).

6.3.2 Enhancement of Secondary Metabolite Through Induction of Elicitor Molecules

Plant produces certain molecules after induction of various biotic and abiotic factors, these factor known as elicitor's molecule. These molecules trigger plant defence mechanism. Most of biotic elicitors are produced by pathogenic microorganism or release by cell wall of microorganism after interaction with plant enzyme, while abiotic elicitors are metallic compound. However these pathogenic microorganisms also promote liberation of endogenous elicitor from plant cell. Endogenous elicitors such as methyl jasmonate and jasmonic acid mediate plant response against abiotic stresses (Ghorpade et al. 2011; Wang and Wu 2013). Several studies have been reported for the enhancement of secondary metabolite production through the elicitors. Among various elicitors methyl jasmonate and yeast extract stimulate 1.53- and 1.41-fold asiaticoside production in *Centella asiatica* (Kim et al. 2004a, b). Used yeast extract, methyl jasmonate, $CdCl_2$ and $CuCl_2$ for enhancement of secondary metabolite in CA, 1 mM methyl jasmonate was used for maximum asiaticoside (116.8 mg/l) content. The highest asiaticoside (342.72 mg/l) is obtained at 36th day of elicitation culture using 0.1 mM MJ and 0.025 mg/l 1-phenyl-3-(1,2,3-thidiazol-5-yl)urea (TDZ). Methyl jasmonate enhances secondary metabolite production as well as influences senescence in *Centella*. TDZ does not affect production of asiaticoside but increases shoot growth in *Centella asiatica* (Kim et al. 2004a, b). In a study, methyl jasmonate may be applied as inducer for triterpenoid synthesis from 2,3-oxidosqualene in *Centella asiatica* plant species. One hundred fifty-two

times higher triterpenoid content were obtained after the treatment of methyl jasmonate in comparison with untreated culture. The result showed methyl jasmonate also inhibits sterol synthesis from cycloartenol (Mangas et al. 2006). Root produces a very few quantity of secondary metabolite; a highly efficient protocol has been optimized for transformation in *Centella asiatica* root through the *Agrobacterium rhizogenes* followed by enhancement of secondary metabolite production elicited by methyl jasmonate (Kim et al. 2007). Using *Agrobacterium rhizogenes* strain R1000 that harbours pCAMBIA1302 having hygromycin phosphotransferase (hpt) and green fluorescence protein, (mgfp5) genes act as marker, and transformation was confirmed by PCR and southern blot analysis. Transformed root did not produce asiaticoside till 5 weeks of inoculation. However treatment of .1 mM methyl jasmonate for 3 weeks produced maximum asiaticoside of 7.12 mg/g drywt. The expression of gene CabAS (*C. asiatica* putative b-amyrin synthase) was significantly different from control after 12 h of MJ treatment in *C. asiatica*. It has shown fungal elicitors are dose and culture age dependent in multiple shoot cultures in *Centella asiatica*. Fungal elicitors culture filtrate (CF) such as *Trichoderma harzianum* (3% v/v), *Colletotrichum lindemuthianum* (1.5%v/v) and *Fusarium oxysporum* (0.5–1.5% v/v) were used for production and enhancement of secondary metabolite. *Trichoderma harzianum* was added on the 10th and 35th day of culture and yielded 9.63 mg/g dry weight content and 1.15 mg dry weight/culture enhanced 2.53- to 2.35-fold asiaticoside content with growth index of 7.67. *Colletotrichum lindemuthianum* was added on day 0th yielded 1.10 mg/g dry weight reduced 3.5- to 8.7-fold asiaticoside content with growth index of 16.10, and *Fusarium oxysporum* was added on day 0th and 30th and yielded 0.18–0.42 mg/g dry weight/culture, and 0.18–0.94 mg/g dry weight/culture show poor asiaticoside content with growth index of 4.85–8.45, respectively, as compared to untreated control. All the above results showed that the *Trichoderma harzianum* has the potential to upregulating asiaticoside production in *Centella asiatica*. (Archana et al. 2013) In another study, it has shown that elicitor concentration and elicitation day effect enhancement of asiaticoside in *C. asiatica* species. The 2-hydroxybenzoic acid is better than yeast extract for the enhancement of asiaticoside production in *Centella asiatica*. Addition of 100 μM 2-hydroxybenzoic acid on day tenth enhances production secondary metabolites fivefold, while in the case of yeast extract, 4 g/l enhances 3.5-fold in *Centella asiatica* (Loc and Giang 2012). The important role of methyl jasmonate in the upregulation of secondary metabolite in *C. asiatica* has also been identified. They were analysed for metabolic profiling of asiaticoside and madecassoside acid as well as aglycones, asiatic acid and madecassic acid. About 0.2 mM methyl jasmonates were added on 2, 4 and 6 days of cell suspension culture. Liquid chromatography along with mass spectrometry revealed both quantitative and qualitative variability between control and treated sample (James et al. 2013). In vivo asiatic acid content of different plant parts and in vitro elicitation of asiatic acid applying organic elicitor have been evaluated in *Centella asiatica*. HPLC analysis revealed in vivo highest asiatic acid (190.2 μg/g fresh wt.) present in root part, while addition of organic elicitor produced (258.3 μg/g fw.) asiatic acid in leaf callus (Mohammadparast et al. 2014). Different concentrations of chitosan and its

derivatives such as elicitor effects, wet weight of hairy root as well as asiaticoside content have been studied in *Centella asiatica*. The results showed addition of 30 ppm of chito-oligosaccharide yielded maximum fresh weight of hairy root (551, 68 mg/g) and asiaticoside content (5, 97 mg/g), fresh weight in *C. asiatica* (Zahanis et al. 2016).

6.4 Analysis in Secondary Metabolites in *Centella asiatica*

Secondary metabolites are those compounds which plants secrete in stress condition. Total secondary metabolite such as flavonoids, phenols and tannins has been estimated using standard protocols. Quantitative as well as qualitative analysis of secondary metabolite is essential for identifying compound available in medicinal plants. Several studies have been reported in quantitative and qualitative analysis of secondary metabolite in *C. asiatica*. Separation of triterpenes such as asiaticoside, madecassoside and asiatic and madecassic acid has been established by using reverse phase HPLC employing acetonitrile/water on RP 18 columns on detection wavelength of 205 nm. Quantification of triterpenoid is accomplished by calibration curves having correlation coefficient near to one in *Centella asiatica* (Gunther and Wagner 1996). Suspension culture and analysis of asiaticoside by TLC and HPLC in vitro cultured leaves, callus and cells of suspension culture have been explored (Nath and Buragohain 2005). Four principal triterpenoids have been identified by the used of TLC and mass spectrometry. Combination of ethyl acetate and methanol showed best for separation of this compound from the rest of the component found in the main extract. Further separation of compound was confirmed by employed MALDI-TOF mass spectrometry (Bonfill et al. 2006). It was shown that behaviour of active constituent of two different accessions as well as different parts of *Centella asiatica* species varied in amount of phytochemical by using TLC and HPLC. HPLC analysis revealed leaf that showed higher phytochemical content in comparison with petiole as well as second accession contained maximum asiaticoside (2.56 ug/ml), madecassoside (5.30 ug/ml) and asiatic acids 3421.60 ug/ml (Zainol et al. 2008). Antioxidant properties of *C. asiatica* have been studied and obtained in phytochemical compounds such as reducing sugar, alkaloids, flavonoids, phenols, saponins, tannins, anthraquinone, steroids, terpenoids and cardiac glycosides. TLC were performed for the analysis of phytochemical compound which showed phenolic compound having one band, and Rf was 0.83 which is similar to standard gallic acid, while in case of antioxidant, one band also obtained and Rf was 0.63 similar to standard Vit. C in *C. asiatica*. HPLC analysis revealed methanolic extract of phenols which contains three peaks which was similar to gallic acid, whereas nine peaks were obtained from hexane extract (Desai et al. 2013). Methods of qualitative and quantitative analysis of ethanolic extract of root, stem and leaf have been established in *C. asiatica* by using TLC and standard chemical protocol, respectively. They were isolated and different components were identified, viz. alkaloids, saponin, flavonoids, terpenoides, and phenol and tannin by employing TLC as well as

the Rf values of these contents in different solvent systems were noted (Biradar and Rachetti 2013). An efficient protocol has been documented to analyse micropropagated nodal explants and bioactive molecules from the root, stem and leaf by employed TLC in *C. asiatica*. The effects of different nitrogen sources on shoot regeneration and malt extract on elicitation for enhancement of asiaticoside production as well as profiling fatty acid methyl ester (FAME) have been reported. Using four different nitrogen compounds such as $NH4NO_3$ (1.65 g/l), KNO_3 (0.8 g/l), $NaNO_3$ (1.65 g/l), Ca $(NO_3)_2$ (0.825 g/l) and employed on five different accession of *C. asiatica.* It was observed that accession number 347492 (14.66 ± 2.4) showed maximum shoot regeneration with ammonium nitrate. In RP-HPLC analysis revealed in accession, 347,492 malt extracts are enhanced six times in asiaticoside production in comparison with control. GC-MS analysis has showed the five accessions of *C. asiatica* enriched in pentadecanoic acid; 9, 12 octadecadienoic acid (Linoleic acid); and 9, 12, 15 octadecatrienoic acid (linolenic acid) and fatty acid methyl esterase (Roy et al. 2016).

6.5 Conclusion and Future Prospect

C. asiatica gained attention for several years because of its huge medicinal properties as well as cosmetic application. Secondary metabolites produced from this medicinal plant contain pharmaceutical properties which has been significant for curing various diseases. Uncontrolled overexploitation of medicinal plant depleted wild stock; therefore it has been listed in the threatened species by International Union for conservation of nature and natural resources. In vitro culture offers option to endurable exploitation of plant and production of secondary metabolite such as triterpenoid along with enhancement through the elicitor's molecules. However, hairy root culture enhances secondary metabolite formation in aerial part of plant, but several researchers resolve this problem by designing hairy root culture along with elicitation. Besides their pharmacological importance, triterpenoid also is used in cosmetic industries. Large-scale secondary metabolite production operated through the bioreactor, till date only one report describes cell suspension culture of *C. asiatica* in bioreactor. So there is a need to construct an appropriate bioreactor having low shear impeller and optimize the culture condition by using statically techniques which enhance secondary metabolite production. Elicitor's molecule plays a key role in the enhancement of secondary metabolite but how they altered their secondary metabolite machinery in vitro remains limited. However, recent studies genomics of *C. asiatica* revealed the various genes overexpressed under the elicitation, although several genes have been sequenced and cloned by the employed transcriptomic, genomics and metabolomics. Efforts towards metabolic pattern of secondary metabolite enhancement altered by the elicitor molecule need to be strengthened. In this way researcher will explore pathway altered through the elicitor and identified genes which encode key enzyme.

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Chapter 7
Characterization of a Secondary Metabolite from *Aegle marmelos* (Vilva Tree) of Western Ghats

Vellingiri Manon Mani and Arockiam Jeyasundar Parimala Gnana Soundari

Abstract The current world is emphasized to create new therapeutic drugs from natural sources to compete the various life-threatening diseases. This investigation has mainly focused to develop prospective metabolite to treat cancer. The bioactive metabolite has been targeted to be produced by a medicinal tree *Aegle marmelos* (Vilva tree) for anticancer potentiality. The stated medicinal tree secretes several metabolites which have been used extensively in traditional medicine to treat various diseases and disorders. This current research aimed to extract a single bioactive metabolite through preliminary analysis such as antimicrobial and antioxidant assessment against different clinical bacterial and fungal pathogens. The crude metabolites extract from branch sample of Vilva tree explored the maximum activity, so it was taken for purification process by chromatographic techniques. On purification through HPLC analysis, about seven different fractions were eluted, and those were determined for antioxidant and antimicrobial assessment. MF4 fraction explored its maximum activity at minimum concentration for both assessment. This MF4 compound on chemical characterization was found to be 5-acetoxytridecane, a potential compound and it evinced good anti-angiogenic activity through HET-CAM testing which manifested a strong anticancer potentiality.

Keywords *Aegle marmelos* · Angiogenesis · Anticancer · HET-CAM · Secondary metabolites

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Authors Vellingiri Manon Mani and Arockiam Jeyasundar Parimala Gnana Soundari have been equally contributed to this chapter.

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7.1 Introduction

The increasing emergence of lead drugs for the resistance produced by the pathogenic strains and arrival of new diseases has initiated the need for searching novel metabolites with best anticancer and antimicrobial properties than the existing one. The leading research has focused on antioxidants for curing and/or preventing diseases or disorders from potential plant metabolites especially contained with antioxidants. Antioxidants are gaining a lot of importance as a panacea for a large number of lifestyle diseases like aging, cancer, diabetes, cardiovascular, and other degenerative diseases owing to our sedentary way of life and stressful existence. In order to employ the production of antioxidants as lead drugs, it is essential to know about the properties of these agents that may be explored with antimicrobial, anticancer, antiviral, antihemorrhagic and anti-angiogenic properties. Many of the antioxidants are produced by the plants and microbes as by-products or metabolites, and they are found to have the biological properties in protecting against these diseases. These metabolites possess mainly with biological properties in a prospective manner.

Metabolites are the substances or the compounds which are produced by the living organisms such as plants, animals, and microorganisms. Owing to the technical improvements in the screening programs and separation and isolation techniques, the number of natural products or the compounds has been discovered which exceeds a million; among them about 50–60% are produced by plants (alkaloids, flavonoids, steroids, terpenoids, carbohydrates, etc.), and 5% is of microbial origin. A number of drugs made synthetically today are derived from the structures of plant metabolites. Plants have formed the basis of sophisticated traditional medicine systems among which are Ayurvedic, Siddha, Unani, and Chinese. These systems of medicine have given rise to some important drugs still in use today. The search for new molecules, nowadays, has taken a slightly different route where the science of ethno botany and ethnopharmacognosy is being used as guide to lead the chemist toward different sources and classes of compounds. Plants supply major of the active ingredients from traditional medicinal products, and plant extracts have long been used in screening program in pharmaceutical companies. It might be thought that most of the plant kingdom has been thoroughly examined in the search for biologically active molecules. There are estimated to be 250,000 species of plants in the world and probably 10% of these species have been tested for biological activity. In traditional medicine systems in countries such as India and China (Baker et al. 2000), plants have formed the basis for novel drug discovery. In a study it has been shown that at least 119 chemical substances derived from 90 plant species can be considered as important drugs that are in use in one or more countries. Of these 119 drugs, 74% were discovered as a result of chemical studies directed at the isolation of the active substances from plants used in traditional medicine. There are several medicinal plants and trees used for medicinal purposes from traditional age to till date. On the contrary, few medicinal plants/trees were under endangered list but still the medicinal world need for curing several diseases. With this view, this research explores the investigation on a potential medicinal plant, *Aegle marmelos* (Vilva tree) – a traditional medicinal and religious tree.

Aegle marmelos (Linn) Correa, commonly known as bael (or bel or Bilva), belonging to the family Rutaceae, is a moderate-sized, slender, and aromatic tree. It is indigenous to India and is abundantly found in the Himalayan tract, Bengal, and Central and South India. It is extensively planted near Hindu temples for its wood and leaves which are generally used for worship. The plant has been used in the Indian traditional medicines from time immemorial. It is associated with various important medicinal properties. Chemical investigation of the different parts of the plant has resulted in the isolation of a large number of novel and interesting metabolites. Some of the compounds have been screened for bioactivity.

Extensive investigations have been carried out on different parts of *A. marmelos*, and as a consequence, varied classes of compound, viz., alkaloids, coumarins, terpenoids, fatty acids, and amino acids, have been isolated from its different parts. Notably, majority of reports on the isolation and compound characterizations have been reported by many Indian workers. Broadly, *A. marmelos* leaves contained γ-sitosterol, aegelin, lupeol, rutin, marmesinin, β-sitosterol, flavone, glycoside, O-isopentenyl halfordiol, marmeline, and phenylethyl cinnamamides. Besides these potential metabolites from this tree, this paper mainly targets the research to focus on production and characterization of a prospective metabolite from the Vilva tree and assessing its biological properties as antioxidants to treat cancer.

7.2 Materials and Methods

7.2.1 Plant Sample Collection and Preparation

Mature healthy, asymptomatic plant materials (bark, branches, leaves, and root) were collected by sampling different parts of the trees of *A. marmelos* growing randomly in the Western Ghats region (Nilgiris cluster, Tamil Nadu, India) (Mani et al. 2015). Sampling was performed on five trees of *A. marmelos* collected from foot hills of Vellingiri and Marudhamalai [Coimbatore, Tamil Nadu, India (11.0183° N, 76.9725° E)]. Bark samples were obtained by cutting tree bark at 150 cm above the ground level from a depth of 1–1.5 cm inward with the help of sterile machete. Small discs of leaves (0.5 cm diameter) were cut using sterile pinch cutter. Root samples were obtained by digging the soil at least 1 m away around the main trunk and 2 ft. in depth. Fifteen samples were taken from each tree and five each from root, inner bark, inner branches, and leaves (Mani et al. 2015). All the segments were shade dried, powdered, and assessed for antioxidant, antimicrobial, and phytochemical analyses in five different solvents such as methanol, ethyl acetate, acetone, dimethyl sulfoxide (DMSO), and hexane. On which, branch sample from Vellingiri Hills showed maximum activity at least concentration in ethyl acetate extract when compared to other samples from different collection areas. This crude methanolic branch sample was taken for further studies to separate a bioactive metabolite.

7.2.2 Purification of the Bioactive Compound

The concentrated crude pigmented extracts were subjected to preliminary thin-layer chromatography (TLC) and then to silica gel column chromatography. The partially purified pigmented metabolites were further purified through high-performance liquid chromatography (HPLC), and the fractions were taken for chemical characterization.

7.2.2.1 Thin-Layer Chromatography

The movement of the metabolites from crude methanolic extract (ME) in specific solvent systems was detected following thin-layer chromatography (TLC). Using a micropipette, 5 microliters of the crude extract was applied 1 cm above from the lower edge of the thin-layer chromatography slides and then air-dried. It was immersed to a depth of 1 cm in the solvents. The chromatogram was developed in a saturated chamber. Different combinations of polar and nonpolar solvents were tested for separation of the metabolites. Solvent systems used were chloroform/methanol, butanol/acetic acid/water, and petroleum ether/ethyl acetate. The solvent system that can separate maximum number of compounds from two different crude extract was taken as the best solvent system and was used for the further studies. The solvent front was marked and R_f value was calculated.

7.2.2.2 Silica Gel Column Chromatography

The crude extracts were subjected to fractionation by column chromatography. The sample was made into slurry with activated silica gel 60–120 mesh (activation at 105 °C for 6 h). The column was packed with dry silica gel (60–120 mesh). Elution was performed by the linear gradient of petroleum ether/methanol and petroleum ether/ethyl acetate for two different extracts (ME and EAE). The fractions were collected at specific intervals, and its purity was determined using TLC solvent system for the respective extracts as mobile phase. Bands were detected using iodine chamber and UV illumination at 250 nm.

7.2.2.3 High-Performance Liquid Chromatography (HPLC)

Analysis was carried out in HPLC (Shimadzu-1100 series), manual injector with quaternary pump, and photodiode array detector equipped with C_{18} column (4.6 × 250 mm) with 5 μL of pore size. The mobile phase used in this analysis was solvent A (acetonitrile 80%) and solvent B (HPLC water 20%). The sample of about 20 μL was injected, and the flow rate was 0.5 mL/min at the wavelength of 449 nm

as the highest peak in crude methanolic extract exhibited at the same nanometer. All fractions were checked for its antimicrobial activity and antioxidant activities. The fractions were eluted at respective wavelength from methanolic extract (ME) and assessed for antimicrobial and antioxidant properties.

7.2.3 Antimicrobial Activity

Antimicrobial activity of the purified pigment compound was determined by well diffusion method (Bauer et al. 1966; Barry et al. 1970) against procured test pathogens such as *Staphylococcus aureus*, *Staphylococcus epidermidis*, *Escherichia coli*, *Pseudomonas aeruginosa*, *Klebsiella* sp., *Salmonella* sp., *Proteus* sp., *Shigella* sp., *Bacillus* sp., and *Candida albicans*.

7.2.4 Antioxidant Activity

7.2.4.1 DPPH Radical Scavenging Activity

DPPH radical was determined along with 1 ml of purified fractions (concentrations 20 µg/ml to 100 µg/ml) from ME; 5 ml of 0.1 mM methanol solution of DPPH was added and vortexed, followed by incubation at 27 °C for 20 min. 0.1 mM methanol solution of DPPH alone served as the control, and absorbance of sample was measured at 517 nm using methanol (Blank) to set 0 (Szabo et al. 2007). The ability of the sample to scavenge DPPH radical was calculated by the following formula:

$$\text{DPPH radical scavenging activity}(\%) = \frac{\left[\left(\text{Abs}_{\text{control}} - \text{Abs}_{\text{sample}}\right)\right]}{\left(\text{Abs}_{\text{control}}\right)} \times 100$$

7.2.4.2 Reducing Power

Total reducing power was determined as described by Oyaizu (1984). 1 ml of sample solution at different concentrations was mixed with 2.5 ml of phosphate buffer (0.2 mol/l, pH 6.6) and 2.5 ml of potassium ferricyanide (1%). The mixture was incubated at 50 °C for 20 mins; 2.5 ml of trichloroacetic acid (10% TCA) was added to the mixture and centrifuged at 3000 g for 10 min. The supernatant (5 ml) was mixed with 1 ml of ferric chloride (0.1%), and the absorbance was measured at 700 nm in a spectrophotometer. Increased absorbance of the reaction mixture indicated increased.

7.2.4.3 Superoxide Anion Radical Scavenging Activity

Measurement of the superoxide anion radical scavenging capacity of the purified fractions from methanol extract was performed according to the method of Liu et al. (1997) using a minor modification. The principle of this method is that superoxide radicals are generated in phenazine methosulfate (PMS) – nicotinamide adenine dinucleotide (NADH) systems by oxidation of NADH and reduction of nitroblue tetrazolium (NBT). In this experiment, the superoxide radicals were generated with 3.0 mL of Tris–HCl buffer (16 mM, pH 8.0) containing 1.0 mL of NBT (50 μM) solution, 1.0 mL NADH (78 μM) solution and purified fractions in different concentrations (20 μg/mL to 100 μg/mL) in methanol. The reaction was initiated by adding 1.0 mL of phenazine methosulfate (PMS) solution (10 μM) to the mixture. The absorbance at 560 nm was measured against a blank, and inhibition activity was calculated using the formula given. Ascorbic acid was used as a standard.

$$\text{Superoxide radical scavenging activity}(\%) = \frac{\left[\left(\text{Abs}_{\text{control}} - \text{Abs}_{\text{sample}}\right)\right]}{\left(\text{Abs}_{\text{control}}\right)} \times 100$$

7.2.4.4 Hydroxyl Radical Scavenging Activity

The scavenging activity for hydroxyl radicals recommended by Yu et al. (2004) was followed with minor modifications. Reaction mixture contained 0.6 mL of 1.0 mM deoxy ribose, 0.4 mL of 0.2 mM phenyl hydrazine, and 0.6 mL of 10 mM phosphate buffer (pH 7.4). It was incubated for 1 hour at room temperature. Then 1 mL of 2% TCA, 1 mL of 1% TBA, and 0.4 mL of purified fractions ME (at different concentrations 20 μg/ml to100 μg/ml) were added and kept in water bath for 20 min. The absorbance of the mixture at 532 nm was measured using spectrophotometer. From the readings, the hydroxyl radical scavenging activity was calculated using the following formula:

$$\text{Hydroxyl radical scavenging activity}(\%) = \frac{\left[\left(\text{Abs}_{\text{control}} - \text{Abs}_{\text{sample}}\right)\right]}{\left(\text{Abs}_{\text{control}}\right)} \times 100$$

7.2.4.5 Metal Chelating Activity

The method of Dinis et al. (1994) has been used to estimate the chelating effect on ferrous ions with some modifications. The Ferrozine solution (3-[2-pyridyl]-5,6-diphenyl-1,2,4-triazine-4,4′-disulfonic acid Na salt) (0.6 mM) was prepared in ultrapure water and stored in the dark place at room temperature. 0.5 mL of various concentrations (20 μg/mL to 100 μg/mL) of all eluted fractions from ME was mixed

with 0.5 mL of $FeSO_4$ (0.12 mM) and with 0.5 mL of Ferrozine (0.6 mM). The mixture was allowed to stand for 10 min at room temperature. After incubation, the absorbance was measured at 562 nm. Ultrapure water was used as a blank, and reaction mixture without sample served as control. EDTA-Na_2 was used as reference standard. The ability of the sample to chelate ferrous ion was calculated relative to the control (consisting of iron and Ferrozine only) using the formula:

$$\text{Ferrousion chelating ability}(\%) = \left[\left(\text{Abs control} - \text{Abs sample}\right) / \text{Abs control}\right] \times 100$$

7.2.5 Characterization of the Bioactive Compound

The purified fractions which explored maximum antioxidant activities were taken for structural elucidation and characterization studies. The bioactive compounds were dissolved in acetone/acetonitrile for spectroscopic analysis using UV-Vis scanning spectrophotometer (Lab India). Scanning was performed between 200 nm–700 nm wavelengths. 1 mg of purified bioactive fractions was dried and analyzed for identifying functional groups using FT-IR spectroscopy (Brucker). The important IR bands of symmetric and asymmetric stretching and stretching frequencies were studied to determine the functional groups present. The mass of the purified compound was found through GC-MS analysis. Determination of the nuclear magnetic resonance (NMR) and the bioactive fractions were dissolved in acetone, and the spectra were recorded on a Bruker Avance III 500 MHz instrument fitted with an inverse triple resonance CryoProbe (TCI). The nuclear magnetic resonance (NMR) spectrum was observed for $^{1}\boldsymbol{H}$ and $^{13}\boldsymbol{C}$.

7.2.6 In Vitro *Studies*

7.2.6.1 MTT Assay

The cytotoxic effect of the bioactive compound was studied using cancer cell lines. The HT-29 cell line was obtained from the National Centre for Cell Sciences (NCCS), Pune. The cells were maintained in minimal essential medium supplemented with 10% FBS, penicillin (100 U/mL), and streptomycin (100 µg/mL) in a humidified atmosphere of 50 µg/mL CO_2 at 37 °C. Cells (1×10^5/well) were plated in 24-well plates and incubated in 37 °C with 5% CO_2 condition. After the cell reaches the confluence, the various concentrations of the samples were added and incubated for 24 h. After incubation, the sample was removed from the well and washed with phosphate-buffered saline (pH 7.4) or MEM without serum. 100 µL/well (5 mg/mL) of 0.5% 3-(4, 5-dimethyl-2-thiazolyl)-2, 5-diphenyl-tetrazolium bromide (MTT) was added and incubated for 4 h. After incubation, 1 mL of DMSO

was added in all the wells. The absorbance at 570 nm was measured with UV-Vis spectrophotometer using DMSO as the blank. Measurements were performed, and the concentration required to inhibit 50% of cells (IC_{50}) was determined graphically. The % cell viability was calculated using the following formula:

$$\%\,\text{cell viability} = \left(A_{570}\ \text{of treated cells} / A_{570}\ \text{of control cells} \times 100\right)$$

Graphs were plotted using the % cell viability as Y-axis and concentration of the sample in X-axis. Cell control and sample control was included in each assay to compare the full cell viability in cytotoxicity assessments.

7.2.7 Determination of HET-CAM Test (Luepke 1985 and Valdes et al. 2001). Hen's Egg Test on the Chorioallantoic Membrane (HET-CAM) of Chick Eggs

In order to understand the inflammatory tissue reactions of metabolite coated materials on the live tissues, the materials were placed on the surface of chorioallantoic membrane (CAM) of embryonated chick eggs. The inflammatory response on CAM was evaluated by direct evaluation method. The eggs of 9th day incubated were taken for the study according to the reference of Valdes et al. (2001). Insertion of test sample in different concentrations such as 10, 25, 50, 75, 100 and 200 µg/ml of MF4; positive control (1 N NaOH), solvent control (methanol) and negative control (0.9% NaCl) were taken for the determination assay. After 2 and 18 h of inoculation with the above-stated materials the eggs were opened to evaluate the blood vessels recorded for each egg (one time value for each endpoint).

7.3 Results

7.3.1 Purification of the Crude Extract

7.3.1.1 TLC

The methanolic crude extract was run in thin-layer chromatography using five different solvent systems. The R_f value calculated for different solvent systems, and the best solvent system was petroleum ether: ethyl acetate (25%) for methanolic crude extract (ME). Maximum of five bands got separated using these solvent systems, and the Rf value for ME was given in the Fig. 7.1.

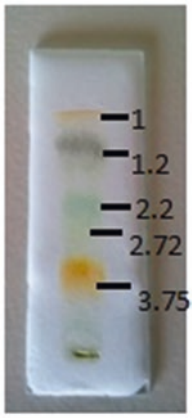

Fig. 7.1 TLC of methanolic crude extract

7.3.1.2 Silica Gel Chromatography

The concentrated methanolic crude extract was run in silica gel column chromatography, and it was eluted with the linear gradient of solvent system for purification of metabolites. An effective separation of the compounds was carried out by increasing a steady concentration of petroleum ether/ethyl acetate for ME. Nine fractions have been obtained from methanolic crude extract, and these were compared with HPLC fractions eluted.

7.3.1.3 High-Performance Liquid Chromatography (HPLC)

Further the crude extract of ME was subjected to HPLC analysis, and the mobile phase was acetonitrile/water. The fractions were eluted at the wavelength of 449 nm. The highest peak was found at a retention time of 72.1 mins for ME. The eluted compounds were determined for the yield, antioxidant profile, and antimicrobial activity.

7.3.2 Antimicrobial Activity for Purified Compounds

The crude methanolic extract from branch sample was active against ten pathogens such as *S. aureus*, *S. epidermidis*, *Klebsiella* sp., *Shigella* sp., *S. typhi*, *P. aeruginosa*, *E. coli*, *V. cholerae*, *Proteus* sp., and *C. albicans* in preliminary study. This may be due to active components which were present in the tree extracts. On comparing the fractions eluted from ME, fraction 4 was found to be highly susceptible to pathogens and, it formed highest zone of inhibition (Table 7.1). These results indicated the potential bioactive fractions from ME which contained different

Table 7.1 Antimicrobial activity for purified compounds

	Methanolic extract (ME)						
Pathogens	F1	F2	F3	**F4**	F5	F6	F7
S. aureus	2	1.3	1.7	**1.2**	2	1.2	2
S. epidermidis	2.1	1.5	2	**2.1**	2.1	2.2	2.1
Klebsiella sp.	1.2	1.9	2.1	**2.4**	1.8	2.3	2.2
Shigella sp.	1.5	1.5	1.9	**1.9**	1.2	1.6	2.8
S. typhi	2.1	1.5	2	**2**	1.9	1.9	1.2
P. aeruginosa	2.9	2.1	2.9	**3.2**	2.7	2	2.9
E. coli	2.5	2.5	1.8	**3**	1.8	1.2	1.8
V. cholerae	1.9	1.5	2.2	**3**	1.6	2.8	2.1
Proteus sp.	2.6	1.4	1.6	**2.9**	1.5	2	1.8
C. albicans	2.1	1.6	1.2	**2.8**	1.2	1.6	1.5

<0.5 cm indicates no inhibition activity

antibacterial substances and reflect the variety of secondary metabolites. The result showed the methanolic extract fractions possess a strong antimicrobial activity against both Gram-positive and Gram-negative bacteria.

7.3.3 Antioxidant Analysis of Purified Compounds

7.3.3.1 DPPH Radical Scavenging Activity

DPPH assay is one of the most widely used methods for screening antioxidant activity of natural product. In this study, seven fractions from ME were taken. MF4 were found to exhibit highest activity, and the IC_{50} percentage was 58.5 μg/ml (Fig. 7.2). The scavenging activity was found to be increased with the increasing concentrations, and this proved the past findings of Vilva which had a strong support in scavenging mechanisms.

7.3.3.2 Reducing Power Assay

The reducing properties were generally associated with the presence of reductones. The antioxidant action of reductones is based on the breaking of the free radical chain by donating a hydrogen atom (Gordon, 1990). In this investigation, the reductive power was increased accordingly at that concentration in MF4. Increased reductive power indicates the highest activity. Among the eluted compounds, MF4 had steady increasing OD units which indicated that they have the highest reductive capacity (Table 7.2).

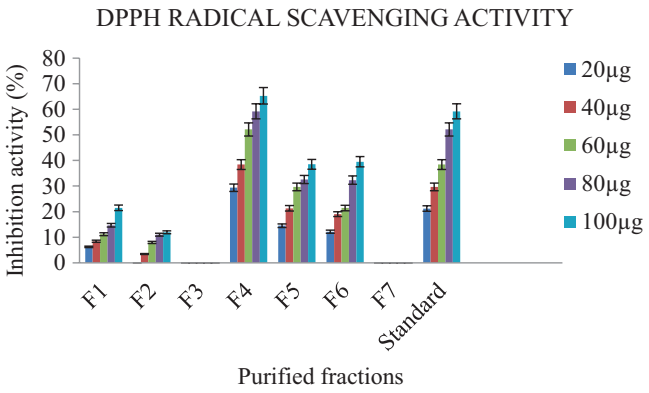

Fig. 7.2 DPPH radical scavenging activity of different fractions. F1–F7: Fractions from methanolic crude extract. Data represented as mean value ± SE (n = 3)

7.3.3.3 Superoxide Radical Scavenging Activity

It is well known that superoxide anions damage biomolecules directly by forming H_2O_2, OH, peroxynitrite, and singlet oxygen during aging. The superoxide anion radical scavenging activity of purified fractions assayed by PMS-NADH system for purified fractions ME given in Fig. 7.3. Superoxide is harmful because it reduces iron – III to iron II – and it gives rise to strong oxidant like singlet oxygen and interacts with the other compounds such as nitric oxide radical or hydrogen peroxide gives rise to the hydroxyl radical and nitrogen dioxide. The superoxide scavenging activity of plant fractions increased markedly with increase in concentration. Thus higher inhibitory effects of the fractions on superoxide anion formation noted here possibly renders them as a promising antioxidants, MF4 showed highest activity among the eluted fractions from ME, and the IC_{50} percentage was 74 µg/ml.

7.3.3.4 Hydroxyl Radical Scavenging Activity

Hydroxyl radical scavenging assay showed the ability of the eluted fractions in inhibiting hydroxyl radical-mediated deoxyribose degradation in a Fe^{3+} EDTA ascorbic acid and H_2O_2 reaction mixture. The samples were added to the reaction mixture as it remove the hydroxyl radicals from the sugar and prevent the reaction. The samples showed the activity by eliminating hydroxyl radicals in a dose-dependent manner. Among the eluted fractions of ME, MF4 explored the highest scavenging activity at least concentration. The IC_{50} percentage was found to be 76 µg/ml for MF4, and the results were graphed in Fig. 7.4.

Table 7.2 Reducing power assay for purified fractions

	Methanol extract							
Concentration (µg/ml)	F1	F2	F3	**F4**	F5	F6	F7	Standard
20	0.041 ± 0.03*	0.027 ± 0.5	0.031 ± 0.06	**0.043 ± 0.02**	0.039 ± 0.5	0.044	0.037 ± 0.06	0.078 ± 0.5*
40	0.039 ± 0.5	0.035 ± 0.06	0.030 ± 0.06*	**0.132 ± 0.03***	0.044 ± 0.06	0.038 ± 0.03*	0.039*	0.146 ± 0.5
60	0.091 ± 0.06	0.027 ± 0.03*	0.033 ± 0.5	**0.154***	0.056 ± 0.03*	0.040 ± 0.5	0.047 ± 0.03	0.234 ± 0.03*
80	0.081 ± 0.5	0.046 ± 0.02	0.037 ± 0.03	**0.162 ± 0.5**	0.055 ± 0.02	0.048 ± 0.06	0.053 ± 0.5*	0.186 ± 0.06
100	0.093 ± 0.02	0.027	0.027 ± 0.02	**0.178***	0.053	0.052 ± 0.02	0.069 ± 0.03	0.223 ± 0.03

Data represented as mean value ± SD (n = 3). Standard: ascorbic acid. *$p < 0.05$: significant

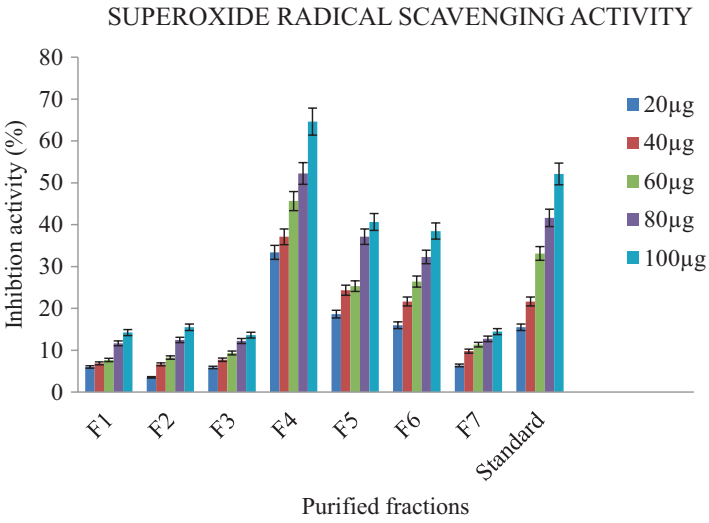

Fig. 7.3 Superoxide radical scavenging activity. F1–F7: Fractions from methanolic crude extract. Data represented as mean value ± SE (n = 3)

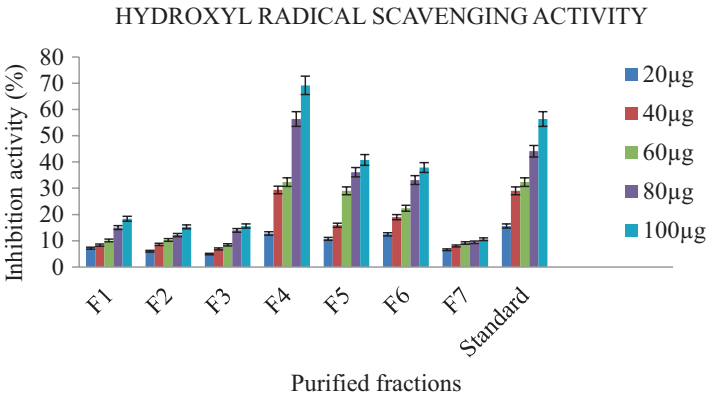

Fig. 7.4 Hydroxyl radical scavenging activity. F1–F7: Fractions from methanolic crude extract. Data represented as mean value ± SE (n = 3)

7.3.3.5 Metal Chelating Activity

The chelating ability of ferrous ions by ME fractions were estimated by the method of Dinis et al. (1994). The capacity of antioxidant for chelating metal is strongly dependent on the number of hydroxylic groups in ortho-position. Metal chelating activity of the purified fractions of ME at various concentrations was graphed in Fig. 7.5. MF4 showed the metal chelating activity and the IC_{50} value was found to be 66 µg/ml concentration and it represents the greatest inhibition activity and it

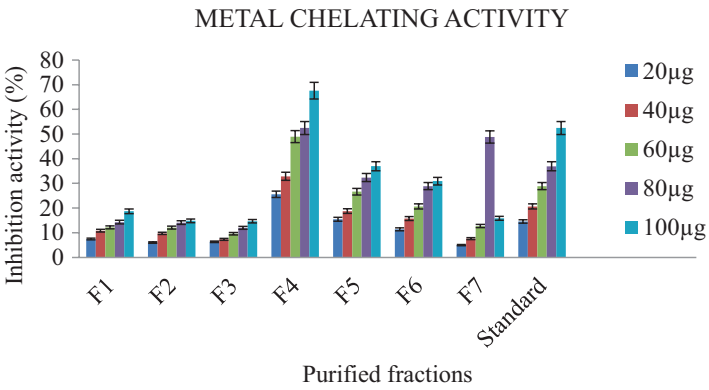

Fig. 7.5 Metal chelating activity of purified fractions. F1–F7: Fractions from methanolic crude extract. Data represented as mean value ± SE (n = 3)

placed a promising activity. From these results we can conclude that the fraction MF4 was found to be the bioactive compound.

7.3.4 Chemical Characterization of the Compound

7.3.4.1 UV-Spectra

UV-visible spectra analysis was done for MF4 in the range of 300 nm to 700 nm. A1 and C1 were taken as control solvents, viz., ethyl acetate and methanol. MF4 (D1) was absorbed at 3.24 and this was the highest peak at the wavelength of 410 nm. Relatively, crude methanolic extract (B1) peak was absorbed in 3.22 OD at the wavelength of 417 nm. The absorbance wavelength for MF4 corresponded to the wavelength of carotenoids. Hence it may be concluded that the bioactive compound may fall in the category of carotenoid groups.

7.3.4.2 NMR Spectra

The ^{1}H NMR spectrum of the compound MF4 displayed CH_3–0.96, CH_2–1.23, CH_2–1.29(triplet), CH-3.95,CH_3–2.01 protons were present (Fig. 7.6a). The ^{13}C NMR spectrum of the compound signals corresponding to the presence of –O-C=O group carbon atoms value at 171, respectively, for C_1, C_2, C_3, C_4, C_5, and C_6 atoms was marked in MF4 (Fig. 7.6b).

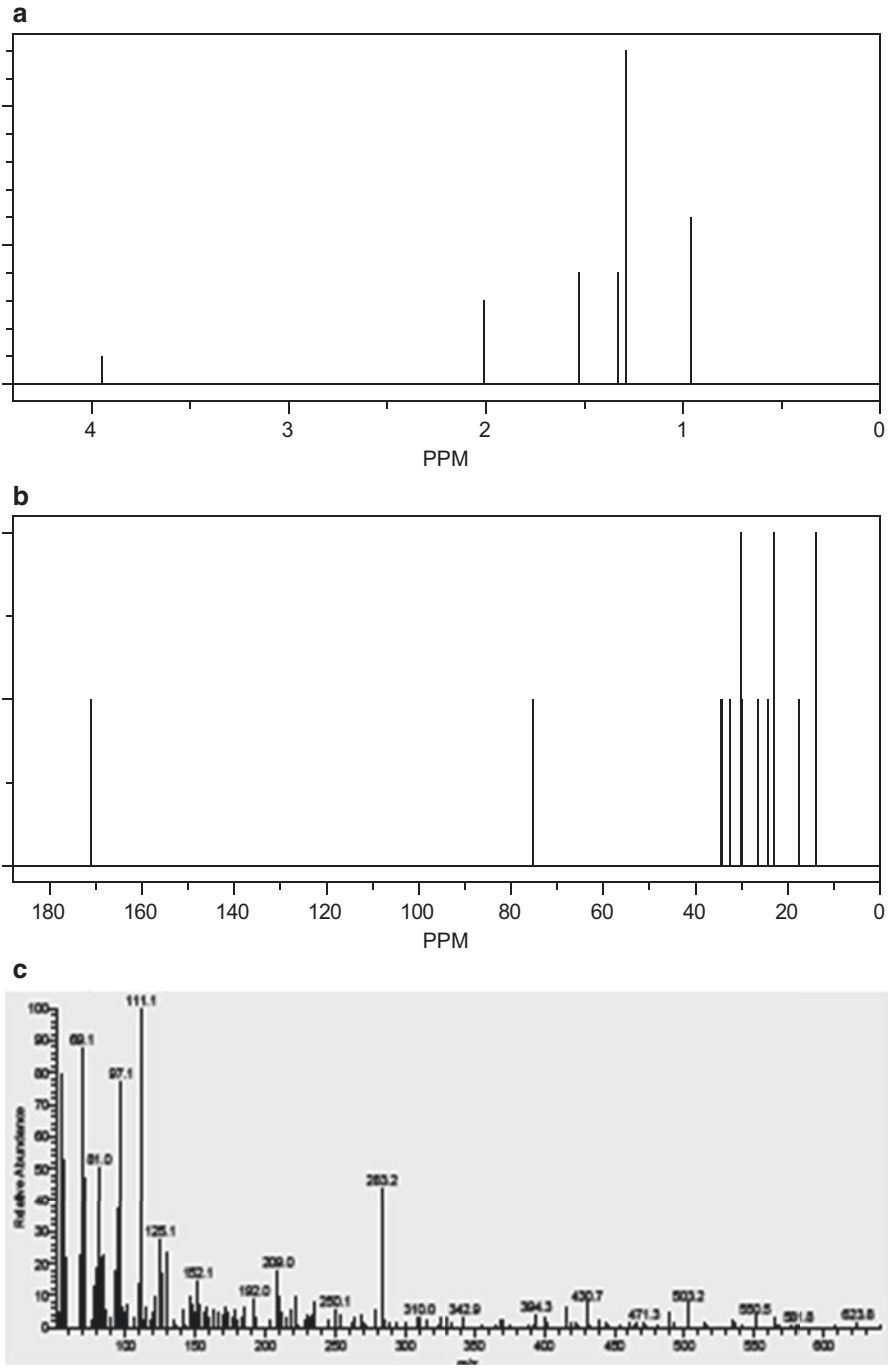

Fig. 7.6 NMR and FT-IR data analysis of fraction MF4 (**a**) ^{1}H NMR for MF4, (**b**) ^{13}C NMR for MF4, (**c**) chromatogram of the compound MF4, (**d**) FT-IR for MF4, (**e**) structure of the compound 5-acetoxytridecane

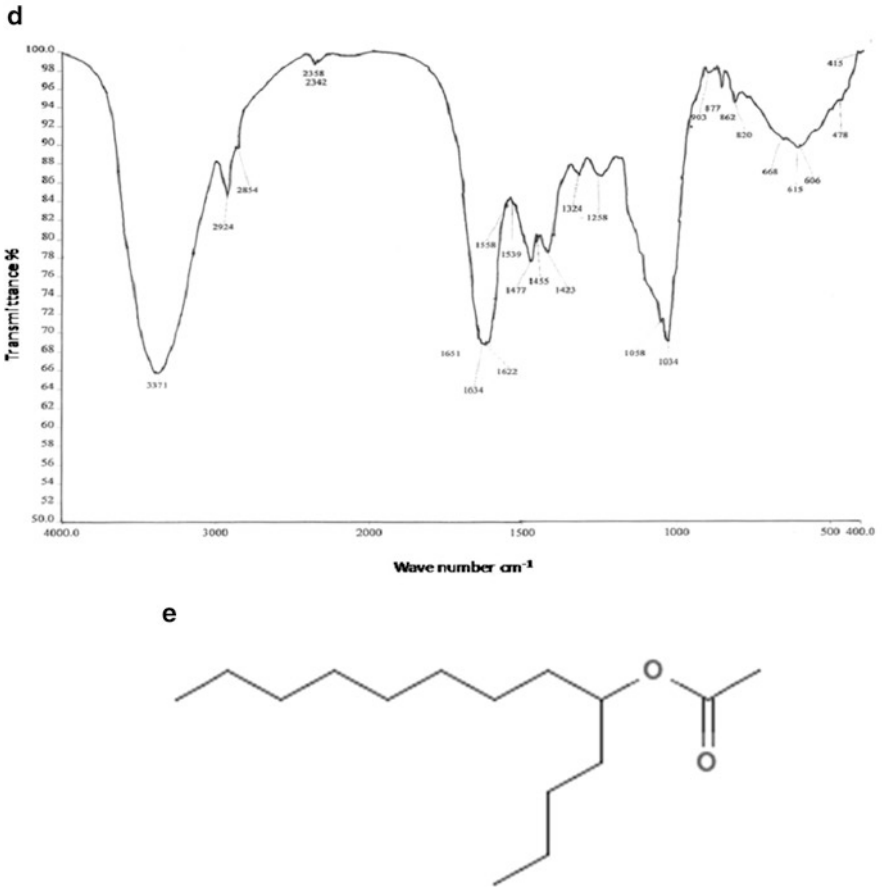

Fig. 7.6 (continued)

7.3.4.3 GC-MS Analysis

The gas chromatography–mass spectrometry (GC-MS) was analyzed for purified fractions of MF4. The chromatogram was obtained with three major peaks in MF4. The highest peak was with the retention time 30.51, and R_f value was 6 with relative abundance 111.1 for MF4. The total ion chromatogram was given in the Fig. 7.6c. The molecular mass was 242 for MF4.

Finally from the results of UV-spectra, NMR, and GC-MS analysis, we conclude the compound MF4 was found to be 5-acetoxytridecane.

7.3.4.4 FT-IR Spectrum for Purified Compound

The FT-IR spectrum has proven to be the most effective way to give the information about the functional groups present in the compound. The FT-IR spectra of MF4 were depicted in Fig. 7.6d, which marked the stretching frequencies recorded at

3371 and 3408 which correspond to O-H, C-H, and N-H (hydroxyl and polysaccharide) stretching. The regions 1651, 1634, 1622, and 1662, 1653, and 1645 denote the regions C=O stretching and N=O asymmetric groups (ester) of compounds, respectively.

5-Acetoxytridecane (Fig. 7.6e) was a potential pale green-colored compound. This compound was found to be nontoxic and has been used extensively in chemical replacement for methyltridecane. This compound was not an aromatic or taste enhancer, but they have been used in pharma industries as packaging materials. This compound has been extensively used in traditional medicines to treat oral- and gastro-related problems like ulcers and wounds created by bacteria. With this short view, this product can be implemented for further anticancer-related assays for the confirmation as anticancer drug.

7.3.5 In Vitro *Studies*

7.3.5.1 MTT Assay

The present investigation has been carried out for bioactive compound MF4. The studies have been performed on HT – 29 colon cancer cell lines with the control of triton X 100. The 50% viability was found to be 57.47% for MF4 and the results were tabulated (Table 7.3). The present study has showed that methanolic fraction could extensively inhibited cell proliferation architecture in dose-dependent manner. MF4 was found to be inhibited maximum number of cells. So MF4 was taken for further anti-angiogenesis analysis.

7.3.6 Anti-angiogenic Activity by HET-CAM Test

The CAM assay is a perceptive, easily feasible, and cheap in vivo check for enquiries of the anti-angiogenic promise of individual compounds. The compound 5-acetoxytridecane exhibited a promising anti-angiogenesis. The compound MF4 inhibited the angiogenesis at an interval of 2 h and 18 h (Table 7.4) (Fig. 7.7). The solvent acetone explored its potentiality by inhibiting the blood vessels for 2 hrs and

Table 7.3 Viable cells of HT29 for MF4

Sample concentration (µM)	Average OD at 540 nm	Percentage viability
Control	0.3725	–
6.25	0.3144	84.40
12.5	0.2611	70.1
25	0.2312	62.1
50	0.2141	57.47
100	0.1641	44.05

Table 7.4 Anti-angiogenesis effect of MF4 in HET-CAM test

Sample	For 2 h		For 18 h	
	No. of vessels in untreated CAM	No. of vessels in treated CAM	No. of vessels in untreated CAM	No. of vessels in treated CAM
Negative control – acetone	18	10	18	05
Sample – 1-200 µl	12	10	12	08
Sample – 1-400 µl	10	05	13	03
Positive control – NaOH	11	11	11	11

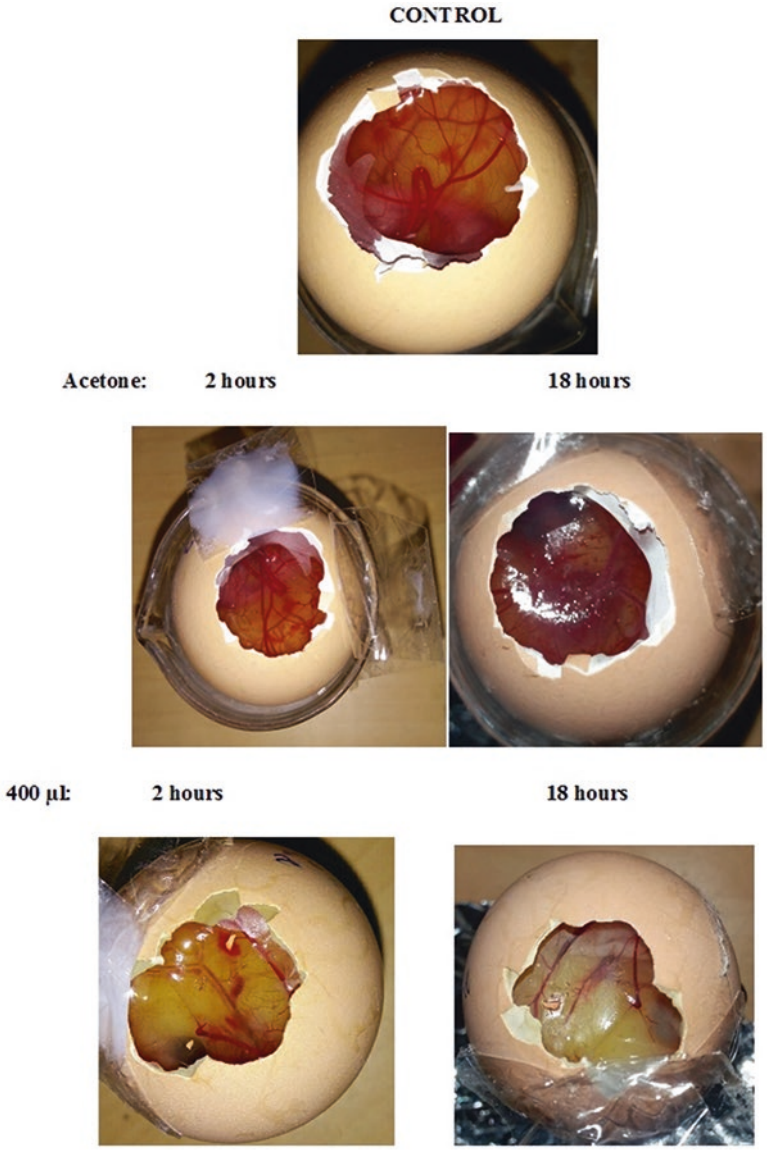

Fig. 7.7 HET-CAM test on chick embryo

18 hrs, respectively. This evinced that the compound has the good anti-angiogenic potentiality which could be explored as anticancer agents.

The same investigation was carried out by Krishna et al. (2014) who studied the anti-angiogenic potential in CAM for prodigiosin. Yildiz et al. (2013) investigated the anti-angiogenic effects for commercial anticancer agents such as diltiazem, imatinib, and bevacizumab. Pyripyropene A isolated from *Aspergillus niger* and hypocrimins (A and B) extracted from *Hypocrea vinosa* have good anti-angiogenic effects; similarly a synthetic analogue pinabulin was derived from *Aspergillus* sp., and fumagillin from *A. fumigatus* has been exhibiting the anti-angiogenic effects.

7.4 Discussion

The current investigation deals with the bioactive secondary metabolite from Vilva tree for a potential anticancer drug. The research focused to target the metabolite to be therapeutic agents which should be explored with anticancer properties. The tree samples were collected from Western Ghats regions as this region consists of a diversified nature with different taxonomical wealth. The samples were completely organized for the purification of a metabolite was done according Mani et al. (2015). The tree *A. marmelos* is a traditional medicinal tree which has been extensively used to treat various disorders in Siddha and Unani medicinal system. The tree evinced its properties through their potential antioxidants contained do explored with antiviral, antibacterial, antifungal, and anticancer properties in great grail. Phenolic and flavonoid compounds obtained from a medicinal plant/tree source have the potential of acting as antioxidants or free radical scavengers. These mainly possess an ideal structural chemistry for free radical scavenging activity in a biological system that could probably save the organism from a set of diseases. Antioxidative properties of flavonoids and polyphenols arise from their high reactivity as hydrogen or electron donors which can stabilize and delocalize the unpaired electron (chain-breaking function) and from their potential to chelate metal ions (Bristy et al. 2017). The samples from tree was normally followed to get the crude extract by using different polar and nonpolar solvents on which branch methanolic crude extract evinced its preliminary antioxidant activity when compared to other samples. This was subjected to purification techniques by chromatography, and it eluted about seven different fractions. These fractions were separated at the wavelength of 449 nm, and the analytical HPLC graph exhibited that these fractions majorly comes under flavonoids. Flavonoids are the best therapeutic agents currently used in pharmaceutical and medicinal fields. The antioxidative properties of flavonoids from plant sources or microbial sources are due to several different mechanisms, such as scavenging of free radicals; chelation of metal ions, such as iron and copper; and inhibition of enzymes responsible for free radical generation. Depending on their structure, flavonoids are able to scavenge practically all known reactive oxygen species (Gracia et al. 1997). These eluted fractions from branch methanolic crude extract were analyzed for antimicrobial and antioxidant properties. Among the seven

fractions from HPLC analysis, MF4th fraction exhibited the highest inhibition activity against the clinical pathogens. The clinical pathogens were selected on the basis of diseases causing mainly in gastrointestinal tract. This was similar to the research of Rani and Khuller (2004) who reported the methanolic extract of *A. marmelos* showed inhibition against 54 pathogens. Pattnaik et al. (1996) reported the essential oils extracted from *A. marmelos* showed antibacterial activity against 22 bacteria (including Gram-positive cocci and Gram-negative rods) and 12 fungi (3 yeastlike and 9 filamentous). Further his report stated that *A. marmelos* essential oil inhibited the 21 bacteria and all 12 fungi. On contrary, the antioxidant assays such as DPPH, superoxide, metal chelation, reductive power ability, and hydroxyl radical scavenging were assessed for HPLC fractions to determine the efficient prospective secondary metabolite. DPPH assay is an efficient and preliminary activity for the detection of compound to be potential radical scavenger. DPPH accepts an electron donated by an antioxidant compound and become decolorized, which can be quantitatively measured from the changes in absorbance. In this investigation of purified fractions, MF4 exhibited the maximum scavenging mechanism at least concentration in a dose-dependent manner. This suggests that this compound can be taken for other antioxidant assessment. This was similar to the results of Bristy et al. (2017), and their investigation reported that the whole fruit, ripe fruit, branch, and leaf samples exhibited maximum activity at minimum concentration. Similarly, other antioxidant results of eluted fractions were analyzed, and the results on comparing to other fractions, MF4, were found to be the highest one with the maximum activity at minimum concentration. So this fraction MF4 was taken for in vitro analysis using HT-29 colon cancer cell lines, and it was determined through MTT assay. The cell viability at 50% was estimated to be 57.47%, and this was confirmed to be inhibitory concentration. Rajadurai and Prince (2005) reported that pretreatment with *A. marmelos* leaf extract at different doses showed a significant effect on the activities of marker enzymes, lipid peroxides, lipids, lipoproteins, and antioxidant enzymes in animals (mice). Further it was confined to be basic concentration for future biological determination activities, and this laid a foundation for the anticancer studies. Reports of Costa-Lotufo et al. (2005) showed the anticancer potential of 11 plants, but only the extracts of *Oroxylum indicum*, *Moringa oleifera*, and *A. marmelos* showed potential anticancer activity. He also reported that *A. marmelos* (L.) inhibits the proliferation of transplanted Ehrlich ascites carcinoma in mice. The anticancer effect of hydroalcoholic extract of *Aegle marmelos* (AM) was studied in the Ehrlich ascites carcinoma bearing Swiss albino mice. The spatial effect of various Vilva tree administration schedules exhibited after 6-day administration which increased the survival of tumor-bearing mice. On starting with the anticancer studies, it is must to determine the anti-angiogenesis activity. This is done to prevent the formation of blood vessels in tumor cells which is the basic mechanism for the next stage metastasis. Angiogenesis is the physiological process through which new blood vessels form from preexisting vessels. Angiogenesis is a complex process that is tightly regulated by pro- and anti-angiogenic growth factors. In some diseases,

the body loses the ability to control angiogenesis, and new blood vessel growth is either excessive (e.g., cancer) or inadequate (e.g., coronary artery disease) (Folkman 1971; Kerbel 1991; Staton et al. 2009). Angiogenesis occurs in a series of complex and interrelated steps. First, diseased or injured cells release pro-angiogenic growth factors, such as VEGF, FGF, or PDGF, into the surrounding tissue. Angiogenic growth factors are released in response to secreted proteins in the microenvironment (e.g., epidermal growth factor, fibroblast growth factor, insulin-like growth factor, interleukins, platelet-derived growth factor) (Pugh and Ratcliffe 2003), hypoxia, hypoglycemia, mechanical stress (e.g., increased pressure caused by proliferating tumor cells), release of inflammatory proteins (e.g., cyclooxygenase-2, prostaglandins, mast cell activation), and genetic alterations (Blancher et al. 2001). The released growth factors bind to, thereby activating, endothelial cells that form the walls of nearby blood vessels (Engelman 2009). In order to control the growth factors in a normal regulation for the tumor cells, it is must to arrest the angiogenesis in tumor. In this paper, we have determined the anti-angiogenic process through hen's egg test on chorioallantoic membrane (HET-CAM). Different concentration of MF4 was taken on which 75 µg/ml to 100 µg/ml exhibited slow degradation process in the embryonated eggs after 2 and 18 h of incubation. The compound MF4 of 100 µg/ml to 200 µg/ml manifested its activity by completely arresting the blood vessels formation in the growing embryo. From this preliminary assay, we conclude that the bioactive metabolite MF4 has the potentiality of arresting the newly formed blood vessels and also act as anti-angiogenic agent. Further, the compound MF4 will be implemented on anti-angiogenic markers from commercial supplements and comparative study will be assessed.

7.5 Conclusion

The present study has led a new impact to assess the bioactive metabolite produced by a medicinal tree *A. marmelos* for anticancer potentiality. The tree produces several metabolites, and those have been used extensively in traditional medicine to treat various diseases. And this research focused to extract a single bioactive metabolite through preliminary analysis such as antimicrobial and antioxidant determination. Further the crude extract from branch sample exhibited the highest activity, so it was taken for purification process by chromatographic techniques. On purification about seven different fractions were eluted, and those were determined for antioxidant and antimicrobial assessment, and MF4 fraction explored its maximum activity at minimum concentration. This MF4 compound on chemical characterization was found to be 5-acetoxytridecane, and it exhibited good anti-angiogenic activity. Further research will be carried on to determine the anticancer property in in vivo models.

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Chapter 8
Role of Secondary Metabolites for the Mitigation of Cadmium Toxicity in Sorghum Grown Under Mycorrhizal Inoculated Hazardous Waste Site

Prasann Kumar, Shweta Pathak, Mukul Kumar, and Padmanabh Dwivedi

Abstract Apart from the many primary metabolites like carbohydrate, proteins, fats, and hormones, a number of organic compounds in plants are not synthesized in the principle stream, but they are most important for the plant functions. These compounds are secondary metabolites broadly classified in terpenes, alkaloids, and phenols. Nowadays, heavy metal contamination is the greatest concern worldwide. The role of secondary metabolites is well acquainted with facts that it imparts the significant effect in the mitigation of heavy metal toxicity in the plants. The FTIR data of the experiments indicate clearly that phenols, aldehyde, and ketones will play the critical role in the regulation of heavy metal toxicity in plants.

Keywords Cadmium · Phenol · Secondary Metabolites · Sorghum · Toxicity

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8.1 Introduction

Deprivation of accepted resources is perhaps one of the lethal lapses mankind has ever prepared in its voyage of progress and civilization. All the natural resources are contaminated with lethal lapses. Among them, the land and water resources are worst affected and under continuous stress with both biotic and abiotic, due to anthropogenic interventions. If we talk about the soul of infinite life, i.e., soil, then it seems to appear the primary recipient by design or accident of a myriad of waste products and chemicals used in modern society. Soil contamination can be defined as the addition of any substance to the soil that may exert adverse effects on its functioning and capacity to yield a crop. Contamination of heavy metal is of special worry due to well-known reports emanating both from India and abroad. Various diseases and disorders are observed both in human and livestock due to metal toxicity. A scientist reported that the greatest problems most likely involve mercury, cadmium, lead, chromium, arsenic, nickel, etc. To a greater or lower degree, all of these elements are toxic to humans and any other animals.

Cadmium is extremely toxic, causing heart and kidney disease and bone embrittleness; Cr, Ni, and Pb are moderately responsible for mutagenic, lung cancer, convulsion, and brain damage like some deadlier diseases. We have tested several plant species for their capacity of scavenging heavy metals from soil and sludge, and finally we reach to the conclusion that among the tested plants, *Sorghum vulgare* L is more adapted to grow on contaminated sites with respect to other plant and able to mitigate the heavy metal toxicity from hazardous waste site or cultivated site. Compared to organic pollutants, the remediation of toxic metals in porous matrices (soil and sediments) requires a specific approach since metals cannot be mineralized and hence require for their removal. Treatments make necessary metal extraction to avoid their dissemination in the environment and/or the food chain contamination. Soil washing is a well-known method that uses various chemical extracts. However, this method can only be applied to soils after their excavation contrary to soil flushing, an in situ soil remediation (Mulligan et al. 2001). Both soil remediation methods are only suitable for point source contaminations. Nonpoint source contamination, i.e., moderate metal concentration, but wide surface contaminated is less studied. Typically, many agricultural soils are concerned by these contaminations as a result of repeated application of both fertilizers and pesticides containing trace metals at various concentrations, along with atmospheric deposition. Metal concentration encounters are lower than those recorded in industrial sites but are sometimes high enough to generate a risk for the environment and human beings, through the food chain. Additionally, nonpoint source contaminations can be turned into point source ones by means of concentration phenomena, depending on several parameters, e.g., the metal species. Phytoextraction goes down under the class of current technology popularly called green cure or phytoremediation. It has been estimated that this technology is reserved for nonpoint source pollution nowadays. A scientist reported that this technology can be applied for the removal of the very low concentration of the contaminants present in the soil. Blaylock (2000) has

Fig. 8.1 (**a**, **b**) Root of 1-month-old sorghum plant grown in pots with contaminated soils of 1500 ppm cadmium nitrate

Fig. 8.2 (**a**, **b**) Sorghum plant of two-leaf stage grown in pots with contaminated soils of 700 ppm cadmium nitrate

quantified the fact that those areas where lead is one of the significant contaminants, where Pb extraction by *Brassica juncea* is feasible only for sites whose metal concentration did not exceed 1500 mg/kg. We have tested the sorghum plant for the level of tolerance along with the mitigating effect of polyamines, and we found sorghum can tolerate 1500 ppm of the cadmium toxicity (Figs. 8.1, 8.2, 8.3, and 8.4). These findings indicate that if it is cultivated in industrial areas where cad-

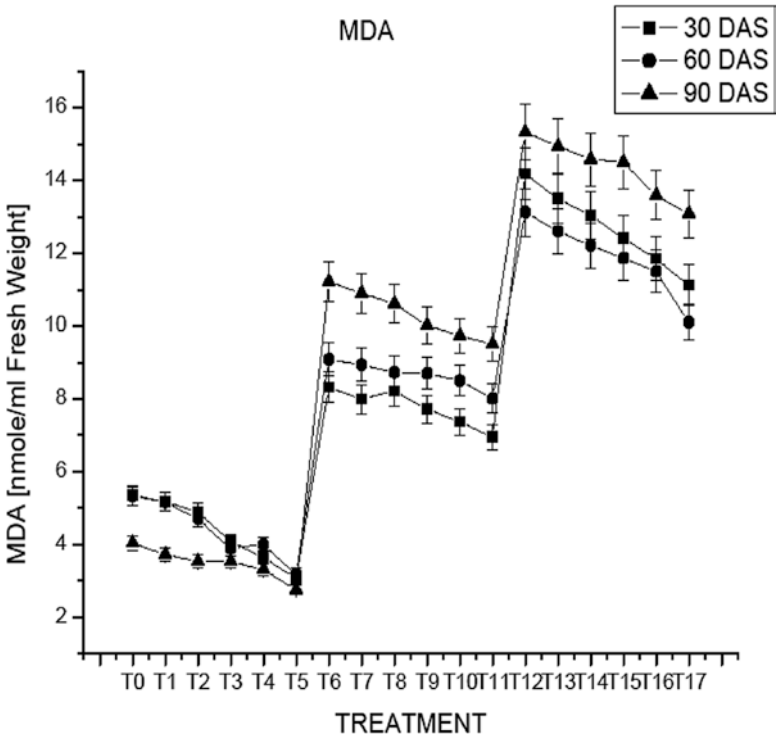

Fig. 8.3 MDA concentration in the leaves of sorghum treated with cadmium nitrated, mycorrhiza, and putrescine

mium toxicity is one of the major problems then, definitely, it will work efficiently. On the basis of our finding, we recommend the sorghum plant in a contaminated area with mycorrhizal application as well as polyamines.

8.2 Phytoremediation: Tool for Green Cure

Phytoremediation is eco-friendly approach for the removal of contamination of agricultural soils. After the treatment, the soil health is going to improve, and it allows sustaining crop production. If you have been considering the economics incurred in the treatment, then these are supposed to be generally cheaper than physical ones. There are many limitations of phytoremediation: [1] low soil thickness that can be treated by plants depending on the depth of the settling which generally does not exceed half a meter and [2] low translocation rate of metals from roots to shoots. Besides this one, the major limit is slowing of the treatment (Baker et al. 2000) as a consequence of the low availability of metals at a given time. The reasonable period of remediation is considered to be less than 5 years (Khan et al.

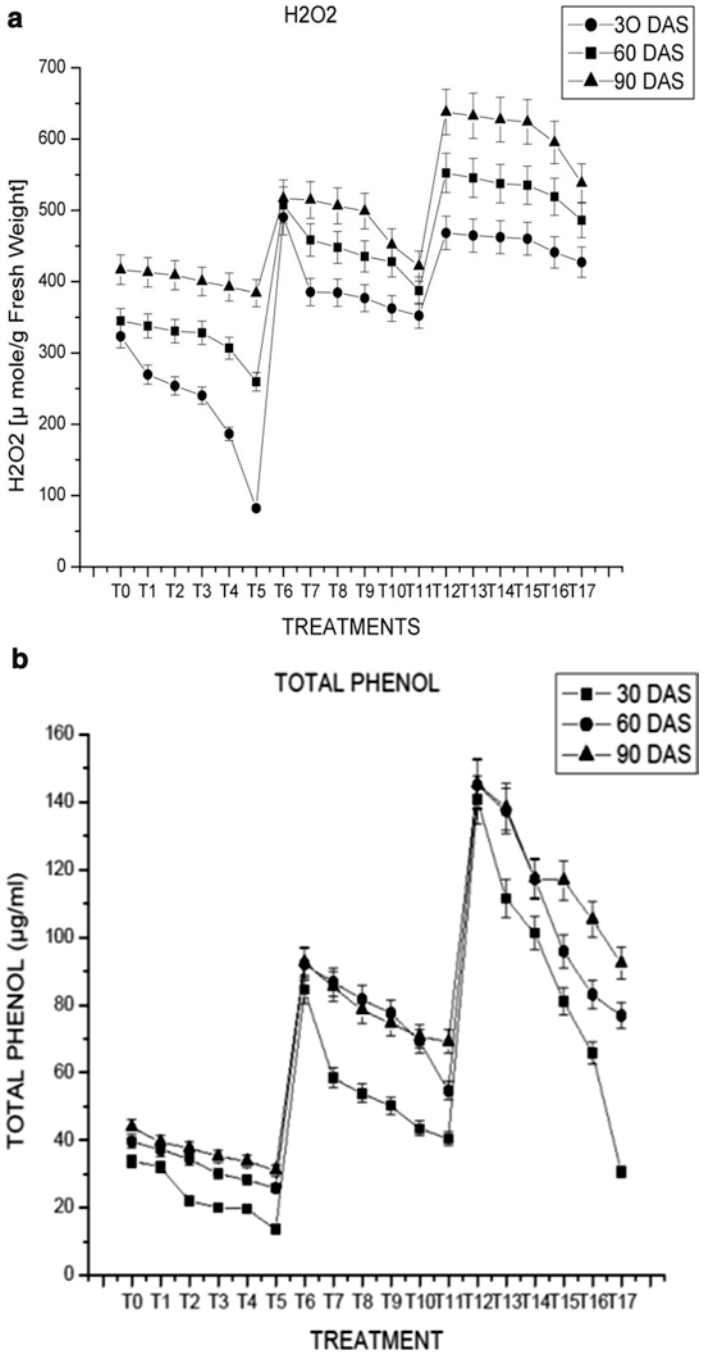

Fig. 8.4 (**a**, **b**) H_2O_2 concentration and total phenol concentration in the leaves of sorghum treated with cadmium nitrate, mycorrhiza, and putrescine

2000), while much more time is usually required to clean the soil (Baker et al. 2000; Dickinson and Pulford 2005). To enhance the concentration of hazardous, toxic metals at the plant's disposal, various synthetic chelating compounds such as diethylenetriamine pentaacetic acid (DTPA) and ethylenediaminetetraacetic acid (EDTA) have already been used. Regrettably, the biodegradability of these compounds is rather low or can be considered as one of the limiting factors for the same (Lombi et al. 2001), and they may be toxic to plants, microorganisms (biomass and diversity), and nematodes (Lasat 2002; McGrath et al. 2002; Romkens et al. 2002; Bouwan et al. 2005) present in the soil. If we think about the soil health, then definitely it acts as one of the threats. Moreover, faster mobilization of Pb by EDTA than by plants has been shown (Shen et al. 2002; Chen et al. 2004) with a risk of Pb lixiviation in the soil suggested that plants cannot extract high amount of metals in a short period (Barona et al. 2001). Finally, these compounds are expensive, on the basis of the amount applied per hectare (Barona et al. 2001). A potential alternative option consists of optimizing the synergistic effect of plants and microorganism (Glick 2003) by coupling phytoextraction with soil bioaugmentation, also called rhizoremediation (Kuiper et al. 2004). This technique has been widely developed for the remediation of soils contaminated by organic pollutants (Barac et al. 2004; van Aken et al. 2004) but not for metals. Overall the uptake of metals by plants can be enhanced by two harmonizing ways:

1. Amplification of mobility of metals in soil and sediments, resulting in elevated metal concentrations in plants. In this condition, we certainly convey thanks to microorganism which produces biosurfactant (Herman et al. 1995; Mulligan et al. 1999, 2001), siderophores (Diels et al. 1999; Dubbin and Louise Ander 2003), and organic acids (Di Simine et al. 1998; Majewska and Kurek 2005). These compound are treated as the natural chelating agent and this combined with the metal and forms organo-metallic coordinate compounds.
2. Enhancement of biomass of plants by associating them with Plant Growth Promoting Rhizobacteria (PGPR) (Zhuang et al. 2007) and/or ArbuscularMycorrhizal Fungi (AMF) (Khan 2006).

In case of sorghum, we have applied the *Glomus* EM fungi for enhancement of uptake of cadmium from contaminated soils, and results were found significant. Plants rising in metal polluted soils anchorage a significant group of microorganism that are capable of tolerating high concentration of metal and giving a number of benefits to both the soil and the plant of concern. In our study related to the microbe-assisted remediation approaches, we find that, among the microorganism involved phytoremediation of heavy metal, the rhizosphere fungus deserves special attention because they can directly improve the phytoremediation process by changing the metal bioavailability through altering the soil pH, release of chelators (organic acid and siderophores), and oxidation/reduction reaction. Similarly, the metal-tolerant mycorrhizal fungi have been frequently reported in hyperaccumulators growing in

metal polluted soils indicating that these fungi have evolved a heavy metal tolerance and that they may play important role in phytoremediation of the site. Extraction of heavy metals goes down under the class of current technology popularly called green cure or phytoremediation. It has been estimated that this technology is reserved for nonpoint source pollution nowadays. Scientist reported that this technology can be applied for the removal of the very low concentration of the contaminants present in the soil.

8.3 Antioxidant-Mediated Mitigation of Heavy Metal Toxicity in Sorghum

We have tested the sorghum plant for the level of tolerance along with the mitigating effect of mycorrhiza, and we found sorghum can tolerate the widest range of the cadmium toxicity. These findings indicate that if sorghum is cultivated in industrial areas where cadmium toxicity is one of the major problems, then, definitely, it will work efficiently, and we recommend sorghum plant in a contaminated area with mycorrhizal application. *Sorghum vulgare* L. plants when exposed to cadmium nitrate with the concentration of 0.07% and 0.15% for 90 days exhibit phytotoxic responses along with tolerance. The observations of specific responses were depending on treatment combinations. The significant hazardous effect and oxidative damage of cadmium were evident by increase in MDA content and hydrogen peroxide content. The metal tolerance and detoxification strategy adopted by the plant were investigated with reference to a nonenzymatic antioxidant system, and the synthesis of proline and total soluble sugar and antioxidant enzymes, viz., peroxidase (POD, EC 1.11.1.x), ascorbate peroxidase (APX, EC 1.11.1.11), catalase (CAT, EC 1.11.1.6), and polyphenol oxidase (PPO, EC 1.14.18.1), shows induction for treating concentration of cadmium. The FTIR spectrum shows the picks of alcoholic, aldehyde, and ketone functional groups were sharper and strong in plants grown in higher concentration of cadmium. Dispersion scanning calorimetric data shows the decreased trend of stability with increasing concentration of cadmium. This was expressed in terms of glass transition phase temperature. The plants treated with putrescine and Glomus showed the decreasing trend of sharpness of pick with respect to plants not treated. The picks obtained after Powder XRD were stronger in plants grown in higher doses of cadmium-treated soil with respect to plants treated with putrescine and mycorrhiza. These picks were measured at 2Θ value of 20 to 30.Due to metal accumulation coupled with detoxification potential, the sorghum appears to have potential for its use as hyperaccumulator and can be used as phytoremediator species in contaminated environments having toxic pollution of cadmium (Figs. 8.1a, b, 8.2a, b, 8.3, 8.4a, b, 8.5a, b, and 8.6a, b).

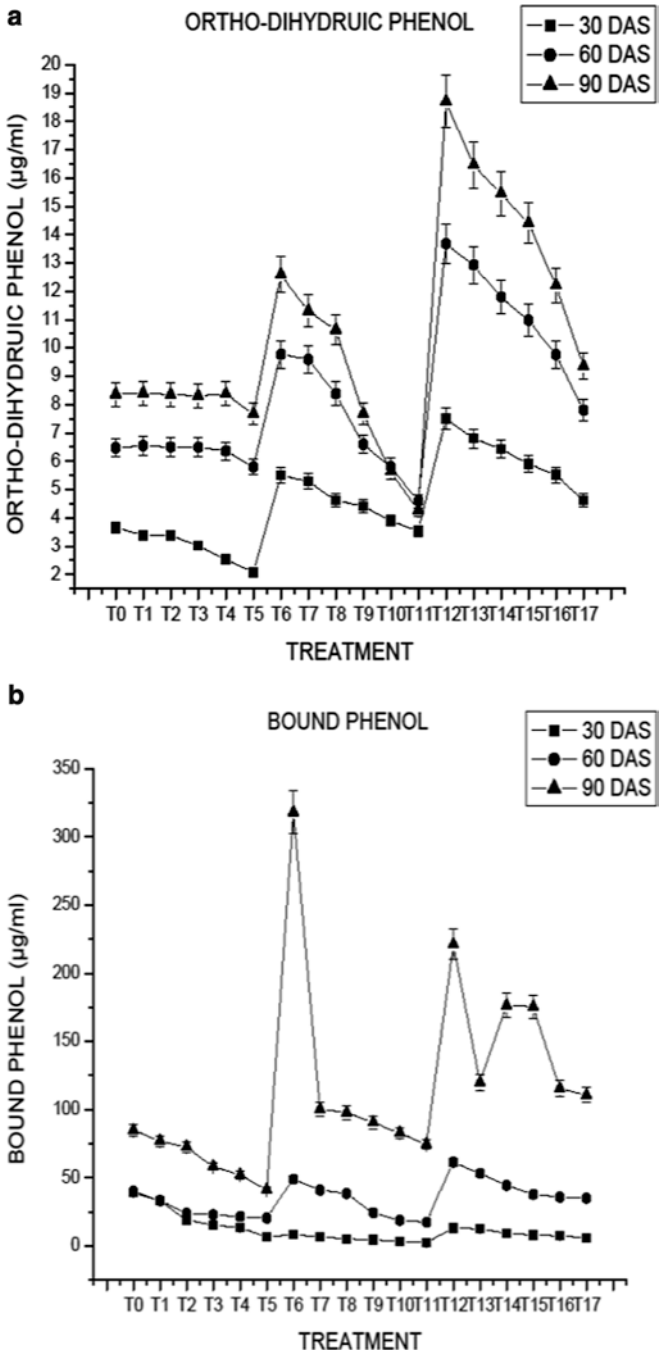

Fig. 8.5 (**a**, **b**) Ortho-dihydric phenol and bound phenol concentration in the leaves of sorghum treated with cadmium nitrate, mycorrhiza, and putrescine

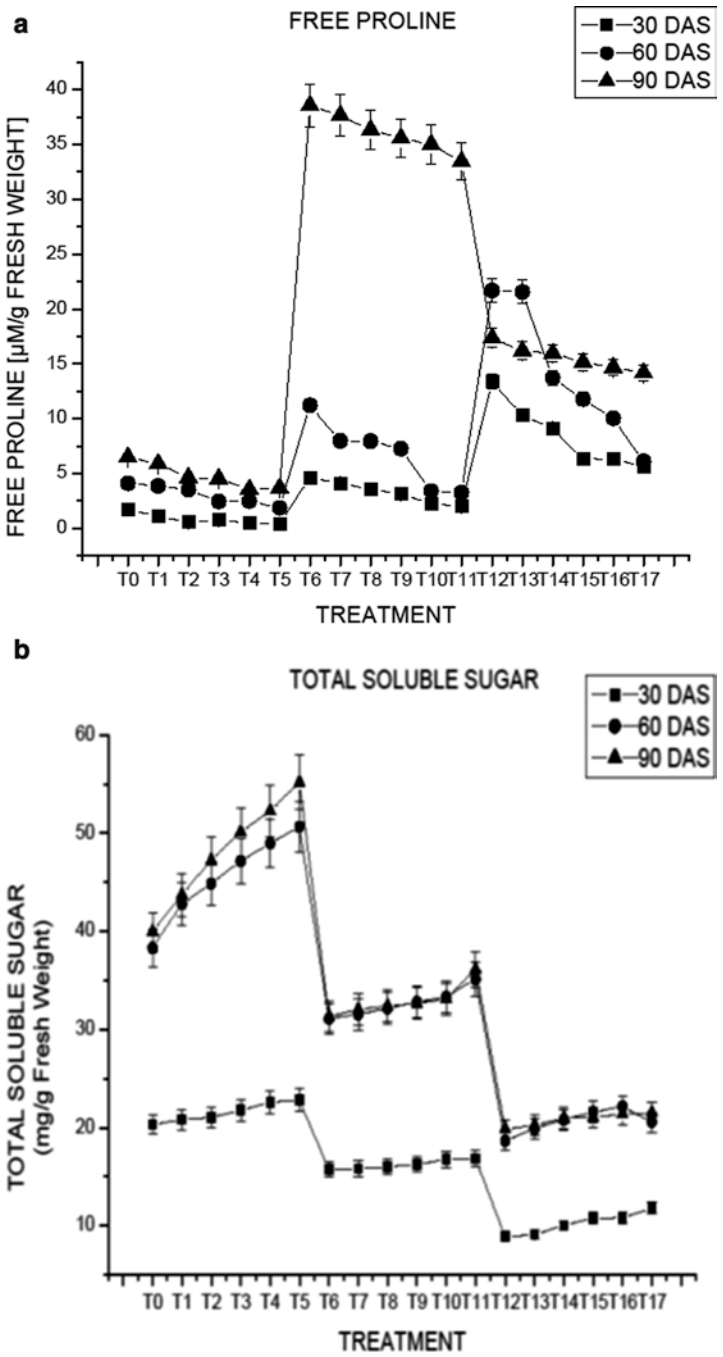

Fig. 8.6 (**a**, **b**) Free proline and total soluble sugar concentration in the leaves of sorghum treated with cadmium nitrate, mycorrhiza, and putrescine

8.4 Bioaugmentation of Metals from Contaminated Waste Site

Plant-associated microbes can also immobilize the heavy metals in the rhizosphere through metal reduction reaction. For example, inoculation of Cr-resistant bacteria *Cellulosimicrobium cellulans* to seeds of green chili grown in Cr (VI)-contaminated soils decreased Cr uptake into the shoot by 37% and root by 56% compared with uninoculated controls. This study indicated that bacteria reduced the mobile and toxic Cr (IV) to nontoxic and immobile Cr (III) in the soil. (a) Plant-associated microbes improve plant nutrients and water uptake. Microbial metabolites reduce metal toxicity, (b) metal biosorption, and (c) metal reduction and complexation reactions. Plant-associated microbes reduce heavy metal stress in plants through (d) increasing antioxidants defense and/or producing ACC deaminase and (e) improve the plant growth by producing plant growth regulators. Certain plant-associated microorganism has the potential to alter the mobility of heavy metal through oxidation or reduction reaction. Metal oxidation by rhizosphere microbes is particularly interesting from a phytoextraction point of view. For instance, the sulfur-oxidizing rhizosphere bacteria have been reported to enhance Cu mobilization in contaminated soils and its uptake in plant tissue (Shi et al. 2011). The result of this experiment shows that the sulfur-oxidizing bacteria reduce the rhizosphere soil pH via conversion of reduced sulfur to sulfates, thus making Cu available for plant uptake. Similarly, Chen and Lin (2001) have also pointed out that Fe-/S-oxidizing bacteria have the potential to enhance metal bioavailability in the soils through acidification reaction (Rajkumar et al. 2012). The plant-associated microbes may also contribute in plant metal uptake through biosorption mechanism (Rajkumar et al. 2012). Biosorption can be defined as the microbial adsorption of soluble/insoluble organic/inorganic metals by a metabolism dependent, active process (Ma et al. 2011). Several authors have pointed out that bacterial biosorption mechanism accounted for reduced plant metal uptake. For instance, Madhaiyan et al. (2007) observed the inoculation of metal-binding bacteria *Magnaporthe oryzae* and *Burkholderia* sp. reduced Ni and Cd accumulation in roots and shoots of tomato. These studies indicate that the metal-binding bacteria can reduce the metal bioavailability and/or restricts its entry into the plant root/shoot (Rajkumar et al. 2012). The mycorrhizal fungi can also act as filter barrier against the translocation of heavy metals from plant roots to shoots (Rajkumar et al. 2012). Experiment with pine seedlings revealed that the inoculation with the EMF *Scleroderma citrinum*, *Amanita muscaria*, and *Lactarius rufus* reduced translocation of Zn, Cd, or Pb from roots to shoots compared with the controls. This effect was attributed to the increased metal biosorption by outer and inner compounds of the increased metal biosorption by outer and inner components of the mycelium (Krupa and Kozdrój 2007). Since mycorrhizal fungi have large surface area, which endows mycorrhizal fungi with a strong capacity at adsorbing heavy metals from soil. The fungal cell wall

components and intracellular compounds may also immobilize/arrest the metals in the interior of plant roots (Meharg 2003). Although these studies suggest that inoculation of plants with metal-binding microbes could be suitable approach for plant protection against heavy metals and phytostabilization of metal polluted soils, several authors have pointed out that microbial biosorption/bioaccumulation mechanism was not solely responsible for the decreased metal accumulation and translocation in plants (Babu and Reddy 2011; Vivas et al. 2003). All these reports clearly indicate that the plant-associated microorganism differ in their capacity to alter the heavy metal bioavailability and its uptake by plants through metal mobilizing/immobilizing metabolites actions (Rajkumar et al. 2012). However, survival and colonization potential of these microbes greatly influence the quality of metal accumulation in plants growing in metal-contaminated field soils, because adverse physicochemical-biological properties of soils including metal toxicity, indigenous microbial communities, adverse pH, nutrient deficiency, etc. reduce the survival, activity, and colonization potential of inoculated microbes and thus potentially lead to alter the metal mobilization and/or immobilization (Rajkumar et al. 2012). Since each pollutes soil and has a specific profile, the potential of plants to uptake metals can vary to a large extent, depending on which metal is involved, its concentration, the microbial partner and their survival and colonization potential, plant type, and its growth conditions (Rajkumar et al. 2012). Ever since plant linked microbes possess the capacity of plant growth promotion and/or metal mobilization/immobilization, there has been growing interest in the occasion of manipulating plant-microbe interactions in metal-contaminated soils (Aafi et al. 2012; Azcón et al. 2010; Braud et al. 2009; Dimkpa et al. 2008; Hrynkiewicz et al. 2012). Microbial metabolites/processes promote plant growth and metal mobilization/immobilization in vivo but are incapable to confer useful traits on their host in metal-contaminated soils (Rajkumar et al. 2012). In addition the isolation of a variety of plant-associated microbes and description of its beneficial metabolites/processes are time-consuming since it requires the analysis of more than thousands of isolates (Rajkumar et al. 2012). Thus, the strong molecular research effort is required in order to find a specific biomarker associated with the beneficial microbes for efficient microbe-assisted phytoremediation (Rajkumar et al. 2012).

Since the activity of inoculated microbes is necessary to exhibit beneficial traits for improving the plant growth and overall phytoremediation process in metal-contaminated soils, the colonization and survival in metal stress field environment are considered as important factors. Thus advancing the knowledge on multiple metal resistances, survival and compatibility of microbes may be important to utilize their potential as inoculants for phytoremediation purpose (Rajkumar et al. 2012). The noteworthy advances have been made in accepting the role of plant-associated microbes in metal mobilization/immobilization and in the claim of these processes in heavy metal phytoremediation (Braud et al. 2009). In the future, the additional advantages are expected.

8.5 Conclusion

It is concluded that the polyamines like putrescine and mycorrhiza *Glomus* impart significant mitigation of cadmium-induced toxicity in sorghum mediated through the defensive role of enzymatic and nonenzymatic antioxidants in plants.

With the aim of adopting this cure technology, the soil should be taken as bioreactor supported by the means of engineering of the rhizosphere. The wide-scale expansion of this technique will thus depend on:

[A] The consistency of the procedure which still requires to be established at the field scale
[B] The ease of implementation just like microbial-coated seeds of legumes that are usually used in agriculture
[C] The opportunity to clean up contaminated soil of diverse toxicity such as inorganic and organic pollutant
[D] The economic value behind the cleanup technology with the help of microorganism-assisted plant

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Chapter 9
In Vitro Production of Some Important Secondary Metabolites from *Zingiber* Species

Sanatombi Rajkumari and K. Sanatombi

Abstract Many higher plants are major sources of natural products which are used as pharmaceuticals, flavor and fragrances, dye and pigments, pesticides, and food additives. The search for new plant-derived chemicals has become a priority in current and future efforts toward sustainable conservation and rational utilization of biodiversity. In the recent years, the evolving commercial importance of secondary metabolites has led to a great interest in the production and enhancement of bioactive plant metabolites by means of tissue culture technologies. Plant cell culture systems represent a potential renewable source of valuable medicinal compounds which are not limited by the low yields associated with natural harvest or the high cost associated with complex chemical synthesis as well as provide more resistance to pathogens and adverse environmental and climatic conditions. Different strategies, using an in vitro system such as undifferentiated cell cultures and hairy root culture, have been extensively studied to improve the production of plant chemicals as they are more genetically stable. Among the medicinal plants, *Zingiber* is also considered as an important genus comprising many plant species that has received much attention in food and medicinal industry due to the presence of different secondary metabolites that contribute to its diverse biological activities. Based on this limelight, the present chapter focusses on several studies of in vitro production of important secondary metabolites from different *Zingiber* species. Moreover, the applications and strategies for the enhancement of these valuable metabolites by using in vitro technology are also discussed in this chapter.

Keywords Elicitors · In vitro culture · Secondary metabolites · *Zingiber* species

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9.1 Introduction

Plants are the most excellent designers and producers of a variety of low molecular weight compounds, secondary metabolites, that help in plant defense and are beneficial to mankind as pharmaceuticals, food additives, flavor and fragrance ingredients, and industrial products (Murthy et al. 2014). Secondary metabolites are often synthesized from primary metabolites and have no direct implication on the growth and development of plants. Also, the distribution of these metabolites is sometimes confined to particular tissues of genus or species, where these are often accumulated in high concentrations and may show marked biological effect as well as high toxicity. Mixtures of volatiles and essential oil isolated from plants represent the important constituents of plants where the volatiles are mostly terpenoid compounds (monoterpenoids, sesquiterpenoids, and diterpenes) and are among the most valuable compounds produced by plants, besides alkaloids, phenolics, and phenylpropanoids.

Within the past few decades, there has been a revival of interest in folk medicine, especially in herbal remedies, and in its conjunction with modern medicine. The use of medicinal plants with secondary metabolites for the treatment of many health diseases, disorders, and infections dates back to ancient history of mankind (Wyk and Wink 2004). During the last hundred years, most of the natural products have been partly replaced by synthetic drugs, for which plant structures were chemical models for new drugs. It should, therefore, be of priority to search for new plant-derived chemicals in current and future efforts toward sustainable conservation and rational utilization of biodiversity (Philipson 1990). Biotechnological approaches, using plant tissue culture, have emerged as a potential alternative tool for the production of desirable medicinal bioactive compounds from plants with the possibilities of supplementing the conventional method (Ramachandra and Ravishankar 2002). The deliberate stimulation of defined metabolites within carefully regulated in vitro cultures provides an excellent opportunity for in-depth study of biochemical and metabolic pathways (Karuppusamy 2009). Cell cultures are promising technologies to obtain plant-specific valuable metabolites in large scale rather than in vivo in the intact plant due to a series of advantages which includes (a) continuous and reliable source for the production of natural desirable compounds without seasonal constraints; (b) compounds produced in vitro can directly parallel those in the whole plant; (c) isolation of compounds can be rapid and efficient, as compared to extraction from complex whole plants; (d) interfering compounds that occur in the field-grown plant can be avoided in cell cultures; (e) potential model to test elicitation; and (f) cell cultures can be radiolabeled, such that the accumulated secondary products, when provided as feed to laboratory animals, can be traced metabolically (Karuppusamy 2009).

Recent advances in molecular biology, enzymology, and fermentation technology of plant cell cultures have opened new perspectives for high-volume commercial production of pharmaceuticals which have led to the isolation of the bioactive

substances of cultured cells, achieved by optimizing the culture conditions, selecting high-producing strains, and employing precursor feeding, transformation methods, and immobilization techniques (Dicosmo and Misawa 1995; Hansen and Wright 1999). Genome manipulation and transgenic hairy root cultures have revolutionized the role of plant tissue culture, thereby resulting in relatively large amounts of desired compounds produced by plants infected with an engineered virus, whereas transgenic plants can maintain constant levels of production of proteins without additional intervention (Sajc et al. 2000). The demand for using natural products in medicinal applications has been increasing, and metabolic engineering can alter the production of pharmaceuticals and help to design new therapeutic drugs with anti-inflammatory, antitumor, antiviral, hypoglycemic, antiparasite, antimicrobial, tranquilizing, and immunomodulating activities (Marden et al. 1997; Wongsamuth and Doran 1997; Doran 2000).

Several plant families such as Apiaceae, Asteraceae, Cupressaceae, Hypericaceae, Lamiaceae, Lauraceae, Leguminosae, Liliaceae, Malvaceae, Myrtaceae, Oleaceae, Pinaceae, Rosaceae, and Rutaceae are reported to be rich in secondary metabolites, which are present in volatile and essential oils (Figueiredo et al. 2008). Among the various medicinal plants used in both traditional and modern medicine systems, genus *Zingiber* (family Zingiberaceae), which comprises approximately 85 plant species, are also invariably considered as nutritionally rich food products as well as rich source of valuable medicines with commercial importance (Mabberley 1990). These plant species are important natural resources which are found in wild or widely cultivated and also have a wide spectrum in terms of traditional medicinal uses (Ammon 1991; Jansen 1999; Chamratpan and Homchuen 2012; Devi et al. 2014). In the recent years, several reports concerning the isolation and identification of various secondary metabolites from these plant species along with their related pharmacological properties have been published (Riyanto 2007; Karnchanatat et al. 2011; Matsuda et al. 2011; Sivasothy et al. 2012; Wiart 2012; Saifudin et al. 2013; Ogawa et al. 2014; Khrimian et al. 2015). Most of these plant species also produce essential oil and novel bioactive compounds which possess several important pharmacological properties such as anti-inflammatory, antioxidant, antimicrobial, antiviral, insecticidal, anticancer, anti-genotoxic, immunomodulatory, and anticholinesterase activities (Okonogi and Chaiyana 2012; Farhath et al. 2013; Sivasothy et al. 2013; Jegannathan et al. 2016; Lai et al. 2016; Moirangthem et al. 2016; Priprem et al. 2016).

During the last decade, several promising findings for a variety of medicinally valuable substances and exploration of the biosynthetic capabilities of various cell cultures of *Zingiber* species have been reported (Jansen 1999; Riyanto 2007; Ogawa et al. 2014). The aim of the present chapter is to focus on the importance of tissue culture technology in the production of some important secondary metabolites from *Zingiber* species reported earlier. Additionally, the successful research on various enhancement strategies for improved and mass-scale production of these metabolites for commercial application has also been reviewed here.

9.2 In Vitro Cultures for Secondary Metabolite Production in *Zingiber* Species

Advance researches in the area of plant tissue culture technology have resulted in the production of a wide variety of pharmaceutical products of interest like alkaloids, terpenoids, steroids, saponins, phenolics, flavonoids, and amino acids. Successful attempts on the production of some important secondary metabolites by in vitro cultures of *Zingiber* species are discussed below and shown in Table 9.1.

Table 9.1 Secondary metabolites produced by in vitro cultures of *Zingiber* species

Secondary metabolites	Type of in vitro cultures	Plant species	References
Gingerols (6,8 and 10-gingerol)	Callus, mini-rhizomes, adventitious roots and regenerated plants	*Zingiber officinale*	Zarate and Yeoman (1996)
	Microrhizomes and regenerated plants	*Zingiber officinale*	Dehghani et al. (2011), Aly et al. (2013), Ma and Gang (2006)
	Multiple shoots	*Zingiber officinale*	Cafino et al. (2016)
	Tertraploid clones and shoot tip cultures	*Zingiber officinale*	Sanwal et al. (2010)
	Callus	*Zingiber officinale*	Anasori and Asghari (2008), El-Nabarawy et al. (2015)
Shogaols	Callus, mini-rhizomes and regenerated plantlets	*Zingiber officinale*	Zarate and Yeoman (1996)
Zerumbone	Microrhizomes	*Zingiber zerumbet*	Idris et al. (2007), Shinija et al. (2009)
	Cell suspension cultures	*Zingiber zerumbet*	Jalil et al. (2015)
Zingiberene	Callus	*Zingiber officinale*	Anasori and Asghari (2008)
	Regenerated plantlets	*Zingiber officinale*	Dehghani et al. (2011)
Phenolic compounds	Callus	*Zingiber zerumbet*	Stanly et al. (2011)
	Shoot tip cultures, microrhizomes	*Zingiber officinale*	Sakamura et al. (1986)
Geranial	Rhizomes	*Zingiber officinale*	Iijima et al. (2014)
β-Eudesmol	Rhizomes	*Zingiber zerumbet*	Yu et al. (2008)
α-Humulene or α-Caryophyllene	Rhizomes	*Zingiber zerumbet*	Yu et al. (2011), Alemdar et al. (2016)
Monoterpenes	Rhizomes and leaves	*Zingiber cassumunar*	Bua-in et al. (2014)

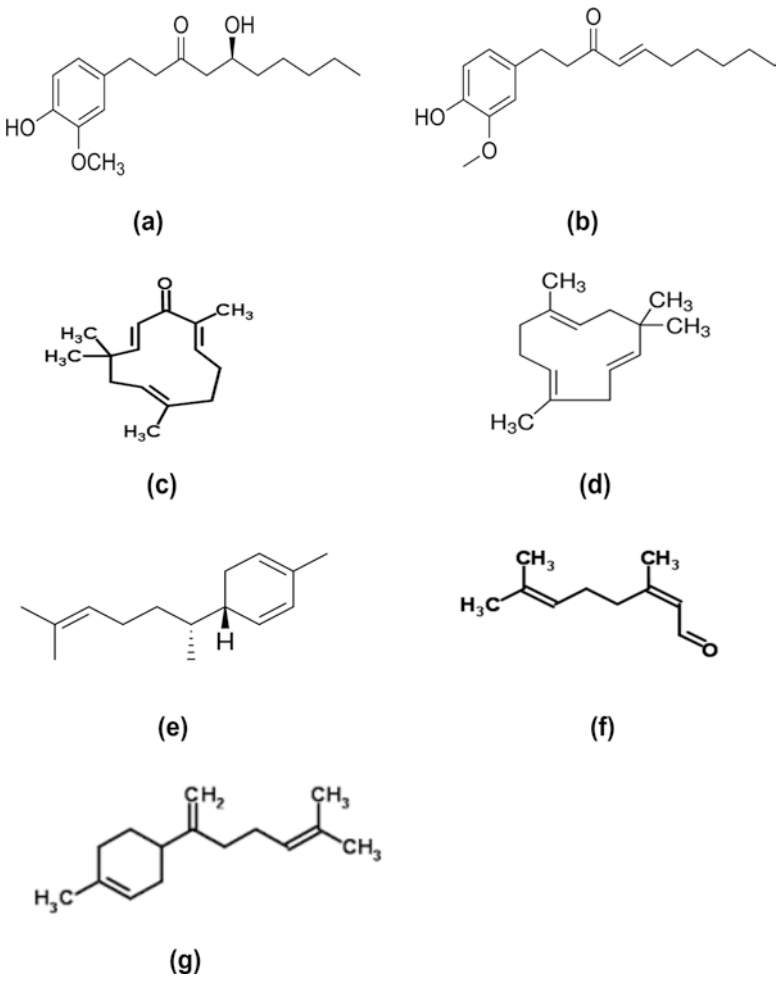

Fig. 9.1 Chemical structures of some important secondary metabolites produced from different *Zingiber* species by in vitro cultures: (**a**) gingerols, (**b**) 6-shogaol, (**c**) zerumbone, (**d**) α-humulene, (**e**) α-zingiberene, (**f**) geranial, (**g**) ß-eudesmol

The chemical structures of some important secondary metabolites produced from *Zingiber* species are represented in Fig. 9.1.

9.2.1 Gingerols

Gingerols and their homologues consist of a series of aldols, each with a phenolic group, and they form the pungent principles of ginger. The characteristic odor and flavor of ginger are due to a mixture of volatile oils like shogaols and gingerols

(Harold 2004). The homologous series are 4-gingerol, 6-gingerol, 8-gingerol, and 10-gingerol, of which, 6-gingerol [5-hydroxy-1-(4-hydroxy-3-methoxy phenyl) decan-3-one] is considered as the main pungent ingredient (Zarate and Yeoman 1996). 6-gingerols have been reported to be a pungent yellow oil but can also form a low-melting crystalline solid and are distributed predominantly within the rhizome but to a much lesser extent in the adventitious roots. There is also a positive correlation between the number of pigmented cells and the amount of 6-gingerol present in the plant material (Zarate and Yeoman 1994). The content of the active principle is not uniform and can vary significantly depending upon the plant varieties, seasonal conditions, and geographical regions in which ginger is grown (Gruenwald 2004).

Gingerols are biologically active components with antioxidant and anti-inflammatory properties that may make a significant contribution toward medicinal applications of ginger (Dugasani et al. 2010). Due to this reason, there has been an increase in the number of commercial products of ginger containing gingerols and shogaols as main active ingredients (Kiuchi et al. 1982). 6-gingerols also possess various pharmacological and physiological effects including analgesic, antipyretic, gastroprotective, cardiotonic, and antihepatotoxic activities as well as act as potent inhibitor on prostaglandin biosynthesis (Govindarajan 1982; Suekawa et al. 1984; Shoji et al. 1982; Hikino et al. 1985; Mustafa et al. 1993). It has also been found to show a strong correlation with the antioxidant activity in in vitro studies of ginger (Sanwal et al. 2010; Pawar et al. 2015). In vitro cultures of *Zingiber officinale* are being investigated as an alternative means for the production of gingerols. Production of 6-gingerol in morphologically undifferentiated callus cultures, mini-rhizomes, adventitious roots, and regenerated plants of *Z. officinale* has been reported for the first time by Zarate and Yeoman (1996). In the study, the amount of 6-gingerol produced was found to be highest in mini-rhizome (6.58 μg per explant) followed by regenerating explants (2.29 μg per explant), in vitro adventitious roots (2.16 μg per explant), and callus (1.22 μg per explant). Similarly, the presence of 6-gingerol in in vitro rhizomes and regenerated plants of *Z. officinale* was also detected by thin-layer chromatography (TLC) method in a study by Dehghani et al. (2011). An HPLC (high-performance liquid chromatography) method for the analysis of gingerols from both the field-grown rhizomes and in vitro-grown microrhizomes of ginger has been developed by Aly et al. (2013) where in vitro rhizomes harvested from in vitro-propagated plantlets and in vitro microrhizomes were found to produce higher 6-gingerol with the amount of 45.37 and 28.11 mg/g, respectively, than that of in vitro calli, shoots, and roots (7.89, 7.46, and 6.40 mg/g, respectively). Recently, another improved yet simple HPLC method was developed to analyze 6-gingerol production from in vitro-grown multiple shoots of ginger cultured under different light wavelengths (Cafino et al. 2016). In their study, it was found that different wavelengths of light-affected 6-gingerol production and red-light irradiation significantly improved the accumulation of 6-gingerol with the yield of 191.07 μg per culture bottle in multiple shoot cultures. In another study by Sanwal et al. (2010), the production of gingerols in tetraploid clones of ginger, derived from their respective diploid clones by in vitro shoot tip culture, was studied. They reported

that the total gingerol content, especially 10-gingerol in tetraploid clones, was found to be remarkably higher as compared to that of diploid clones. The production of gingerol in in vitro cultures is also known to be affected by different factors as well as found to be a differentiation-dependent process. In a study by Anasori and Asghari (2008), the effects of light and differentiation stages of callus cultures of *Z. officinale* were reported to influence the production of gingerol. The presence of gingerol was detected by TLC in semi-differentiated and differentiated cells cultured under light condition. Furthermore, the effect of precursors and biotic elicitors on the production of gingerols in callus cultures of *Z. officinale* was studied by El-Nabarawy et al. (2015). In the study, it was found that 6-gingerol production (5–30 μg/ 100 mg fresh weight) was positively influenced by the addition of mevalonic acid (1 and 5 mg/l) followed by yeast extract (250 and 500 mg/l) and *Aspergillus niger* (250 mg/l), while the other levels of precursors and biotic elicitors did not succeed in producing either of 8-gingerol or 10-gingerol in the cultures studied. Metabolic profiling of in vitro-micropropagated and conventionally greenhouse-grown ginger has been done by Ma and Gang (2006), and gingerols (6-gingerol, 8-gingerol, and 10-gingerol) were produced in in vitro-propagated plants with content of 1.740–2.247, 0.482–0.924, and 0.832–1.154 mg/g fresh weight, respectively. In a very recent work of Min et al. (2017), the effects of tissue culture and mycorrhiza applications in organic farming on concentrations of phytochemicals and antioxidant capacities in ginger rhizomes and leaves were studied. The study reported that the combined application of tissue culture and mycorrhiza significantly increased concentrations of total flavonoids and gingerols as well as antioxidant activity of the rhizome, thus suggesting their synergistic effects. Moreover, considerable amounts of phenolic compounds were also found in the bound fractions of the rhizomes, and 6-gingerol, ferulic acid, and lutein were predominant ones among gingerols, phenolic acids, and carotenoids, respectively, in ginger rhizomes.

9.2.2 Shogaols

Shogaols form the pungent constituents of ginger along with gingerols, and among them, 6-shogaol [(4E)-1-(4-hydroxy-3-methoxyphenyl)-4-decen-3-one] is the major biologically active compound found in large amounts in dried ginger powder (Bhattarai et al. 2001). 6-Shogaol is formed when β-hydroxy group of the gingerol undergoes catalytic dehydration at high temperature (80 °C) and in acidic condition (pH 1) (Govindarajan 1982; Baranowski 1985). 10-shogaol, the only nonpungent compound among the gingerols and shogaols, has the ability to stimulate the increase of adrenaline secretion (Iwasaki et al. 2006). A number of in vitro and in vivo studies have found that 6-shogaol exhibits many medical and biological effects including antipyretic, analgesic, antitussive, and anti-inflammatory activities as well as inhibitory effect on inflammatory nitric oxide synthase, cyclooxygenase-2 (COX-2) gene expression, and nuclear factor, NF-kappa B transcription (Suekawa

et al. 1984; Pan et al. 2008). Moreover, it has also been reported to exert neuroprotective effects on dopaminergic neurons in in vitro and in vivo Parkinson's disease models (Park et al. 2013).

The first in vitro production of 6-shogaol in two culture systems (callus inducing and regenerating) of ginger was reported by Zarate and Yeoman (1996). It was found that the accumulation of this metabolite was much higher in culture systems where morphological differentiation was apparent, although unorganized callus tissue also seems to possess the necessary biochemical machinery to produce and accumulate some phenolic pungent principles. The content of 6-shogaol in callus and regenerated plants as well as mini-rhizomes in their study was found to be in the range of 50–200 μg per gram fresh weight and more than 6 μg per gram explant, respectively. Additionally, a clinical trial study of 6-shogaol and gingerols (6-, 8-, and 10-gingerol) in order to examine their pharmacokinetics and tolerance in humans has been conducted by Zick et al. (2008). The study concluded that 6-gingerol, 8-gingerol, 10-gingerol, and 6-shogaol are quickly absorbed and detected in the serum as glucuronide and sulfate conjugates, with the majority detected as glucuronide metabolites. These constituents, at normal concentrations (0.5%–2.5%), are detectable in the serum starting at a 1.0 g dose with the exception of 6-gingerol, which is detectable at a 250 mg dose with maximum concentrations ranging from 0.1 μg/ml to 1.7 μg/ml.

9.2.3 Zerumbone

Zerumbone [(2E,6E,10E)-2,6,9,9- tetramethylcycloundeca-2,6,10-trien-1-one] is a naturally occurring monocyclic sesquiterpene containing a cross-conjugated dienone moiety found in large quantities in rhizome of *Z. zerumbet* and also in many natural foods we consume (Murakami et al. 1999). Other *Zingiber* plant species with zerumbone among their constituents include *Z. amaricans* (Riyanto 2003), *Z. ottensii* (Sirat and Nordin 1994), *Z. aromaticum* (Muhammad 2009), and *Z. montanum* (Kishore and Dwivedi 1992).

It has been reported that the zerumbone yield from tissue-cultured plantlets and cell cultures is generally low, and further optimization in the methods is required. As microbes lack the native zerumbone biosynthesis pathway, metabolic engineering also required not only the introduction of several genes of the pathway but also precursor feeding and optimization of gene expression to increase their production (Nandagopal et al. 2017). The production of zerumbone from in vitro-derived rhizome of *Z. zerumbet* was reported earlier by Idris et al. (2007). In the study, the solvent extracts from the tissue culture-derived plants and cultivated rhizomes were analyzed using capillary GC and GC-MS (gas chromatography-mass spectrometry), and results showed comparable amount of this metabolite in both the extracts. Furthermore, in another study, GC-MS analysis of in vitro rhizome of *Z. zerumbet* revealed the presence of zerumbone with relative percentage of 40.25% (Shinija et al. 2009). Recently, the production of zerumbone in response to different

physiochemical parameters (carbohydrate substrate, light regime, agitation speed, and incubation temperature) has been studied in cell suspension cultures of *Z. zerumbet* (Jalil et al. 2015). The study reported that the production of zerumbone was significantly affected by different carbohydrate source, and the highest zerumbone content (3.73 mg/l) was recorded in cells cultured in sucrose-based medium followed by maltose, glucose, and fructose. On the contrary, the production of zerumbone was not significantly affected by the different concentrations of sucrose studied, and hence, the highest amount of the metabolite (3.70 mg/l) was detected on medium containing 3% sucrose. Similarly, culture medium with different pH conditions did not affect zerumbone production significantly, but it was higher in media with initial pH 5.2 with the concentration of 4 mg/l. It was also proved that irradiation showed a remarkable effect on zerumbone production in the cell suspension cultures, where cells incubated under 16:8 (light/dark) condition were found to produce the highest amount of zerumbone (3.42 mg/l and 2.53 mg/l) under dark condition. Additionally, cells which were agitated continuously at 70 and 100 rpm showed the production of more than 3.60 mg/l zerumbone. It was also found that low temperature (18 °C) negatively affected zerumbone production (only 1.87 mg/l), and at 24°C, there was increased production (3.90 mg/l). The study concluded that although the zerumbone production was not abundant as compared to conventionally grown rhizome of *Z. zerumbet*, the possibility of producing zerumbone during early stage could serve as a model for subsequent improvement (Jalil et al. 2015). In another study, the metabolic changes in rhizomes of *Z. zerumbet* after infection with soft rot-causative necrotrophic phytopathogen, *Pythium myriotylum*, were analyzed by GC-MS. It was found that infection triggered a considerable enhancement in the relative content of zerumbone (up to 81.59% increase) (Keerthi et al. 2014).

9.2.4 Zingiberene

Zingiberene [5-(1, 5- dimethyl-4-hexenyl)-2-methyl-1, 3-cyclohexadiene] is a sesquiterpene hydrocarbon which forms a major aromatic component of rhizome oil of *Z. officinale* plant (Gong et al. 2004; Govindarajan and Connell 1983). Earlier in vitro studies have shown this compound to possess a considerable spectrum of biological activities such as antiviral, antiulcer, and antifertility activities as well as cytotoxicity and genotoxicity on different neuron cell lines (Togar et al. 2015). Recently, α-zingiberene has also been shown to have an antiproliferative effect in a study by Lee (2016), and the study indicated that the pathway for apoptosis by α-zingiberene exists due to cytochrome C release and caspase activation, resulting in apoptosis.

Isolation of zingiberene from ginger essential oil has been reported earlier, but the results were of low purity or in low yield only after several tedious sequential purification steps (Denyer et al. 1994). Thus, a rapid and simple isolation of this compound from ginger essential oil has been performed by Millar (1998), which resulted in good yield with >99% purity. The production of zingiberene in callus

culture of ginger has been reported earlier, and it has been found to be affected by light and differentiation of cells (Anasori and Asghari 2008). In the study, zingiberene was produced only on semi-differentiated and differentiated callus cultures grown under light conditions. No zingiberene was detected on TLC plates of the dedifferentiated callus grown in light or dark environment. Also, in vitro zingiberene production in in vitro-regenerated plants of ginger has been reported by Dehghani et al. (2011).

9.2.5 Phenolic and Other Volatile Compounds

Phenolic compounds are important plant antioxidants which exhibit considerable scavenging activity against free radicals (Zheng and Wang 2003; Chinnici et al. 2004; Huang et al. 2009). This antioxidant activity of phenolic compounds is due to their redox properties which help them to behave as reducing agents, metal chelators, and hydrogen atom donors along with the ability to quench the singlet oxygen (Rice-Evans et al. 1995). The in vitro production of phenolic compounds was reported in callus cultures of *Z. zerumbet* by Stanly et al. (2011), and it was estimated to be 9 mg gallic acid equivalent/g of the extract.

Earlier study by Sakamura et al. (1986) has reported the volatile oil constituents in ginger rhizomes produced by in vitro shoot tip culture, in which plantlets with rhizomes were produced in vitro from shoot tips of ginger grown in both modified Gamborg's B5 and Murashige-Skoog media supplemented with various levels of growth regulators. Moreover, in the study, prevalence of similar volatile oil constituents as those formed in ex vitro rhizomes was observed in the in vitro rhizomes. Among the volatile oil present, acyclic oxygenated monoterpenes predominated in the oil obtained from the rhizome grown in the modified B5 medium, while the oil from the rhizome grown in the modified MS medium consisted mainly of sesquiterpene compounds.

9.3 Metabolic Engineering and Production of Secondary Metabolites in *Zingiber* Species

Metabolic engineering involves the targeted alteration of metabolic pathways found in an organism, and when this technique is applied to plants, it results in the manipulation of endogenous biochemical pathways in which the range, scope, or nature of a plant's existing natural products are modified to provide beneficial commercial and agronomic traits (Lessard 1996; Kinney 1998). Over the last few years, plant cell cultures have been intensively investigated as a possible tool for the production of commercial plant secondary metabolites, including fine chemicals such as pharmaceuticals, agrochemicals, flavors, insecticides, fragrances, and cosmetics (Whitmer et al. 2002). In spite of these efforts, few limited number of industrial

processes have been developed for the production of secondary products, such as shikonin, berberine, ginsenosides, and paclitaxel (Ramachandra and Ravishankar 2002). As in many cases, production is too low for commercialization, and hence, metabolic engineering becomes an emerging technique that provides various strategies to improve productivity by increasing the number of producing cells and carbon flux through a biosynthetic pathway or by overexpression of genes coding for rate-limiting enzymes or blocking the mechanism of feedback inhibition and competitive pathways and decreasing catabolism (Karuppusamy 2009).

In the recent years, the involvement of genetic engineering in the production of cells to perform particular metabolic tasks is in consideration. Moreover, research work on how these techniques might contribute to the development of a new strategy to enable the production of useful secondary metabolites from plants on a commercial scale is the major focus nowadays. Studies related to molecular cloning of genes involved in the synthesis of bioactive compounds from *Zingiber* species, which were published earlier, are discussed below.

9.3.1 Zingibain

Zingibain or ginger protease (EC3.4.22.67) is a cysteine protease extensively used in food industry for cheese-making or meat tenderization. This enzyme has been isolated and purified from *Z. officinale* and *Z. ottensii* rhizomes and is known to show antiproliferative activities against fungi and human malignant cell lines (Karnchanatat et al. 2011). Recently, this protein has also been purified and characterized by using three-phase partitioning method, and also the effects of temperature, salts, and organic solvents on the efficiency of this method were also studied (Gagaoua et al. 2015, 2016). It was found that the use of ammonium sulfate salt and t-butanol as organic solvent on partitioning of zingibain increases its relative enzyme activity up to 100% (Gagaoua et al. 2016).

9.3.2 Geranial

The fresh lemony aroma of ginger is mainly due to the presence of monoterpenes (e.g., geranial, geranyl acetate, and geraniol), sesquiterpenes (e.g., zingiberenes), and phenylpropenes (e.g., eugenol) (Gong et al. 2004; Govindarajan and Connell 1983). Among these, geranial, which is synthesized from geraniol diphosphate (GDP) by geraniol synthase, was reported as the most olfactory aroma-active compound in ginger (Nishimura 1995). In a study by Iijima et al. (2014), molecular cloning of the geraniol dehydrogenase gene, ZoGeDH, from ginger rhizome (*Z. officinale* cv. Kintoki) was conducted. Further, the recombinant ZoGeDH protein was functionally characterized, and its expression corresponding to the accumulation of geranial in ginger tissues was confirmed in their study.

9.3.3 β-Eudesmol

A new sesquiterpene synthase gene, β-eudesmol synthase gene, has been isolated and functionally characterized from *Z. zerumbet* (Yu et al. 2008). In the study, functional expression in *Escherichia coli* and in vitro assay showed that the encoded enzyme catalyzed the formation of β-eudesmol and five additional by-products. Further quantitative reverse transcription polymerase chain reaction (RT-PCR) analysis also revealed that β-eudesmol gene transcript accumulated in rhizomes. To further confirm the enzyme activity and assess the potential for metabolic engineering of β-eudesmol production of this gene, gene cluster encoding six enzymes of the mevalonate pathway was introduced into *E. coli* and co-expressed it with β-eudesmol synthase gene. When supplemented with mevalonate, the engineered *E. coli* also produced β-eudesmol with a yield of 100 mg/l.

9.3.4 α-Humulene

α-Humulene or α-caryophyllene [(1E,4E,8E)-2,6,6,9-tetramethylcycloundeca-1,4,8-triene] is a monocyclic sesquiterpene derived from farnesyl diphosphate (FPP) and is being studied for its potential anti-inflammatory effects (Passosa et al. 2007; Fernandes et al. 2007). In a study by Yu et al. (2011), a family of cytochrome P450 gene expressed in rhizomes of *Z. zerumbet* was cloned and functionally expressed in yeast using a PCR-based cloning strategy. It was found that one of these P450s converts α-humulene into 8-hydroxy-α-humulene, a proposed intermediate of zerumbone biosynthesis. The gene was designated as CYP71BA1, a new member of the CYP71 family, and its transcripts were detected almost exclusively in rhizomes and expressed during rhizome development. Recently, the heterologous expression, purification, and biochemical characterization of α-humulene synthase have also been investigated by Alemdar et al. (2016). In the study, α-humulene synthase from *Z. zerumbet* was expressed as a polyhistidine-tagged protein in an *E. coli* strain. Bioactivity assays were conducted with the natural substrate farnesyl diphosphate (FDP) in a two-phase system with in situ extraction of products, and gas chromatography-flame ionization detection (GC-FID) was used to monitor the conversion of FDP to α-humulene and β-caryophyllene.

9.3.5 Chalcone Synthase

Chalcone synthase is the rate-limiting enzyme of 6-gingerol biosynthesis, and its association with variation of 6-gingerol content in contrasting landraces of ginger due to single nucleotide polymorphism (SNP) in this gene has been demonstrated by Ghosh and Sen-Mandi (2015). The study revealed high chalcone synthase gene

expression in the high 6-gingerol containing landraces as compared to those low 6-gingerol containing landraces. Sequencing of chalcone synthase cDNA (complementary deoxyribonucleic acid) and subsequent multiple sequence alignment revealed seven SNPs between high and low 6-gingerol containing landraces. Converting this nucleotide sequence to amino acid sequence, alteration of two amino acids becomes evident; one amino acid change (asparagine to serine at position 336) is associated with base change (A → G), and another change (serine to leucine at position 142) is associated with the base change (C → T). Since asparagine at position 336 is one of the critical amino acids of the catalytic triad of chalcone synthase enzyme responsible for substrate binding, landraces with a specific amino acid change, viz., asparagine (found in high 6-gingerol containing landraces) to serine, cause low 6-gingerol content.

9.3.6 Monoterpene Synthase

Monoterpene synthases or monoterpene cyclases are the key enzymes that are involved in monoterpene biosynthesis by catalyzing the cyclization of the ubiquitous geranyl diphosphate (GPP/GDP) to the specific monoterpene skeletons (Croteau 1987; El-Tamer et al. 2003; Schwab et al. 2001). The monoterpene synthase gene involved in the synthesis of essential oil has been successfully isolated from *Z. cassumunar* by Bua-in et al. (2014). This gene was reported to encode a protein of 590 amino acids, which showed 40–74% identical with known sequences of other angiosperm monoterpene synthases belonging to the isoprenoid biosynthesis C1 superfamily. Moreover, this gene transcript was detected almost exclusively in the leaves which might have control over the biosynthesis of some monoterpenes such as sabinene and β-pinene in the leaves of *Z. cassumunar.*

9.3.7 Other Synthase Gene

Several sesquiterpene synthases such as (S)-β-bisabolene synthase (Fujisawa et al. 2010) and germacrene D synthase (Picaud et al. 2006) from ginger have also been characterized and reported. Moreover, other terpene synthases such as (+)-germacrene D synthase and (S)-β-bisabolene synthase from ginger rhizome and α-humulene synthase and β-eudesmol synthase from *Z. zerumbet* rhizome have also been isolated by Koo and Gang (2012). In addition, 25 monoterpene synthase genes from ginger have been identified, out of which 13 are functionally characterized. Other novel terpene synthases such as (−)-caryolan-1-ol synthase, α-zingiberene, and β-sesquiphellandrene synthase have also been discovered. All these enzymes are reported to be responsible for the formation of majority of sesquiterpenoids in ginger rhizomes.

9.4 Elicitors for Improvement of Secondary Metabolite Production in *Zingiber* Species

Plant cell cultures, in response to various physical, chemical, or microbial factors, show different physiological and morphological changes. These physical, chemical, or microbial factors are collectively known as elicitors, and elicitation is a process of inducing the synthesis of secondary metabolites by the plants to ensure their survival, persistence, and competitiveness (Namdeo 2007). Elicitors can stimulate the accumulation of secondary metabolites in plant tissue cultures, as a major response to biotic and abiotic stress. Therefore, the type of elicitor, dose, and treatment are major factors in determining the production of secondary metabolites. The most common and effective elicitors are fungal cell extracts, heavy metal salts, and polysaccharides from fungal and plant cells. In addition to the chemical agents, UV radiation, hyperosmotic stress, and temperature shift have been shown to be effective for metabolite production in some plant species (Wang and Wu 2013). The accumulation, yield, and composition of secondary metabolites in plants are found to be affected in various ways by several factors, from their formation to their final isolation which includes physiology, environment, genetic makeup, evolution, and the amount of plant material and manual labor needs. In addition to the commercial importance of the variability in yield and composition of the secondary metabolites, the possible changes are also important when these are used as chemotaxonomic tools. Thus, knowledge of the factors that determine the chemical variability and yield for each species are very important (Figueiredo et al. 2008).

In context to these, several studies related to the effectiveness of different factors and elicitors on secondary metabolite production in *Zingiber* species have also been reported. Ghasemzadeh et al. (2010) have studied the effect of glasshouse light intensities (310, 460, 630, and 790 μmol m^{-2} s^{-1}) on the production of total phenolics, total flavonoids, and antioxidant activities in ginger varieties. They found that total flavonoid content and antioxidant activity were observed to increase significantly under a light intensity of 310 μmol m^{-2} s^{-1}, whereas total phenolic biosynthesis was highest under light intensity of 790 μmol m^{-2} s^{-1}. Another study of Ghasemzadeh and Jaafar (2011) has reported that elevated CO_2 concentrations alter secondary metabolite accumulations in all parts of the ginger varieties and resulted in significant increase in total flavonoids, total phenolics, and total soluble carbohydrates and starch. Similar effectiveness of CO_2 levels (800 μmol·mol^{-1}) in significantly increasing anthocyanin, rutin, naringenin, myricetin, apigenin, fisetin, and morin contents in ginger leaves was further reported by Ghasemzadeh et al. (2012). Additionally, the combined effect of salicylic acid and CO_2 enrichment was found to enhance anthocyanin and flavonoid production along with highest chalcone synthase (CHS) activity and antioxidant activity compared with single treatment effects. Moreover, the influence of α-amylase, viscozyme, cellulase, protease, and pectinase enzymes on the yield of volatile oil and secondary metabolites in ginger has been investigated by Chari et al. (2013). In the study, pre-treatment of ginger with α-amylase or viscozyme followed by extraction with acetone and ethanol afforded

higher yield of oleoresin and gingerol up to 20% and 12.2% as compared to control. Also, ethanol extract of cellulase pre-treated ginger had the maximum polyphenol content (37.5 mg/g). Similar significant impact of enzymatic pre-treatments and ultrasound on the yield of oleoresin and its phytoconstituents (gingerols, shogaols, and other nonvolatiles) in ginger has been recently reported by Varakumar et al. (2017). In their study, enzymatic pre-treatment of ginger rhizome extract using accellerase increased the yield of 6-, 8-, and 10-gingerols and 6-shogaol by 64.10, 87.8, 62.78, and 32.0% within 4 h. In the same study of Varakumar et al. (2017), maximum oleoresin yield (69 and 64 g kg^{-1}) was obtained with enzyme and ultrasound pre-treatment, respectively, from ginger rhizome powder with three-phase partitioning system consisting of ammonium sulfate concentration of 10% w/v, 0.5:1 ratio of t-butanol to slurry, and at 5% (w/v) solid loading in the slurry.

The effects of phosphate fertilization and inoculation with isolates of arbuscular mycorrhizal fungi *Scutellospora heterogama*, *Gigaspora decipiens*, *Acaulospora koskei*, and *Entrophospora colombiana* and an assemblage of all four isolates on growth, development, and oleoresin production of micropropagated *Z. officinale* were investigated by da Silva et al. (2008). Inoculation with *S. herogama* and *G. decipiens* resulted in larger yields of oleoresin, corresponding to 3.48% and 1.58% of rhizome fresh biomass, respectively. Based on retention index and mass spectrometry, constituents such as ar-curcumene, zingiberene, γ-cadinene, bisabolene, δ- or α-cadinene, and farnesol were also characterized in in vitro rhizomes. Apart from these, two other constituents were characterized as possible members of the gingerol class. Hence, the study showed that the screening and inoculation of arbuscular mycorrhizal fungi in ginger plants consequently increase the oleoresin production as well as the aggregate value of ginger rhizome production.

9.5 Conclusion

Medicinal and aromatic plants produce different valuable products with variable composition. In the recent years, in vitro plant cell culture methodologies have progressed and found to be highly effective for selective and production of pharmaceuticals from various medicinal plants on commercial scale. These technologies are built on advances in biotechnology field and thus, served as a tool to enhance the production of metabolites and extend the continued use of these plants as renewable sources of pharmaceuticals. This present chapter provides an overview and examples of some important secondary metabolites produced by *Zingiber* species in vitro systems. Some of these metabolites produced from *Zingiber* species are being commercialized, and different elicitors were found to enhance the production of metabolites in these species also. However, knowledge related to biosynthetic pathways of desired bioactive compounds in in vitro cultures of the plants is often still in its primary stage, and hence, strategies are consequently needed to develop more information based on cellular and molecular level. Also, introduction of newer techniques of molecular biology and increased use of genetic tools, so as to produce

transgenic cultures and to study the structure and regulation of biosynthetic pathways, will provide a significant step toward making cell cultures more generally applicable to the commercial production of many secondary metabolites. Thus, intense and extensive efforts in this field are necessary that will further lead to the successful biotechnological production of unknown yet specific and valuable phytochemicals.

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Chapter 10
Hairy Root Culture for In Vitro Production of Secondary Metabolites: A Promising Biotechnological Approach

Ravi Shankar Singh, Tirthartha Chattopadhyay, Dharamsheela Thakur, Nitish Kumar, Tribhuwan Kumar, and Prabhash Kumar Singh

Abstract Hairy root culture (HRC)-based in vitro production system has become a promising biotechnological approach in recent years. The hairy root formation is the result of *Agrobacterium rhizogenes*-induced pathogenesis in plants, characterized by high growth rate, growth in hormone-free media besides genetic stability. These roots can imitate intact plants in the production of secondary metabolites and also amenable to upscaling in the bioreactor. HRCs are being harnessed as one of the methods of choice in tissue culture for high yield of valuable secondary metabolites of medicinal and other commercial importance. Many secondary metabolites, which were earlier extracted from wild plants from their natural habitat, are now being produced using plant cell cultures including HRCs. This book chapter is focussed mainly on the development of hairy root culture in different medicinal plants and its application, challenges, and prospects in the production of valuable secondary metabolites.

Keywords Hairy root culture · *Agrobacterium rhizogenes* · Secondary metabolites

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10.1 Introduction

Hairy root cultures (HRCs) are a promising biotechnological approach and one of the methods of choice for the in vitro production of valuable secondary metabolites. These can imitate intact plants in the production of secondary metabolites, as many of the secondary metabolites are extracted from wild plants from their natural habitat and are often found scarce. Hairy root formation, as the result of genetic transformation by *Agrobacterium rhizogenes*, has several attractive properties including secondary metabolite production, faster growth rate than plant cell cultures, and high genetic stability and growth in hormone-free media (Charlwood and Charlwood 1991; Eapen and Mitra 2001; Zhao 2014). HRCs are being harnessed in tissue culture for high production and productivity of valuable secondary metabolites such as pharmaceuticals, coloring, and flavoring agents. HRCs' genetic stability and potentiality of mimicking the parent plants for biosynthesis of metabolites over plant cell/callus and suspension cultures make it a unique system. These root cultures are becoming a very attractive and cost-effective choice for mass-producing desired plant metabolites and expressing heterologous proteins. Several studies demonstrated the feasibility of scaling-up hairy root-based processes while maintaining their biosynthetic potential besides its immense potential for applications in phytoremediation (Georgiev et al. 2012).

The cell suspension and HRC-based in vitro productions were reported for bioactive molecules such as shikonins and azadirachtin and several others (Satdive et al. 2007; Chaudhury and Pal 2010; Singh et al. 2010). HRCs have been successfully employed also in cases where secondary metabolites are found only in the aerial part of an intact plant. For example, lawsone and artemisinin both normally accumulate only in the aerial part of the plant, but hairy roots shown to produce these (Liu et al. 1999). Many of valuable secondary metabolites are synthesized in roots, and their synthesis is linked to root differentiation (Flores et al. 1999). Genetic transformation of plants using *A. rhizogenes* has emerged as an important alternative to intact plants as well as cell cultures for the production of secondary metabolites (Giri and Narasu 2000; Christey 2001). However, the major limiting factor in using cell cultures is their genetic instability during long-term cultures/subculture which leads to low product yields. In this regard, hairy roots offer unique advantages in their genetic and biosynthetic stability (Bapat and Ganapathi 2005). More importantly, it could be the continuous source of various valuable secondary metabolites and reduce the dependency on natural resources. Furthermore, recent advances in genetic engineering could be aptly applied for modulation of biosynthetic pathways in hairy root culture system for desired yield and are an achievable proposition.

The preference for hairy roots is also due to their genetic stability, and selection of good hairy root lines is a prerequisite, and plants producing high levels of secondary metabolites usually generate high-producing cell lines (Kim et al. 2002). Hence HRC could be considered as more biosynthetically efficient for secondary metabolite production compared to their mother plants (Kittipongpatana et al. 1998).

This book chapter is focussed mainly on the development of hairy root culture in different medicinal plants and its application, challenges, and prospects in the production of valuable secondary metabolites.

10.1.1 A. rhizogenes *and Ri T-DNA Genes*

Just like *A. tumefaciens*, *A. rhizogenes* could be considered as a natural genetic engineer, owing to its capacity to send and integrate in plant genome a precise piece of its own DNA, present in a specialized plasmid, called Ri (root-inducing) plasmid. This gram-negative soil bacterium was identified in around 1930s (Riker et al. 1930; Hildebrand 1934) as the causal agent of "hairy root" or "root mat" disease in plants. In the wounded plant parts, the bacteria move through chemotaxis to cause infection followed by the transfer and integration of its genetic material in the plant genome, resulting in the development of hairy roots in the infected part. The development of these adventitious hairy roots is the manifestation of genomic integration and expression of specialized *Agrobacterium*, present in the Ri plasmid and present in *A. rhizogenes*. Before further detailed discussion on this bacterium, a concise description of these genes is presented below.

Similar to the Ti plasmid present in *A. tumefaciens*, *A. rhizogenes* contains the Ri plasmid. The transfer DNA (T-DNA) part of these two plasmids as well as the *vir* regions share a good amount of similarity, indicating a similar gene transfer mechanism to be operative in both *A. tumefaciens* and *A. rhizogenes* (Moriguchi et al. 2001). For example, both the Ti and Ri plasmids have been found to contain T-DNA, flanked by 24 bp direct repeat called border sequence (Yadav et al. 1982). Both the plasmids have been found to contain genes responsible for T-DNA processing, attachment, and transfer to the plant cell, as well as genes for opines' catabolism in transformed plant parts (Moore et al. 1979; White and Nester 1980; Chilton et al. 1982; Spanò et al. 1982; Costantino et al. 1984; Tepfer 1990). Depending upon the type of opines produced, *A. rhizogenes* has been classified into different subgroups. These include agropine-, mannopine-, cucumopine-, and mikimopine-type strains (Veena and Taylor 2007). Interestingly, the agropine-type strains of *A. rhizogenes* contain Ri plasmid with two T-DNAs (termed as split T-DNA); these are symbolized as T_L (left) and T_R (right) T-DNAs. While the T_L T-DNA part has been found to contain the hairy root-inducing *root oncogenic loci (rol)* genes (White et al. 1985; Estramareix et al. 1986; Slightom et al. 1986), the T_R T-DNA has been found to contain the genes for auxin and opine biosynthesis genes (Christey 2001), which serves auxiliary role in hairy root development (Cardarelli et al. 1987a, 1987b; Vilaine et al. 1987). It has been documented that though both T_L and T_R T-DNAs are independently transferred in the plant genome, integration of the T_L T-DNA in the plant genome is essential for induction of hairy roots (Phelep et al. 1991; Nilsson and Olsson 1997; Sevon and Oksman-Caldentey 2002).

The other strains of *A. rhizogenes* are characterized by the presence of Ri plasmids containing single T-DNA part. Keeping their role in hairy root development in

mind, the *rol* genes of *A. rhizogenes* have been studied in detail. The agropine-type Ri T-DNAs have been examined to reveal the presence of 18 open reading frames (ORFs, Slightom et al. 1986). Among these ORFs, the ORF10, ORF11, and ORF12 are the *rolA*, *rolB*, and *rolC* genes, respectively. The ORF15 corresponds to the *rolD* gene, which is found only in the Ri T-DNA of agropine-type strains (Meyer et al. 2000). More focus has been directed to the *rolA*, *rolB*, and *rolC* genes, as they have been reported to be essential for hairy root development (White et al. 1985).

The *rolA* gene, as analyzed in different strains of *A. rhizogenes*, ranges in size from 279 bp to 423 bp (Meyer et al. 2000) and encodes a protein with DNA-binding motif (Suzuki 1989), indicating its role as a transcription factor (Levesque et al. 1988). Transgenic expression of the *rolA* gene in plants has resulted in varied phenotypes, including wrinkled and dark green leaves, dwarfism/semi-dwarfism, delayed senescence, interference with gibberellin and polyamines, higher sensitivity to auxin, and functional imbalance in phytohormone level (van Altvorst et al. 1992; Schmülling et al. 1993; Dehio and Schell 1993; Dehio et al. 1993; Prinsen et al. 1994; Sun et al. 1991; Martin-Tanguy et al. 1996; Maurel et al. 1991).

The *rolB* gene size has been found to range from 762 bp to 837 bp, encoding a protein with 259–279 amino acids (Meyer et al. 2000). Interestingly, the function of *rolA* and *rolB* genes has been reported to have antagonistic effect on each other. Longer transcripts (2.1–2.8 kb) of the *rolA* gene have been observed (Durand-Tardif et al. 1985), which spans the entire *rolB* sequence. Thus, an antisense RNA for *rolB* gene might be generated, which would lead to the antagonistic effects of these two important genes on each other in the transformed plant part. Transgenic overexpression of the *rolB* gene has been observed to lead to suppression of adventitious root induction (Spena et al. 1987) and necrosis (Schmulling et al. 1988). The *rolB* protein has been documented to possess β-glucosidase activity, which might play a role in the release of active auxins through hydrolysis of inactive β-glucosides (Estruch et al. 1991a). In corroboration to this idea, *rolB* expression has been observed to change auxin perception and sensitivity (Shen et al. 1990; Maurel et al. 1994; Meyer et al. 2000).

The *rolC* gene is ~540 bp in size and encodes protein with 179–181 amino acid residues (Meyer et al. 2000). It has been observed to have cytokinin-β-glucosidase activity (Estruch et al. 1991b); however transgenic expression of the *rolC* gene has been found not to increase cytokinin level in plant (Nilsson et al. 1993a). Interestingly, the *rolC* gene has been observed to affect the balance of phytohormones and polyamines in plant cells (Estruch et al. 1991c; Nilsson et al. 1993b; Martin-Tanguy 2001).

In case of the *rolD* gene, transgenic expression has been observed to result in early flower induction and increased number of flowers. Enzymatically, the *rolD* protein containing 344 amino acid residues coded by the 1032-bp-long *rolD* gene (Meyer et al. 2000) has been documented to be ornithine cyclodeaminase, the enzyme involved in conversion of ornithine to proline (Trovato et al. 2001). This feature has made *rolD* a potent candidate gene for addressing abiotic and biotic stress resistance in plants (Mauro et al. 1996; Bettini et al. 2003). Furthermore, for better understanding of induction of hairy root and its application, a pictorial representation is shown in Fig. 10.1.

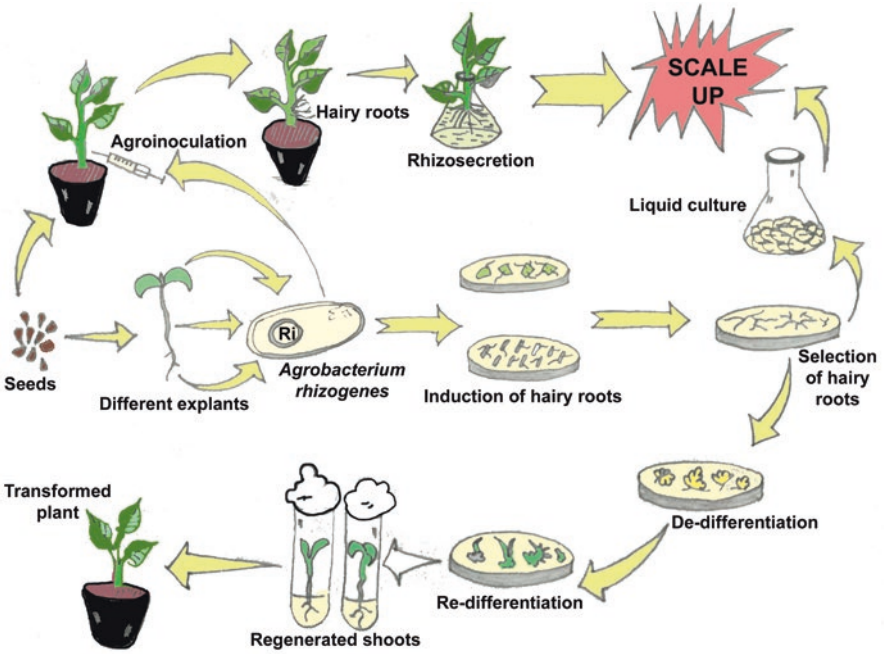

Fig. 10.1 Induction of hairy root and its application

10.1.2 HRCs for In Vitro Production of Secondary Metabolites

Recent progress made in the scale-up of the hairy root cultures has paved the way for industrial exploitation of this system (Guillon et al. 2006). Several groups are working on hairy root culture for optimizing the protocols for high yield by modulating cultural conditions and limiting factors in biosynthetic pathways. Biotechnological approaches including HRCs have been reported for the production of anti-cancerous compounds like resveratrol, podophyllotoxin, and zerumbone (Nandagopal et al. 2017).

HRCs have been developed in several plants and in some of cases being successfully exploited (Table 10.1). Some of the notable examples are being discussed in this chapter. The artemisinin and sugar accumulation in *Artemisia vulgaris* and *Artemisia dracunculus* "HRCs" were reported by Drobot et al. (2017). It was found that the sucrose content was 1.6 times higher in hairy root lines of *A. vulgaris*, while the fructose content was 3.4 times higher in hairy root cultures of *A. dracunculus* than in the control roots. The development of HRCs of *A. vulgaris* and *A. dracunculus* having higher yield of artemisinin and its derivatives and sugars than the mother plant is remarkable for in vitro production of valuable secondary metabolites. Moharrami et al. (2017) suggested that oxide nanoparticles (FeNPs) could be an effective elicitor in hairy root cultures of *Hyoscyamus reticulatus* for increasing the production of hyoscyamine and scopolamine (tropane alkaloids). They used FeNPs

Table 10.1 Secondary metabolites produced using HRCs in different plants

Secondary metabolite	Plant species	Reference
Betalains	*Beta vulgaris*	Georgiev et al. (2010)
Drimartol A	*Artemisia annua*	Zhai and Zhong (2010)
Glucosinolates	*Brassica rapa*	Kastell (2009)
Resveratrol	*Arachis hypogaea*	Abbott et al. (2010)
Anisodamine	*Brugmansia candida*	Cardillo et al. (2010)
Triterpenoids	*Centella asiatica*	Kim et al. (2010a)
Catharanthine	*Catharanthus roseus*	Wang et al. (2010)
Alkaloid	*Catharanthus roseus*	Zhou et al. (2010)
Indole glucosinolates	*Chinese cabbage*	Zang et al. (2009)
Rosmarinic acid	*Coleus blumei*	Bauer et al. (2009)
Hyoscyamine	*Datura stramonium*	Amdoun et al. (2010)
Rutin	*Fagopyrum esculentum*	Kim et al. (2010b)
Gentiopicroside	*Gentiana macrophylla*	Zhang et al. (2010)
Flavonoid	*Glycyrrhiza uralensis*	Zhang et al. (2009)
Gossypol	*Gossypium hirsutum*	Verma et al. (2009)
Ginsenoside	*Panax quinquefolius*	Mathur et al. (2010)
Plumbagin	*Plumbago indica*	Gangopadhyay et al. (2011)
Daidzein and genistein	*Psoralea corylifolia*	Shinde et al. (2010)
Psoralen	*Psoralea corylifolia*	Baskaran and Jayabalan (2009)
Tanshinone	*Salvia miltiorrhiza*	Yan et al. (2011), Kai et al. (2011)
Diterpenoid	*Salvia sclarea*	Kuzma et al. (2009)
Paclitaxel	*Taxus x media* var. Hicksii	Syklowska-Baranek et al. (2009)
Hydroxybenzoates	*Daucus carota*	Sircar and Mitra (2009)
Phenolic acids	*Salvia miltiorrhiza*	Yan et al. (2006), Xiao et al. (2010)
Glucosinolates	*Tropaeolum majus*	Wielanek and Urbanek (2006)
Tropane alkaloids	*Anisodus acutangulus*	Kai et al. (2012)
Tropane alkaloids	*Atropa baetica*	Jaber-Vazdekis et al. (2008)
Triterpenoid saponins	*Panax ginseng*	Choi et al. (2005), Kim et al. (2009)
Camptothecins	*Ophiorrhiza pumila*	Yamazaki et al. (2004)
Monoterpene indole alkaloids	*Catharanthus roseus*	Zhou et al. (2010), Goklany et al. (2010)
Phytoecdysteroids	*Ophiorrhiza pumila*	Okuzumi et al. (2003)
Dolichols	*Coluria geoides*	Skorupinska-Tudek et al. (2008)
Camptothecins	*Ophiorrhiza pumila*	Sirikantaramas et al. (2007)
Hydroxybenzoates	*Daucus carota*	Sircar and Mitra (2009)
Proanthocyanidins	*Vitis vinifera*	Terrier et al. (2009)
Tropane alkaloids	*Datura metel*, *Hyoscyamus muticus*	Moyano et al. (2003), Häkkinen et al. 2016
Isoflavonoids	*Glycine max*	Yi et al. (2010)
Pyridine alkaloids	*Nicotiana tabacum*, *Nicotiana glauca*	Kajikawa et al. (2009), De Boer et al. (2011)
Lignans	*Linum corymbosum*	Bayindir et al. (2008)

(continued)

Table 10.1 (continued)

Secondary metabolite	Plant species	Reference
Flavonoids	*Glycine max*	Jiang et al. (2010)
Triterpenoid saponins	*Panax ginseng*	Jung et al. (2003)
Tanshinones	*Salvia miltiorrhiza*	Gao et al. (2009), Yang et al. (2012)
Anthocyanins	*Medicago truncatula*	Pang et al. (2008)

at different concentrations (0, 450, 900, 1800, and 3600 mg L^{-1}) for exposure times (24, 48, and 72 h). The highest hyoscyamine and scopolamine production was nearly fivefold increase over the control with 900 and 450 mg L^{-1} FeNPs at 24 and 48 h of exposure time, respectively.

Rauwolfia serpentina and *Solanum khasianum* are two important medicinal plants that contain alkaloids like ajmaline, ajmalicine, solasodine, and α-solanine. Enhanced production of secondary metabolites in response to biotic and abiotic elicitors in HRCs of *R. serpentina* and S. *khasianum* was reported by Srivastava et al. (2016). In this report, NaCl was used as abiotic elicitor in these two plants, while cellulase from *Aspergillus niger* was used as biotic elicitor in *S. khasianum*, and mannan from *Saccharomyces cerevisiae* was used in *R. serpentina*. Ajmalicine production was stimulated up to 14.8-fold at 100 mM concentration of NaCl, while ajmaline content enhanced 2.9-fold at 100 mgL^{-1} dose of mannan after 1 week of treatment. Solasodine content was enhanced up to 4.0-fold and 3.6-fold at 100 mM and 200 mM NaCl, respectively, after 6 days of treatments.

The hairy roots of *Ophiorrhiza pumila* produce camptothecin (CPT), a monoterpenoid indole alkaloid used as a precursor in the synthesis of chemotherapeutic drugs, and are considered a promising alternative source of CPT; however, the knowledge about the biosynthetic pathway and regulatory mechanism is still limited (Udomsom et al. 2016). In this study, five genes, namely, OpERF1–OpERF5 (AP2/ERF transcription factors), were studied involving RNAi techniques in hairy root lines of *O. pumila.* The analysis of transcriptome and metabolome of suppressed hairy roots showed no significant changes in the metabolome, including CPT and related compounds; further the suppression of OpERF2 resulted in reduced expression of genes in the 2-C-methyl-d-erythritol 4-phosphate and secologanin-strictosidine pathways, supplier of strictosidine, a precursor, for CPT biosynthesis. In case of *Withania somnifera* (Indian ginseng), hairy (transformed) roots were induced by *A. rhizogenes* strain R1601 from cotyledons and leaf explants for the production of withanolide A, a steroidal lactone of medicinal and therapeutic value (Murthy et al. 2008). Four clones of hairy roots are identified that differed in their morphology. The biomass of faster-growing cultures doubled in 8–14 days that further increased to a fivefold after 28 days compared with cultured, non-transformed seedling roots. The content of withanolide A in transformed roots (157.4 μg/g dry weight) was 2.7-fold more than in non-transformed cultured roots (57.9 μg/g dry weight).

Hairy root lines were also developed in grapevine (*Vitis vinifera* cv Pinot Noir 40024), wherein its ability to produce various stilbenes and elicitation was demon-

strated, which proved it as potentially valuable system for producing resveratrol derivatives (Tisserant et al. 2016). In this case, stilbenes' constitutive production was observed in roots, which also is elicited by methyl jasmonate leading to accumulation of ε-viniferin, δ-viniferin, resveratrol, and piceid after 18 days of growth. In *Isatis tinctoria* 24-day-old HRCs yielded 438.10 μg/g dry weight of total flavonoid (rutin, neohesperidin, buddleoside, liquiritigenin, quercetin, isorhamnetin, kaempferol, and isoliquiritigenin), which was significantly greater (341.73 μg/g) than that of 2-year-old field-grown roots (Gai et al. 2015). Production of tropane alkaloids, which are important natural compounds used as pharmaceuticals ingredients, was also reported to have produced from HRCs of *Datura stramonium* (Ling et al. 2011).

10.1.3 Advantages of HRCs

High branching and high growth rate of hairy roots make it more suitable for commercial upscaling in the bioreactor (Hu and Du 2006). HRCs of *Lithospermum erythrorhizon*, *Harpagophytum procumbens* (Ludwig-Muller et al. 2008), and *Scopolia parviflora* (Min et al. 2007) were studied in bubble column bioreactors to obtain shikonin, harpagide, ginsenosides, and alkaloids, respectively. Furthermore, HRCs can produce secondary metabolites over successive generations without losing genetic or biosynthetic stability (Giri and Narasu 2000). On the other hand, though adventitious roots, cell suspension culture, organ culture, etc. were successfully induced in many plant species and used for the production of high-value secondary metabolites, their genetic stability and slow growths are major limitation. In *Hyoscyamus muticus* transgenic hairy roots, a very long-term subculturing effect on the production capacity of hairy roots was studied (Häkkinen et al. 2016). In this study, hairy roots producing high tropane alkaloid levels were subjected to 16-year follow-up in relation to genetic and metabolic stability. Also, the cryopreservation method was followed to preserve the hairy roots of *H. muticus* to replace laborious subculturing. Though in this case the post-thaw recovery rates declined to low level, the expression of transgene is observed to be unchanged in cryopreserved roots with some fluctuation in the metabolite yields.

10.1.4 Challenges in Developing and Maintaining HRCs

Induction of hairy roots depends upon the genotype of the plant, bacterial strain, and culture condition, among other factors. Hence, developing a suitable protocol for hairy root induction is a prerequisite for HRCs. Scaling-up of culture for large volume production is one of the major challenges due to the branched, delicate, and sensitive nature of hairy roots. Designing appropriate bioreactors suitable to culture hairy root lines is required for the commercial exploitation. Reactors used for mass production of hairy roots can roughly be divided as liquid-phase, gas-phase, or

hybrid kind of reactors (Srivastava and Srivastava 2007). A number of bioreactors with varying designs for the growth of hairy roots have been attempted for in vitro production using HRCs like conventional airlift, bubble column, stirred tank, and nutrient mist bioreactors.

Understanding the biosynthetic pathways of secondary metabolites is required for aptly modulation of biosynthetic pathways through metabolic engineering. HRC systems have a great potential for commercial production of a number of secondary metabolites and recombinant proteins. Furthermore the recent development in high-throughput genomics tools helps in a big way to understand the regulation of secondary metabolism. Mehrotra et al. (2010) pointed out that the global information from various "-omic" platforms in relation to pathway architecture, structural, and functional aspects of important enzymes and genes that can support the design of sets of engineering, resulting in the generation of wide-ranging views of DNA sequence-to-metabolite passageway networking and their control to obtain desired results.

10.1.5 Prospect of HRCs

HRC-based in vitro production system has shown its potential in the production of the scarce or high-value plant bioactive molecules of pharmaceutical and commercial importance. These systems with the aid of genetic engineering approaches open new possibilities for further enhancing the productivity. The rational modulation of pathways is feasible now, be it redirecting the metabolic flux or improving the rate-limiting steps of pathway or inactivating the inefficient pathway(s) for by-product formation. Development of large-scale culture methods using bioreactors has made production of secondary metabolites feasible at the industrial scale (Murthy et al. 2008). Sophisticated bioreactor-derived bioactive molecules hold the prospect of providing remedies for improving human health (Sivakumar 2006). The benefit of HRCs is that these can be easily upscaled from small-scale cultures to the level of bioreactors for larger production of the desired metabolites.

HRCs have potential for the production of recombinant proteins as well as by virtue of its properties like fast growth, genotypic and phenotypic stability, and hormone-free growth. HRC-based biotransformation systems are being considered as highly effective in case of a range of plants, exogenous substrates, and diverse chemical reactions, and it has come to a new stage of functional research in developing pharmaceutical lead compounds by making chemical modification with the help of its inherent enzyme resources (Banerjee et al. 2012). The cryopreservation methods developed could be very helpful in preserving the elite hairy root lines.

In conclusion, HRC system developed so far in several plants for in vitro production of secondary metabolites is one of the methods of choice and promising to harness it to the commercial level. But like other approaches of in vitro production, it also has limitation that needed to be looked into, so that HRC becomes an economically viable proposition for in vitro production of secondary metabolites.

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Chapter 11
Ocimum gratissimum: A Review on Ethnomedicinal Properties, Phytochemical Constituents, and Pharmacological Profile

Chaudhary Priyanka, Sharma Shivika, and Sharma Vikas

Abstract In the present chapter, an effort was made to compile the ethnomedicinal, phytochemical, and pharmacological properties of *Ocimum gratissimum* commonly known as Camphor basil or Ram Tulsi. It is a herbaceous plant that belongs to family Lamiaceae. The plant is about 1–2 feet long, and its leaves have cloves-like flavor that leads to its use in vegetables seasoning. This plant has immense therapeutic uses. *O. gratissimum* have chemopreventive, ant carcinogenic, free radical searching, radio defensive, and various other pharmacological uses. Plants like *O. gratissimum* deliver different bioactive constituents that are utilized generally as sustenance added substances, nourishment colorants, pharmaceuticals, pesticides, and aromas. Biotechnology acquired plant cell culture innovations and has been considered for long, an alluring option for the extraction and utilization of their important secondary metabolites. In vitro culture methods provide an attractive alternative for the protection of uncommon, debilitated, or imperiled therapeutic plants as well as effective means of their rapid clonal micropropagation of critical plants and also permit the creation of hereditarily steady and consistent source. The present chapter underlines the conventional utilizations and clinical possibilities of *O. gratissimum*.

Keywords Bioactives · Ethnomedicinal · Lamiaceae · Micropropagation · *Ocimum gratissimum* · Pharmaceuticals

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11.1 Introduction

Basil is the well-known name given to any aromatic herb belonging to the genus *Ocimum* of Lamiaceae family (Pushpangadan and Bradu 1995) known broadly for their pharmaceutical, culinary, and sweet-smelling properties (Chandrappa et al. 2010). "Basil" is gotten from the Greek word "Basilica" which implies the imperial plant (Pushpangadan and Bradu 1995).

The therapeutic properties of plants have been found numerous hundreds of years prior before now and had been the foundation of all treatments for quite a long while until the point that the intercession of manufactured medications. In spite of the fact that the manufactured medications have been viable in treatment, the world is currently seeing an arrival to natural cures due to the various and unsafe reaction going with the activity of engineered drugs (Amar 2000). Natural pharmaceutical can be utilized as another option to some business drugs (Anyamene and Ezeadila 2010). The act of home grown pharmaceuticals in Asia implies a long relic of human associations with the earth (Sasidharan et al. 2011).

The therapeutic values of plants lie in their Phytochemicals, which deliver distinct physiological activities on the human body. Phytochemicals are compounds present in plants that are utilized as nourishment and medication to drugs to shield against unhealthiness and to take care of human health (Afolabi et al. 2007). Medicative plants are found to be helpful in curing numerous diseases and have invariably promoted the hunt for totally different extracts from plants that might act as a possible supply of recent antimicrobial agent (Bushra and Ganga 2003). The utilization of plant extracts and phytoproducts is gaining awareness because of their accessibility, cost adequacy, demonstrated nature of specificity, biodegradability, low danger, and least leftover poisonous quality in the biological system (Ogbo and Oyibo 2008). According to the World Health Organization, more than 80% of the total populace depends on restorative plants as wellspring of drugs for their essential social insurance in provincial territories in creating nations, as well as in created nations also where present day meds are overwhelmingly utilized (WHO 2005). Simply, a medicinal plant is any plant which in one or more of its organ contains substances that can be utilized for remedial purposes or which contains substances which can be utilized as precursors for the production of significant medications (Sofowora 1993).

Plant tissue culture techniques currently play vital role in the clonal propagation and quantitative development of the medicinally important plant. It overcomes the barriers in conventional vegetative propagation and fulfills the demand for huge-scale cultivation in a very short time by quick multiplication. Thus far we are able to speed up the assembly rate of the common plant by 10,000 times, and an outsized range of productive plants may be increased consistently through tissue culture (Rao et al. 1996).Nature has been a supply of medicative agents for thousands of years, and a powerful range of contemporary medicine is isolated from natural sources, which primarily supported their use in ancient medication. These plant-based ancient drugs systems still play a significant role in health care, with

concerning eightieth of the world's inhabitants relying primarily on ancient medicines for their primary health care (Dubey et al. 2004). Plants are important wellspring of an extensive variety of secondary metabolites, which are used as pharmaceuticals, agrochemicals, flavors, scents, hues, biopesticides, and food additives. More than 80% of accessible medications roughly 30,000 known natural merchandise are of plant origin (Fowler and Scragg 1988).

11.1.1 Distribution

The variety Ocimum is hierarchally high among the herbs and is an extremely versatile group comprising of around 160 species (Balyan and Pushpagandan 1988) with a geographic distribution spread over tropical, subtropical areas of Asia, Africa, and South and Focal America and hotter parts of temperate districts of the world (Simon et al. 1990; Pushpangadan and Bradu 1995; Janick 1999). The plant is indigenous to tropical regions particularly India and it is likewise in West Africa. In Nigeria, it is found within the Savannah and waterfront ranges. It is also cultivated in Ceylon, South Ocean Islands, and furthermore inside Nepal, Bengal, Chittagong, and Deccan (Nadkarni 1999). Plants belonging to this genus are dominatingly bushes and herbs and are perpetual, triennial, or biennial in habit and rich wellsprings of significant secondary metabolites, for example, phenols, flavonoids, alkaloids, terpenoids, and essential oils (Pushpangadan and Bradu 1995). *O. gratissimum* is a standout among the most utilized therapeutic plants as an overall wellspring of flavors and furthermore as a consolidated supply of extracts with strong antioxidant and antibacterial properties.

11.1.2 Botanical Description

O. gratissimum (Fig. 11.1) is a perennial plant that is woody at the bottom. It has an average height of 1–3 m high. The leaves are wide and chiefly ovate, usually 5–13 cm long and 3–9 cm wide. The leaves measure over 10 × 5 cm and are ovate to ovate-lanceolate, sub-acuminate to acuminate at apex, and simple and decurrent at base with a coarsely crenate, serrate margin, pubescent and dotted on both the sides. The leaves show the presence of covering and glandular trichomes. Stomata are rare or absent on the upper surface, whereas they are present on the lower surface. Ordinary trichomes are few, while the long ones up to six celled are present on the margins mostly; the short ones which are two celled are generally found on the lamina. Petioles are up to 6 cm long and racemes up to 18 cm long. The peduncles are thickly pubescent. Calyx is up to 5 mm long, campanulate and 5–7 mm long, and greenish-white to greenish-yellow in color (Bhat 2003).

Fig. 11.1 *Ocimum gratissimum*

Scientific Classification

Kingdom: Plantae
Order: Lamiales
Family: Lamiaceae
Genus: *Ocimum*
Species: *Gratissimum*

11.1.3 Medicinal Properties

Among all groups of the plant kingdom, individuals from the Lamiaceae have been utilized for quite a long time in people prescription drug to treat various diseases, for example, upper respiratory tract infections, diarrhea, migraine, ophthalmic, skin sicknesses, pneumonia, cough, high fever, stiffness, loss of motion, epilepsy, sunstroke, flu, gonorrhea, emotional sickness, abdominal pains, sore eyes, ear diseases, barrenness, tooth rinse, and conjunctivitis (Onajobi 1986, IIori et al.,1996, Dhawan et al. 1977; Abdulrahman 1992; Sofowora 1993; Sulistiarini et al. 1999). *O. gratissimum* is antiseptic (Nakamura et al. 1999; Pessoa et al. 2002; Holets et al. 2003) and has discovered wide use in toothpaste and mouth wash and additionally some topical treatments. It is utilized as an amazing wash for sore throats and tonsillitis. It is likewise utilized as an expectorant and a cough appetite suppressant. The plant extricate is employed against gastrointestinal helminths of creatures and man (Fakae et al. 2000; Chitwood 2003). Carminative properties of *O. gratissimum* make it a decent decision for furious stomach. It is utilized as an emetic and for hemorrhoids. The blossoms and the leaves of this plant are rich in basic oils, so it is utilized as a part of arrangement of teas and imbuement. The leaves have solid fragrant scent and are famously used to season soup and zest meat. The entire plant and the volatile oil

are utilized as a part of conventional medication particularly in Africa and India (Sen 1993; Gupta et al. 2002).

Recent investigations on *O. gratissimum* demonstrated that the plant extract can be a resource of a pharmaceutical for individuals living with human immunodeficiency infection (HIV) and acquired immunodeficiency syndrome (AIDS) (Elujoba 2005). The *Ocimum* oil is likewise dynamic against numerous species of microscopic organisms (*Staphylococcus aureus*, *Listeria monocytogenes*, *Escherichia coli*, *Shigella*, *Salmonella*, and *Proteus*) and fungi (*Trichophyton rubrum*, *T. mentagrophytes*, *Cryptococcus neoformans*, *Penicillium islandi*, and *Candida albicans*) (Akinyemi et al. 2004, Lemos et al. 2005, Lopez et al. 2005). Extracts of *O. gratissimum* have antimicrobial (Adebolu and Oladimeji 2005), antibacterial (Nakamura et al. 1999), antifungal (Lemos and Paula 2005), antimalarial (Ezekwesili et al. 2004), and antiprotozoal activities (Holets et al. 2003); chemopreventive, anticarcinogenic, and radioprotective activities; and various other pharmacological uses (Gupta et al. 2002).

11.1.3.1 Pharmacological Properties

11.1.3.1.1 Anticonvulsant Activities

It has been discovered that leaves of *O. gratissimum* contain constituents which have anticonvulsant and anxiolytic activities and furthermore secured mice against tonic seizures induced with the aid of maximum electroshock but became not powerful toward pentylenetetrazol (PTZ)-precipitated seizures (Freire et al. 2006). Anticonvulsant and anxiolytic activities of leaf extracts and fraction of *O. gratissimum* L. (Lamiaceae) were contemplated by utilizing seizures instigated by pentylenetetrazol and open-field tests in mice. It has been demonstrated that the concentrates and part expanded the inactivity of tonic and tonic-clonic seizures and passing and evoked half security against mortality. Intense danger test in mice gave an oral LD50 more prominent than 5000 mg/kg for the methanol extricate (Okoli et al. 2010). It has been explored that concentrates of the leaves showed anticonvulsant movement by postponing the beginning of PTZ-instigated seizures and shielding treated mice from mortality induced by seizures. PTZ actuates shaking by estranging the γ-aminobutyric corrosive (GABA) receptor chloride (Cl)- channel complex (Corda et al. 1990) to weaken GABA-subordinate hindrance.

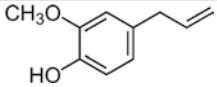
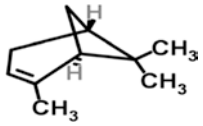
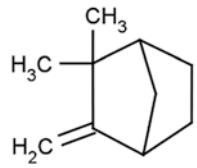
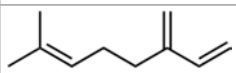
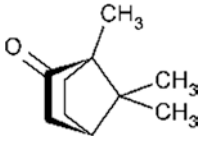
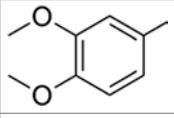
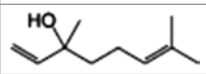
11.1.3.1.2 Antiprotozoal Activities

Previous screening of unrefined extracts of plants utilized as a part of conventional drug demonstrated that the essential oil of *Ocimum gratissimum* hindered the development of *Herpetomonas samuelpessoai* (Holets et al. 2003). *H. samuelpessoai* is a nonpathogenic trypanosomatid that offers critical antigens with *Trypanosoma cruzi*, the agent of Chagas' disease (Souza et al. 1974). *H. samuelpessoai* can be

effectively developed in a characterized medium at 28 °C and 37 °C and can initiate a humoral and cell-intervened invulnerable reaction (Roitman et al. 1972). The impact of the essential oil of *Ocimum gratissimum* has been accounted for on *Herpetomonas samuelpessoai*, a nonpathogenic trypanosomatid (Table 11.1).

It has been discovered that when parasites were developed at 28 or 37 °C, in artificially characterized or an intricate medium containing essential oil obtained from *Ocimum gratissimum*, at that point fundamental oil dynamically repressed the development of protozoan at fixations from 20 to 250 µg/ml. Additionally it has been accounted for that IC_{50} in characterized and complex media at 28 °C were 100 and 91 µg/ml, individually. Cells developed in synthetically characterized medium were more delicate to essential oil at convergence of 50, 62.5, and 100 µg/ml in connection to those refined in complex medium at 37 °C. Ultrastructural and enzymatic changes of the trypanosomatid were likewise assessed. *H. samuelpessoai* was treated with 100 µg/ml of essential oil in artificially characterized medium at 28 °C for 72 h, and it has been observed that modification happen in the internal mitochondrial layer with a critical increment in the quantity of cristae, and mitochondrial grid

Table 11.1 Chemical structures of the active compounds present in *Ocimum gratissimum*

Name of the compound	Chemical structure	Classification	Activity	References
Eugenol		Phenylpropene	Antibacterial activity	Chopra et al. (2003), Gupta et al. (2002)
Pinene		Monoterpene	Antibacterial activity	Imelouane et al. (2009)
Camphene		Monoterpene	Wound healing activity	Kashyap et al. (2011)
β-Myrcene		Monoterpene	Antimicrobial activity	Kpadonou et al. (2012)
Camphor		Terpenoid	Antioxidant activity	Hsu et al. (2012)
Methyl eugenol		Phenylpropanoid	Antioxidant activity	Joshi (2013)
Linalool		Terpene alcohol	Antimicrobial activity	(Duman et al. 2010)

turn out to be less thick in a few cells. It has been accounted for that cells developed within the sight of 100 µg/ml of essential oil demonstrated a diminishing activity of the succinate cytochrome c reductase catalyst, a commonplace mitochondrion marker, when contrasted with untreated cells (Holets et al. 2003).

11.1.3.1.3 Antifungal Activities

It has been recommended that essential oils of *Ocimum gratissimum and Plectranthus glandulosus* may be helpful operators for control of *Aspergillus flavus* growth and AFB1 synthesis. It has been discovered that MIC of *Ocimum gratissimum* was 800 mg/l while that of *Plectranthus glandulosus* was 1000 mg/l. Essential oils of *Ocimum gratissimum* or *Plectranthus glandulosus* may limit starch catabolism in *A. flavus* by following up on some key enzymes, and this may bring about the diminishing of its capacity to synthesize AFB1. These recommended essential oils of *Ocimum gratissimum* and *P. glandulosus* can be intense fungicides for the control of *A. flavus*. For the pragmatic utilization of these oils as novel parasitic control operator, further research is needed on wellbeing issues for human wellbeing (Tatsadjieu et al. 2008).

Earlier examination has demonstrated that *O. gratissimum* has action against other fungi *Trichophyton rubrum* and *T. mentagrophytes* (Lima et al. 1993; Nwosu and Okafor 1995). It has been established that thymol, a constituent of *O. gratissimum* essential oil, was profoundly dynamic against *T. rubrum*, *T. mentagrophytes*, *C. neoformans*, *C. albicans*, and *Malassezia pachydermatis* (Dubey et al. 2000). It has been reported that ethanolic crude extract, ethyl acetate, hexane, and chloroformic portions of *O. gratissimum* indicated activity toward *Cryptococcus* neoformans. It was found that chloroformic fraction of *Ocimum gratissimum* at concentration of 62.5 µg/ml repressed 92% of Cryptococcus isolates and the hexanoate division restrained eight (40%) of these same isolates in a concentration of 250 µg/ml, eugenol hindered the growth of 16% of isolates of *Cryptococcus neoformans* at concentration of 0.9 µg/ml, and the essential oil showed an inhibition of 8% of isolates at concentration of 125 µg/ml. This proposed chloroformic portion, eugenol, and essential oil constituents of *O. gratissimum* leaves are vital wellsprings of new antifungal agents (Lemos et al. 2005).

It likewise has been accounted for those essential oil elements of *O. gratissimum* which became active against *Botryosphaeria rhodina*, *Rhizoctonia* sp., and two strains of *Alternaria* sp. It has been found that the development of *Alternaria* sp. isolated from tomato was most sensitive with a zone measurement of hindrance of 9 mm at the lowest concentration (0.01 mg/disc) tested. *Botryosphaeria* sp. and *P. chrysogenum* demonstrated zone of restraint 23 mm and 45 mm at 4.12 mg/disc; *Aspergillus niger and Rhizoctonia* sp. indicated zone of hindrance of 10 mm at 0.08 mg/disc. The negligible inhibitory groupings of eugenol were 0.16 and 0.31 mg/disc for *Alternaria* sp. and *P. chrysogenum*, individually (Terezinha et al. 2006). Antifungal activity of *O. gratissimum* essential oil has been explored against *Aspergillus (flavus and tamarii)*, *Fusarium (poea and verticillioides)*, *and*

Penicillium (citrinum and griseofulvum) species isolated from conventional cheese wagashi. It has been discovered that essential oil of *Ocimum gratissimum* had extensive fungistatic action against all the species with MIC values ranged from 800 to 1000 mg/L. *Penicillium* species and *Fusarium poae* were the most sensible to this essential oil with MIC equivalent to 800 mg/L (Philippe et al. 2012).

11.1.3.1.4 Hepatoprotective Effect

It has been discovered that serum catalase and DPPH levels were expanded and diminished in stress proteins including HSP70, and iNOS was diminished in CCl_4-administrated rats that were treated with OGAE or silymarin when contrasted with those rats that were treated with saline, or CCl_4 significant abatements of MMP-9/MMP-2 proportion, uPA, phosphorylated ERK (p-ERK), and NF-κB (p-P65) were likewise identified in livers of CCl_4 administrated rats that were regarded with OGAE or silymarin when contrasted with those rats that were treated with saline or CCl_4. Because of these discoveries, it has been accounted for that OGAE can productively restrain CCl_4-actuated liver wounds in rats and consequently be a potential nourishment or herb for avoiding liver wounds (Chiu et al. 2012).

It has been revealed that ethanolic concentrate of *O. gratissimum* leaf likewise uncovered noteworthy chemopreventive impacts on compound initiated papilloma beginning by adjusting processing catalysts, for example, cytochrome P450, glutathione-s-transferase, and aryl hydrocarbon hydroxylase (Prashar et al. 1994; Karthikeyan et al. 1999). It has been explored that oral organization of watery concentrate of *O. gratissimum* leaf lessens oxidative and toxicant activity and improves particular exercises of hepatic cancer prevention agent chemicals in rats (Ighodaro and Ebuehi 2009). The hepatic poisonous quality of CCl_4 is essentially through the era of trichloromethyl free radical in liver microsomes and thus actuates lipid peroxidation (Recknagel 1983). Likewise it has been demonstrated that *O. gratissimum* leaf watery concentrate (OGAE) might be imperative in shielding H9c2 cells from H_2O_2-prompted cell passing by hindering the mitochondrial subordinate apoptosis pathway (Lee et al. 2011).

11.1.3.1.5 Antidiabetic Activity

The hypoglycemic impacts of the fluid leaves concentrate of *O. gratissimum* were explored in streptozotocin-initiated diabetic rats. The concentrate was directed once at the measurements of 250, 500, and 1000 mg/kg body weight. The watery concentrate at the measurement of 500 mg/kg fundamentally brought down the blood glucose level ($P < 0.05$) of the diabetic rats by 81.3% after 24 h of concentrate organization. Preparatory phytochemical screening uncovered the nearness of decreasing sugars, cardiovascular glycosides, gum, tannins, saponins, glycosides, flavonoids, glycerin, and steroids. The middle deadly measurement (LD50) in rats was computed to be 1264.9 mg/kg body weight. The leaves concentrate of *Ocimum*

gratissimum was accounted for to have antidiabetic movement in streptozotocin-instigated in diabetic rats (Mohammed et al. 2007).

11.1.3.1.6 Antidiarrheal Activity

The diarrhea is described by expanded recurrence of gut sound and development, wet stool, and stomach torment. It is the main source of lack of healthy sustenance and demise among kids in the creating nations of the world today. The castor oil test has been a broadly utilized pharmacological test to screen and assess antidiarrheal properties of medications in rats. Within 1 h of oral organization of the oil the creatures start to empty watery stools (Victoria et al. 2000). The antidiarrheal property of aqueous extract of *O. gratissimum* was examined in Wistar albino rats. It has been examined that watery leaf concentrate of *O. gratissimum* at different dosages tried (25, 50, and 100 mg/kg body weight) demonstrated against diarrheal movement by the lessening in the rate of defecation and consistency of feces in albino rats. It has been demonstrated that this plant has antidiarrheal activity against castor oil-induced diarrhea in rats. Therefore it has been inferred that *O. gratissimum* might be utilized as a part of the treatment of gastrointestinal issue especially looseness of the bowels (Ezekwesili et al. 2004). It has been showed that this plant possesses antidiarrheal activity against castor oil-induced diarrhea in rats; therefore it has been concluded that *O. gratissimum* may be used in the treatment of gastrointestinal disorders particularly diarrhea (Ezekwesili et al. 2004).

11.1.3.1.7 Antibacterial Activity

The vital oil of *O. gratissimum* repressed *S. aureus* at a concentration of 0.75 mg/ml. The essential oil was likewise dynamic against individuals of the family Enterobacteriaceae. The insignificant inhibitory concentrations (MICs) for *Shigella flexneri*, *Salmonella enteritis*, *Escherichia coli*, *Klebsiella* sp., and *Proteus mirabilis* were at concentrations extending from 3 to 12 μg/ml. The minimum bactericidal concentration of the crucial oil changed into inside a twofold dilution of the MIC for this organism. The compound that showed antibacterial action in the essential oil of *Ocimum gratissimum* was distinguished as eugenol (Nakamura et al. 1999).

Antibacterial profile and phytochemical screening of ethanolic extract of crisp leaves of *Ocimum gratissimum* was examined on *E. coli* and *Listeria monocytogenes*. It has been demonstrated that the ethanolic extract of *O. gratissimum* repressed the growth of *E. coli* and *L. monocytogenes* at different concentrations. The concentration of 250 mg/ml inhibited the isolate with most noteworthy measurement zone of hindrance going from 22 mm to 25 mm. The MIC effects showed that ethanolic extract of the fresh leaf on test living being had MIC of 9.25 mg/ml, while MBC had 2.15 mg/ml for *L. monocytogenes* and 2.50 mg/ml for *E. coli* (Koche et al. 2012). It has been validated that eugenol isolated from *O. gratissimum* have antimicrobial action (Iwalokun et al. 2003).

It also has been investigated that *Ocimum gratissimum* oil has antibacterial action against *Staphylococcus aureus*, *Streptococcus mutans*, *Enterococcus faecalis*, and *Staphylococcus epidermidis.* The most extreme zone of hindrance was seen in *Staphylococcus aureus* (16.0 mm), followed by *Streptococcus mutans* (14.0 mm), *Enterococcus faecalis* (10.0 mm), and *Staphylococcus epidermidis* (8.0 mm) (Verma et al. 2011). The antibacterial action of various extracts (such as cold water extract, hot water extract and steam distillation extract) from the leaves of *O. gratissimum* has been assessed against S*taphylococcus aureus*, *Escherichia coli*, *Salmonella typhi*, *and Salmonella typhimurium* pathogenic microbes that reason looseness of the bowels (diarrhea). It has been demonstrated that only steam distillation extract hindered the development of the life forms with width of zones of restraint ranging from 30.0 mm for *S. typhi* to 39.0 mm for *S. typhimurium*. Minimum inhibitory concentration (MIC) of the steam distilled oil ranged from 0.1% for *S. aureus* to 0.01% for *E. coli* and *S. typhimurium* and 0.001% for *S. typhi*. Accordingly this extract can be utilized to treat the runs that are caused by these life forms in infected individuals (Adebolu and Oladimeji 2005).

Hydro-refined volatile oils from the leaves of *O. gratissimum* L. (Lamiaceae) from Meru region in Eastern Kenya were likewise assessed for antimicrobial action. The antimicrobial actions of the essential oils were assessed against both Gram-positive (*Staphylococcus aureus*, *Bacillus* spp.) and Gram-negative (*Escherichia coli*, *Pseudomonas aeruginosa*, *Salmonella typhi*, *Klebsiella pneumoniae*, *Proteus mirabilis*) bacteria and a pathogenic fungus *Candida albicans*. The oil had articulated antibacterial and antifungal activities on all the microbes. The MIC of oil for Gram-negative bacteria ranged from 107 to 750 mg/ml and 93.7 to 150 mg/ ml for Gram-positive bacteria. The MIC for the fungus *C. albicans* was 50 mg/ml. The MIC values for chloramphenicol range from 22.5 to 31.3 mg/ml. The minimum inhibition concentration (MIC) for the oil was more prominent than that of reference antibiotic (Matasyoh et al. 2007). It has been showed that *O. gratissimum* oils have properties that can hinder the development of *psychrophiles* and heat-resistant organisms and proposed there were requirement for the utilization of this plant and its subsidiaries for the basic role of enhancing sustenance and antimicrobial actions (Mbata and Saikia 2005).

11.1.3.1.8 Antioxidant Activity

The greater part of the antioxidative capability of herbs and flavors is because of the redox properties of their phenolic compounds, which allow them to act as reducing agents, hydrogen contributors, and singlet oxygen quenchers (Caragay 1992). Plant phenolic compounds are for the most part secondary metabolites having high antioxidant activity and are common in the species of Lamiaceae (Chang et al. 1977). Antioxidant compounds may work as free radical scavengers, complexing specialists for prooxidant metals, reducing agents, and quenchers of singlet oxygen formation (Canj and Poganaga 1997). It has been uncovered that the extract from the leaves of *O. gratissimum* has great cell reinforcement potential probably on account

of its phytochemical constituents such as phenolic-eugenols, thymol, flavonoids, phenylpropanoids, linalool, and citral (Thabrew et al. 1998; Jedlickova et al. 1992). These active agents confer on it its antioxidative, chemotherapeutic, antispasmodic, and analgesic actions (Aziba et al. 1999; Rabelo et al. 2003).

The antioxidant activity of *O. gratissimum* has been evaluated utilizing different examine systems, for example, coupled response of beta-carotenoid linoleic acid (Leal et al. 2006), ferric thiocyanate method, DPPH scavenging activity (Akinmoladun et al. 2007), and reducing power assay (Oduksoya et al. 2005). The impacts of *O. gratissimum* on the basal and damaged (cadmium-induced) serum phosphatase (ALP, ACPT and ACPP) levels in male guinea pigs were researched, and dose- and time-dependent impacts of the unrefined extracts of *O. gratissimum* on basal phosphatase levels were likewise examined. It has been reported that oral administration of 20 mg of *O. gratissimum* caused a critical dose-dependent decline ($P < 0.05$) in the basal serum levels of ACPT and ACPP. Basal serum concentrations of ACPT and ACPP changed from 23.50 ± 1.04 and 7.50 ± 0.29 to 8.25 ± 0.75 and 2.25 ± 0.29 IU at 20 mg, representing 65 and 70% diminishes in their actions individually. This demonstrated that *O. gratissimum* hinders the oxidative phosphatase enzyme activity and accordingly avert oxidative anxiety/harm that could happen amid typical metabolic procedures (Aprioku and Obianime 2008). It additionally has been investigated that 0.25–8 mg/kg of cadmium (Cd) given intraperitoneally caused a noteworthy dose-dependent raise ($P < 0.05$) in the basal serum levels of ALP, ACPT, and ACPP, thus inducing oxidative anxiety which is steady with previous works on cadmium (Kara et al. 2005; Obianine and Aprioku 2008).

11.1.4 Phytoconstituents

O. gratissimum Linn (Labiatae) is grown for the essential oils in its leaves and stems. Phytochemical analysis confirmed the presence of alkaloids, tannins, phenolics, saponins, glycoside, resins, steroids, and terpenoids (Lapido et al.; 2010; Koche et al.; 2012). GC/MS of its essential oil (ocimum oil) demonstrated the presence of eugenol as the most important component, whereas thymol, germacrene, bisabolene, citral, geraniol and linalool, monoterpenes-1, 8-cineole, β-pinene, cis-Ocimene, trans-Ocimene, camphor, methyl eugenol, caryophyllene, germacrene-D, xanthones, lactones, and other volatiles present as minor components (Sulistiarini et al. 1999; Nadkarni 1999; Keita et al. 2000; Lemos et al. 2005; Vieira et al. 2001; Matasyoh et al. 2007). Essential oils from the plant have been accounted for to have a fascinating range of antifungal properties (Dubey et al. 2000).

11.2 Micropropagation

With the quick increment in total populace, outrageous weight on the accessible cultivable land, and quick vanishing of characteristic living spaces for therapeutic plants together with ecological and geopolitical dangers, it is progressively hard to get plant-derived compounds (Mulabagal and Tsay 2004). Plant cell cultures are an alluring option source to entire plant for the creation of secondary metabolites (Dornenburg and Knorr 1997). Plant cell cultures have a few preferences over traditional agrarian creation, viz., autonomous of topographical and ecological varieties, characterized production system with consistent supply of items, uniform quality, yield, velocity of creation, and creation of novel compounds.

The real trouble in the utilization of Lamiaceae species for pharmaceutical reasons for existing is its individual inconstancy, because of hereditary and biochemical heterogenicity (Vieira et al. 2001; Dode et al. 2003). *Ocimum* is traditionally propagated through seeds. Poor seed germination potential confines their increase, and seed determined offspring of all *Ocimum* species demonstrate inconstancy because of cross-pollinating nature of the plant (Heywood 1978). In vitro culture system offers an amazing choice for the investigation and protection of uncommon, debilitated, or imperiled restorative plants and is a viable option which implies for fast augmentation of species when high consistency of descendants is required for which traditional strategies have restrictions (Ajitkumar and Seeni 1998; Sahoo and Chand 1998; Prakash et al. 1999). In this way enthusiasm for utilizing these procedures for quick and vast scale engendering of therapeutic and fragrant plants has been essentially expanded (Vincent et al. 1992; Purohit et al. 1994; Patnaik and Debata 1996; Sahoo et al. 1997). Numerous in vitro have been directed on Lamiaceae species, including *Ocimum basilicum* L. (Sahoo et al. 1997; Begum et al. 2002), *Ocimum kilimandscharicum* (Saha et al. 2010), and *Ocimum sanctum* (Singh and Sehgal 1999; Begum et al. 2000; Banu and Bari 2007). There is an expanding worry all through the world about the uncontrolled misuse and consumption of earth's common assets. Current advance in hereditary qualities and biotechnology is very received upon the utilization of in vitro culture. In this manner the use of plant tissue culture has increased major industrial significance in the generation of secondary metabolites from organ or fluid cell cultures and furthermore utilized as model frameworks for the investigation of plant cell hereditary qualities, physiology, natural chemistry, and pathology (Zhong 2001).

11.3 Enhanced Production of Secondary Metabolites

Secondary metabolites or natural products are usually reserved for natural compounds of natural origin that are unique to one organism or common to a small number of closely associated organisms (Mann 2005). Secondary metabolites play a chief function in the adaptation of plants to the surroundings and in overcoming stress conditions. Plant secondary metabolites are referred to as compounds that

have no essential function in the maintenance of life processes in the plants; however they are critical for the vegetation to have interaction with its surroundings for adaptation and protection. In higher plants a wide range of secondary metabolites are integrated from primary metabolites, e.g., carbohydrates, lipids, and amino acids (Ramakrishna and Ravishankar 2011).

Plants deliver a substantial assorted variety of natural products which are typically sub-isolated in classes as indicated by metabolic pathways at polyketides, lignans, coumarins, flavonoids, terpenoids, steroids, alkaloids, and so forth. These are of high-quality importance for the plant for their communication with the earth because of their parts as pollinator attractants, for advantageous interaction and for barrier against assaults by microorganisms, other flora, or animals. Moreover, they're economically vital to man as a supply of prescription drugs, flavors, aromas, bug sprays, colors, sustenance added substances, poisons, and so on. (Zarate et al. 2003). The decent variety of secondary metabolites from flora might be commercially explored as herbal extracts or immaculate compounds after extraction and isolation by phytochemical process.

Advances in the range of tissue culture for the assembly of secondary metabolites by callus culture have made it feasible for the expanded yield of a wide assortment of pharmaceuticals, including alkaloids, terpenoids, steroids, saponins, phenolics, and flavonoids. In the course of the most recent decades, plant cell societies have been seriously explored as a conceivable device for the creation of business plant optional metabolites, including fine chemicals, for example, pharmaceuticals, agrochemicals, flavors, bug sprays, scents, and beauty care products. Biotechnological approaches particularly plant tissue culture assumes an imperative part in the creation of fascinating healthful compounds from plants (Ramachandra and Ravishankar 2002). Different techniques are utilized for the production of secondary metabolites from plants such as optimization of cultural conditions like medium, temperature, pH, light and oxygen supply, choice of high-producing strains, addition of precursors, biotransformation, elicitor treatment, utility of immobilized cells, and product secretion into the culture media. Precursors are naturally occurring compounds, intermediates originating from biosynthetic pathways, or related synthetic compounds. Addition of precursors or related compounds to the culture media stimulates the production of secondary metabolites. This approach is wonderful if the precursors are inexpensive. Plant cell culture holds much promise as a method for generating complex secondary metabolites in vitro (Nasim et al. 2010).

It has been reported that in *Taxus baccata*, expansion of essential amino acid phenylalanine accelerated the Taxol quantity (Khosroushahi et al. 2006). So as to boost the blend of secondary metabolites, a few natural compounds can be added to the way of life medium (Namdeo et al. 2007). Supplementation of phenylalanine lifted level of target compound. The impact of antecedent nourishing (phenylalanine) on the generation of isoflavones in *Psoralea corylifolia* bushy root culture exhibited that phenylalanine expanded the generation of daidzein and genistein. Genetic transformation can be an effective tool for boosting the productiveness of novel secondary metabolites, specifically through *Agrobacterium rhizogenes* brought about furry roots (Shinde et al. 2009).

With the expanding interest for the crude medications, the vegetation is being overexploited, threatening the survival of numerous uncommon species. To keep up with the required demand of the critical secondary metabolites and their sources, progressed biotechnological instruments are utilized, for example, culturing of plant cells, hereditary control planning to reestablish the germplasm, and addition of enthusiasm of qualities for the generation of vital dynamic rule (Nasim et al. 2010). Plant cell culture holds much guarantee as a strategy for creating complex secondary metabolites in vitro. Tissue culture innovation is a brand new molecular device for plant metabolic engineering to extend the assembly of treasured compounds (Junaid et al. 2010).

To enhance yield of secondary metabolites, metabolic designing offers promising points of view, however, requires the expertise of the direction of the secondary metabolite pathways. In vitro proliferation of restorative plants with advanced bioactive standards and cell culture systems for specific metabolite generation is observed to be exceptionally valuable for business generation of therapeutically important compounds (Rahimi et al. 2012). In vitro culture systems speaks to a unique choice for the investigation and preservation of uncommon, undermined, or jeopardized therapeutic plants (Ajitkumar and Seeni 1998; Prakash et al. 1999) and in addition device for proficient quick clonal proliferation of critical plants permitting generation of hereditarily steady and consistent with genuine sort offspring (Hu and Wang 1983). Thus, the enthusiasm for utilizing these methods for quick and expansive scale engendering of restorative and sweet-smelling plants has been fundamentally expanded (Vincent et al. 1992; Bhat et al. 1995; Sahoo et al. 1997).

11.4 Conclusion

The different advantages of *Ocimum gratissimum* made it a genuine marvel of nature. *O. gratissimum* demonstrated expansive range of antimicrobial, antifungal, and cell reinforcement exercises against a few pathogens because of the nearness of phytoconstituents like methyl eugenol, camphene, myrcene, pinene, and so forth. More broad inquiries are important to scan dynamic standards in charge of these activities. In vitro spread of therapeutic plants with improved bioactive standards and cell culture techniques for specific metabolite creation is observed to be exceptionally helpful for business generation of restoratively vital mixes. Huge scale plant tissue culture is observed to be an appealing option way to deal with traditional techniques for estate as it offers an autonomous supply of biochemical accessibility of plants. The request of *O. gratissimum* is expanding because of its high restorative esteem. To take care of the expanding demand for pharmaceuticals, plant tissue culture is exceptionally helpful for increasing and saving this species, which are hard to recover by traditional techniques and spare them from annihilation. Additionally research and preservation of plant species is expected to protect nature's common medications.

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Chapter 12
Phytochemical Analysis with Special Reference to Leaf Saponins in *Gnidia glauca* (Fresen.) Gilg.

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Abstract *Gnidia glauca* (Fresen.) Gilg. belongs to Thymelaeaceae family and has been found to possess a variety of traditional phytomedicinal and agrochemical applications. The leaves of *G. glauca* are a rich source of phytochemicals like alkaloids, phenols, flavonoids, tannins, glycosides, saponins, terpenoids, and steroids. Among these phytochemicals, saponins were extracted into 80% methanol. *G. glauca* leaf saponin fraction isolate has been subjected for qualitative analysis by hemolytic assay and quantitative analysis by conventional chromatography like TLC and spectroscopy methods. Results confirm that *G. glauca* leaf saponin fraction isolate contains triterpenoid saponins. Further characterization has been carried out by RP-HPLC, LC/MS-ESI, and FTIR. Results suggest that *G. glauca* leaf saponin fraction contains triterpenoid saponins that belong to basic skeletons of oleanane and ursane types of triterpenoid saponins. The investigations provided in the present chapter provide a comprehensive account of knowledge regarding phytochemical analysis of saponins present in *G. glauca* leaves.

Keywords Saponins · *G. glauca* (Fresen.) Gilg. · TLC · RP-HPLC · LCMS-ESI · FTIR

12.1 Introduction

Plants are promising sources of phytochemical compounds and naturally originate in nature. Generally, any part of the plant contains various bioactive phytocompounds such as phenolics, alkaloids, tannins, saponins, steroids, flavonoids, and terpenoids. Phytochemicals serve in plant defense mechanisms against microorganisms, insects, and herbivores. They are the most of complex and absorbing chemical

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structures and represent natural biological and pharmacological activities either as individual compounds or as complex mixtures.

Saponins are naturally occurring triterpenoids/steroid glycosides that are found in particular abundance in various plant species and reported to be identified in 500 genera from 80 plant families. The most common sources of saponins are the higher plants, but increasing numbers are being found in lower marine animals also. Saponins are molecular substances which can be found in higher plants, herbs, vegetables, beans, and other legumes. The word saponin is derived from the "sapo" (Latin sapo = soap), indeed found in soapwort (*Saponaria officinalis*), soaproot (*Chlorogalum pomeridianum*), soapbark (*Quillaja saponaria*), soapberry (*Sapindus saponaria*), soapnut (*Sapindus mukorossi*). Commercial saponins are extracted mainly from *Yucca schidigera* and *Quillaja saponaria*.

Saponins are polar in nature and are freely soluble in water but insoluble in nonpolar solvents. A typical saponin molecule consists of a triterpene (C30) or steroid (C27) aglycones (sapogenins) attached to one or more oligosaccharide side chains by glycosidic linkage. The carbohydrate moiety consists of either pentoses, hexoses, or their respective uronic acids. The glycone (sugar) and aglycone (triterpene/steroid) groups provide saponins with strong surface active agents that produce lather when the combination of a hydrophobic sapogenin and a hydrophilic sugar parts, just like a detergent. Thus saponins can be detected by their ability to cause foaming and to hemolyze blood cells by interactions with membrane glycoproteins, phospholipids, and cholesterol of erythrocytes. Saponins affect the permeability of the small intestinal mucosal cells and thus have effect on active nutrient transport. Saponins have also been shown to inhibit the protein degradation by forming saponin-protein complexes. Cardiac glycosides are also saponins that have the ability to reduce the risk of death due to heart failure. Some of the saponins cause toxicity to cattle and sheep, and some are used as arrow poisons.

Gnidia glauca (Fresen.) Gilg. is a large shrub belongs to Thymelaeaceae family (Fig. 12.1). It is widely distributed in Western Ghats of India, Sri Lanka, and Tropical Africa. *G. glauca* is used in traditional medicine and exhibits several benefits like as a treatment option for a number of human ailments, cancer, sore throat, abdominal pain, wounds, burns, and snake bites (Amarajeewa et al. 2007). Leaves are applied to treat contusions, swelling, backache, and joint aches (Kareru et al. 2005). It is considered as a powerful vesicant. The roots of this plant are used as antiviral agent against rabies in Ethiopia (Teklehaymanot and Giday 2007). *G. glauca* is used for agrochemical applications as a molluscicide, insecticide, pesticide, and even larvicidal agent (Borris and Cordell 1984; Franke et al. 2002; Javaregowda and Naik 2007).

G. glauca is the rich source of valuable phytocompounds like alkaloids, steroids, saponins, emodins, phytosterols, tannins, diterpenes, phenolics, and cardiac glycosides (Ghosh et al. 2013; Parixit et al. 2013; Syed et al. 2013; Ashvin et al. 2015). Herein we report the isolation of *G. glauca* leaf saponin fraction and characterization for the presence of triterpenoid saponins by TLC, HPLC, LC/MS-ESI, and FTIR analyses.

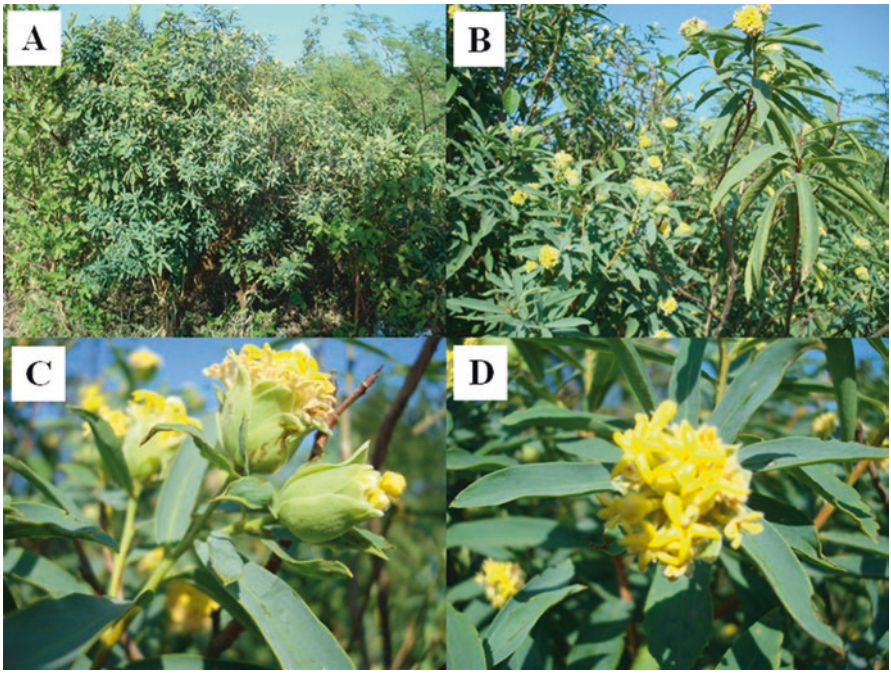

Fig. 12.1 *Gnidia glauca* (Fresen.) Gilg plant with (**A**) Bushy tree; (**B**) Branch; (**C**) Flower bud; (**D**) Flower bunch

12.2 Materials and Methods

12.2.1 Chemicals and Reagents

Organic solvents used in the present study are of either analytical grade or HPLC grade. Solvents such as n-hexane, petroleum ether, dichloromethane (DCM), *n*-butanol, di-methyl sulfoxide (DMSO), and methanol (MeOH) and other chemicals such as silica gel-G, sodium chloride (NaCl), agar-agar, vanillin, saponin from *Quillaja* bark, diosgenin, and anisaldehyde were procured from either Sigma-Aldrich (USA) or HIMEDIA (Mumbai, India) or Sisco Research Laboratories (Mumbai, India). Millipore (Milli-Q) water was used throughout all the investigations. All other chemicals and reagents used unless and otherwise mentioned were of analytical grade.

12.2.2 Plant Material

The plants collected from the wild should be authenticated by expert plant taxonomists. For the present study, *Gnidia glauca* (Fresen.) Gilg. plants from the forests of Western Ghats in Karnataka (India) were collected, got authenticated by expert plant taxonomists of Department of Botany, Sri Jagadguru Chandrashekara Bharathi Memorial College, Sringeri, Chikmagalur District, Karnataka. Fresh plant tissues (leaves) were used for phytochemical analysis, and drying process was carried out under controlled conditions to avoid too many chemical changes. Leaves were gently brushed to remove soil and debris, washed under running tap water, dried under shade, and made into coarse powder using analytical mill to improve the subsequent extraction by rendering the sample more homogenous and increasing the surface area and penetration of solvent into the cells.

12.2.3 Extraction of Phytochemicals from Leaf Material

Leaves containing complex matrices produce a range of phytochemicals with different functional groups and polarities. Organic solvents of different polarities are selected in extraction of leaves to exploit the various solubilities of plant constituents. Soxhlet extraction is widely used for initial/bulk extraction, as this method consumes less time and solvent.

12.2.3.1 Soxhlet Extraction

Leaf powder was subjected for defatting with lipophilic solvents, such as n-hexane, di-ethyl ether, or ethyl acetate, followed by successive extractions with organic solvents with increasing polarity. Most saponin extractions are performed on defatted powdered tissue using methanol (MeOH), ethanol (EtOH), water, or aqueous alcohol as extracting solvents. Extracts were filtered and concentrated by recovering the solvents using rotary evaporator (Medica, India; Model: Evator) under reduced pressure at a temperature below 40 °C to minimize the degradation of saponin compounds and vacuum dried at −45 °C using freeze dryer (Lark, India). Parallely aqueous alcohol/water extracts were dried using freeze dryer (Lark, India).

12.2.3.1.1 Removal of Chlorophyll

Solvent extracts of *Gnidia glauca* leaves prepared in methanol, ethanol, and aqueous alcohol contain large amount of chlorophyll and when concentrated form a dark-green viscous paste; hence, chlorophyll should be removed. Chlorophyll is more soluble in nonpolar solvents and so can be removed by solvent partitioning

using separating funnel. Aqueous alcoholic extract was diluted to about 20% v/v methanol/ethanol with water. Then extract was partitioned with several aliquots of diethyl ether, so as to see that the chlorophyll should go into the nonpolar phase.

12.2.3.1.2 Isolation of Saponin Fraction

The obtained partitioned aqueous layer was reduced to 40 mL and then treated with 60 mL of *n*-butanol. The combined *n*-butanol extract was washed twice with 10 mL of 5% sodium chloride, and aqueous part afford the dark brown saponin fraction was obtained in separating funnel. Thus isolated brown saponin solution was concentrated by rotary evaporator, and freeze-dried in a freeze dryer (lyophilizer) until a constant weight of the powder was obtained (Edeoga et al. 2005). The percent recovery of saponins was calculated, and the dried extract is stored in a brown glass vial in a freezer (−20 °C) until required, to minimize degradation at room temperature. Soxhlet extraction is used when the desired phytochemical has a limited solubility in a solvent and the impurity is insoluble, then it allows for unmonitored and unmanaged operation while efficiently recycling a small amount of solvent to dissolve a larger amount of material.

12.2.4 Preliminary Screening of Saponins

G. glauca leaf saponin fraction was subjected to qualitative tests and screened for the confirmation and identification of saponins using standard methods as described by Harborne (1984), Sotheeswaran 1988Mohamed et al. (2004), Edeoga et al. (2005) and Sonam (2015).

12.2.4.1 Foam Test

Dissolve 100 mg of saponin fraction in 20 mL of distilled water. The resultant suspension is shaken in a graduated cylinder for 15 min.

Formation of persistent froth layer indicates the presence of saponins. The foaming ability of saponin is causative by the combination of a lipid soluble sapogenin and a water soluble sugar chain.

12.2.4.2 Salkowski Test

Dissolve 2 mg of saponin fraction in few mL of chloroform and slowly add few drops of concentrated sulfuring acid along the walls of test tube.

Formation of a brownish red ring at the junction of two layers and lower layer turns to yellow color on standing indicates the presence of triterpenoid saponins.

12.2.4.3 Hemolytic Activity

Some of the saponins are capable of lysing the erythrocytes by dissolving their membranes, a process called hemolysis. Erythrocytes are particularly susceptible to this form of chemical attack because they have no nucleus and therefore cannot effect membrane repair (Bruneton 2012). This property is used as a screening test for saponins.

The hemolytic activity of *G. glauca* saponin fraction was performed following the modified method of Sotheeswaran 1988 and Mohamed et al. (2004). Blood-coated agar plates were used for the screening test. Commercially available agar powder (4 gm) and 100 mL of distilled water were sterilized, cooled to 45 °C, to this 5% of erythrocyte suspension was added and mixed thoroughly to make it homogenous, poured carefully into sterilized Petri dishes avoiding air bubbles. Subsequently, the suspension was allowed to solidify at room temperature for 20 min. Whatman no.1 filter paper was punched to obtain 5 mm discs and dipped into the different concentrations (10, 50, 100, and 150 μg/mL) of saponin fraction prepared in methanol, and saponin from *Quillaja* bark (final concentration 0.5 mg/mL; dissolved in D.W) was used as standard, prepared in a similar manner as that of test sample to obtain the blood agar plates, and incubated for 24 h at room temperature, and then the agar plates were observed for any hemolytic clear zones. If a hemolytic zone was present, zone diameter was measured and recorded.

12.2.4.4 Determination of Total Saponins

Total saponin content in the *G. glauca* leaf saponin fraction isolate was estimated (Hiai et al. 1976). Concentration of standard saponin from *Quillaja* bark was prepared 0.5 mg/mL in 80% aqueous methanol (w/v), while the test sample *G. glauca* saponin fraction isolate was taken at 10 mg/mL in 80% methanol (w/v) for the analysis. *Quillaja* bark saponin standard solution (0, 25, 50, 75, 100, 125, and 250 μL μg/mL) was taken into test tubes, and volume of each is made up to 0.250 μL with 80% aqueous methanol. To each tube 0.250 μL vanillin reagent was added, and then 2.5 mL of 72% (v/v) sulfuric acid was added slowly along the inner side of the test tube walls; contents were mixed well, kept in a boiling water bath at 60 °C for 10 min, and then cooled in ice-cold water bath for 3–4 min. Absorbance was measured at 544 nm against the reagent blank; a calibration curve was prepared with the help of standard saponin from *Quillaja* bark. The total saponin content of test sample was expressed as saponin from *Quillaja* bark equivalents (*Qs* μg/mg extract).

12.2.5 Chromatography

The separation and purification of phytochemicals are mainly carried out using chromatographic methodology such as paper chromatography (PC), thin-layer chromatography (TLC), high-performance liquid chromatography (HPLC), and mass spectroscopy (MS).

12.2.5.1 Thin-Layer Chromatography

Saponins are much more polar than the sapogenins because of their glycosidic linkage, and they are easily separated by TLC. The separation and analysis of saponin fraction were done by TLC on 5 × 20 cm silica gel-G-coated TLC plates and the TLC plates were prepared with 1:2 of aqueous silica gel-G and thickness maintained between 0.25 to 0.5 mm. The validated mobile phase mixture is acetic acid-*n*-butanol-dichloromethane (1:5:4). The mobile phase was developed by PRISMA optimization system proposed as described by Nyiredy et al. 1985. Solvent mixture was poured into a thin-layer chromatography tank and allowed for saturation. *G. glauca* saponin fraction 5 µL (5 mg/mL in distilled water) was loaded on plate, and separation was carried out. After drying the plates, the separated solutes were observed by various methods. Colored substances could be seen directly or under UV chamber or detected by spraying anisaldehyde-sulfuric acid reagent, followed by heating at 100 °C for 5–10 min. Green, brown, pink-reddish, and yellowish colored spots appeared on the plates, indicating the presence of saponins (Fig. 12.1). Separated solutes could be identified by comparing their retardation factor (R*f*) with suitable standards (Harborne 1984).

$$\mathrm{Rf} = \frac{\text{Distance traveled by the solute front}}{\text{Distance traveled by the solvent front}}$$

12.2.5.2 RP-HPLC

Reverse-phase high-performance liquid chromatography (RP-HPLC) is used in the analysis of saponins. *G. glauca* saponin fraction (1 mg/mL in Milli-Q water) was applied to Zorbax SB-C-18 column (4.6 × 250 mm 5 µm), using methanol:water (35:65 ratio) as mobile phase for separation and the separated analytes were detected at 210 nm using diode array detector (DAD). Similarly standards such as saponin from *Quillaja* bark (1 mg/mL in Milli-Q water) and diosgenin (1 mg/mL was dissolved in methanol or ethanol). Samples are filtered through 0.45 µm PVDF membrane prior to column injection. Injection volume was 10 µL, flow rate was maintained at 1 mL/min, and column temperature was maintained at 35 °C. The phytoconstituents present in the separated peaks were identified based on their retention times that match with the retention times of relative authentic standards.

12.2.5.3 Mass Spectrometry (MS)

MS analysis of molecules measures the "mass-to-charge ratio" of molecular ions, and the number of ions representing each mass/charge unit is recorded as spectrum. Mass spectrometry coupled with liquid chromatography is used in the analysis of unknown compounds. Compatibility of mass spectra is convincing evidence for

identification of saponins present in the saponin fraction isolate. Silverstein et al. (2005) suggested that ionization and ion separation are convenient for selecting tentative molecular formulae and fragments on the basis of unit mass peaks.

Mass profiles of saponins present in the *G. glauca* leaf saponin fraction isolate were determined by LC/ESI-MS. The effluent was allowed to directly unite into mass analyzer that exhibit the positive and negative modes and set to scan range m/z 100–600 spectral data were recorded. The column effluent molecules were given spectral masses based on the retention times of chromatogram. Saponins present in the sample were identified with the help of their probable masses obtained by ESI mass and data interpreted using METLIN and HMDB databases.

12.2.5.4 Fourier-Transform Infrared Spectroscopy (FTIR)

Fourier-transform infrared spectroscopy (FTIR) is a method of obtaining infrared spectra by first collecting an interferogram of a sample signal using an interferometer and then performing a Fourier transform (FT) on the interferogram to obtain the spectrum. Fourier-transform infrared spectroscopy (FTIR) is a powerful tool for identifying the type of functional groups present in the given sample. *G. glauca* leaf saponin fraction (1 mg) was mixed with 2–4 mg of potassium bromide salt (KBr), compressed into a thin pellet, and subjected to FTIR spectroscopy analysis between the range 4000 and 400 cm^{-1}. Infrared spectrum was recorded, and functional groups were identified as described by Silverstein et al. (2005).

12.3 Results and Discussion

Through extraction 8.6% crude saponin fraction was obtained from *G. glauca* leaves. Phytochemical analyses of *G. glauca* leaf saponin fraction gave positive results for saponins. The foam and Salkowski test results revealed the presence of saponin terpenoids (Fig. 12.2 A and B). These are capable of destructing erythrocytes by dissolving their membranes as saponins have hemolytic activity, which is also one of the confirmation tests for hemolytic saponins. The clear inhibited zones on blood agar plates against the erythrocyte area indicate the presence of saponins (Fig. 12.2 C).

In the assay for determination of total saponins, steroidal saponins have double bond at C-5; triterpenoid sapogenins have an OH group at their C-3 position react with vanillin reagent in acidic solution to give a colored complex with the absorbance maxima at 544 nm. The linear regression data produced by saponin from *Quillaja* bark as standard provide linear relationship between absorbance at 540 nm (Y) and concentration (X) of standard content (Table 12.1 and Fig. 12.3). The value of regression of triterpenoid saponin content was obtained to be 392 μg/mL equivalents to saponin from *Quillaja* bark.

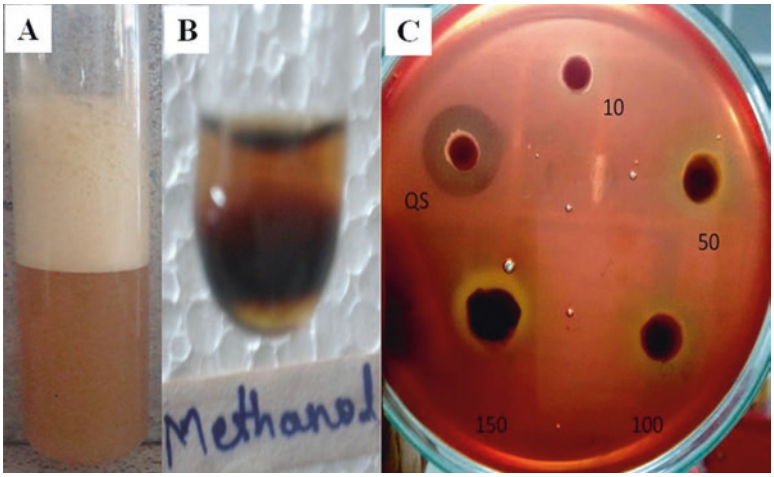

Fig. 12.2 Preliminary screening of saponin fraction: (**A**) Foam Test (**B**) Salkowski Test and (**C**) Blood hemolysis Test

Table 12.1 Quantitative estimation of total triterpenoid saponin content in *G. glauca* leaf saponin fraction isolate

Sl. no.	Concentration in µg	OD at 540 nm
1	0	0
2	25	0.096667±0.015
3	50	0.196667±0.023
4	75	0.276667±0.020
5	100	0.353333±0.015
6	125	0.446667±0.030
7	Sample	0.456667±0.025

Standard deviation ±0.018 and standard error ±0.0098

Optimum resolution of saponin fraction on silica gel-G TLC plate was observed by PRISMA optimization system. A final acetic acid-*n*-butanol-dichloromethane ratio of 1:5:4 (v/v) provided a good resolution and was found to be the most suitable mobile phase for the identification of saponins present in the *G. glauca* leaf saponin fraction isolate by TLC. The characteristic of colored spots of separated saponins were prominent on TLC plates as observed under UV light, and also on TLC plates sprayed with chromogen locating agent (anisaldehyde-sulfuric acid reagent), the separated solutes appeared as yellow-, green-, brown-colored spots and were found to be developed at Rf values that are matched with the *Quillaja* saponin and diosgenin standards (Fig. 12.4). The Rf values separated saponin molecules were recorded and listed in the Table 12.2. The TLC results of *G. glauca* leaf saponin fraction isolate were comparable with standards and also confirmed the presence of saponins in the extract.

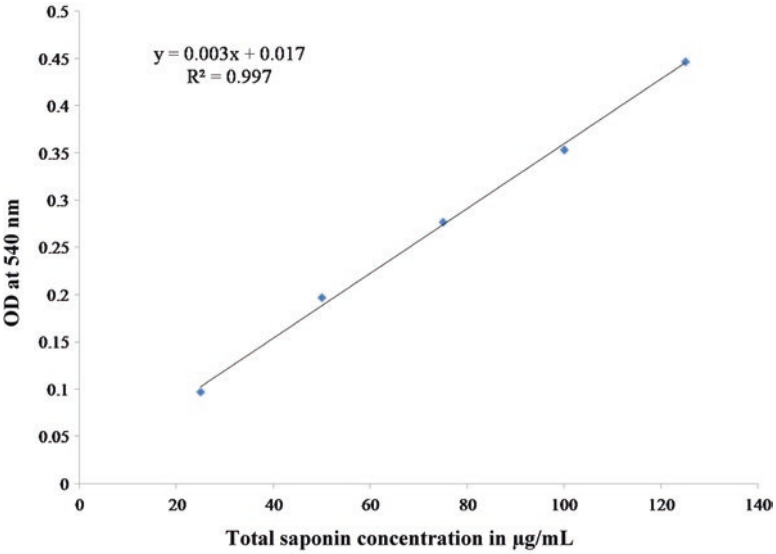

Fig. 12.3 Saponin from *Quillaja* bark (Triterpenoidsaponin) standard calibration curve. (Standard deviation ±0.0185 and standard error ±0.0097)

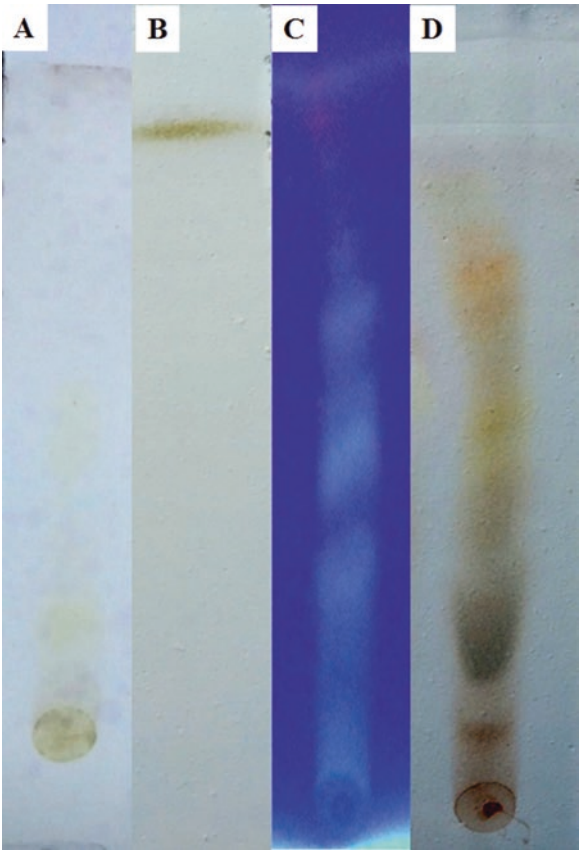

Fig. 12.4 Thin-layer chromatographic separation of *Gnidia glauca* leaf saponin fraction isolate performed with optimized mobile phase obtained by PRISMA model. (**A**) *Quillaja* Saponin, (**B**) Diosgenin, (**C**) View under UV light, (**D**) Spryed with chromogen locating reagent

Table 12.2 Retardation factor values of thin layer chromatograms (TLC)

Separated bands	Rf value
1	0.95
2	0.81
3	0.76
4	0.58
5	0.47
6	0.42
7	0.31
8	0.14
QS (5mg/ml)	0.13
DG (5mg/ml)	0.96

Rf Retardation factor, *QS* Quillaja bark saponin, *DG* Diosgenin

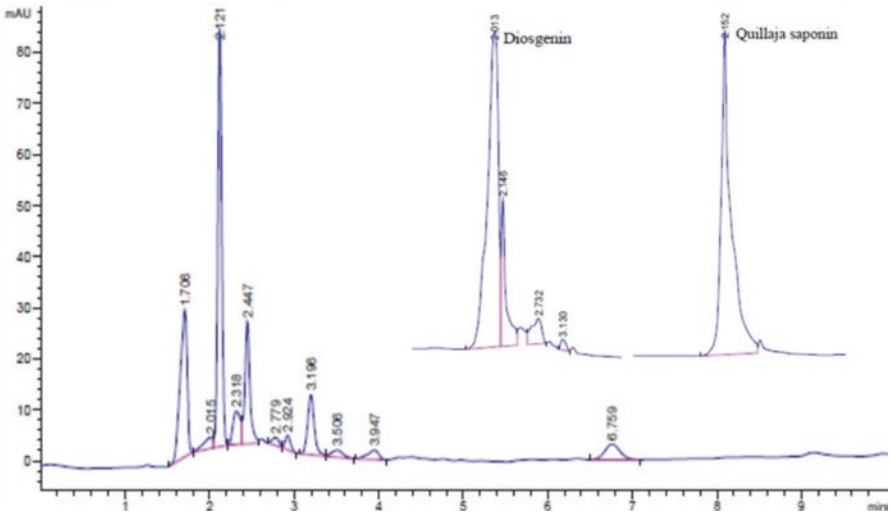

Fig. 12.5 HPLC chromatogram of *G.glauca* leaf saponin fraction isolate

The RP-HPLC method developments for saponin analysis that were available in the published literature were tried to separate saponins present in the *G. glauca* leaf saponin fraction isolate, but none of them was found suitable for separation by any single method. Hence, a suitable RP-HPLC method was developed and optimized for the analysis of *G. glauca* leaf saponin fraction isolate. In this method, methanol-water (35:65) was used as mobile phase, flow rate maintained at 1 mL/min, wavelength was maintained at 210 nm, and Zorbax SB-C-18 column (4.6 × 250 mm, 5 μm) was used for separation and identification of saponins. Results of RP-HPLC chromatograms are shown (Fig. 12.5). The retention times for separated saponins of *G. glauca* leaf saponin fraction isolate were recorded as 1.78, 2.01, 2.12, 2.31, 2.44,

2.77, 2.92, 3.19, 3.50, 3.94, and 6.75 min, while standard saponin from Quillaja bark is 2.15 min, and diosgenin is 2.01 min, respectively. The retention times of *G. glauca* leaf saponin fraction isolate are comparable to that of retention times of standards, i.e., saponin from *Quillaja* bark and diosgenin (Fig. 12.5).

An ESI-MS spectrum was performed in both positive and negative modes were examined in this study. ESI process generates vapor phase ions that can be analyzed for mass-to-charge ratio within the mass spectrometer. Charge separation is the primary method by which ions are formed for organic and biological molecules with acidic or basic functional groups.

All saponins present in the *G. glauca* leaf saponin fraction isolate gave intense molecular ion $[M+H]^+$ in their mass spectra. On the basis of LC/ESI-MS spectra shown in Fig. 12.6, the saponins present in the *G. glauca* saponin fraction isolate were identified and confirmed as triterpenoid saponins that belong to oleanane/ursane (MW 412.40) class of saponins, i.e., α-amyrin/β-amyrin (MW 426.38), oleanolic acid/ursolic acid (MW 456.36), and 3,21-dihydroxy-23-oxo-12-oleanen-28-oic acid and 6,19-dihydroxyurs-12-en-3-oxo-28-oic acid (MW 485.48). Representative phytochemicals were confirmed as basic skeletons of oleanane and ursane types of triterpenoid saponins (Fig. 12.7). The mass spectra of analytes with retention times such as 1.83, 2.03, 3.33, and 3.46 are probable triterpenoid saponins observed at m/z 411.20, 425.42, 486.48, and 456.85, respectively (Table 12.3). Protonated and de-protonated fragmentation information was useful for the elucidation of the structures of known or unknown compounds. LC/ESI-MS spectra have been successfully applied for the identification of saponins in *G. glauca* leaf saponin fraction isolate.

The presence of saponins in *G. glauca* leaf saponin fraction isolate was also confirmed by infrared absorption spectrum and compared with the respective standards. The FTIR spectrum of saponin fraction (Fig. 12.8) revealed the probable basic functional groups such as -OH, -C=O, C-H, C=C, and C-O-C absorption bands (Table 12.4) that are characteristics of saponins, as reported in the published literature (Toshiyuki et al. 2001; Da Silva et al. 2002; Almutairi and Ali 2015). The FTIR spectra of standard saponin from *Quillaja* bark asserted saponin absorption spectrum reported in the Almutairi and Ali (2015). The similarity between the FTIR spectrum of the *G. glauca* leaf saponin fraction isolate and the standard saponin from *Quillaja* bark revealed the presence of triterpenoids saponins in the *G. glauca* leaf saponin fraction isolate. Thus the saponins present in the *G. glauca* leaf saponin fraction isolate are confirmed as triterpenoids saponins that belong to oleanane/ursane basic skeleton type (Fig. 12.7).

12.4 Conclusion

Saponins are widely distributed in plant kingdom with diverse functions. The leaves of *Gnidia glauca* (Fresen.) Gilg. are the rich source of valuable phytochemicals. The isolated leaf saponin fraction, which is rich in triterpenoid saponins, could be

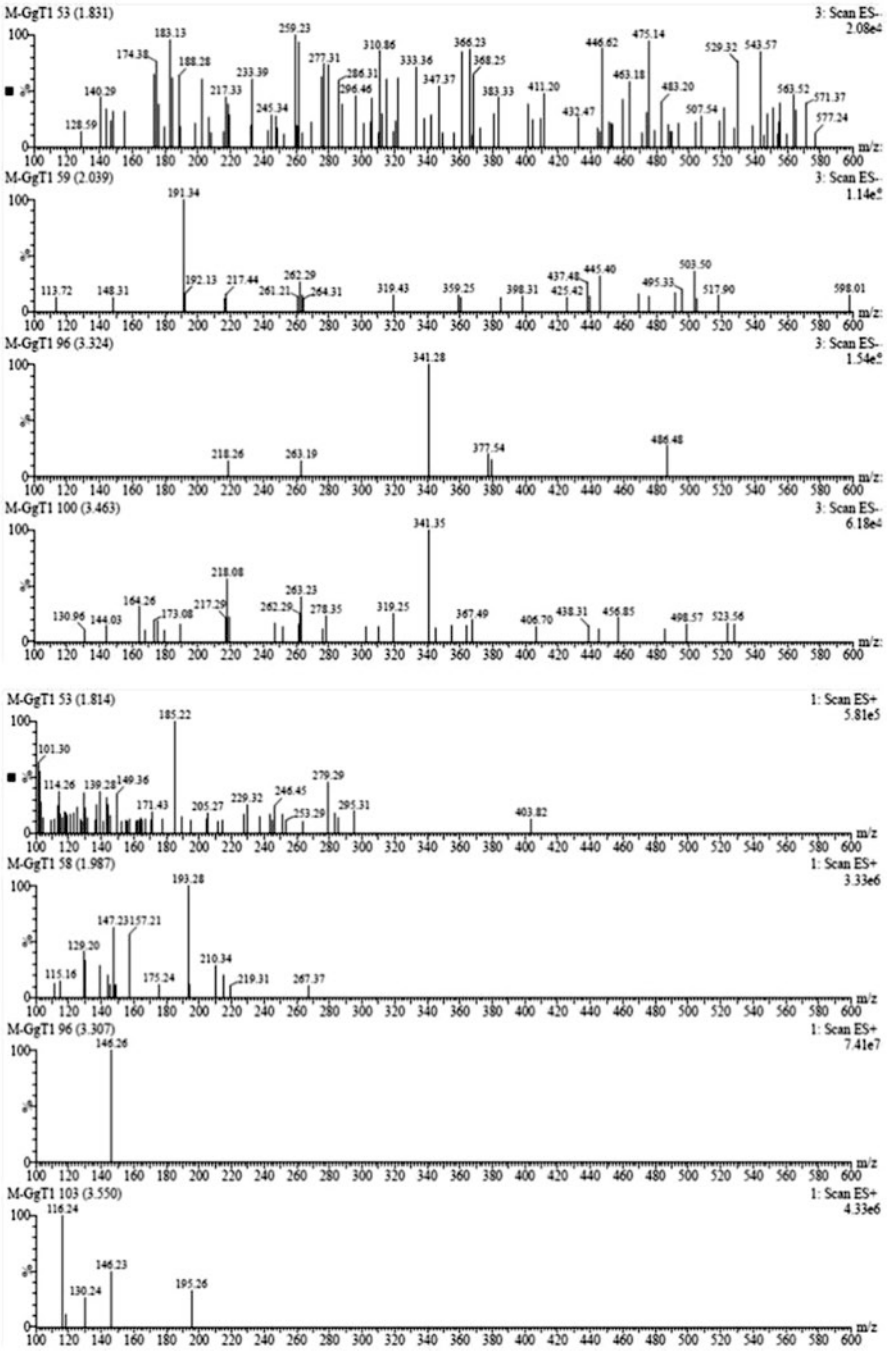

Fig. 12.6 LC/MS-ESI (+ve and -ve) spectrum of *Gnidia glauca* leaf saponin fraction isolate

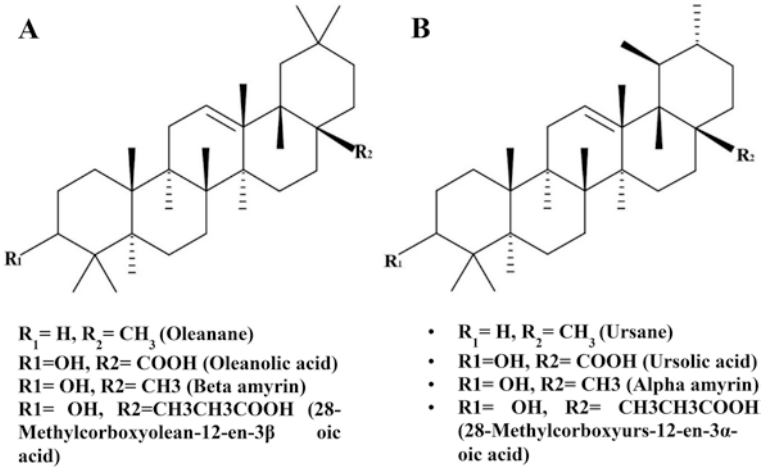

Fig. 12.7 Molecular structure of Oleanane (**A**) and Ursane (**B**) type of Triterpenoid saponins

Table 12.3 Mass-based search for triterpenoids in the *Gnidia glauca* leaf saponin fraction isolate by MS spectrum

Sl. No.	Compound name	Retention time in min	Mass in *m/z*	Molecular formula	Other fragments
1	Oleanane, ursane	1.8	412.40	$C_{30}H_{52}$	577.34, 571.37, 543.57, 529.32, 507.54, 483.20, 475.14, 463.18, 446.62, **411.20**, 368.25, 365.23, 347.37, 333.36, 310.86, 286.31, 277.31, 259.23, 233.39, 217.33, 188.28, 183.13, 174.38, 140.29
2	Olean-12-en-3β-ol (β-amyrin), urs-12-ene-3β-ol (α-amyrin)	2.0	426.38	$C_{30}H_{50}O$	598.01, 517.90, 503.50, 495.33, 445.40, 437.48, **425.42**, 398.31, 319.43, 262.29, 217.44, 191.34, 148.31
3	β-amyrin/α-amyrin+ acetic acid ($[M + CH3COO]^-$)	3.3	485.40	$C_{32}H_{53}O_3$	**486.48**, 377.54, 341.28, 263.19, 218.26
4	Oleanolic acid, ursolic acid	3.5	456.36	$C_{30}H_{48}O_3$	523.56, 493.57, **456.85**, 438.31, 367.49, 341.35, 319.25, 263.23, 218.08, 164.26

Note: M = 425.42
Sources: EMBL, METLIN, and HMDB

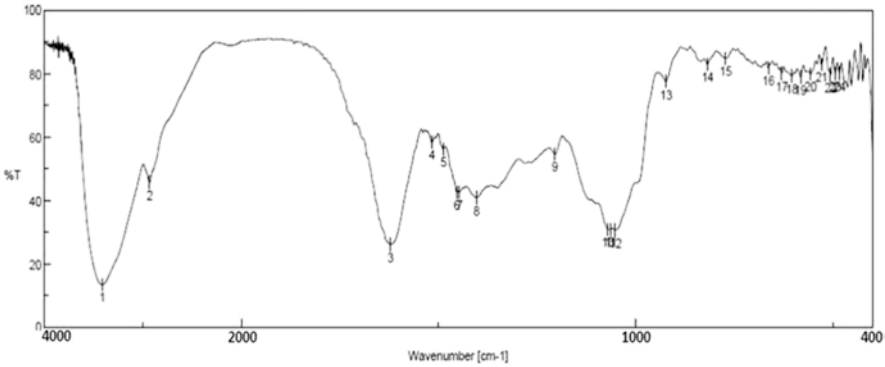

Fig. 12.8 FTIR absorption spectral data of *Gnidia glauca* leaf saponin fraction isolate

Table 12.4 Functional groups identified from the *Gnidia glauca* leaf saponin fraction isolate by FTIR absorption spectrum

Peak no.	Wave number in cm^{-1}	Bond	Functional groups
1	3410.5 (s, b)	O–H stretch, H–bonded	Alcohols, phenols
2	2929.3 (m)	C–H stretch	Aromatics
3	1621.8 (m)	C=C stretch	Aromatic hydrocarbons
4	1516.7 (s)	C=O stretch	Carbonyls (general)
5	1487.8 (m)	C=C stretch	Aromatic hydrocarbons
6	1454.1 (m)	C–C stretch	Aromatic hydrocarbons
7	1447.3 (m)	C–C stretch	Aromatic hydrocarbons
8	1403.9 (m)	O–H bend	Alcohols
9	1205.3 (s)	O–H bend	Alcohols
10	1071.3 (s)	C–O–C stretch	Carbon-oxygen (alcohols, esters)
11	1063.5 (s)	C–O–C stretch	Carbon-oxygen (alcohols, esters)
12	1052.0 (s)	C–O–C stretch	Carbon-oxygen (alcohols, esters)
13	923.7 (m)	O–H bend	Alcohols
14	818.6 (s)	C–H bend	Aromatic hydrocarbons
15	772.4 (s)	C–H bend	Aromatic hydrocarbons

m medium, *s* strong, *b* broad

extracted into aqueous methanol. Further characterization of saponins could be carried out by TLC, RP-HPLC, LC/MS-ESI, and FTIR analyses. The results of present study confirm that the triterpenoid saponins are present in *G. glauca* leaves that belong to basic skeletons of oleanane and ursane types of triterpenoid saponins. The protocols described in this chapter are best suitable for the isolation and characterization of saponins not only from *G. glauca* leaves but also from other sources.

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Chapter 13
In Vitro Production of Bacosides from *Bacopa monnieri*

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Abstract *Bacopa monnieri* (L.) Wettst. (Plantaginaceae) is an important Ayurvedic medicinal herb commonly known as brahmi, growing in the region of Indian subcontinent. Bacosides are the major chemical component having the major role in the biological and pharmacological field. *Bacopa* cultivation is time-consuming, requires labor team, and needs great efforts to maintain the quality of bacosides as growths are affected by environmental factors such as soil, water, temperature, climate, pests, and pathogens. To solve these problems, organ and cell cultures have been adopted for swift and efficient production of *Bacopa* biomass and bacosides. In the current chapter, various parameters, such as types of media, media composition, elicitors, salinity, drought, types of vessels used, and effect of heavy metals, were investigated against the in vitro production of bacosides from *Bacopa monnieri*.

Keywords Adventitious shoot · *Bacopa monnieri* · Bacoside A · Biomass · Cell suspension · Medicinal plant · Secondary metabolites

13.1 Introduction

Bacopa monnieri (L.) Wettst. (water hyssop), which belongs to the family Plantaginaceae, is an annual creeping plant found in wet, damp, and marshy areas and is locally known as brahmi or jalabrahmi (Rajan et al. 2015). In the ancient Indian system of medicine, Ayurveda, *B. monnieri* has been classified under Medhya Rasayana and described in ancient Ayurvedic medical encyclopedias, namely, *Charaka Samhita*, *Sushruta Samhita*, and *Astanga Hrdaya*, as cure for mental disorders and loss of intellect and memory (Singh and Dhawan 1997; Kongkeaw et al. 2014). Commercially available preparations of brahmi improve

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brain function, increase the ability to concentrate, and improve memory in all the population groups. Clinical studies have shown that brahmi is involved in the reconstruction of mental functions in children suffering from attention deficit hyperactivity disorder (ADHD) and improves cognitive functions in patients after strokes and in epilepsy (Jyoti and Sharma 2006; Calabrese et al. 2008; Kamkaew et al. 2013). Most of these effects are due to the ability of the plant extracts to modulate the cholinergic system (Peth-nui et al. 2012). Most of the pharmacological activities attributed are due to the presence of bacosides and triterpenoids belonging to the saponin group (Rastogi et al. 2012). Brahmi is also used in the treatment of neurodegenerative diseases like Alzheimer's or Parkinson's disease (Srivastav et al. 2017). The plant also exhibits most of the pharmacological activities like antioxidant, anti-inflammatory, antipyretic, anti-ulcer, cardioprotective, cooling, laxative, and adsorbing effects (Biswas et al. 2012; Muszynska et al. 2016; Nemetchek et al. 2017). It is also used in treating dermatitis, anemia, diabetes, cough, swelling, fever, arthritis, anorexia, and dyspepsia. Alcoholic extract and aqueous-alcoholic extracts activate detoxification processes, support processes of the renewal and regeneration of tissues, intensify the processes of protein synthesis, stabilize the structure of cell membranes, and prevent the overgrowth of the prostate (Muszynska et al. 2016). Brahmi also acts as diuretic and cardiotonic and strengthens and also stimulates menstruation, and it also functions as a metal chelating agent in the bloodstream (Shah 2012; Wasnik 2012). The herb is used as an adaptogen, where it helps the body to adapt to the adverse environmental conditions and has a normalizing effect on the human body, and it is also a good source of elements (Łojewski et al. 2014; Muszynska et al. 2016).

Plant cell and organ cultures are promising technologies to obtain plant-specific valuable metabolites (Verpoorte et al. 2002). Cell and organ cultures have a higher rate of metabolism than field grown plants because the initiation of cell and organ growth in culture leads to fast proliferation of cells/organs and to a condensed biosynthetic cycle (Rao and Ravishankar 2002). Further, plant cell/organ cultures are not limited by environmental, ecological, and climatic conditions, and cells/organs can thus proliferate at higher growth rates than whole plant in cultivation. Several biotechnological advances have been developed in tissue culture that improve secondary metabolite production such as optimization of cultural conditions, selection of high-producing strains of lines, precursor feeding, elicitation, metabolic engineering, transformed root cultures, micropropagation, and bioreactor cultures, among others (Sarin 2005).

Due to various pharmacological effects, brahmi is having extensive demand, and various commercial preparations are available in the market (Pravina et al. 2007). Almost the entire commercial requirement is met solely from the wild natural populations resulting in listing this plant as threatened plant (Tiwari et al. 2001).

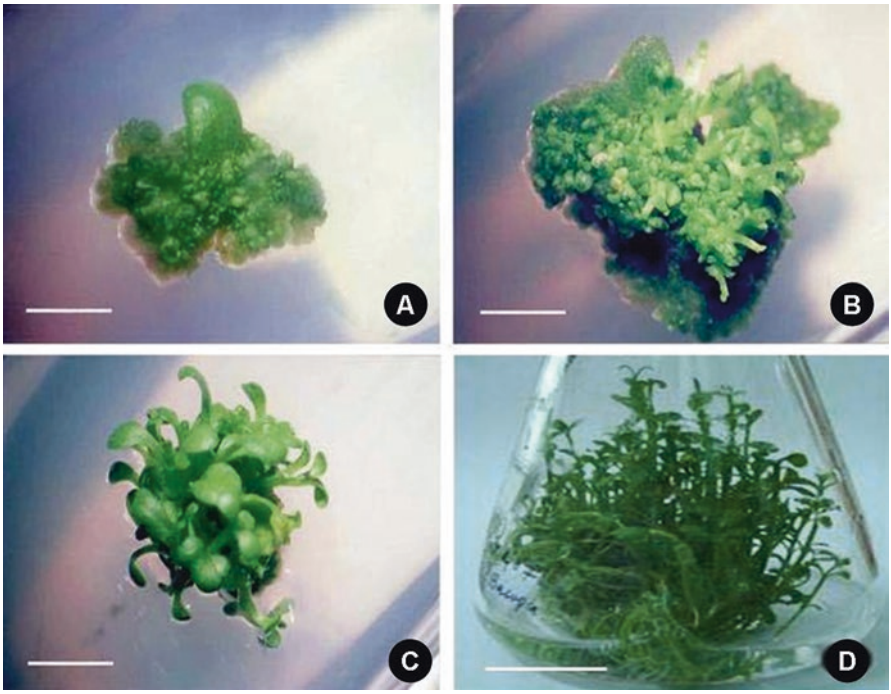

Fig. 13.1 Induction and multiplication of adventitious shoots of *Bacopa monnieri* on semisolid and liquid cultures. Adventitious shoots developed on leaf explant after 3rd week (**A**) and after 4th week (**B**) (bar = 0.2 cm). (**C**) Shoots developed on leaf explant on semisolid medium (bar = 0.4 cm). (**D**) Shoots developed on leaf explant in liquid medium (bar = 2.5 cm)

13.2 In Vitro Production of Bacosides

13.2.1 Effect of Semisolid and Liquid Medium

Praveen et al. (2009) established the rapid protocol for the mass propagation of adventitious shoots using semisolid and liquid medium and assessment of bacoside A content in both the cultures (Fig. 13.1). In this they have established the cultures on Murashige and Skoog (MS) semisolid medium supplemented with different concentrations (0.5, 1.0, 1.5, 2.0, and 2.5 mg/L) of cytokinins [6-benzylaminopurine (BAP), kinetin (KN), and thidiazuron (TDZ)] using leaf as explants and observed that cultured supplemented with 2 mg/L KN yielded highest number of shoots and was also responsible for the maximum production of bacoside A content and liquid cultures were found to be superior for the propagation of brahmi. The number of shoots produced in the liquid cultures was about 155.6 shoots per explant with the biomass of 8.60 g fresh and 0.35 g dry biomass. The fresh and dry biomass was

3-fold and 1.8-fold increments, respectively, when compared to semisolid shoot cultures. The amount of bacoside A was highest in the shoots regenerated in liquid medium (11.92 mg/g DW), and it was 2.2-fold higher compared to shoots grown on semisolid cultures.

13.2.2 Effect of Sucrose and pH

Naik et al. (2010) worked out on the potential for increasing biomass and the concentration of bacoside A in the in vitro regenerated shoots by varying the levels of sucrose and pH in the shoot regeneration medium. They had cultured the leaf explants on MS medium supplemented with 2 mg/L KN with varying concentration of sucrose (0, 1, 2, 3, 4, 5, and 6% at pH of 5.8) and pH (4.5, 5.0, 5.5, 6.0, and 6.5 with 2% sucrose) to study their effect on shoot regeneration, biomass accumulation, and bacoside A production, and they found that shoot biomass increased (150.50 ± 2.84 shoots per explant, fresh wt 6.31 ± 0.12 g, and dry wt 250 ± 5.00 mg) on the medium supplemented with 2% sucrose and pH which was set at 4.5. The increase in the concentration of carbon source had a negative impact on the bacoside A production. Their study clearly provided the evidence that stressful condition of carbon source elevated the synthesis of bacoside A in brahmi shoots.

13.2.3 Effect of Macroelements and Nitrogen Source

Naik et al. (2011) further studied the effects of varied concentrations of macroelements and the overall nitrogen source in the form of either ammonium salt or as nitrate ions for the production of biomass and accumulation of bacoside A content and found that double strength of NH_4NO_3 was favorable for the biomass accumulation and metabolite production from different concentrations of macroelements studied. The number of adventitious shoot biomass and bacoside A content was optimum when the NO_3^- concentration was higher than that of NH_4^+. The results suggest that biomass accumulation and bacoside A content (27.10 mg/g DW) was favored with increase in the concentration of nitrogen source in the form of nitrate with the NH_4^+/NO_3^- ratio of 14.38/37.60 mM.

13.2.4 Effect of Elicitors

Methyl jasmonate (MJ) is one of the signaling molecule that is responsible for the upregulation of the key genes involved in the biosynthetic pathway of secondary metabolites so as to increase the levels of production (Cheong and Choi 2003;

Sharma et al. 2013). The shoot cultures were established in liquid medium, and the same were elicited with different concentrations of MJ (50–200 μM) for different intervals of time. The cultures treated with 50 μM of MJ for 1 week was found to be optimum for the production of bacoside A with 4.4 mg/g DW basis with an increase of 1.8-fold as compared with the control (2.27 mg/g DW), and all other concentrations of MJ incubated for different intervals of time (Sharma et al. 2013).

Sharma et al. (2015a) studied the effect of different abiotic elicitors [jasmonic acid, copper sulfate, and salicylic acid (SA)] on the shoot cultures of brahmi for the enhanced production of bacoside A content. The shoot cultures treated with varied concentrations of elicitors showed varied response for different durations of the elicitor treatments. Cultures treated with jasmonic acid concentration of 1 mg/L induced higher bacoside yield (8.46 mg/g DW) which was around threefold higher than the control cultures (2.74 mg/g DW) at 6 days of elicitation, whereas cultures treated with 45 mg/L concentration of copper sulfate and 50 μM SA increased the bacoside yield to 8.73 mg/g DW and 8.14 mg/g DW, respectively, after 9 days of exposure time.

13.2.5 Effect of Salinity and Drought

Salinity in soils and ground water has become a major environmental issue, which restricts the growth and productivity of plants (Shannon et al. 1994; Munns and Tester 2008). Salinity affects every aspect of plant physiology at cellular and complete plant through osmotic and ionic stresses and, therefore, the productivity of the crop plants (Zhu 2001; Ahire et al. 2013). Ahire et al. (2013) studied the effect of different concentrations (0–200 mM) of sodium chloride-induced salt stress to know the effects on growth, membrane damage, osmotic adjustment, ion concentrations, antioxidative defense mechanism, and the accumulation of principle medicinal component bacoside A in the shoot cultures of *Bacopa monnieri*. With the increase in the concentration of NaCl, both K^+ and Ca^{2+} concentrations decreased. Further reduction in the shoot number per culture, fresh weight, dry weight, and tissue water content were also observed with the varied concentrations of NaCl as compared with the control. Higher amounts of free proline, glycinebetaine, and total soluble sugars accumulated in NaCl-stressed shoots indicating that it is a glycinebetaine accumulator. The antioxidant enzyme activities (superoxide dismutase, catalase, ascorbate peroxidase, and guaiacol peroxidase) also increased with the rise in NaCl level. The bacoside A content also increased with increase in the concentration up to 100 mM NaCl (1.32 mg/g DW), and thereafter the levels of bacoside A content reduced with increase in the concentration of NaCl up to 200 mM.

The stress factors such as salt, drought, low temperature, flooding, heat, oxidative stress, heavy metal toxicity, and pathogenic stress adversely affect the plant growth and productivity, and these stresses affect almost every aspect of plant physiology, which leads to a series of morphological, physiological, biochemical, and

molecular changes. Potassium (K^+) plays an important role in the regulation of osmotic potential of plant cells, and calcium (Ca_2^+) is the structural element essential in the synthesis of cell wall. The calcium is said to antagonize the uptake of excess K^+. Many soils have an excess of certain elements, particularly sodium, potassium, or calcium (Taiz and Zeiger 2010; Ahire et al. 2014) which results in severe stress and subsequently decline in productivity. A study conducted by Ahire et al. (2014) shows that with the increase in the concentration of KCl and $CaCl_2$ (0–200 mM), Na^+ concentrations decreased. Accumulation of K^+ increased significantly in KCl (50–100 mM)-stressed shoots as compared to control, while it decreased in $CaCl_2$-treated shoots indicating that it prevents the uptake of K^+ ions. Ca^{2+} accumulation significantly increased with increasing concentrations of $CaCl_2$ up to 150 mM but decreased at higher concentrations. Further reduction in the shoot number per culture, fresh weight, dry weight, and tissue water content was also observed with the varied concentrations of KCl and $CaCl_2$ (0–200 mM) as compared with the control. Higher amounts of free proline, glycinebetaine, and total soluble sugars accumulated in KCl- and $CaCl_2$ (0–200 mM)-stressed shoots indicating that it is a glycine betaine accumulator. The antioxidant enzyme activities (superoxide dismutase, catalase, ascorbate peroxidase, and guaiacol peroxidase) also increased with the rise in KCl and $CaCl_2$ up to 100 mM level, but KCl suppressed the activities at higher concentrations. The bacoside A content also increased with increase in the concentration up to 100 mM KCl and $CaCl_2$, and thereafter the content of bacoside A reduced with increase in the concentration of KCl and $CaCl_2$ (0–200 mM). The increase in the bacoside A content might be due to increased water stress which can regulate large number of transcripts including phenylpropanoid metabolic pathway genes. Calcium may act as a second messenger and thus trigger the expression of several genes associated with the biosynthetic pathway of bacoside A.

13.2.6 Enriched Nutrient Medium

Łojewski et al. (2014) worked on the enrichment of the nutrient media with MS media enriched with Mg ions, serine, and anthranilic acid and estimated the elements present in the in vitro-raised plantlets and the overall bacoside A content. The elements found in the in vitro-regenerated plantlets are magnesium, zinc, potassium, calcium, copper, sodium, and iron. The highest accumulation of magnesium was determined in the plant tissues cultured in media doped with 0.1 g/L serine and 0.5 g/L Mg and 0.5 mg/g of anthranilic acid and 1.0 g/L Mg. The highest concentration of bacosides (37.3 mg/g dry weight) was found in the media supplemented with 0.25 g/L serine and 0.1 g/L Mg and 0.5 g/L serine and 0.5 g/L Mg.

13.2.7 Hairy Roots

Agrobacterium rhizogenes, a soil bacterium of Rhizobiaceae family, induces neoplastic growth of plant cells that differentiate to form "hairy roots" at the sites of infection (Largia et al. 2016). Hairy roots are induced by the integration of a segment of bacterial DNA called transfer of T-DNA into the chromosome of the plant cell and its expression (Veena and Taylor 2007; Largia et al. 2016). The hairy roots offers an interesting property of the ease of multiplication in the absence of growth regulators and also easy regeneration of whole plants, avoiding the intermittent callus induction and thus circumventing problems associated with somaclonal variations. Hairy roots were induced by the infection of leaf explants with four different strains of *A. rhizogenes* (MTCC 532, MTCC 2364, A4, and R 1000). Later, these hairy roots were used for the complete regeneration of the plantlets. The hairy roots induced from the strains of A4 and MTCC 532 were able to regenerate into complete plantlets without any hormonal supplementation, while the hairy roots induced from MTCC 2364 regenerated with the supplementation of plant growth regulators, but the hairy roots induced from R1000 failed to regenerate into the entire plantlet. The integration of the rol A gene was confirmed by polymerase chain reaction (PCR) and Southern hybridization. One of the lines of hairy roots was found suitable for the highest biomass growth, and also it produced highest total phenolic and flavonoid content. Further on elicitation with 10 mg/L chitosan for 2 weeks, these particular lines produced 5.83% of bacoside A content which increased fivefold and threefold when compared with the untransformed and transformed unelicited controls, respectively (Largia et al. 2016).

13.2.8 Effect of Different Vessels

Sharma et al. (2015b) cultivated the shoot on different vessel types, viz., shake flask (1 L), Growtek® (1 L), and modified bench top air lift bioreactor (ALB), to know the suitable vessel type for production of shoots and bacoside A content and found that ALB was found to be the most suitable vessel type for production of shoots, where the number of shoots produced in this vessel was 443.33 shoots as compared to that in Growtek® (42.67) and shake flasks (23.33), respectively. The bacoside production in shoot cultures cultivated in the ALB was 1.75-fold higher as compared to cultures in shake flask.

13.2.9 Effect of Heavy Metals

Naik et al. (2015) studied the effect of heavy metals on in vitro adventitious shoot production and bacoside A content in *Bacopa monnieri*. The cultures were treated with different concentrations of heavy metals such as manganese ($MnSO_4.H_2O$, 0.1 mM normal content in MS medium served as a control, 0.2, 0.4, 0.6, 0.8, and 1.6 mM), zinc ($ZnSO_4.7H_2O$, 0.03 mM normal supplement in MS medium as control, 0.06, 0.12, 0.24, and 0.48 mM), and copper ($CuSO_4.5H_2O$, 0.0001 mM normal supplement in MS medium served as control, 0.02, 0.05, 0.1, and 0.2 mM). The cultures were grown for 2 months to study the effect. Optimum number of adventitious shoots (123.50 shoots/explant), fresh weight (3.826 g), and dry weight (0.226 g) were obtained in the medium supplemented with 0.12 mM Zn concentration (Table 13.1), and the highest production of bacoside A content was observed in the medium treated with 0.2 mM copper where 28.09 mg/g DW was recorded (Fig. 13.2).

13.2.10 Effect of Different Media, Medium Strength, and Carbon Sources

The levels of secondary metabolites produced by in vitro cultures can vary dramatically. Most reports focus on the composition of the medium nutrients to achieve optimized accumulation of metabolites in cultured cells (Rao and Ravishankar 2002). Naik et al. (2017) studied the effect of different media, medium strength, and carbon sources on adventitious shoot cultures and production of bacoside A. The leaf

Table 13.1 Various concentrations of heavy metals effect on shoot regeneration and biomass accumulation

Metals	Concentration (mM)	Mean no. shoots/explants	Fresh weight (g)	Dry weight (g)
Control	Mn, 0.10; Zn, 0.03; and Cu 0.0001	70.75 ± 1.10c	2.344 ± 0.030d	0.166 ± 0.002d
Mn	0.20	28.25 ± 1.43^{f}	0.916 ± 0.022^{j}	0.070 ± 0.002^{i}
	0.40	29.50 ± 1.04^{f}	1.072 ± 0.058^{i}	0.083 ± 0.002^{h}
	0.80	57.75 ± 1.03^{e}	2.222 ± 0.046^{e}	0.155 ± 0.003^{e}
	1.60	54.25 ± 1.43^{e}	2.084 ± 0.029^{f}	0.145 ± 0.002^{e}
Zn	0.06	76.00 ± 1.95^{b}	2.997 ± 0.040^{c}	0.184 ± 0.002^{c}
	0.12	123.50 ± 3.30^{a}	3.826 ± 0.055^{a}	0.226 ± 0.003^{a}
	0.24	63.75 ± 1.31^{d}	2.441 ± 0.075^{d}	0.154 ± 0.002^{e}
	0.48	56.25 ± 1.75^{e}	1.400 ± 0.024^{h}	0.106 ± 0.006^{g}
Cu	0.02	122.00 ± 1.58^{a}	3.170 ± 0.028^{b}	0.198 ± 0.001^{b}
	0.05	64.75 ± 1.49^{d}	1.157 ± 0.027^{i}	0.089 ± 0.001^{h}
	0.10	75.50 ± 1.65^{b}	1.729 ± 0.035^{g}	0.118 ± 0.006^{f}

Mean values in a column followed by the different letters are significantly different according to Duncan's multiple range ($p \leq 0.05$) test. (Source: Naik et al. (2015))

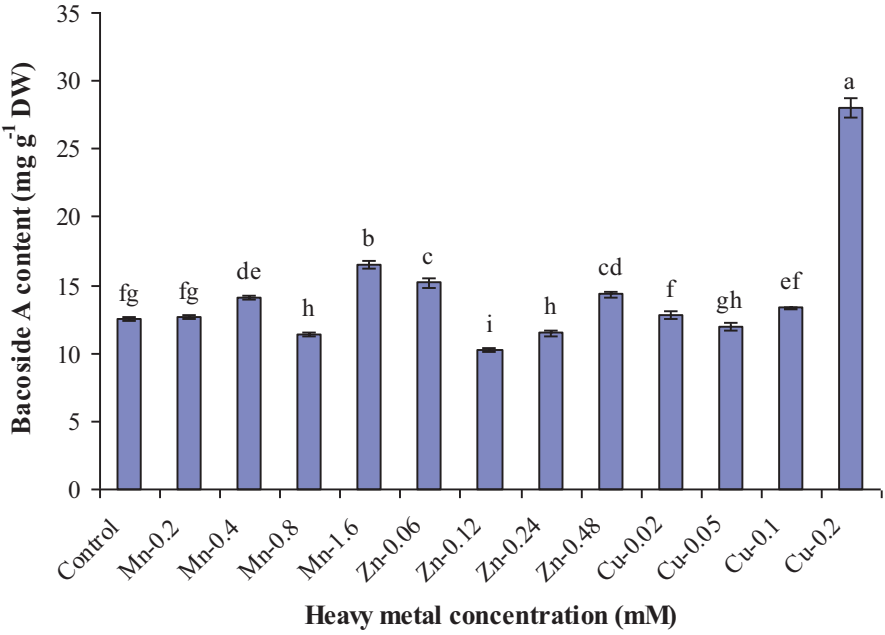

Fig. 13.2 Different concentrations of heavy metals effect on bacoside A accumulation. (Source: Naik et al. (2015))

explants were cultured in different media, viz., MS (1962), Gamborg's (B5) 1968, Nitsch and Nitsch (NN) 1969, and Chu's (N6) 1978, for the growth of adventitious shoots and bacoside A content and found that optimum number of shoots (70.75 shoots/explant), fresh weight (2.344 g), dry weight (0.166 g), and bacoside A content (13.052 mg/g DW) were obtained from the cultures grown in MS medium (Fig. 13.3). Different concentrations of MS media (0.25, 0.50, 0.75, 1.0, 1.5, and 2.0×) were also tested for the regeneration of the shoots and found that full strength MS medium was optimum for the production of shoot biomass and bacoside A content (Fig. 13.4). Similarly, different carbohydrate sources were studied to check the regeneration efficiency and bacoside A production and found that 2% sucrose was favorable for the production of shoot biomass and combination of glucose + fructose was found suitable for the highest accumulation of bacoside A content [15.58 mg/g DW (Fig. 13.5)].

13.2.11 Cell Suspension Culture

Rahman et al. (2002) established the induction of callus from leaf explants of tissue culture-raised plants, and the same was maintained on MS medium augmented with BAP (2 mg/L) and indole-3-acetic acid [IAA (0.1 mg/L)]. From this callus, the cell suspension cultures were established using MS medium supplemented with NAA

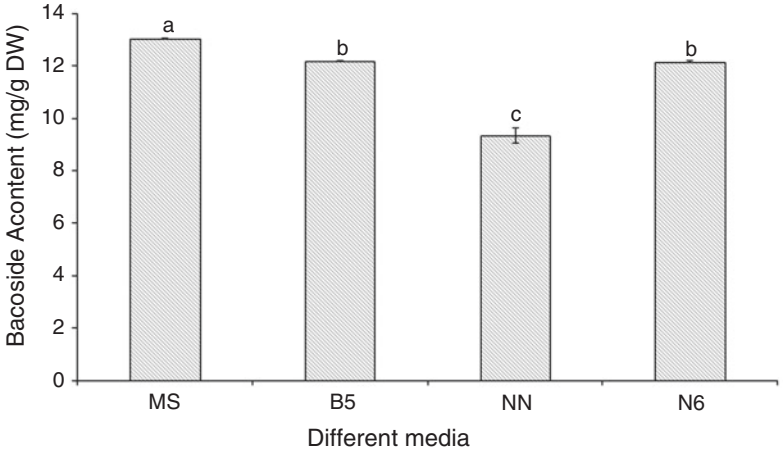

Fig. 13.3 Different media effect on bacoside A accumulation. (Source: Naik et al. (2017))

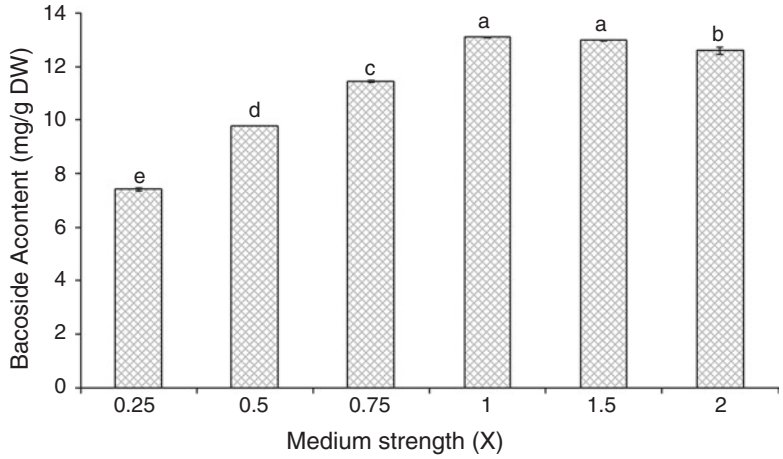

Fig. 13.4 Various medium strength effect on bacoside A accumulation. (Source: Naik et al. (2017))

(1 mg/L), KN (0.5 mg/L), casein hydrolysate (1 g/L), and sucrose 30 g/L), and 10 different lines were established. Among them BM5 and BM9 were found to be the best lines for the growth of biomass and production of bacoside content.

Monica et al. (2013) established the callus cultures from leaf explants on B5 medium supplemented with lower concentrations of 2,4-dichlorophenoxyacetic acid [2,4-D (0.25–0.5 mg/L)]. The established callus was later cultured in the liquid medium to establish the cell suspension cultures. They used MS medium fortified with 1-naphthaleneacetic acid [NAA (1 mg/L)], KN (0.5 mg/L), casein hydrolysate (1 g/L), and sucrose (30 g/L). The quantification of the total saponins was done using spectrophotometrically and found that 166% increment of saponins was observed when compared to the natural plant system.

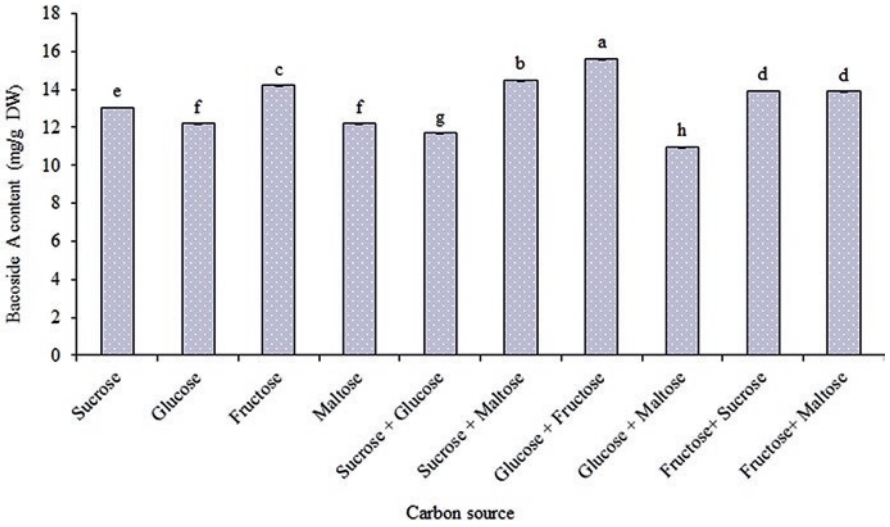

Fig. 13.5 Various carbon sources effect on bacoside A accumulation. (Source: Naik et al. (2017))

13.3 Conclusions

The optimization of *Bacopa* organ and cell culture for the production of bacosides involves different parameters such as types of media, media composition, elicitors, salinity, drought, types of vessels used, and effect of heavy metals. Worldwide extensive research work has been carried out to optimize these cultural conditions, which may be emerged as alternative to the field cultivation. In addition to the production of bacosides, these protocols may be applied for the production of saponins, alkaloids, terpenoids, and other phenolic contents. The present work can be utilized for the industrial level bioreactor-scale production of bacosides from the in vitro culture of *Bacopa monnieri*.

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Chapter 14
Production of the Anticancer Compound Camptothecin in Root and Hairy Root Cultures of *Ophiorrhiza mungos* L.

B. Wetterauer, E. Wildi, and M. Wink

Abstract Camptothecin (CPT), a modified monoterpene indole alkaloid, is a well-known antineoplastic agent. A comparison of 25 different transformed root culture lines of *Ophiorrhiza mungos* (hairy roots transformed by *Agrobacterium rhizogenes*), which produce CPT, showed a total CPT content of 890–3200 µg/g dry weight. Untransformed root cultures of *O. mungos* accumulated approx. 2000 µg CPT/g root tissue, or 2500 µg CPT of DW in total, respectively. Both transformed and untransformed root organ cultures of *O. mungos* showed high yields of CPT and therefore represent an interesting system for the feasible and sustainable bioproduction of CPT as raw material for the pharmaceutical industry. CPT production in other systems will be reviewed and compared. In the present study, comprehensive comparative kinetics of transformed and untransformed root organ cultures of *O. mungos* in relation to culture conditions, growth parameters, nutrient utilization, and CPT formations were determined. The absolute CPT content showed a positive correlation with growth; thus a constitutive CPT biosynthesis is assumed. CPT was released to the culture medium to a substantial degree. Media CPT content seemed to be actively regulated by the roots themselves, at least in the untransformed line, and exhibited a specific level for each line. The influence of the nitrogen source in relation to growth and CPT biosynthesis was investigated. Ammonium was preferred as a nitrogen source, but nitrate seemed to be beneficial and essential for pH value regulation and consequently for root growth and CPT biosynthesis.

Keywords Camptothecin · *Ophiorrhiza mungos* · Root organ culture · Kinetics · Nutrition · CPT production

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Abbreviations

CPT Camptothecin
DW Dry weight
FW Fresh weight
rpm Revolutions per minute

14.1 Introduction

Camptothecin (CPT), a modified monoterpene indole alkaloid, is a well-known antineoplastic agent. CPT and the derivatives topotecan and irinotecan act as inhibitors of DNA topoisomerase I, thus preventing cell division. Another target, which would enhance the cytotoxic effect of DNA topoisomerase inhibition, was found recently in our group. CPT and topotecan can bind to tubulin and inhibit microtubule formation in dividing cells (Wang et al. 2016).

CPT, topotecan, and irinotecan are widely used in the chemotherapy of several cancers and thus of high demand. Although a chemical synthesis is possible (Winterfeldt et al. 1972), the drug still derives from plant sources. Many tropical plants have the ability to produce CPT, such as members of the families Nyssaceae/Cornaceae, Rubiaceae, Icacinaceae, Apocynaceae, and Loganiaceae (Lorence and Nessler 2004). An important source for CPT is *Camptotheca acuminata*, which is cultivated in tropical parts of China.

CPT is a valuable drug with market prices per kilogram between US $ 3500 and 350,000 (Table 14.1). Because of its commercial value, we considered it interesting, to search for alternative ways to produce CPT, such as using plant cell and tissue cultures (Wink et al. 2005). We focussed on root and hairy root cultures, since they consist of differentiated tissues in which the biosynthesis of plant secondary metabolites is still active (Flores et al. 1987) and not reduced as in undifferentiated callus or cell suspension cultures (Wink 1987). Roots and root and suspension cultures of *Camptotheca acuminata* do not produce interesting amounts of CPT (Table 14.2). Therefore, we decided to work on *Ophiorrhiza mungos* L. (Rubiaceae) instead, which produces CPT also in roots. As expected, CPT production was low in callus and cell suspension cultures. Methoxy CPT was only found in green photosynthetically active tissues (Table 14.2).

Ophiorrhiza mungos L. (Rubiaceae), a shrubby plant with a distribution restricted to Japan, Malaysia, Indonesia, Micronesia, the eastern regions of China and Vietnam, and the western coasts of India and Sri Lanka, is one of the known plants of the genus *Ophiorrhiza* producing CPT and CPT derivatives. The morphologically diverse genus of *Ophiorrhiza* comprises around 150 described species, which are distributed in the tropical Indo-Pacific region and Australia with partly complex classifications and many synonyms (Bremekamp 1966; Darwin 1976; Botanical Nomenclature data base of the Botanical Gardens of Missouri 2017). Some of them are known to produce other secondary metabolites, such as ophiorines (*O. japonica*;

Table 14.1 Available market size data, demands, and raw material prices of CPT and CPT derivatives[a]

Information	Source
Demand of CPT in 1993 was estimated at about 350 kg to 1050 kg only for the USA and only for cancer treatment (calculated on given data). Further demands based on other fields of application are not included	Li and Adair (1994)
CPT and derivatives are in the price range of US $ 5000–25,000/kg	Verpoorte et al. (1998)
Worldwide market size of irinotecan and topotecan in 1999 was estimated at about US $ 520 million and of CPT derivatives in 2002 at US $ 1 billion. About 200 kg CPT as raw material would be needed for the production of CPT derivatives	Sudo et al. (2002)
1 kg CPT US $ 432,000	Ramachandra Rao and Ravishankar (2002)
Projected sales of pharmaceutical alkaloids derived from plant for 2002: US $ 4045 million [b]	Raskin et al. (2002)
The worldwide market of irinotecan and topotecan was estimated at about US $ 750 million in 2002 and at US $ 1 billion by 2003 [c]	Lorence and Nessler (2004)
The worldwide market of irinotecan and topotecan has reached US $ 1 billion per year, which represents 1000 kg of CPT in terms of raw material, approximately	Asano et al. (2004)
Market of irinotecan and topotecan has reached US $ 1 billion, which represent approximately one tonne of CPT in terms of raw material	Watase et al. (2004)
Chinese supplier (http://www.push-herbchem.com): 1 kg of CPT (99%) costs about US $ 8500, and for bulk order >1 kg, it becomes cheaper; delivery capacity: 50 kg/month; 1 kg of 10-hydroxy-CPT costs about US $ 26,400	Personal communication (2009)
Reported trade of *Nothapodytes nimmoniana* woodchips in the last decade from Maharashtra was around 400 metric tonnes. Current demand in the Indian market for woodchips of *N. nimmoniana* is around 500–700 metric tonnes; the supply being 50% less than the current demand	Gharpure et al. (2010)
Sale of FDA approved topotecan reached US $ 72 million in 2009	Rajan et al. (2013)
Cheapest Chinese supplier for inner-Chinese market (Wuhan Bangshunda technology Co., Ltd.): 1 kg of CPT costs about 3500 € (US $ 4740) (purity unknown), delivery capacity: 1000 kg/month; 1 kg of 10-hydroxy-CPT costs about 8100 € (US $ 10,960)	Personal communication (2013)
The annual CPT production throughout the world is only 600 kg, far from meeting the demand of the market	Kai et al. (2014)
At present, approximately 3000 kg of CPT is needed internationally per year [d]	Cui et al. (2015)
Estimation of the US Agency for Healthcare Research and Quality (AHRQ), the cancer treatment in the USA in 2011 costs US $ 88.7 billion, with 11% for prescription medication	Lalaleo et al. (2016)
World market value should have reached US $ 2.2 billion in 2008 [e]	Deepthi and Satheeshkumar (2017a)

[a]See also Isah and Mujib (2015b), but data cannot be verified in relation to given references or literature is not available

[b]Cited in wrong way in Suhas et al. (2007)

[c]Source Oberlies and Kroll (2004) – also cited in Deepthi and Satheeshkumar (2017a) – referring to "sales figures of camptothecin analogues come from the Annual Report of GlaxoSmithKline (for Hycamtin ca. US $ 140 million) and the 2002 sales figures for the top 400 prescription drugs reported by Med. Ad. News in May 2003 (for Camptosar ca. US $ 574 million)"

[d]Source unknown

[e]The given source Sankar-Thomas et al. (2008) gave no comparable data, so the source is unknown

Table 14.2 CPT contents in different tissues and culture systems of *Ophiorrhiza mungos* and *Camptotheca acuminata* (Wink et al. 2005; Wetterauer 2008)

Plant species	Tissue	Specificity	Σ CPT (μg/g DW)	Σ CPT (μg/g FW)	CPT (μg/g DW)	MCPT (μg/g DW)
Ophiorrhiza mungos	Callus [1]		7.3 ± 3	0.45 ± 0.2		
	Cell suspension culture [1]	With hormones	4 ± 2	0.3 ± 0.1		
		Without hormones	122 ± 3	22 ± 1		
	Untransformed root organ-shaking cultures [1]	Total culture [1]	2500 ± 250	220 ± 20		
		Root tissue [1]	2060 ± 200	180 ± 20		
		Young tissue [2]	1780 ± 25	297 ± 4		
		Old tissue [2]	1362 ± 25	206 ± 4		
		Culture medium [1]	436 ± 40	38 ± 4		
	Green untransformed root organ-shaking culture [1]	Root tissue	1361 ± 34	126 ± 3	1183 ± 33	178 ± 8
	Transformed root organ-shaking cultures [1]	Different lines total	893 ± 100–3211 ± 200	73 ± 10–243 ± 20		
		Different lines root tissue	719 ± 80–2856 ± 160	59 ± 7–216 ± 10		
		Different lines medium	175 ± 20–355 ± 60	14 ± 2–27 ± 5		
	Young shoots from plates [1]		2050 ± 350	254 ± 39		
	Sterile plant culture [2]	Root	750 ± 16	47 ± 1.4	604 ± 10	146 ± 12
		Aerial part [b]	637 ± 16	87 ± 2	47 ± 1	590 ± 16
	Plant	Young leaves [2]	834 ± 14	130 ± 2	206 ± 12	627 ± 7
		Old leaves [b,2]	633 ± 42	125 ± 8	82 ± 6	551 ± 42
		Old leaves [1]	672 ± 190	152 ± 40	54 ± 35	618 ± 160
		Root [2]	1245 ± 5	160 ± 1	1245 ± 5	n.d.
	Seeds [a,3]		103			

Camptotheca acuminata	Callus [1]		43 ± 0.2	0.184 ± 0.07		
	Cell suspension culture [1]	With hormones	55.2 ± 6	3.5 ± 0.4		
		Without hormones	103 ± 2	9.9 ± 0.2		
	Transformed root organ cultures from plates [1]	Line 517	44 ± 3	5 ± 0.4		
		Line 801	47	6		
	Plant roots [1]		375 ± 20	63 ± 3		
	Seeds [a,3]		943			

Abbreviations: *MCPT* 9-methoxy-CPT, *n.d.* not detectable
[a]Seeds were not differed into dry or fresh weight ($n = 1$)
[b]Traces of 10-hydroxy-CPT were determined as well
[1]Measurements by HPLC, [2]Measurements by LC-MSMS, [3]measurements spectroscopically

Aimi et al. 1985), harman (*O. communis*; Hamzah et al. 1994), or ophiohayatones (*O. hayatana*; Chan et al. 2005) instead of CPT or CPT derivatives.

Very often root organ cultures, so-called "hairy roots", have been generated out of roots and other plant material of *Ophiorrhiza* species after transformation with *Agrobacterium rhizogenes*, trying to use them for CPT production (Saito et al. 2001; Sudo et al. 2002; Asano et al. 2004; Sirikantaramas et al. 2007a), for elucidation of the CPT biosynthesis and its regulation (Yamazaki et al. 2003a, b, 2004, 2013; Kitajima 2007; Sirikantaramas et al. 2007b), or for screening of CPT derivatives (e.g., Kitajima et al. 2002). In most instances, transformed root cultures of *Ophiorrhiza* species have been used for these investigations, because they seemed to be the only stable root organ system, or promised higher yields of CPT as well as higher growth rates (Flores et al. 1987).

In this communication, we report the production of CPT and derivatives in untransformed root and transformed "hairy root" organ cultures of *O. mungos*. We were able to generate stable untransformed root organ cultures without hormone treatment. We established the kinetics of CPT production in roots and its export into the culture medium in relation to nitrogen and sugar sources. CPT yields in root, and "hairy roots" of *O. mungos* are compared to production rates in other systems, indicating that the *O. mungos* cultures offer an economically interesting and sustainable possibility for CPT production.

14.2 Material and Methods

14.2.1 Plant Material

Untransformed root cultures were established from primary roots of surface-sterilized germinating seeds obtained from the Old Botanical Garden of Göttingen, Germany. Primary roots were excised and transferred to culture medium.

Several transformed root culture lines of *O. mungos* were generated from different plant tissues and *Agrobacterium rhizogenes* strains. Primary roots were mainly transformed by low pressure infiltration with different bacterial concentrations and time regimes. Newly growing side roots were separated and cultured. Adventitious roots were excised and cultured as well.

When the cultivation was initialized, 0.5 g/l cefotaxime (Claforan®, Aventis Pharma Germany GmbH, Frankfurt on Main, Germany) was added to the culture medium to reduce the remaining free *Agrobacteria*. Transformation controls were carried out by PCR to check for the presence or absence of the *rolC*- and *virC*-gene (according to Yang and Choi 2000). In successfully transformed cultures, only the *rolC*-gene could be detected by PCR. As a positive control, the DNA of the respective bacterial strain was used and, as a negative control, the DNA of the untransformed root culture line (details in Wetterauer 2008).

14.2.2 Culture Conditions

Sterile transformed and untransformed roots of *O. mungos* were maintained as shaking cultures in 50 ml modified Gamborg B5 medium (Gamborg et al. 1968) with 1% sucrose, ½ MS vitamins, without hormone addition, at a pH value of 5.7, at 21 °C, and 60 rpm in the dark. The culture medium was exchanged every 14 days. Every 28 days the older tissues were cut away and discarded. For propagation, the cultures were divided after an extended growing period of approx. 56 days.

14.2.3 CPT Production and Growth Kinetics of Untransformed and Transformed Root Culture Lines

For a general comparison, CPT content of the 25 established transformed root culture lines of *O. mungos* was measured. For this purpose, three culture flasks of each line with 3.5 ± 0.5 g cell weight (FW) were transferred into new culture medium and cultivated for 14 days under standard conditions. At the end of the incubation time, the roots were harvested, and CPT content and weight were determined. Based on these results, the transformed root organ line 607 with an average CPT content and good vitality was selected for further, more detailed physiological analysis (Fig. 14.4). This line 607 was established by transformation of primary roots with the *Agrobacterium rhizogenes* strain LBA 9402.

About 24 replicate cultures of the untransformed line and the selected transformed line 607 were prepared for general growth characterizations. The roots were partitioned to an inoculum size of 2 g ± 0.5 g, washed with sterile water, wiped, transferred into fresh culture flasks, and studied over a cultivation time of 28 days (two culture periods of 14 days with a single medium exchange) under standard conditions. After 0, 1, 2, 4, 6, 10, 14, 15, 16, 18, 20, 24, and 28 days, two culture flasks of each line were harvested, and the parameters shown in Fig. 14.5 determined such as the kinetics of ammonium, nitrate, phosphate, sucrose, glucose, and fructose in the culture medium and in addition to pH and conductivity using standard methods described in detail in Wetterauer (2008).

Three kinds of root tissue weights were determined at the time of harvest. First, the wet weight of the root culture with the interstitial medium was measured. Afterward the roots were washed with tap water and pressed by the hand three times, dried between paper towels, and the FW was then determined. The fresh roots were frozen at −80 °C and lyophilized, and the DW was determined. The dry roots were stored at room temperature in airtight closed plastic bags until further treatment.

14.2.4 CPT Determination of Root Tissue and Culture Medium

The lyophilized roots were milled to fine powder by mortar and pestle. Ten to twelve mg of the root powder were dissolved in 1 ml of methanol, shaken, incubated for 30 min at room temperature, and centrifuged for 10 min at 13000 rpm, and the supernatant was transferred to a new vial. The same procedure was repeated a second time, and supernatants of both extractions were united. Extractions were carried out in duplicates.

To 150 µl of the methanol extract, 500 µl of 50 µM KH_2PO_4 solution (pH 2.6) and 500 µl CH_2Cl_2 were added. After rapid shaking and 10 min of incubation, the CH_2Cl_2 phase was separated, and the procedure was repeated a second time by again adding 0.5 ml CH_2Cl_2. The united solvent phases were reduced to dryness under a nitrogen stream, and the residue CPT was dissolved in 1 ml of methanol for quantitative measurements.

The quantitative determination of the CPT content of culture media and root tissues was based on the method of van Hengel et al. (1992). CPT contents were measured spectroscopically by measuring the fluorescence of 100 µl of the methanol root extracts or culture media (direct as harvested) in a 96-well-plate reader GENIOS (Tecan Austria GmbH, Grödig/Salzburg, Austria) with the software Magellan 6 and 96-well-plates, black, FluoroNunc, F96, CC (Nalge Nunc International, Wiesbaden, Germany). Qualitative and quantitative validations of this method had been carried out by HPLC and lambda scans. The following parameters were used: excitation wavelength filter, 360 nm; emission wavelength filter, 465 nm; manual amplification factor, 50 (determined by integrated optimizing function); number of flashes, 3; delay time, 0 µs; and integration time, 40 µs. For quantification, external calibration curves with CPT solutions in modified Gamborg B5 culture medium and methanol in the concentration range of 0–20 µg CPT/ml (Sigma-Aldrich Chemie GmbH, Steinheim, Germany) were established. The calibration curve was linear up to 10 µg CPT/ml for methanol and 5 µg CPT/ml for Gamborg B5 culture medium.

After the measurements, the CPT content of media and root tissues was calculated per flask, g FW, and g DW.

14.2.5 LC-MS/MS Analysis

LC-MS/MS analysis was performed on a Finnigan LCQ-Duo ion trap mass spectrometer with an ESI source (ThermoQuest) coupled to a Finnigan Surveyor HPLC system (MS pump plus, autosampler, and PDA detector plus) with a EC 150/3 Nucleodur 100-3 C18ec column (Macherey-Nagel). A gradient of water and acetonitrile (ACN) (with 1% formic acid each) was applied from 10 to 50% ACN in 20 min at 30 °C. The flow rate was 0.5 ml/min. The injection volume was about

20 µl. All samples were measured in the positive mode. The MS was operated with a capillary voltage of 10 V, source temperature of 240 °C, and high purity nitrogen as a sheath and auxiliary gas at a flow rate of 80 and 40 (arbitrary units), respectively. The ions were detected in a mass range of 50–2000 m/z. Collision energy of 35% was used in MS/MS for fragmentation. Data acquisitions and analyses were executed by Xcalibur™ 2.0.7 software (Thermo Scientific), respectively.

14.3 Results

14.3.1 Phytochemical Analysis

Using HPLC and LC-MSMS, we could unambiguously identify CPT and 9-methoxy CPT (Fig. 14.1). For rapid and routine CPT measurements, we used photometric determinations, since CPT shows a unique blue fluorescence at the excitation wavelength of 366 nm (Fig. 14.2).

14.3.2 CPT Content of Transformed Root Cultures Lines of O. mungos

CPT was detected in all our root organ cultures of *O. mungos* (Fig. 14.3). Eighty to ninety percent of the total CPT was stored in the root tissues of 25 transformed root culture lines, and 10–20% were detected in the corresponding culture media (Fig. 14.4). The range of the total CPT content (culture medium + root tissue) was between 890 and 3200 µg/g DW (0.08–0.32%). The variations in CPT content between the lines were based mainly on different CPT contents in the root tissues. The media CPT content seemed to be quite stable, especially in view of the absolute CPT content per flask. The DW/FW ratios varied between 6.5 and 8.1% but did not correspond to the CPT levels. CPT production was apparently specific for each line.

14.3.3 Kinetics of Growth Parameters, Nutrient Utilization, and CPT Formation of Untransformed and Transformed (Line 607) Root Cultures

Growth kinetics of an untransformed and transformed root cultures are illustrated in Fig. 14.5. The relative growth of the untransformed line was comparatively slow for the first 10 days, rising to 150% by the end of the first culture period, and continuing to rise irregularly up to 360% in the second culture period. In contrast, the transformed line 607 showed a continuously rising relative growth until the end of the

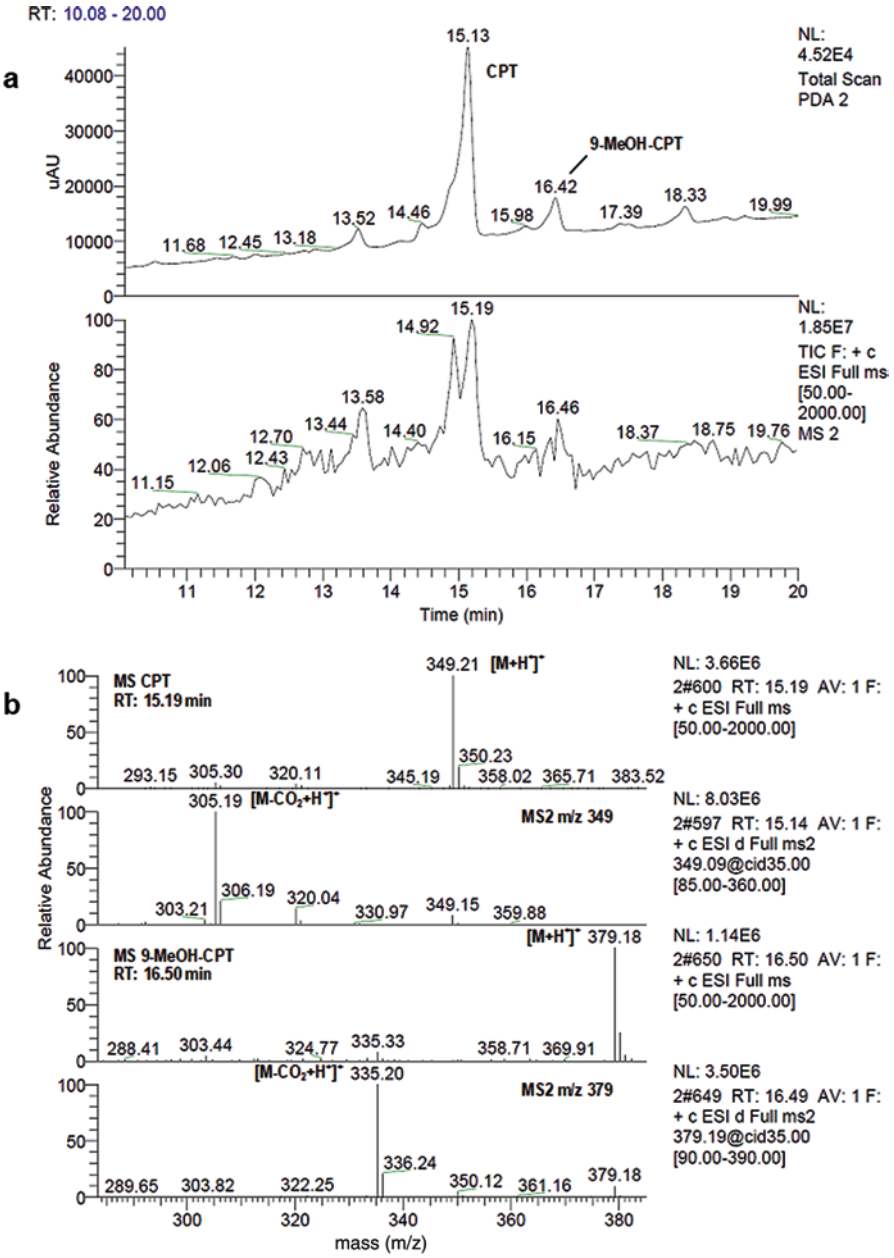

Fig. 14.1 (**a**) Profile of CPT and 9-methoxy-CPT in root organ cultures by LC-MS (TIC and PDA), (**b**) MS, and MS2 mass spectra of CPT and 9-methoxy-CPT

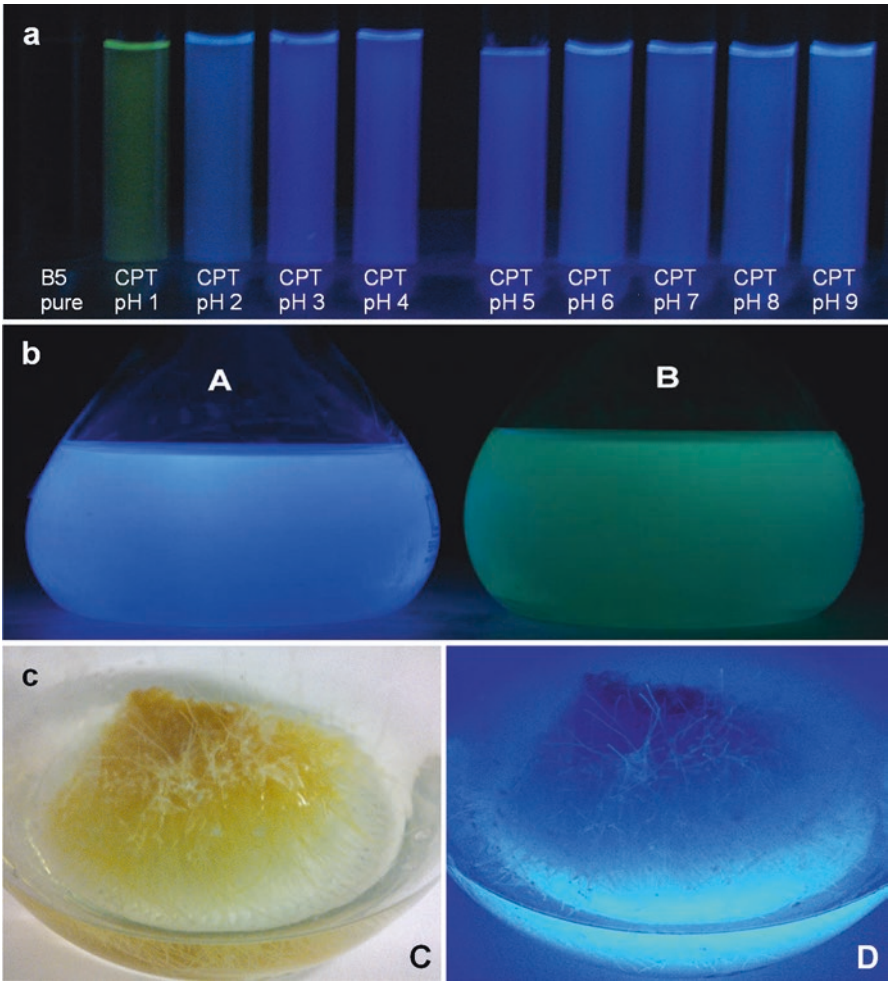

Fig. 14.2 (**a**) CPT fluorescence at pH values from one to nine in B5 culture medium (excitation wavelength 366 nm; CPT concentration 7 μg/ml), (**b**) fluorescence of CPT (*A*) and 9-methoxy-CPT (*B*) in methanol (excitation wavelength 366 nm; concentration 20 μM each), (**c**) untransformed root organ-shaking culture of *Ophiorrhiza mungos* by day light (*C*) and by CPT fluorescence at excitation wavelength 366 nm (*D*)

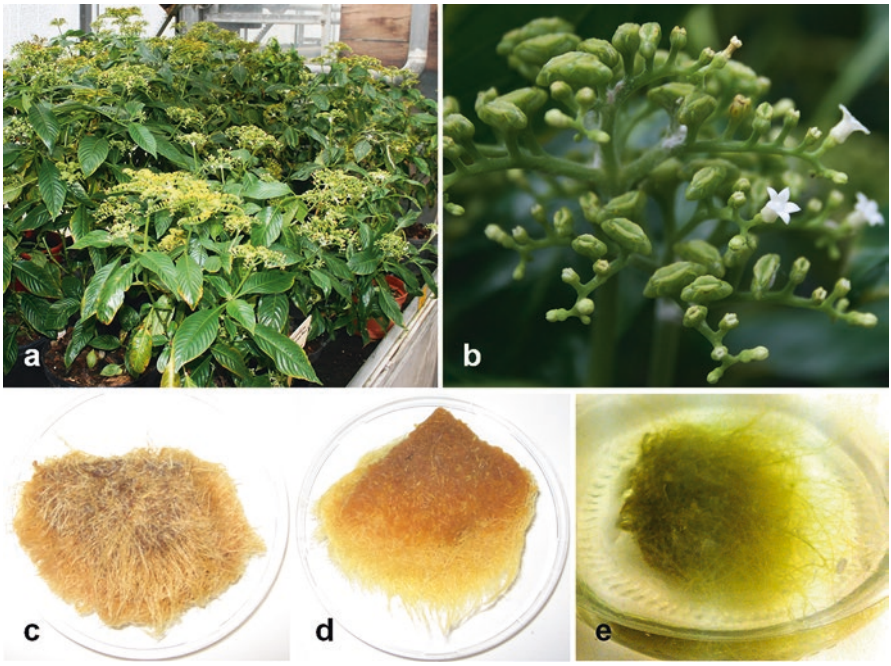

Fig. 14.3 *Ophiorrhiza mungos* plants and organ cultures. (**a**) Plants in the greenhouse, (**b**) inflorescence with flowers and young seed capsules, (**c**) transformed root organ-shaking culture line 607, (**d**) untransformed root organ-shaking culture, (**e**) green untransformed root organ-shaking culture

first cultivation period of up to 270% and only a slight relative growth of up to approx. 350% in the second culture period (Fig. 14.5).

In the first cultivation period, the untransformed cultures showed no uptake of nitrate, but they absorbed nearly half of the nitrate (13 mM) in the second culture period. The transformed cultures absorbed half of the nitrate (~15 mM) in both culture periods. The nitrate uptake seemed to be directly correlated with the relative and the absolute growth.

Both lines absorbed the ammonium totally in the first 100 h. The nitrate uptake did not start until the ammonium uptake was completed. Consequently, ammonium was preferred as a nitrogen source by both root lines. The uptake of ammonium, nitrate, and phosphate was usually faster in the transformed than in the untransformed line.

In the first cultivation period, the sucrose uptake was slower and incomplete, whereas in the second period, total sucrose was taken up or metabolized faster to glucose and fructose. Again the transformed cultures showed an accelerated uptake and metabolism rate as compared to the untransformed line. The glucose content of media was consistently lower than the fructose content. We assume that glucose was preferentially used as a carbon source.

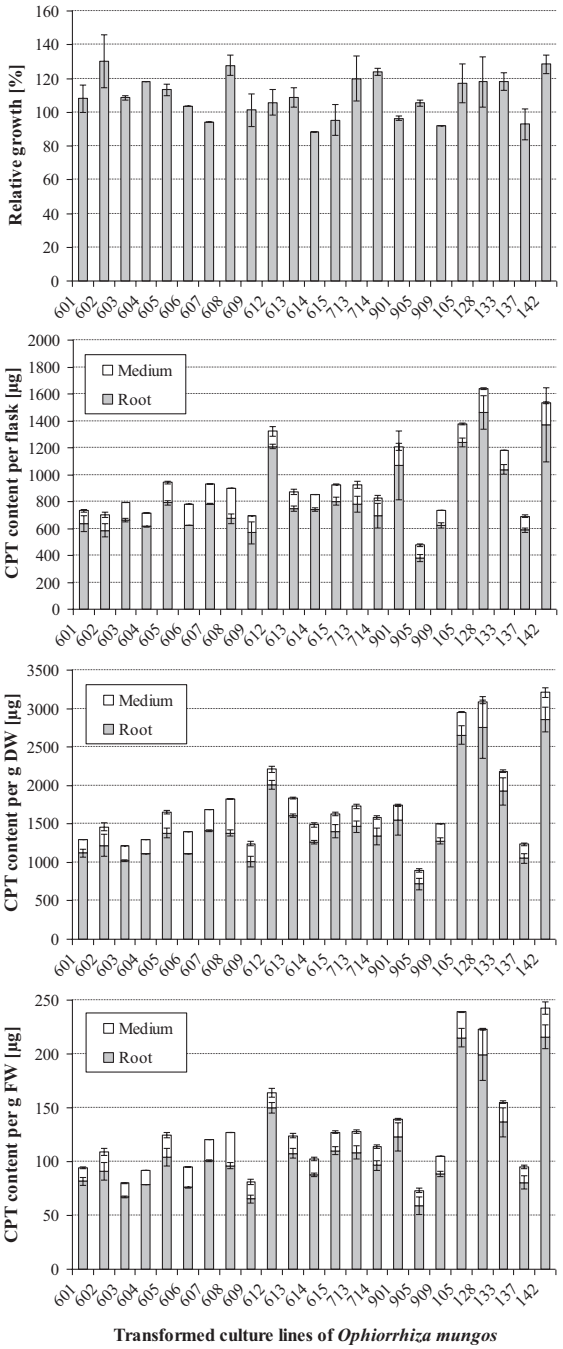

Fig. 14.4 Comparison of relative growth and CPT contents of different transformed root-shaking culture lines of *Ophiorrhiza mungos* after 14 days of cultivation ($n = 3$)

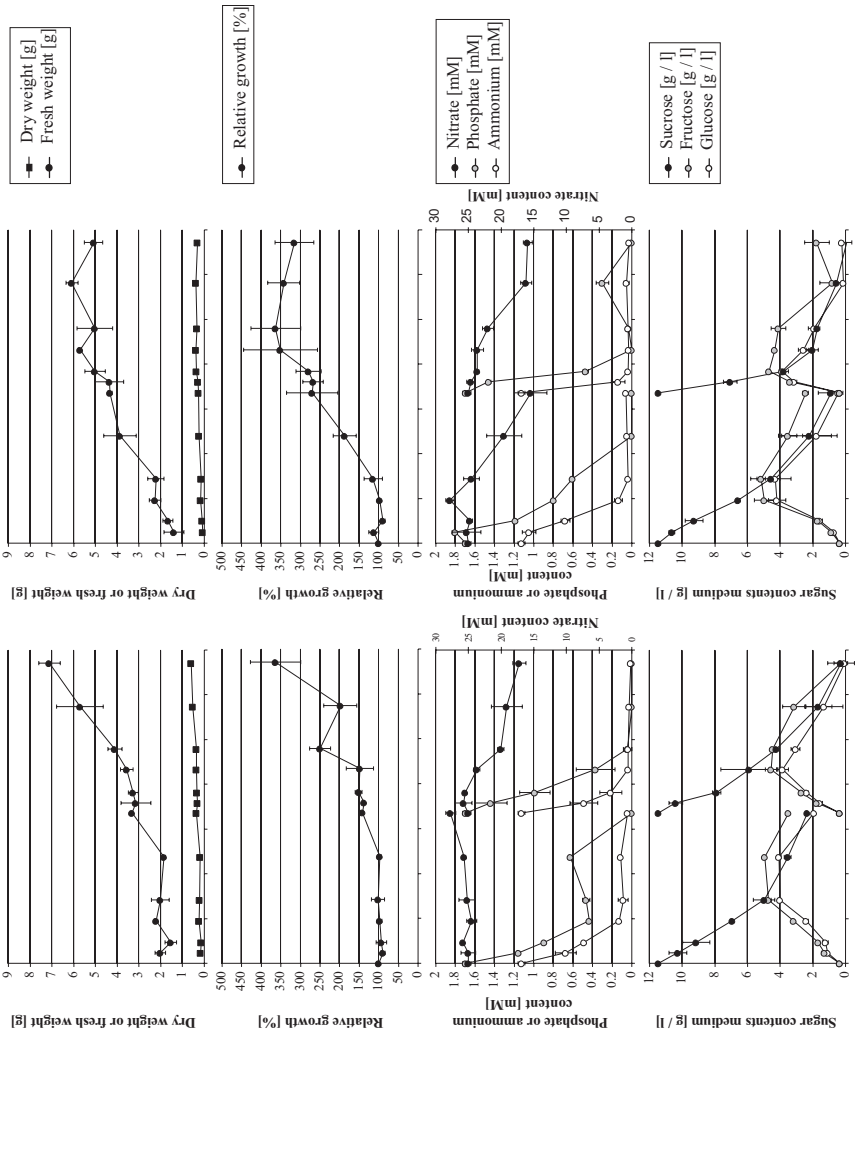

Fig. 14.5 Comparison of untransformed and transformed (line 607 transformed with LBA 9402) root organ-shaking cultures of *Ophiorrhiza mungos* in terms of growth behavior, nutrition uptake, physical parameters, and CPT contents ($n = 2$). Roots were cultured at 60 rpm in the dark at 21 °C for 672 h. Parent organ root cultures were cut to equal inoculum cultures of 2 g ± 0.5 g FW at 0 h. After 336 h the test cultures were transferred to fresh culture medium (modified B5 medium with MS vitamins, 1% sucrose, pH 5.7)

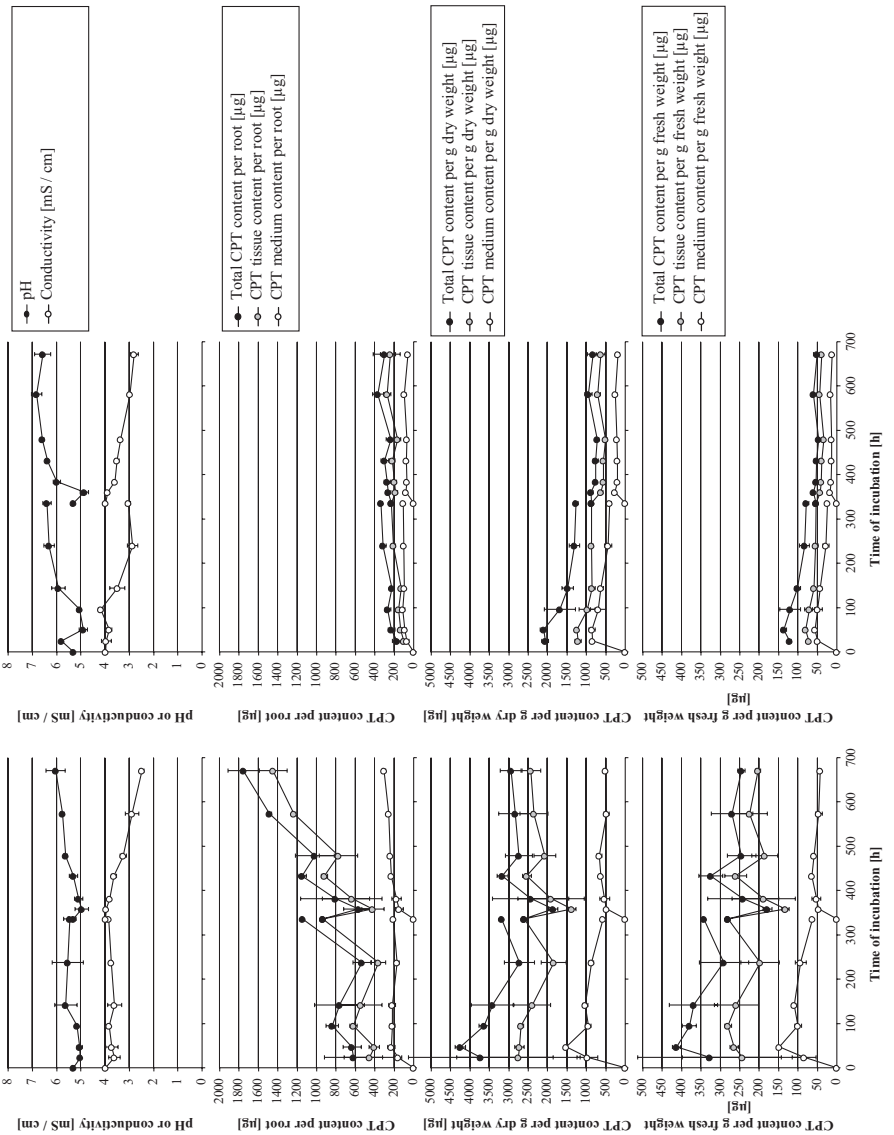

Fig. 14.5 (continued)

The nitrogen sources, ammonium, and nitrate apparently had the biggest influence on the pH values in the culture medium. As described in the literature (e.g., Neumann and Römheld 2002), the pH became more acidic during ammonium uptake (owing to an ammonium-proton antiport) and more alkaline during nitrate uptake (owing to a nitrate-proton symport combined with hydroxide ion release). The variations in proton concentrations became more apparent in the transformed line. Both lines adjusted the pH to a specific optimal value very fast. The course of conductivity apparently followed that of the nitrate, since the nitrate concentration was highest in the culture medium with 30 mM.

Both root organ culture lines, transformed as well as untransformed, only synthesized CPT, but no CPT analogues (only found in green tissues). The increase of total CPT content per root paralleled growth in both lines (FW and relative growth). In the culture medium, the total CPT content was quite constant. In the first 48 h after transfer, with and without partitioning, the CPT content in the culture medium of the untransformed cultures increased to 220 µg per flask (12.6 µM), while CPT increased to 80–100 µg per flask (4.6–5.7 µM) in the transformed cultures and remained stable until the end of the culture periods. However, the main differences in the total CPT content were based on the tissue content, which was five to six times higher in the untransformed line than in the transformed line over the total given period.

The general time course of CPT production, calculated per g FW and g DW, was identical for each line. Both lines showed a primary increase in tissue and media CPT content after partitioning. CPT decreased during the first culture period again and seemed to be adjusted to a fixed level by the second culture period at the latest (untransformed cultures, 0.24% per DW or 0.021% per FW for root tissue and total CPT ~0.3% per DW or 0.025% per FW; line 607, 0.08% per DW or 0.005% per FW and total CPT ~0.09% per DW or 0.006% per FW). Each stress (partitioning, or transfer into a fresh culture medium, or both) was followed by an increase of the CPT content in roots and the culture medium. This increase was transient during the first 150 h. But the relative CPT content in the culture medium and in the root tissue showed distinct ratios for both lines. For the transformed line 607, the CPT root tissue content was one and a half to two times higher than the medium content, and for the untransformed line, it was two to three times higher.

The kind of supply and dosage of nitrogen did not affect the CPT production directly (Wetterauer 2008). Again, this was an indication that the CPT biosynthesis is regulated constitutively, and it seems that the nitrogen, necessary for biosynthesis, is stored in the roots or reactivated out of root material, because no effect in biosynthesis became obvious with different nitrogen supplies or with none at all.

14.4 Discussion

As shown in Table 14.2 and in Figs. 14.4 and 14.5, untransformed and transformed root organ cultures of *O. mungos* produce CPT in amounts, which are similar to CPT contents in plants. In Table 14.3, we have collected all published data for CPT

Table 14.3 Overview of published qualitative and quantitative CPT contents in plants, fungi, and bacteria, so far. Species are shown in alphabetical order, multiple references of one species in chronological order

Species (family)	Kind of tissue resp. plant parts	Contents of camptothecins [µg/g DW or µg/g FW (bold)]		References
1. Plants				
Camptotheca acuminata Decaisne (Nyssaceae)[3]	Cell cultures	CPT	2.5–4	Sakato et al. (1974) and van Hengel et al. (1992)
	Fruits	CPT	300	Hsu et al. (1977) quoted in Li and Adair (1994)
	Branches	CPT	40	
	Stem bark	CPT	100	
	Roots	CPT	100	
	Root bark	CPT	200	
	Branches	CPT	160	Tien et al. (1977) quoted in Li and Adair (1994)
	Leaves	CPT	400	
	Roots	CPT	360	
	Bark	CPT	120	Adamovics et al. (1979)
		DCPT	2	
		HXCPT	0.4	
		HXMCPT	0.2	
	Bark and wood	11-HCPT	Traces	Wall et al. (1986)
	Leaves	CPT	160	Cao et al. (1992)
	Young leaves	CPT	4000–5000	López-Meyer et al. (1994)
		HCPT	20–30	
	Seeds	CPT	3000	
		HCPT	25	
	Bark	CPT	1800–2000	
		HCPT	2–90	
	Root	CPT	400	
		HCPT	13–20	
	Leaves[1a]	CPT	100 ± 15	Liu and Adams (1996)
	Bark[1a]	CPT	620 ± 69	
	Stem[1a]	CPT	490 ± 55	
	Roots[1a]	CPT	520 ± 124	
	Sub terrestrial part[1a]	CPT	420 ± 47	
	Total tree[1a]	CPT	440 ± 45	
	Callus	CPT	2040–2360	Wiedenfeld et al. (1997)

(continued)

Table 14.3 (continued)

Species (family)	Kind of tissue resp. plant parts	Contents of camptothecins [µg/g DW or µg/g FW (bold)]		References
		HCPT	80–100	
	Young leaves	CPT	450–3490	Vincent et al. (1997)
	Cell suspension culture	CPT	0.007!	Song and Byun (1998)
	Young leaves	CPT	2421–3022	Li et al. (2002)
	Old leaves	CPT	482	
	Young fruits	CPT	842	
	Old fruits	CPT	2362	
	Callus culture	CPT	49.7 ± 3.5 max.	Park et al. (2003)
	Seedlings[0] leaves	CPT	210 ± 10	Liu and Reinscheid (2004)
	Seedlings[0] branches	CPT	260 ± 11	
	Tree[1b] young leaves	CPT	450 ± 11	
	Tree[1b] old leaves	CPT	16 ± 1	
	Tree[1b] young branches	CPT	240 ± 12	
	Tree[1b] old branches	CPT	17 ± 1	
	Tree[1b] roots	CPT	480 ± 25	
	"Hairy roots"	CPT	1000 ± 150	Lorence et al. (2004)
		HCPT	150 ± 10	
	Roots	CPT	787!	Pi et al. (2010)
	Stems	CPT	437!	
		MCPT	245!	
	Leaves	CPT/MCPT	n.d.!	
	Young flower buds	CPT	2449!	
		MCPT	1400!	
	Opening flowers	CPT/MCPT	n.d.!	
	Fading flowers	CPT/MCPT	n.d.!	
	Seeds	CPT	2029!	
		MCPT	254!	
	Cell suspension culture[6]	CPT	3.8!	
		MCPT	1.3!	

(continued)

Table 14.3 (continued)

Species (family)	Kind of tissue resp. plant parts	Contents of camptothecins [µg/g DW or µg/g FW (bold)]		References
	Seedling cotyledon	CPT	1192 ± 38!	Kai et al. (2014)
	Seedling hypocotyl	HCPT	1923 ± 77!	
	Seedling root	CPT	1293 ± 15!	
	Seedlings treated with hormone elicitors	HCPT	1138 ± 54!	
		CPT	369 ± 23!	
		HCPT	1908 ± 154!	
		CPT	1810 max.	
		HCPT	2600 max.	
Camptotheca lowreyana Li (Nyssaceae)	Young leaves	CPT	3913–5537	Li et al. (2002)
	Old leaves	CPT	909–1184	
Camptotheca yunnanensis Dode (Nyssaceae)	Young leaves	CPT	2592–4494	Li et al. (2002)
	Old leaves	CPT	590	
Chonemorpha grandiflora Syn. *Chonemorpha fragrans* (Apocynaceae)	Stem with bark	CPT	13	Kulkarni et al. (2010)
	Callus	CPT	3	
	Hairy root cultures	CPT	240–300	Kedari and Malpathak (2014)
	Plant roots	CPT	330	
Dysoxylum binectariferum (Roxb.) Hook (Meliaceae)	Bark	CPT	1045	Jain et al. (2014)
		MCPT		
Ervatamia heyneana (Wall) T. Cook (Apocynaceae)	Wood and stem bark	CPT	1300	Gunasekera et al. (1979)
		MCPT	400	
Mappia foetida Mier (Icacinaceae/ Olacaceae)[1]	Bark, stem, root, leaves	CPT	600–1000	Govindachari and Viswanathan (1972a)
		MCPT	10–20	
		CPT	n/a	
	Stems	MCPT	n/a	
		ACPT	n/a	
	Stem	CPT	600	Govindachari and Viswanathan (1972b)
		MCPT	10	
	Bark	CPT	800	
		MCPT	10	
	Roots	CPT	1000	
		MCPT	20	
	Leaves	CPT	100	
		MCPT	Traces	

(continued)

Table 14.3 (continued)

Species (family)	Kind of tissue resp. plant parts	Contents of camptothecins [µg/g DW or µg/g FW (bold)]		References
Merrilliodendron megacarpum (Hemsl.) Sleumer (Icacinaceae)[5]	Leaves and stem	CPT	530	Arisawa et al. (1981)
		MCPT	170	
Mostuea brunonis Didr. (Loganiaceae/ after Lorence and Nessler (2004) Gelsemiaceae)	Total plant	CPT-20-*O*-*β*-glycoside	100	Dai et al. (1999)
		Deoxypumiloside	100	
		Strictosamide	600	
Nothapodytes foetida (Wight) Sleumer (Icacinaceae)[1,3,4]	Stem wood	CPT	1400–2400	Aiyama et al. (1988)
		dCPT	19	
	Shoot	CPT	750	Roja and Heble (1994)
		MCPT	130	
	Seedlings	MCPT	7	
	Seeds	CPT	500	
		MCPT	600	
	Callus	MCPT	1	
	Stem	ACPT	0.24	Wu et al. (1995)
	Callus	CPT	9.5	Ciddi and Shuler (2000)
		MCPT	Traces	
	Cell culture	CPT	1.1	Fulzele et al. (2001)
		MCPT	0.81	
	Untransformed root organ culture	CPT	100	Fulzele et al. (2002)
		MCPT	16	
	Calli of root organ culture	CPT	1.7	
		MCPT	0.58	
	Callus	CPT/MCPT	Traces	Fulzele and Satdive (2003)
	Embryo	CPT	110	
		MCPT	28	
	Seedlings	CPT	610	
		MCPT	53	
	Root [2a]	CPT	2000	
		MCPT	970	

(continued)

Table 14.3 (continued)

Species (family)	Kind of tissue resp. plant parts	Contents of camptothecins [µg/g DW or µg/g FW (bold)]		References
	Stem [2a]	CPT	1100	
		MCPT	350	
	Leaves [2a]	CPT	450	
		MCPT	130	
	Stem	MACPT	2.5	Srinivas and das (2003)
	Seedlings (4–5 months):	CPT	340	Roja (2006)
	Roots	MCPT	Traces	
	Stem	CPT	270	
		MCPT	Traces	
	Leaves	CPT	340	
		MCPT	Traces	
	Adult plant:	CPT	1000	
	Roots	MCPT	20	
	Stem	CPT	600	
		MCPT	20	
	Leaves	CPT	100	
		MCPT	Traces	
	Bark	CPT	800	
		MCPT	10	
	Seeds	CPT	580	
		MCPT	140	
	Mature seeds	CPT	540	Wu et al. (2008)
		MCPT	380	
	Immature seeds	CPT	400	
		MCPT	210	
	Leaves	CPT	580	
		MCPT	1800	
	Stem	CPT	1780	
		MCPT	1540	
	Roots	CPT	15,590	
		MCPT	3850	
	Callus γ-rays treatment (max. at 20Gy)	CPT	980 ± 3!	Fulzele et al. (2015)
		MCPT	43 ± 4!	

(continued)

Table 14.3 (continued)

Species (family)	Kind of tissue resp. plant parts	Contents of camptothecins [µg/g DW or µg/g FW (bold)]		References
Nothapodytes nimmoniana (J. Grah.) Mabb. (Icacinaceae)[1,3,4,7]	Trees:			Padmanabha et al. (2006)
	Wood	CPT	1420	
	Bark	CPT	2360 ± 1580	
	Root wood	CPT	1820	
	Root bark	CPT	3330 ± 2130	
	Leaves	CPT	810 ± 570	
	Seeds	CPT	1790 ± 710	
	Seedlings [2a]:			
	Root tips	CPT	4000	
	Old leaves	CPT	2100	
	Young leaves	CPT	2050	
	Wood	CPT	2000	
	Bark	CPT	1600	
	Root	CPT	1600	
	Root bark	CPT	2900 ± 2100	Kavitha et al. (2010)
		MCPT	900 ± 600	
	Stem bark	CPT	2400 ± 2100	
		MCPT	900 ± 900	
	Stem	CPT	1200 ± 800	
		MCPT	400 ± 200	
	Leaves	CPT	800 ± 700	
		MCPT	60 ± 20	
	Cell suspension culture Intra- and extracellular under different culture parameters	CPT	51.7 max.	Karwasara and Dixit (2013)
	Young fruits	CPT	3300 ± 600!	Manjunatha et al. (2016)
	Immature fruit stalk	CPT	1600 ± 200!	
	Mature fruits	CPT	2300 ± 800!	
	Young leaves	CPT	1700 ± 100!	
	Mature leaves	CPT	1300 ± 50!	
	Old leaves	CPT	1230 ± 260!	
	Inner bark	CPT	660 ± 210!	
	Outer bark	CPT	300 ± 40!	
	Fruit stalk	CPT	1100 ± 200!	
	Roots	CPT	1760 ± 330!	

(continued)

Table 14.3 (continued)

Species (family)	Kind of tissue resp. plant parts	Contents of camptothecins [µg/g DW or µg/g FW (bold)]		References
[3]				
Ophiorrhiza alata Craib (Rubiaceae)	Plant leaf	CPT	83 ± 21	Ya-ut et al. (2011)
	Plant root	CPT	388 ± 32	
	In vitro culture leaf	CPT	94 ± 15	
	In vitro culture root	CPT	556 ± 43	
	"Hairy roots"	CPT	785 ± 52	
Ophiorrhiza barberi (Rubiaceae)	Plant	CPT	n.d.	Rajan et al. (2013)
Ophiorrhiza caudata (Rubiaceae)	Plant	CPT	n.d.	Rajan et al. (2013)
Ophiorrhiza eriantha (Rubiaceae)	Plant	CPT	0.3 ± 0.01 max.	Rajan et al. (2013)
Ophiorrhiza cf. *ferruginea* (Rubiaceae)	Arial parts	Isomalindine	n/a	Arbain et al. (1993a)
Ophiorrhiza grandiflora (Rubiaceae)	Plant	CPT	1.07–1.34 ± 0.01	Rajan et al. (2013)
Ophiorrhiza filistipula (Rubiaceae)	Leaves	7-MCPT	n/a	Arbain et al. (1993b)
Ophiorrhiza kuroiwai Mak. (Rubiaceae)[2,9]	"Hairy roots"	CPT	219.3 ± 31.4	Asano et al. (2004)
	Sterile plant: shoots	CPT	370 ± 50!	
	Sterile plant: root	CPT	250 ± 100!	
Ophiorrhiza liukiuensis Hayata (Rubiaceae)[9]	"Hairy roots"	CPT	83.0 ± 27.4	Asano et al. (2004)
	Sterile plant: shoots	CPT	40 ± 38!	
	Sterile plant: root	CPT	80 ± 30!	
	Total plant	**CPT**	**126.9**	Kitajima et al. (2005)
		MCPT	**126.2**	
		10-MCPT	**29.66**	
Ophiorrhiza mungos L. (Rubiaceae)	Total plant	CPT	12	Tafur et al. (1976)
		MCPT	10.4	

(continued)

Table 14.3 (continued)

Species (family)	Kind of tissue resp. plant parts	Contents of camptothecins [μg/g DW or μg/g FW (bold)]		References
	Callus culture[10]	CPT	4 ± 2	Wink et al. (2005)[11]
	Cell suspension culture:			
	With hormones[10]	CPT	4 ± 2	
	Without hormones	CPT	122 ± 3	
	Root organ culture:			
	Untransformed tissue	CPT	2060 ± 200	
	Untransformed medium	CPT	436 ± 40	
	Transformed lines tissues	CPT	719 ± 80–2856 ± 160	
	Transformed lines media	CPT	175 ± 20–355 ± 60	
	Roots	CPT	176	Roja (2006)
		MCPT	Traces	
	Shoot	CPT	96	
		MCPT	Traces	
	Plant	CPT	170.1 ± 1.9–538.5 ± 8.8	Rajan et al. (2013)
	Callus culture	CPT	40–100	Deepthi and Satheeshkumar (2016)
	Cell suspension culture	CPT	20–330	
	Cell suspension culture	CPT	1120 ± 80 max.	Deepthi and Satheeshkumar (2017a)
	Root organ culture	CPT	860 ± 15 max.	Deepthi and Satheeshkumar (2017b)
Ophiorrhiza mungos var. *angustifolia* (Rubiaceae)	Plant	CPT	127.9 ± 6.1–476.9 ± 0.1	Rajan et al. (2013)
Ophiorrhiza nairii (Rubiaceae)	Plant	CPT	n.d.	Rajan et al. (2013)
Ophiorrhiza pectinata (Rubiaceae)	Plant	CPT	0.28 ± 0.02–38.65 ± 0.01	Rajan et al. (2013)

(continued)

Table 14.3 (continued)

Species (family)	Kind of tissue resp. plant parts	Contents of camptothecins [µg/g DW or µg/g FW (bold)]		References
Ophiorrhiza prostrata D. Don (Rubiaceae)	Roots with and without elicitation under different conditions and age	CPT	280–1900	Martin et al. (2008)
Ophiorrhiza pumila Champ. (Rubiaceae)[3,9,12]	n/a	Chaboside	n/a	Aimi et al. (1990)
	Seedlings generated out of calli	CPT	333	Kitajima et al. (1997)
		MCPT	232	
		Chaboside	30.4	
		Pumiloside	1310	
		(3 *R*)-Deoxypumiloside	441	
		Strictosamide	118	
	Cell culture	No camptothecins	–	Kitajima et al. (1998a)
	Seedlings generated out of calli	CPT	n/a	Kitajima et al. (1998b)
		GOCPT	n/a	
		GOCPTPA	n/a	
	Leaves	CPT	300–400	Saito et al. (2001)
	Young roots	CPT	1000	
	"Hairy roots"	CPT	1000	
	"Hairy roots"			Kitajima et al. (2002)
	On solid medium	CPT	n/a	
		(3*S*)-Pumiloside	**177.4**	
		(3*S*)-Deoxypumiloside	**52.1**	
		(3*R*)-Deoxypumiloside	**49.5**	
	On liquid medium	**Strictosamide**	**49.5**	
		OPHR-23	**2.1**	
		CPT	**47.2**	
		(3*S*)-Pumiloside	**26.4**	
		(3*R*)-Deoxypumiloside	**3.4**	
		Strictosamide	**26.4**	
		OPHR-23	**0.8**	
		OPHR-17	**1.07**	

(continued)

Table 14.3 (continued)

Species (family)	Kind of tissue resp. plant parts	Contents of camptothecins [μg/g DW or μg/g FW (bold)]		References
	Total plant	CPT	300–510	Yamazaki et al. (2003b)
		MCPT	70–140	
		Chaboside	300–690	
	"Hairy roots"	CPT	240	
	Sterile plant: shoot	CPT	225 ± 45!	Asano et al. (2004)
	Sterile plant: root	CPT	760 ± 50!	
	"Hairy roots"	CPT	790 ± 50	Watase et al. (2004)
	Transgenic "hairy root" lines	CPT	680–1640	Cui et al. (2015)
Ophiorrhiza rugosa (Rubiaceae)	Roots	CPT	20	Roja (2006)
		MCPT	Traces	
	Shoot	CPT	10	
		MCPT	Traces	
Ophiorrhiza rugosa var. *decumbens* (Rubiaceae)	Sterile albino plants under different conditions	CPT	558–1040	Vineesh et al. (2007)
	Sterile plants under different conditions	CPT	37–311	
	Tissue culture plant:			Roja (2008)
	Root	CPT	20	
	Stems	CPT	110	
	Leaves	CPT	900	
	Floral parts	CPT	150	
	Parent plant:			
	Roots	CPT	20	
	Stem	CPT	Traces	
	Leaves	CPT	2	
	Shoot cultures:			
	Tissue	CPT	3–130	
	Media	CPT	25–225 μg/l	
	"Hairy roots"	CPT	90 ± 10	Kamble et al. (2011)
	Regenerated roots from "hairy roots"	CPT	120 ± 25	
	Control roots	CPT	80 ± 5	

(continued)

Table 14.3 (continued)

Species (family)	Kind of tissue resp. plant parts	Contents of camptothecins [µg/g DW or µg/g FW (bold)]		References
	Plant	CPT	0.16 ± 0.02	Rajan et al. (2013)
	Multiple shoot cultures	CPT	560	Gopalakrishnan and Shankar (2014)
Ophiorrhiza rugosa var. *prostrata* (Rubiaceae)	Stem	CPT	800	Gharpure et al. (2010)
	Root	CPT	1600	
	Young leaves	CPT	62	
	Old leaves	CPT	22	
	Fruits	CPT	165	
Ophiorrhiza shendurunii (Rubiaceae)	Plant	CPT	0.05 ± 0.01	Rajan et al. (2013)
Ophiorrhiza trichocarpon (Rubiaceae)	Plant	CPT	19.5 ± 0.2–28.31 ± 4.2	Rajan et al. (2013)
Pyrenacantha klaineana Pierre ex. Exell and Mendica (Icacinaceae)	Stems	CPT	n/a	Zhou et al. (2000)
		MCPT	n/a	
2. Fungi [endophytic in]; see also Gurudatt et al. (2010), Zhao et al. (2011), Pu et al. (2013, 2015), and Venugopalan and Srivastava (2015b)				
Entrophospora infrequens MTCC 5124 [*Nothapodytes foetida* (Icacinaceae)][1,4,8]	Mycelium shake flask culture	CPT	5.75 ± 0.31 max.	Amna et al. (2006a) see also Amna et al. (2006b)
	Mycelium bioreactor	CPT	49.6 ± 7.3 max.	
RJMEF001 (Phycomycetes) [*Nothapodytes foetida* (Icacinaceae)]	Mycelium	CPT	0.018 in chloroform extract	Puri et al. (2005)
	Mycelium precursor study	CPT	5 ± 0.3 max.	Amna et al. (2012)
ZP5SE (Neurospora) [*Nothapodytes foetida*]	Mycelium	CPT	**40,000 max.**!	Rehman et al. (2008)
INFU/Ca/KF/3 *Fusarium solani* [*Camptotheca acuminata* (Nyssaceae)]	Mycelium	CPT	6 ± 2.2 max.!	Kusari et al. (2009)
		MCPT	0.95 ± 0.3 max.!	
		HCPT	n/a	
EU284592 [*Nothapodytes foetida* (Icacinaceae)]	Mycelium bioreactor	CPT	45 max	Rehman et al. (2009)

(continued)

Table 14.3 (continued)

Species (family)	Kind of tissue resp. plant parts	Contents of camptothecins [µg/g DW or µg/g FW (bold)]		References
Xylaria sp. [*Camptotheca acuminata* (Nyssaceae)]	Total submerged fungus culture by elicitation with 0.1 mM salicylic acid	HCPT	5400 µg/l fungus culture	Liu et al. (2010)
MTCC 9667 *Fusarium solani* [*Apodytes dimidiata* (Icacinaceae)]	Mycelium	CPT	0.37	Shweta et al. (2010)
		MCPT	0.383	
MTCC 9668 *Fusarium solani* [*Apodytes dimidiata* (Icacinaceae)][13]	Mycelium	CPT	0.536	Shweta et al. (2010)
		HCPT	0.082	
		MCPT	0.449	
Botryosphaeria dothidea X4 [*Camptotheca acuminata* (Nyssaceae)]	Mycelium	MCPT	n/a	Ding et al. (2013)
Alternaria alternata (Fr.) Keissl (MTCC 5477) [*Miquelia dentata* (Icacinaceae)]	Mycelium	CPT	73.9	Shweta et al. (2013a)
		MCPT	n/a	
		HCPT	n/a	
Fomitopsis sp. P. Karst (MTCC 10177) [*Miquelia dentata* (Icacinaceae)]	Mycelium	CPT	55.49	
		MCPT	n/a	
		HCPT	n/a	
Phomopsis sp. (Sacc.) (JX178957) [*Miquelia dentata* (Icacinaceae)]	Mycelium	CPT	42.06	
		MCPT	n/a	
		HCPT	n/a	
Fusarium nematophilum XSXY09	Mycelium	CPT	37	Su et al. (2014)
Alternaria alternata XSQZ04	Mycelium	CPT	29	
Phomopsis vaccinii XSCY02	Mycelium	CPT	24	

(continued)

Table 14.3 (continued)

Species (family)	Kind of tissue resp. plant parts	Contents of camptothecins [µg/g DW or µg/g FW (bold)]		References
Colletotrichum gloeosporioides XSXY05	Mycelium	HCPT	17	
Fusarium oxysporum NFX06 (KC914432) [*Nothapodytes foetida* (Icacinaceae)]	Mycelium	CPT	0.61	Musavi et al. (2015)
3. Bacteria [endophytic in]				
Bacillus subtilis PXJ-5	Broth	CPT	1.55 µg/ml	Shweta et al. (2013b) + personal communication with prof. Dr. Schaanker
JQ9565522.1 [*Miquelia dentata* (Icacinaceae)]	Broth	CPT	0.46 µg/ml	
Bacillus sp. CPC3 JN700911.1 [*Miquelia dentata* (Icacinaceae)]	Broth	CPT	1.18 µg/ml	
Bacillus cereus strain ChST JF935135.1 [*Miquelia dentata* (Icacinaceae)]	First bacteria pellet resuspended in solvent	CPT	**0.000011 µg/ml**	
Lysinibacillus sp. JN160728 [*Miquelia dentata* (Icacinaceae)]	Bacteria pellet of the first sub-culture generation	CPT	**11.84 µg/100 g**	
		MCPT	**n/a**	
	Bacteria pellet of the second sub-culture generation	CPT	**8.53 µg/100 g**	
Bacillus sp. KP125955 [*Pyrenacantha volubilis* Hook. (Icacinaceae)]	Bacteria pellet	CPT	**0.09 µg/g**	Soujanya et al. (2017)
Bacillus sp. KP125956 [*Pyrenacantha volubilis* Hook. (Icacinaceae)]	Bacteria pellet	CPT	**0.068 µg/g**	

(continued)

Table 14.3 (continued)

Species (family)	Kind of tissue resp. plant parts	Contents of camptothecins [µg/g DW or µg/g FW (bold)]		References
Bacillus subtilis KY741853 [*Pyrenacantha volubilis* Hook. (Icacinaceae)]	Bacteria pellet	CPT	**0.106**	
Bacillus subtilis KY741854 [*Pyrenacantha volubilis* Hook. (Icacinaceae)]	Bacteria pellet	CPT	**0.028**	

Publications of isolated fungi that produce CPT or CPT analogues are sorted in chronological order. Additional lists can be found in Lorence and Nessler (2004), Namdeo et al. (2008), Ramesha et al. (2008, 2013), Uma Shaanker et al. (2008), and Kai et al. (2015)

Abbreviations: ACPT 20-*O*-acetyl-CPT, CPT 20(S)-camptothecin, Chaboside is the name for the CPT glycoside: 9-methoxy-10-*O*-*β*-D-glucosyl-CPT, dCPT (20*S*)-18,19-dehydro-CPT, DCPT 20-deoxy-CPT, DW dry weight, FW fresh weight, GOCPT 9-*β*-D-glucosyloxy-CPT, GOCPTPA 9-*β*-D-glucosyloxy-CPT-pentaacetate, Gy gray, HCPT 10-hydroxy-CPT, 11-HCPT 11-hydroxy-CPT, HXCPT 20-hexanoyl-CPT, HXMCPT 20-hexanoyl-10-methoxy-CPT, MACPT 9-methoxy-20-*O*-acetyl-CPT, max. maximum, 7-MCPT 7-methoxy-CPT, MCPT 9-methoxy-CPT, 10-MCPT 10-methoxy-CPT, n/a not applicable, n.d. not detected

[1]*Mappia foetida* was renamed as *Nothapodytes foetida*, e.g., Das and Madhusudhan (1999) and Puri et al. (2005). After Padmanabha et al. (2006) this plant was newly named as *Nothapodytes nimmoniana*. *Mappia foetida* was attributed to the family of Icacinaceae or Olacaceae according to author

[2]Hybrid *O. pumila* × *O. liukiuensis* after Asano et al. (2004)

[3]Additional literature of time, source, and factor depending investigations in different relations: CPT in *C. acuminata*, Liu and Adams (1996, 1998), Vincent et al. (1997), López-Meyer and Nessler (1997), Liu (2000), Liu et al. (1998, 1999), Li et al. (2002), Park et al. (2003), Zu et al. (2003), Yan et al. (2003, 2005), Pan et al. (2004), Pasqua et al. (2004), Li and Liu (2005), Sankar-Thomas and Lieberei (2011), Kai et al. (2014), Ruan et al. (2014), Liu et al. (2015), Hu et al. (2016); CPT and MCPT content amplifications in calli of *Nothapodytes foetida* by growth regulators in questionably high values and standard deviations, Thengane et al. (2003); CPT profiling of *Nothapodytes nimmoniana*, Suhas et al. (2007); further extensive isolations of several qualitative CPT analogues out of *C. acuminata*, Zhang et al. (2004); CPT in "hairy root" cultures of *O. pumila*, Sudo et al. (2002); relative CPT and MCPT contents of 8 *Ophiorrhiza* ssp., Viraporn et al. (2011)

[4]Further sources of CPT are *Nothapodytes collina*, *N. obscura*, *N. obtusifolia*, *N. piltosporsides*, and *N. tomentosa* described by Puri et al. (2005)

[5]*Merrilliodendron* is a monotypic genus with the synonyms: *Stemonurus megacarpus* Hemsl., *Mangifera xylocarpa* Laut., *Merrilliodendron rotense* Kaneh., and *Peekeliodendron missionariorum* Sleum. Botanically it is close to the genus *Nothapodytes* (Arisawa et al. 1981)

[6]CPT and MCPT concentrations of cell suspension cultures of *C. acuminata* were investigated under many different culture conditions. Here the maxima values were listed each

(continued)

Table 14.3 (continued)

[7]Good overviews with further literature about *Nothapodytes nimmoniana* are given in Uma Shaanker et al. (2008), Isah and Mujib (2015a, b), and Isah (2017)
[8]For MTCC 5124 US Patent 7,378,268 Puri et al. (2008)
[9]See also Asano et al. (2009)
[10]Kinetin, 0.1 mg/l; 2,4-dichlorophenoxyacetic acid, 0.5 mg/l
[11]Further studies of *O. mungos*, see in Wetterauer (2008)
[12]See also Yamazaki et al. (2003a) and Sirikantaramas et al. (2007a)
[13]See also Venugopalan and Srivastava (2015a) and Venugopalan et al. (2016)
[0]3-month-old
[1a]Plants during the first year
[1b]One-year-old plant
[2a]Two-year-old plants
!Approximate values extracted out of diagrams

production in plants, cell, and organ cultures from all sources. An interesting new source is endophytic fungi, which have been isolated from some CPT plants. Surprisingly, fungi and bacteria produce CPT in culture, indicating that they carry the genes for CPT biosynthesis. In most instances, CPT yields are low but could probably be improved by optimizing culture conditions.

In conclusion, root organ cultures of *O. mungos* showed the highest CPT yields detected in root organ cultures of *Ophiorrhiza* species: about 0.25% CPT of DW were recorded for untransformed cultures, and up to 0.32% CPT of DW for transformed ones, with moderate growth rates of 3.5-fold in 4 weeks (for detailed growth behavior, see Bastian et al. 2008). Both culture lines have been maintained in vitro for more than 10 years and remain stable in growth and CPT content. We have demonstrated that these cultures could be grown in special fermenters continuously and CPT can be harvested from the medium (Wink et al. 2005). Therefore, the *O. mungos* organ root cultures are an interesting source for the feasible, sustainable, and profitable bioproduction of CPT by fermenter technology as a raw material for the pharmaceutical industry.

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Part II
In Vitro Propagation, Genetic Transformation and Germplasm Conservation

Chapter 15
In Vitro Approaches for Conservation and Sustainable Utilization of *Butea monosperma* (Lam.) Taub. Var. Lutea (Witt.) Maheshwari: A Highly Valuable Medicinal Plant

Rajesh Yarra, Ramesh Mushke, and Madhu Velmala

Abstract Medicinal plants are a globally valuable source of life-saving drugs. The biotechnology is the key tool to select, reproduce, and conserve the critical and endangered species of medicinal plants. *Butea monosperma* grasps a significant position in the pharmaceutical domain because of its enormous medicinal properties resulting from the presence of several classes of secondary metabolites. Poor seed viability, low seed germination rate, and genetic variability restrict propagation of *B. monosperma.* Overexploitation, severe habitat destruction, and restricted distributions are the major constraints of sustainable production of this important plant. The in vitro tissue culture and micropropagation of *Butea monosperma* – significant medicinal plant – have been well established. The quick and reproducible in vitro response to plant growth regulator treatments has emerged as an indispensable balance of genetic transformation studies for this plant species. In this chapter, in vitro regeneration approaches, advancements, and improvements in genetic transformation of *B. monosperma* are addressed. Finally, we propose conclusions and future prospects for this medicinally important tree species.

Keywords *Butea monosperma* · Micropropagation · Genetic transformation

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15.1 Introduction

Butea monosperma (Lam.) Taub. var. lutea (Witt.) (Fabaceae) is popularly known as flame of forest and bastard teak (Kirtikar and Basu 1935), is utmost medicinally important, and is a medium-sized deciduous tree ranging across India, Bangladesh, Nepal, Sri Lanka, Myanmar, Thailand, Cambodia, Vietnam, Malaysia, and Western Indonesia (Firdaus and Mazumder 2012). This tree is an erect, medium-size height (12–15 m), and all parts of the plant have medicinal as well as economic value. It is unique and the most attractive tree that has been placed to certain beneficial determination. *Butea monosperma* is widely used in Ayurveda, Unani, and homeopathic medicine and has become a pearl of modern medicine. Traditionally, it is reported to possess astringent, bitter, alterative, aphrodisiac, anthelmintic, antibacterial, and antiasthmatic properties. Butea gum, a red exudate obtained from the bark, is rich in gallic and tannic acids and exhibits antidiarrheal (Gunakkunru et al. 2005) and antifungal properties (Samaranayake et al. 1989). Phytochemical compounds such as alkaloids, cyanogenic glycosides, phenolic compounds, flavonoids, terpenoids, tannins, and saponins isolated from different parts of the plant has been reported to possess antimicrobial properties (Thirupathaiah 2007). Seeds of *B. monosperma* is also used to treat various ailments which include inflammation, skin and eye diseases, bleeding piles, urinary stones, abdominal troubles, intestinal worms, and tumor (Kirtikar and Basu 1935; Anonymous 1988; Ambasta 1994), and extracts, fractions, and isolated constituents from seeds exhibited antiviral (Yadava and Tiwari 2005), anthelmintic (Prashanth et al. 2001), anti-implantation (Bhargava 1986), and antimicrobial activities (Mehta et al. 1983). Moreover, the flowers of this tree are also a good source of flavonoids and has shown anticonvulsant (Kasture et al. 2000), antihepatotoxicity (Wagner et al. 1986) properties. This tree species is also used for timber, resin, fodder, and dye (Reddy et al. 2001).

B. monosperma var. lutea is endemic to Deccan plateau of India. It has very fewer population, i.e., equaled or less than 100 plants across the plateau. Conservation Assessment Management Planning Workshop for Medicinal Plant of Andhra Pradesh, India (Jadhav et al. 2001), stated that *B. monosperma* var. lutea is a rare and endangered medicinal plant. Presently, it is under threat due to destructive harvesting of plant parts for medicinal use and for firewood, due to devastation of its natural habitat, and due to lack of knowledge about its scarcity (Mahender et al. 2014). Further, propagation of this plant is through seeds (Tandon et al. 2003), but it is hindered by poor germination percentage and less viability. The increasing demand for *B. monosperma* globally and decreasing availability has stimulated many researchers to propagate this pharmaceutically important plant through tissue culture approach. In vitro clonal propagation for large-scale production may therefore be advantageous for the germplasm conservation and cultivation of elite plants of *B. monosperma.* Understanding these problems and overwhelming curiosity in the techniques of plant tissue culture, the present reproducible and repeatable protocol was developed to produce large-scale desirable true to type plants in a limited time.

15.2 Propagation of *Butea monosperma* Var. Lutea

The propagation frequency of *B .monosperma* by seeds is very low, and viable nature of seeds is low and declared as globally endangered status. The cultivation has also been restricted because of the inherent heterozygosity. Selection of propagation technique for a plant depends on its genetic potential and its intended habit. Stem cutting approaches are also not useful to propagate in large scale. Application of biotechnology on in vitro propagation of *B. monosperma* is vital to increase the productivity of *B. monosperma.*

15.3 Tissue Culture of *B. monosperma* Var. Lutea

Plant tissue culture could allow the rapid multiplication and sustainable use of medicinal plants for forthcoming generations. In vitro propagation approaches have numerous advantages over conventional propagation. The achievement of tissue culture is connected to the best selection of explants. Nodal segments, meristems, shoot, and shoot tips cultures are the most frequently used culture types in micropropagation of plants. Explants from cotyledonary nodal segments are appropriate for improved axillary branching. Micropropagation of *B. monosperma* from cotyledonary nodal explants has been successfully utilized and developed (Rajesh et al. 2016).

15.4 Establishing Aseptic Culture

Aseptic culture methods are operative to eliminate the bacterial, fungal, and insect contaminants. To generate aseptic explant material, seeds of *B. monosperma* var. lutea were washed under running tap water for 15 min and then soaked in sterilized distilled water overnight. The soaked seeds were surface disinfected for 8 min in 1% (v/v) sodium hypochlorite solution (Qualigens, Mumbai, India) containing two drops of Tween-20 and rinsed five times with autoclaved, double-distilled water. The seeds were aseptically blot dried on sterile filter paper and then placed on Murashige and Skoog (MS; Murashige and Skoog 1962) medium supplemented with 3% sucrose (w/v) and 0.8% agar (w/v) (HiMedia®, Mumbai, India) (Fig. 15.1a). The pH of the medium was adjusted to 5.8 ± 0.02 prior to autoclaving at 121 °C and 104 KPa pressure for 15 min. The seeds were incubated at 25 ± 2 °C under 16-h photoperiod with a photosynthetic photon flux density of 50 μmol m − 2 s − 1 provided by cool white fluorescent tubes (40w, Philips, India) and with 50–60% relative humidity.

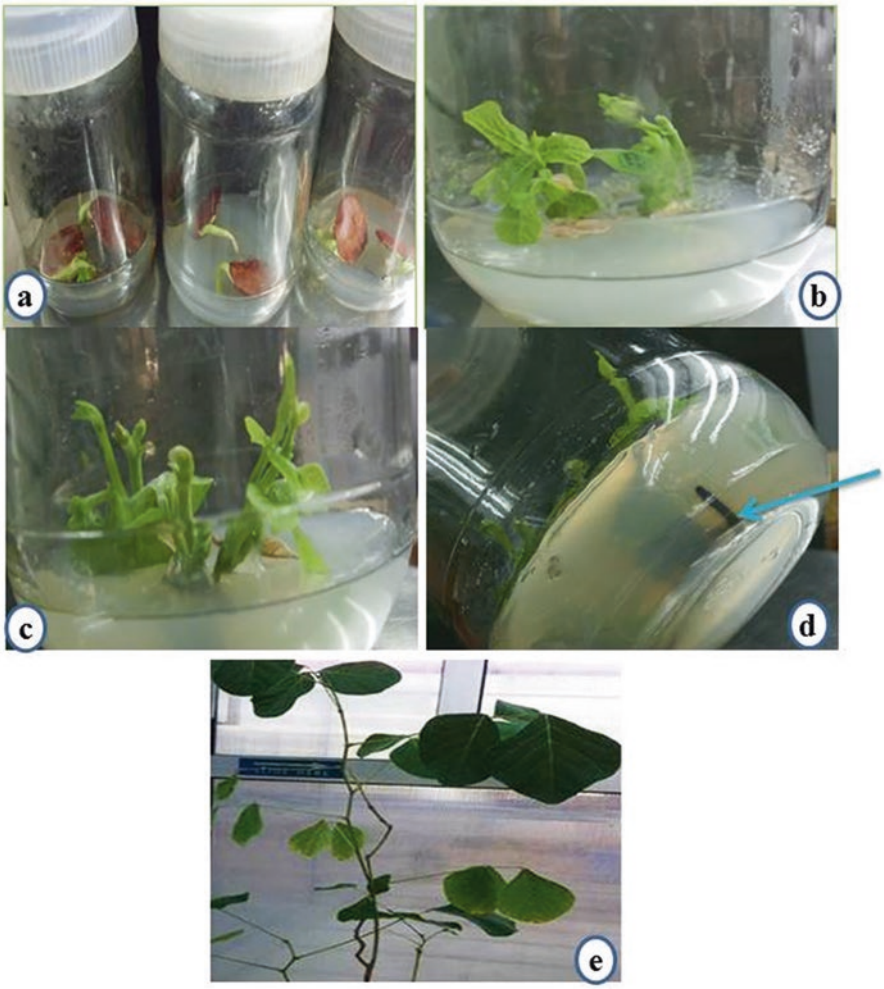

Fig. 15.1 Micropropagation of *Butea monosperma* (Lam.) Taub. var. lutea (Witt.): (**a**) In vitro seed germination on half-strength MS basal medium. (**b**) Multiple shoots induced from in vitro-derived cotyledonary node explants on MS medium augmented with 4.44 μM BAP. (**c**) Shoot elongation on half-strength MS medium augmented with 4.44 μM BAP. (**d**) In vitro rooting of shoots on ½ MS medium augmented with 2.46 μM IBA. (**e**) Well-acclimatized plant in greenhouse

15.5 Culture Medium and In Vitro Regeneration of Plants

Seeds were germinated on half-strength MS basal medium (Murashige and Skoog 1962) (Fig. 15.1a). After 3 weeks of culture on germination medium, cotyledonary nodes were aseptically excised from in vitro grown seedlings and cultured on shoot

induction medium (SIM) containing MS medium supplemented with different concentrations of BAP (2.20, 3.11, 4.44, and 6.66 μM), or Kn (2.32, 3.25, 4.65, and 6.97 μM) alone for 4 weeks. Following 30 d in shoot induction medium, the cultures were subcultured for an additional 3 weeks on the shoot elongation medium (SEM = 1/2 MS augmented with various concentrations of BAP (2.20, 3.11, 4.44, and 6.66 μM) for shoot elongation. Subsequently, they were subcultured for every 2 weeks onto new media comprising the similar composition. After 4 weeks of culture, the average number of shoots per explants and shoot length was noted.

Members of the Fabaceae family have usually been regarded as recalcitrant to in vitro regeneration (Trigiano et al. 1992; Jha et al. 2004). To establish an efficient in vitro regeneration protocol, we first examined the effect of auxin and cytokinins on direct shoot regeneration from cotyledonary node explants of *B. monosperma* var. lutea. The addition of various concentrations of BAP (2.20, 3.11, 4.44, and 6.66 μM) or Kn (2.32, 3.25, 4.65, and 6.97 μM) individually in the culture medium had substantial effects on the in vitro propagation of *B. monosperma*. Among the plant growth regulators that were assessed in the experiment, Kn was not effective for inducing shoots from cotyledonary nodes but BAP effectively induced shoots from cotyledonary explants. Almost all responses were with BAP, demonstrating that this plant growth regulator would be preferred for initiation of shoots. Rates of shoot induction and multiplication varied significantly by BAP concentration alone. Among the tested, 4.44 μM BAP induced a significantly higher number of shoots per explant and higher shoot length (Fig. 15.1b) than other BAP or Kn treatments alone. Increased or decreased concentration of BAP beyond the optimal concentration (4.44 μM) reduced the number of shoots produced, showed necrosis, and had shoot fasciation. In previous reports, BAP proved effective for shoot induction in several plant species (Margaret et al. 2015; Ramesh et al. 2005; Rout et al. 2008).

The reduction in shoot elongation is a common response described in numerous species when the optimal plant growth regulator concentration is added to the culture medium, and we also failed to elongate shoots on MS medium supplemented with optimum BAP (4.44 μM). Shoot length is a significant aspect for the achievement of ex vitro acclimatization since longer shoots may have more reserves to produce roots which promote higher survival rates. The lack of shoot elongation possibly resulted from the hyperhydricity of tissues, which might have been triggered by a hormonal imbalance in the culture medium. Shoot elongation was obtained upon transfer onto shoot elongation medium (SEM) (half MS supplemented with 4.44 μM BAP) and evaluated the shoot elongation by measuring the average shoot length after 3 weeks of culture. This response is similar to what has been reported in previous studies for shoot elongation using BAP (Rasool et al. 2009). The results of this experiment suggested that half-strength MS medium augmented with 4.44 μM BAP. BAP was superior to full-strength MS medium for shoot elongation (Fig. 15.1c). After 5 weeks of transfer to elongation medium, the elongated shoots developed fully expanded 4–5 leaves in healthy condition.

15.6 In Vitro Rooting and Acclimatization

In micropropagation studies, the preceding and critical phase is acclimatization of the rooted seedlings. Productive rooting of in vitro cultured micro-shoots is critical for the fruitful establishment of these shoots, both in the greenhouse and field. Regenerated shoots of 3 cm height with two or three leaflets were separated from the explants and transferred to full- and half-strength MS basal medium augmented with altered levels of indole-3-butyric acid (IBA; 2.46, 4.90, 7.36, and 9.80 μM). In vitro induction of roots from growing shoots has been achieved on half-strength MS medium supplemented with 2.46 μM IBA(Fig. 15.1d), and no rooting was observed on medium without IBA. Superiority of IBA over other auxins in root formation has also been reported (Bulle et al. 2012; Tippani et al. 2013). The rooted plants were removed from culture bottles and washed with water to remove media, then transferred to pots containing autoclaved sterilized soil and vermiculite (1: 1), and covered with polyethylene bags for 1 week to maintain high humidity and subsequently expose to low air humidity for increasing period, and finally polyethylene bags were removed gradually upon the emergence of new leaves in order to acclimatize the plant. These hardened plants are then transferred to the greenhouse (Fig. 15.1e). About 80% of the regenerated plants survived in the greenhouse.

15.7 Genetic Transformation of *Butea monosperma* Var. Lutea

Developments in tissue culture combined with advances in genetic engineering, specifically transformation technologies, have opened up new perspectives for production of pharmaceuticals from medicinal plants. The in vitro regeneration of whole plants from isolated cells and tissues is of great significance in obtaining transgenic plants. Plant genetic transformation technology has become an adaptable platform for cultivar improvement as well as for studying gene function in plants. The achievement in this area represents the result of many years of efforts in tissue culture and plant genetic engineering techniques improvement. Established in vitro regeneration protocol for the production of *B. monosperma* plants from cotyledonary explants has opened new challenge for genetic transformation of this important and valuable medicinal plant. For successful genetic transformation of plants, needs quick and reliable in vitro regeneration protocol. Therefore, this highly efficient and rapid regeneration protocol generates important prospective for engineering of *B. monosperma* with a view to comprehensive biomolecular studies or for further improvement of its medicinal properties. Developing protocols for an efficient genetic transformation of medicinal plants is an important tool to study the molecular basis and regulation of metabolic pathways.

15.8 Conclusions and Future Prospects

Biotechnology and within that in vitro propagation of medicinally important plants like *Butea monosperma* var. lutea appear to propose as boundless and unexploited source of secondary metabolites with high drug potentials. The principal significance of in vitro propagation of rare, critically endangered, and vulnerable species is to produce a large number of plants from single explant, without abolishing the mother plant. Therefore, these can contribute to restore natural habitats and conserve diversity of medicinal plants. Micropropagation is the central dogma for biotechnology because synthesis of various secondary plant metabolites cannot be done chemically. Plant cell and tissue culture could empower the rapid multiplication and sustainable use of medicinal plants for future generations.

Presented data and results in this chapter are intended at enlightening the potential of plant biotechnologies in the protection of valuable medicinal plant *Butea monosperma. Butea monosperma* biotechnology is in the beginning stage. A start toward the development of tissue culture regeneration protocols for this plant species is promising. Previously, we established the in vitro regeneration of this important plant using cotyledonary nodes derived from axenic seedlings. There is an urgent need of extensive research to be carried out for the establishment of regeneration protocols using different explants for the conservation and sustainable use of this valuable medicinal plant. Present improvement in tissue culture and genetic transformation of *B. monosperma* combined with biotechnological applications remain for the development of transgenic plants. Pharmacologically active compounds from *B. monosperma* represent the importance of plant secondary metabolites and also have opened up new perspectives for genetic engineering of this tree species. Tissue culture is a promising substitute for the production of rare and high-value secondary metabolites of medical importance for this tree species. A prerequisite would be to optimize culture conditions from plant tissue like leaf and somatic embryogenesis from different explants that can be easily amenable to tissue culture as well as genetic transformation studies for improvement of secondary metabolites and biomolecule modifications.

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Chapter 16
Cryopreservation of Medicinal Herbs: Major Breakthroughs, Hurdles and Future

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Abstract Throughout the advancement of human civilization, medicinal plants have contributed an immense role. The resources for traditional medicines and most of the modern medicines are obtained from medicinal plants. With the ever-increasing requirement for secondary metabolites, the medicinal plants are exploited all over the world, leading to the threat of their extinction. To combat the situation, there is an urgent need to establish a plan for their long-term conservation. Storage under in vitro cultures, though advantageous, is costly and susceptible to microbial contamination and somaclonal variation. The most competent and economical technique for long-term conservation is cryopreservation (in liquid nitrogen at −196 °C). Traditional techniques of cryopreservation were relied on freeze-induced dehydration and applied for conserving shoot apices and undifferentiated in vitro cultures of cold-tolerant medicinal plant species. However, ice crystal formation usually occurs inside the cell, which is detrimental to cellular structure integrity. As a result, vitrification-mediated new cryopreservation techniques were efficiently employed with all types of the explant. Besides, only small number of medicinal plants could be cryopreserved for their germplasm conservation, and continued efforts to establish cryopreservation protocols for a large array of medicinal plant species are needed. The objective of the present book chapter is to appraise the work carried out in the last one decade employing the diverse cryopreservation methods for the conservation of medicinal plants.

Keywords Cryopreservation · Desiccation · Encapsulation · Long-term conservation · Medicinal plants · Vitrification

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16.1 Introduction

Plants are the origin of numerous medicines or their precursors utilized in the recent medicinal system (Goyal et al. 2008). Effective, trouble-free and broad-spectrum activities are the major advantage of medicinal plant-based drugs with numerous remedial actions. Products obtained from medicinal plants have also proved helpful in lowering the undesirable effects of chemotherapeutic agents (Gómez-Galera et al. 2007; Leonard et al. 2009). Medicinal plants are the existing resource, which is exhaustible if exploited and sustainable if used judiciously. Presently, medicinal plants are mainly harvested from their wild habitat in an exhaustive way, which eventually led to the depletion of the natural population. Industries based on medicinal plants even though are age-old yet run on traditional views and methods. This has caused ineffective, inconsistent and unceremonious promotion of medicinal plants, and, consequently, the supply of raw material situation is untenable and exploitative.

Conservation of the germplasm is presently at the front position of protection acts, and biotechnology has played a significant role (Benson 1999). By tradition, germplasm conservation in the form of seeds at low temperature or in the form of field plantations (field gene banks) was considered as an ideal method. Recently, cryopreservation, i.e. storage of tissues (generally with high meristematic zone and low water content, e.g. shoot tips, seeds, pollen, zygotic or somatic embryos along with callus and cell suspension cultures) at ultra-low temperature in liquid nitrogen (LN; −196 °C), has been recognized as the greatest choice for durable conservation of medicinal plant species (Touchell 2000; Engelmann 2000). This technology is developing speedily over the last three decades and is supposed to be the most thriving for long-term storage. The metabolic activities and cell divisions are arrested at this temperature, thus supporting for long-duration storage. The benefit of this technique is the decrease of cost estimate for in vitro culture, essential space, infectivity and the chances of somaclonal variation. Cryopreservation of embryogenic cells could be an important tool in genetic transformation processes. Furthermore, reducing the number of pathogens is also a significant achievement as proven with *Pelargonium* L'Hér (Gallard et al. 2011). Accordingly, cryopreservation is gaining progressive interest among the researchers and has been effectively employed for the conservation of many medicinal species (Ozden-Tokatli et al. 2007; Popova et al. 2010; Yamamoto et al. 2012a, b; Sharma et al. 2017). The implication of cryopreservation for conserving medicinal plant species is less, though increasing every year. In this chapter, diverse methods of cryopreservation, their successes and their pitfalls during the course of conservation of medicinal species, over the past 10 years, are discussed.

16.2 Importance of Medicinal Plants

Conventional medication system has always remained the affordable and acceptable foundation of the primary health treatment in poor communities to the great extent. Since long, the indigenous people apply herbal medicine that is prepared from the

medicinal plant. The literature reviews reveal that the therapeutic exercise of plants was first applied in China and is as old as 5000–4000 B.C. However, in India, the use of plants as medicine is believed to have started from 3500–1600 B.C. according to the Rig-Veda. Subsequently, the ancient physicians studied the detailed properties of medicinal plants that led to the basic foundation of medical science in India (Prakash and Gupta 2005). Even though modern medicine may exist side by side, in most of the developing countries, a large section of patients depend on the traditional practitioners so as to fulfill their healthcare needs (Vishwakarma et al. 2013). Natural compounds have their own significance based on (1) novel chemical constituents of broad structural diversity, (2) treatment or prevention of many diseases and (3) their disease treatment frequency. Currently, plant research has again started focusing on updating the information on the chemistry of natural products and exploring the applicability of traditional medicine (Newman and Crag 2007). Such insurgence in the use of herbal medicines is occurring due to the requirement of new therapeutics; presence of many natural secondary metabolites having unique diversity in both chemical and biological characteristics; the efficacy of novel natural bioactive compounds functioning as biochemical probes; the development of new techniques to identify biologically active natural products; enhanced techniques for isolation, purification and structural characterization of the active compounds; and progress in solving the requisite supply of the complex natural products. The significance of traditional medicine has been recognized by the World Health Organization (WHO), which has formulated standard guidelines and strategies for botanical medicines. Application of established technologies in the agro-industrial sector is needed to cultivate and process medicinal plants followed by the synthesis of herbal medicines (WHO 1993). Since the contemporary medicines are manufactured from medicinal plants, they are treated as a vital source of new drugs. Thus the research on medicinal plants would help to comprehend plant toxicity besides protecting human and animals alike from natural poisons.

16.3 Requirement of Conservation of Medicinal Plants

The utilization of medicinal plants is rising promptly all over the globe due to the escalating requirement for plant-based medicines and natural health products (Nalawade et al. 2003; Cole et al. 2007). Unfortunately, our planet has been facing a potential drug loss every second year due to the natural destruction rate of medicinal plants being 1000 times greater than the expected trend as per a new conservative estimate. Around 50,000–80,000 species of medicinal plants exist as assessed by the IUCN and the WWF. From there around 15,000 species are already threatened owing to habitat destruction and overharvesting (Bentley 2010). Furthermore, population explosion consumes another 20% of these plant resources (Ross 2005). Based on the exhaustive studies regarding sustainable use and conservation of medicinal plants, several suggestions have been compiled (Larsen and Olsen 2007; Uprety et al. 2012). However, only certain recommendations have been executed for

their conservation and sustainable use such as in situ conservation in botanic gardens and other natural reserves. Biotechnological advancements have offered extensive accumulation of natural products, progresses in plant tissue culture and elicitation technique that eventually is resulting in an efficient synthesis of desirable bioactive compounds. Application of plant cell and genetically transformed hairy root culture has provided an advantageous substitute for the synthesis of atypical and costly secondary metabolites of therapeutic use (Rao and Ravishankar 2002). Higher in vitro regeneration rates, a suitable system of transportation and storage of germplasms have been made feasible using micropropagation and encapsulation of tissue/organ in the form of synthetic seed (Baker et al. 2007; Gantait et al. 2017a, b; Gantait and Kundu 2017). Additionally, the synthetic seed technology is a viable alternative for propagation in case of inadequate production of typical seeds (Lata et al. 2008; Gantait et al. 2015a). But till today, cryopreservation is the sole technology available for conservation of problem species germplasm for a longer duration (Suranthran et al. 2012; Sinniah et al. 2014; Gantait et al. 2015b; Palanyandy et al. 2015; Gantait et al. 2016).

16.4 Factors to Be Considered Before Cryopreservation and Influence of Cryoprotective Agents

For the successful cryopreservation, numerous factors are responsible such as healthy cultures and no signs of microbial infection, cultures at log phase of growth and use of cryoprotectant like glycerol or dimethyl sulphoxide (DMSO) to inhibit the development of intracellular ice crystals. During cryopreservation, cell damage occurs due to two key events, viz., the formation of large ice crystals inside the cell that rupture organelles and ultimately the cell itself, followed by the rise in solute concentration to lethal intensity on account of prefreezing dehydration. Cryoprotectants such as proline amino acid, sucrose, glycols, sugar alcohols, dextrans and polyethylene glycol reduce cryo-injury. Among these chemicals, glycerol, sucrose, proline and DMSO are most commonly used (Fuller 2004). DMSO is an exceptional cryoprotectant reason being its low molecular weight, easy dispersion in solvent, easy penetration inside the cells and ability to be easily washed away. Cryoprotective additives are compounds that minimize cellular damage caused by freezing and thawing. These are used because of their non-toxicity, smooth permeation within the cells and less molecular weight. These additives are allowed to interact with cells in greater concentration during freezing instead of high ionic concentration so as to reduce cell damage. In order to reach an equilibrium in intracellular concentration, cells are incubated in cryoprotective additive prior to freezing, which is a usual practice, and all these compounds have almost the same effect when used in similar concentration. The difference may arise in various cell types in respect to the protective competence presumably owing to the divergent permeability and comparative cellular toxicity in different cell types (Mazur 1984). A cryoprotective substance like DMSO and glycerol when diffused into a cell partially displaces

intracellular water to avoid dehydration as a result of ice crystal formation during freezing (Fuller 2004). Glycerol helps in sustaining the stabilized natural state of proteins and also maintains critical interactions at macromolecular level even below zero degree temperature. The cryoprotective agent and the cell culture media should be combined separately to prepare a cryoprotectant. Optimum levels of cryoprotective agents vary depending on the various cell types but usually range from 5 to 15% (v/v). Always best possible quality of cryoprotective agents should be used, and in order to avoid contaminants, storage of cell/tissue in small quantities is necessary. It has been observed that air quickly oxidizes the DMSO to produce toxic wastes in cells. Hence, preservation is done by placing the material (to be preserved) in a growth medium followed by treating it with a cryoprotectant (5–10%), and then it was transferred to polypropylene ampoules or sterile cryovials closing firmly using screw cap. Non-electrolyte solutes such as sugars are not used frequently since they change the extracellular salt concentration. The presence of impervious solutes in higher concentrations before freezing affects the cellular osmotic stress. But using glycerol, which is a membrane-permeable solute, reduces any such chance because in a hypertonic glycerol medium, water comes out of the cells and glycerol enters on account of change in osmotic pressure. The cells will finally regain their original volume sometime after equilibration due to less osmotic stress enforced by a hypertonic glycerol solution than that of by hypertonic sugar solution. Thus at higher concentrations, glycerol would perform better than sugars without injuring the cells. When the preconditioning medium contains glycerol in significant concentration, some amount of intracellular and extracellular water gets swapped by glycerol, which results in less ice formation. So the extent of cell shrinkage is restricted, along with the unfrozen solution containing less electrolyte concentration in the cells. DMSO and ethylene glycol being membrane permeable also function in a similar manner. Glycerol and several other polyols deliver cryoprotection by additional mechanisms also, such as lipid membrane stabilization facilitated by hydrogen bonding with the polar head groups of membrane lipids (Crowe et al. 1984), a useful mechanism during severely dehydrated period. However, the characteristics of unfrozen portion particularly its glass-forming tendency and viscosity may be affected by these substances. The membrane's relative permeability to both cryoprotectant and water along with the cryoprotectant concentration determines the extent of cell shrinkage and re-swelling.

16.5 Principles of Cryopreservation

Specific genes, when get activated, provide distinct defensive mechanisms that allow various species of plants to endure sub-zero temperatures. But plants at the tropics lack such abilities, and this had been a serious problem for cryobiologists until the end of the twentieth century when some revolutionary discoveries enabled for the application of cryopreservation techniques. In the case of cold-tolerant dormant buds and conventional seeds, direct cryopreservation bypassing pretreatment

is possible as water present is less. Although in many cases, where experimental tools such as embryos, shoot tips, calli and cell suspensions are used for cryopreservation, cells need to be manually desiccated in order to prevent injuries arising when large amount of intracellular water gets crystallized into ice as they are naturally and exceedingly intolerant to freezing. Both the physical mechanisms and techniques involved in the cryopreservation process vary between conventional and the modern approach (Engelmann 1998). Conventional techniques comprise freeze-induced dehydration, where cooling to a set temperature (prefreezing) succeeded by quick immersion in LN is done, while modern techniques involve vitrification, where conversion of water takes place straight from liquid state to an amorphous state bypassing the crystalline phase. Conventional operations are normally complex since these make use of costly and sophisticated programmable freezers. In some cases, this procedure can be eluded using either laboratory or domestic freezer (Kartha and Engelmann 1994). In vitrification-based procedures, samples are either exposed to concentrated cryoprotective media or subjected to air desiccation for cell dehydration, and this step precedes freezing. Then rapid cooling takes place. Hence, the factors responsible for intracellular ice formation are evaded. By applying thermal analysis, glass transitions can be recorded during both cooling and rewarming. There are some practical advantages of vitrification-based procedures when compared to conventional freezing techniques. For complex organs such as shoot tips and embryos containing diverse categories of cells, each of them has exclusive requirements under freeze-induced dehydration state. As the ice formation is impeded in the system itself, the operational intricacy is also reduced in the case of vitrification-based procedures with respect to conventional approach, thus showing greater possibility for large-scale usage, following some small adjustments based on various cell types (Engelmann 1998). A conjoint feature based on the new protocols is the critical step of dehydration instead of freezing step in order to attain higher survival in comparison to conventional protocols. Five types of fundamental procedures have been identified (illustrated in Fig. 16.1) for the conservation of medicinal plants: (1) desiccation, (2) vitrification, (3) droplet-vitrification, (4) encapsulation-dehydration and (5) encapsulation-vitrification (Table 16.1). For selection of the particular method for cryopreservation, several criteria like the survival percentage, rate of regeneration, duration of the procedure and the problems encountered with explant handling must be considered (Gallard et al. 2006).

16.5.1 Desiccation

Sometimes prefreezing open drying of the explants over silica gel or under aseptic airflow for 1–5 h and then osmotic desiccation is the prerequisite for conducting an appropriate cryopreservation procedure as proven with *Paeonia lactiflora* (Seo et al. 2007), *Pistacia* sp. (Ozden-Tokatli et al. 2007), *Ginkgo biloba* (Lu et al. 2009; Popova et al. 2009) and *Coriandrum sativum* (Popova et al. 2010). This method has been applied to a large number of recalcitrant and intermediate seeded species and

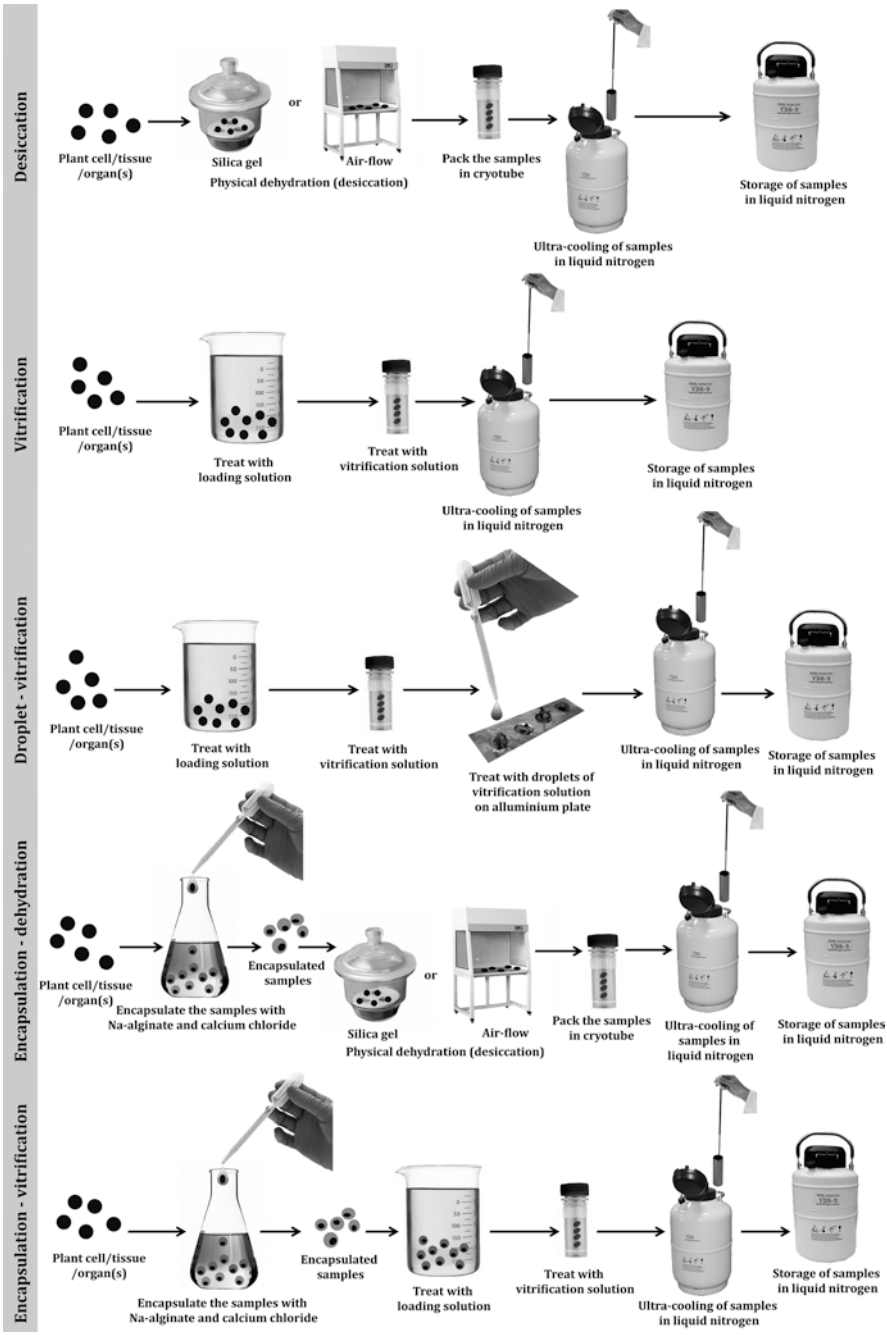

Fig. 16.1 Fundamental procedures (desiccation, vitrification, droplet-vitrification encapsulation-dehydration and encapsulation-vitrification) that have been adopted for successful cryopreservation of medicinal plants

also involved seeds, calluses, somatic embryos or cell cultures (Engelmann 2000). Lu et al. (2009) described the influence of preculture by means of sucrose and abscisic acid (ABA) on desiccation and cryopreservation tolerance of cell cultures of *G. biloba*, whereas a preculture of calli for 21 days on Murashige and Skoog (MS) medium (Murashige and Skoog 1962) added with 10% sucrose and 2 mg l^{-1} ABA followed by drying for 150 min resulted in the highest post-thaw regrowth. Popova et al. (2009) also confirmed the same result and found that the preculture of callus clumps on 100 g l^{-1} sucrose and 2 mg l^{-1} ABA supplemented MS medium for 14 days assisted in the accretion of endogenous soluble sugars that was necessary for post-thaw revival. Preculture of explants in the osmotically active compounds like mannitol, proline, sugars and sugar alcohols helps plant cell desiccation and, therefore, believed to be indispensable for attaining higher regrowth after cryopreservation (Sakai and Engelmann 2007). Additionally, in the induction of tolerance to desiccation, ABA has been known to be crucial for thriving cryopreservation (Garliardi et al. 2003). Ozden-Tokatli et al. (2007) performed the desiccation-cryopreservation employing the seeds of *Pistacia* spp., i.e. *P. terebinthus*, *P. vera* and *P. lentiscus*, which led to different regrowth percentages. Following 8 h drying on silica gel (subsequent to 11.7% moisture content on a fresh weight basis) and direct immersion in LN, a maximum of 90% germination was obtained in *P. vera*. Besides, shorter periods of desiccation (60 min and 15 min) were adequate to decrease their moisture content to about 20% in *P. terebinthus* and *P. lentiscus*, which resulted in seed germination of 16% and 47%, respectively, following cryostorage. Even though the method of desiccation is fast and easy, its application is still inadequate to orthodox seeds, embryos, pollen and shoot tips of desiccation-tolerant plants (Kulus and Zalewska 2014).

16.5.2 Vitrification

The last decade has seen inventions of new methods of cryopreservation on the basis of vitrification (Table 16.1). Earlier, Fahy et al. (1984) defined the term 'vitrification' as 'the solidification of a liquid brought about not by crystallization but by an extreme elevation in viscosity during cooling'. In vitrification, exclusion of maximum or all of the freezable water by mechanical means or through osmotic dehydration is carried out, and then, the subsequent ultra-rapid freezing leads to vitrification of intracellular solutes. The vitrification can be performed easily with high recovery percentage, with increased applicability particularly to the plant species that are susceptible to low temperature. According to Reed et al. (2005), an increase in cellular viscosity occurs during vitrification; thus, a wide range of plant species is capable to endure lethal osmotic and evaporative dehydration stresses. This procedure has effectively been used for an array of medicinal plant species, using shoot tip (Skyba et al. 2010; Brŭnakova et al. 2011; Serrano-Martínez and Casas 2011; Shatnawi 2013; Maki et al. 2015; Sharma et al. 2017), nodal segments (Ray and Bhattacharya 2008; Li et al. 2009), adventitious root (da Silva Cordeiro et al. 2015a, b), seed

Table 16.1 Different cryopreservation techniques and their outcomes, reported during long-term conservation of various medicinal plant species

Scientific name	Explant	Cryopreservation method	Regrowth medium	Regrowth (%)	References
Dioscorea floribunda	NS	Encapsulation-dehydration	MS + 1.5 mg l^{-1} BA +0.2 mg l^{-1} NAA + 0.2 mg l^{-1} GA_3	87	Mandal and Ahuja-Ghosh (2007)
Dioscorea deltoidea	ST	Vitrification	MS + 1.5 mg l^{-1} BA +0.2 mg l^{-1} NAA + 0.2 mg l^{-1} GA_3	83	Mandal and Dixit-Sharma (2007)
		Encapsulation-dehydration		76	
Pistacia vera, *P. terebinthus* and *P. lentiscus*	Seed	Dehydration	MS	90, 16 and 47	Ozden-Tokatli et al. (2007)
Paeonia lactiflora	ST	Dehydration	MS + 1 mg l^{-1} GA_3 + 0.5 mg l^{-1} BA	74.9	Seo et al. (2007)
Gentiana tibetica and *G. cruciata*	Pro-ECa	Encapsulation-dehydration	–	68, 83	Mikuła et al. (2008)
Clitoria ternatea	SE	Encapsulation-dehydration	½MS + 0.5 mg l^{-1} GA_3 + 0.2 mg l^{-1}	55	Nair and Reghunath (2008)
Rauvolfia serpentina	NS	Vitrification	MS + 8.8 µM BA	66	Ray and Bhattacharya (2008)
Colocasia esculenta var. *esculenta*	ST	Droplet-vitrification	MS	73–100	Sant et al. (2008)
Astragalus membranaceus, *Gentiana macrophylla* Pall. and *Eruca sativa* Mill.	HR	Encapsulation-vitrification	½MS	82.9, 75.7 and 100	Xue et al. (2008)
Catharanthus roseus	ECa	Vitrification	MS + 6.62 µM BA +5.37 µM NAA	88.9	Fatima et al. (2009)
Vanilla planifolia	In vitro explant	Droplet-vitrification	MS + 1 mg l^{-1} BA +0.5 mg l^{-1} IBA	10	Gonzalez-Arnao et al. (2009)
Dioscorea bulbifera	Ca	Vitrification	MS + mg l^{-1} kinetin +0.5 mg l^{-1} NAA	60-70	Hong et al. (2009)
Maesa lanceolata	HR	Encapsulation-dehydration	SH	90	Lambert et al. (2009)
M. truncatula			MS	53	
Dioscorea opposita	NS	Vitrification	MS + mg l^{-1} kinetin +0.02 mg l^{-1} NAA	77.1	Li et al. (2009)

(continued)

Table 16.1 (continued)

Scientific name	Explant	Cryopreservation method	Regrowth medium	Regrowth (%)	References
Ginkgo biloba	Ca	Desiccation	MS + 5 mg l^{-1} NAA	54	Lu et al. (2009)
Dioscorea bulbifera	ECa	Encapsulation-dehydration	MS + 2 mg l^{-1} 2,4-D + 0.3 mg l^{-1} BAP	67.8	Mandal et al. (2009)
Indigofera tinctoria	Axillary shoot	Encapsulation-dehydration	MS + 0.1 mg l^{-1} GA_3 + 0.2 mg l^{-1} BA	62.2	Nair and Reghunath (2009)
Panax ginseng	AR	Desiccation	MSL + IBA	67	Oh et al. (2009)
		Vitrification		60	
Ginkgo biloba	Ca	Desiccation	MS + 2 mg l^{-1} ABA	23	Popova et al. (2009)
Dendrobium candidum	PLB	Encapsulation-vitrification	MSL + 0.2 mg l^{-1} NAA + 0.5 mg l^{-1} BA	85	Yin and Hong (2009)
Rubia akane	HR	Vitrification, Droplet-vitrification	½ MS	95	Kim et al. (2010)
Thymus moroderi	ST	Droplet-vitrification	MS + 0.275 μM BA	48.5	Marco-Medina et al. (2010)
Coriandrum sativum	SE	Desiccation	MS + 1 mg l^{-1} GA_3	98.2	Popova et al. (2010)
Alpinia nigra	ST	Vitrification	MS + 1 ppm TDZ	27	Rungjindamai et al. (2010)
Dioscorea bulbifera	ECa	Encapsulation-vitrification	MS + 2 mg l^{-1} kinetin +0.5 mg l^{-1} NAA	70	Yin and Hong (2010)
Hypericum perforatum	ST	Vitrification	RM + 0.5 mg l^{-1} BA	32	Skyba et al. (2010)
Mantisia spathulata	Seed	Dehydration	MS + 4.3 μM GA_3	40	Bhowmik et al. (2011)
M. wengeri			MS + 7.2 μM GA_3	36.6	
Hypericum perforatum	ST	Vitrification	RMB	71.3	Brůnakova et al. (2011)
Tetraclinis articulata	ST	Vitrification	SH	–	Serrano-Martínez and Casas (2011)

Artemisia herba-alba	ST	Encapsulation-dehydration	MS	70	Shatnawi (2011)
Stevia rebaudiana	ST	Vitrification	MS	65.6	Shatnawi et al. (2011)
Panax quinquefolius	Cotyledon	Vitrification	½ SH + 5 mg l^{-1} BA +0.5 mg l^{-1} IBA + 10 mg l^{-1} GA_3 + 1% AC	–	Uchendu et al. (2011)
Dendrobium nobile	Seed	Vitrification	½ MS	68	Vendrame and Faria (2011)
Rhodiola crenulata	Ca	Vitrification	MS + 5 µm BA	72.2	Zhao et al. (2011)
Rabdosia rubescens	ST	Encapsulation-dehydration	MS + 1 mg l^{-1} BA +0.05 mg l^{-1} IAA	85.6	Ai et al. (2012)
Hypericum richeri spp. *Transsilvanicum, H. umbellatum*	Axillary buds	Droplet-vitrification	MS	68, 71	Coste et al. (2012)
Rubia akane	HR	Droplet-vitrification	½ MS	86	Kim et al. (2012)
Dendrobium nobile	PLB	Encapsulation-dehydration	½ MS + 1.0 mg l^{-1} BA +0.1 mg l^{-1} NAA	50.2	Mohanty et al. (2012)
		Encapsulation-vitrification		75.9	
Teucrium polium	ST	Encapsulation-dehydration	MS	20	Rabba'a et al. (2012)
Anemarrhena asphodeloides	ECa	Vitrification	MS +2 mg l^{-1} kinetin +0.5 mg l^{-1} NAA	60	Hong and Yin (2012a)
Artemisia herba-alba	ST	Encapsulation-dehydration	MS	6	Sharaf et al. (2012)
		Encapsulation-vitrification		12	
Kalopanax septemlobus	ECa	Vitrification	MS + 0.1 mg l^{-1} 2,4-D	99.3	Shin et al. (2012)
		Droplet-vitrification		94.2	

(continued)

Table 16.1 (continued)

Scientific name	Explant	Cryopreservation method	Regrowth medium	Regrowth (%)	References
Mentha spp.	ST	V-cryo-plate procedure	MS	73-100	Yamamoto et al. (2012a, b)
Cymbidium eburneum and *C. hookerianum*	PLB	Encapsulation-dehydration	MS	72 and 70	Gogoi et al. (2013)
Saussurea involucrata	Ca	Vitrification	MS + 12.5 μM BA +2.5 μM NAA	40	Guo et al. (2013)
Allium sativum	ST	Encapsulation-dehydration	MS	36	Mahajan et al. (2013)
Dendrobium chrysanthum	PLB	Encapsulation-vitrification	MS + 1 mg l^{-1} BA +0.5 mg l^{-1} NAA	59.9	Mohanty et al. (2013)
Rubia akane	HR	Droplet-vitrification	MS	–	Salma et al. (2014a)
Anemarrhena asphodeloides	ECa	Encapsulation-vitrification	MS + 2 mg l^{-1} kinetin +0.1 mg l^{-1} NAA	80	Hong and Yin (2012b)
Achillea millefolium	ST	Vitrification	MS	80	Shatnawi (2013)
Parkia speciosa	ZE	Desiccation	MS + 2 g l^{-1} AC + 0.1 mg l^{-1} NAA + 0.1 mg l^{-1} BA +0.1 mg l^{-1} GA_3	0	Sinniah and Gantait (2013)
		Vitrification		55.5	
Byrsonima intermedia	ST	Droplet-vitrification	MS	67	Silva et al. (2013)
Ziziphora tenuior	ST	Encapsulation-dehydration	–	20	Al-Baba et al. (2015)
		Encapsulation-vitrification		10	
Clinopodium odorum	ST	V-cryo-plate	MS	71	Engelmann-Sylvestre and Engelmann (2015)
Cleome rosea	AR	Vitrification	MS + 0.25 mg l^{-1} NAA	63.6	da Silva Cordeiro et al. (2015a, b)
	ST	V-cryo-plate	MS + 0.5 mg l^{-1} BA	100	
Wasabia japonica	ST	Vitrification	½ MS	–	Maki et al. (2015)

Atractylodes macrocephala	ST	Droplet-vitrification	MS + 0.3 mg l^{-1} BA +0.02 mg l^{-1} NAA + 0.1 mg l^{-1} GA_3	62	Zhang et al. (2015)
Dioscorea opposita	NS	Encapsulation-vitrification	MS	52	Zhao et al. (2016)
Petiveria alliacea	SE	Vitrification	MS	12 SEs/SE	de Almeida Pettinelli et al. (2017)
		Encapsulation-dehydration		2 SEs/SE	
		D-cryo-plate		19 SEs/SE	
Miconia ligustroides	Seed	Vitrification	MS + 1 mg l^{-1} AC	70	de Oliveira Prudente et al. (2017)
Bacopa monnieri	ST	Vitrification	MS + 0.2 mg l^{-1} BA	20	Sharma et al. (2017)

2,4-D 2,4-dichlorophenoxyacetic acid, AC activated charcoal, AR adventitious root, BA N^6-benzyladenine, Ca callus, ECa embryogenic callus, GA_3 gibberellin A_3, IBA indole-3-butyric acid, Kinetin 6-furfurylaminopurine, MS Murashige and Skoog medium (Murashige and Skoog 1962), MSL Murashige-Skoog liquid medium, NAA α-naphthalene acetic acid, NS nodal segment, HR hairy root, PLB protocorm-like body, RMB revised Linsmaier-Skoog's medium (Linsmaier and Skoog 1965), SE somatic embryo, SH Schenk and Hildebrandt medium (Schenk and Hildebrandt 1972), ST shoot tip, TDZ *N*-phenyl-*N′*-(1,2,3-thidiazol-5-yl) urea or thidiazuron, ZE zygotic embryo, '–' information not available

(Vendrame and Faria 2011), embryogenic calli (Fatima et al. 2009; Hong and Yin 2012a, b) and somatic embryos (de Almeida Pettinelli et al. 2017). During this method, to persuade desiccation tolerance, the explants are generally incubated on nutrient medium containing high sucrose (0.3 M or 0.5 M) concentration and consequently moved to loading solution comprising of 2 M glycerol plus 0.4 M sucrose (Sakai 2000). Fatima et al. (2009) evaluated the different concentrations of sucrose (0.09–0.6 M) and sorbitol (0.2–0.6 M) throughout preculture and found that highest recovery was promoted by 0.4 M sucrose. The explants were then introduced to highly dense cryoprotectant solutions for 15 min up to 2 h, followed by a straight dip into LN (vitrification/one-step freezing). The most accepted plant vitrification solution is PVS2 that consists of 30% (w/v) glycerol, 15% (w/v) ethylene glycol (EG), 15% (w/v) DMSO and 0.4 M sucrose (Hong et al. 2009; Serrano-Martínez and Casas 2011; Shatnawi 2013; Maki et al. 2015; de Almeida Pettinelli et al. 2017; Sharma et al. 2017). PVS3, containing 50% glycerol (w/v), 1.46 M sucrose and devoid of any toxic DMSO, is also known to be used (Brunakova et al. 2011; Skyba et al. 2010; da Silva Cordeiro et al. 2015a, b; Ghaffarzadeh-Namazi et al. 2017). It is important to note that da Silva Cordeiro et al. (2015a, b) found that cryopreserved shoot tips exhibited high survival and regeneration rates when treated with PVS2 than with PVS3. On the other hand, Skyba et al. (2010) also compared both the plant vitrification solutions (i.e. PVS2 and PVS3) by treating the shoot tips of *Hypericum perforatum* L. that revealed an increase in recovery of explants cryoprotected with PVS3. There was an increase in the level of lipid peroxidation in the PVS2-treated regenerants representing membrane damage. Gamez-Pastrana et al. (2011), however, observed that PVS2 removed more freezable water in comparison to PVS3 under the unchanged treatments. da Silva Cordeiro et al. (2015a, b) tested the varied treatment duration (0, 15, 30, 60, 90, 120 or 150 min) in PVS2 solution and noticed a significant raise in the survival rate from 30 min (20%) to 60–150 min (84–97%) of exposure. Similarly, Serrano-Martínez and Casas (2011), Hong and Yin (2012a, b) and Shatnawi (2013) confirmed that the regrowth rate obtained from shoot tips treated with PVS2 at 0 °C for 60–80 min gradually increased from 76 to 80%. The report of de Almeida Pettinelli et al. (2017) depicts that employing the vitrification technique, PVS2-treated somatic embryos, resulted in high post-freezing viability (85%) and considerable proliferation in comparison to the encapsulation-dehydration technique. Ray and Bhattacharya (2008) assessed the impact of size and type of explants, role of exposure duration and concentration of sucrose preculture and vitrification on post-freezing recovery. Preliminary experiments involving PVS1, PVS2 and PVS3 were conducted by them, wherein they found that only PVS2 induced shoot growth. Nodal segments of 0.31–0.39 cm in size were superior to other sizes of nodes or shoot tips. Ghaffarzadeh-Namazi et al. (2017) concluded that cryopreserved callus showed maximum regrowth percentage (98.7%) following PVS3 treatment than in the other three protocols. Generally, PVS2 can be employed with those explants which are tolerant to chemical toxicity and intermediate in size, whereas PVS3 can be employed with large-size explants which are especially sensitive to chemical toxicity or tolerant to osmotic stress (Kim et al. 2009). The optimal duration of exposure for PVS2 varies between 15 and 30 min (de Almeida Pettinelli et al.

2017; Sharma et al. 2017). Moges et al. (2004) and Baghdadi et al. (2011) proposed a solution to the problem of DMSO toxicity, suggesting the use of PVS2 gradient. Further, a diluted (60%) PVS could be used for longer time that also reduces costs (Halmagyi et al. 2004; YaJun et al. 2009). In the same way, substitute to the regular PVS, decreased DMSO or increased sucrose and glycerol concentration can be useful (Kim et al. 2009).

16.5.3 Droplet-Vitrification

Droplet-vitrification is the modified form of the vitrification technique (Table 16.1). The procedure involves pretreated explants in LS and put on aluminium foil strips in a drop of PVS before LN immersion (Gantait et al. 2015c). The technique is carried out on the basis of aluminium's higher ability for thermal conduction, which is responsible for increased pace of freezing/thawing. For accelerating the procedure, explants could be put in the droplets of vitrification solution to perform the dehydration step (Gonzalez-Arnao et al. 2009; Kim et al. 2010, 2012). The handling of explants can be avoided during insertion and extraction of foil strip from the cryovial and thus reducing the chances of tissue injury, which emerges as an advantage. Droplet-vitrification has confirmed faster rates of freezing and thawing comparing to the lone vitrification method (Sakai and Engelmann 2007; Gantait et al. 2015c). Zhang et al. (2015) found as high as 76% and 62% survival and regrowth levels following cryopreservation in the Chinese medicinal plant *Atractylodes macrocephala* Koidz cultivar 'Baizhu'. Thermal analysis by means of differential scanning calorimetry recommended that the treatment with PVS2 serves as a vital part for effective cryopreservation using droplet-vitrification. Kim et al. (2012) reported that *Rubia akane* roots are very sensitive to the preculture in sucrose-supplemented medium (up to 0.5 M sucrose) and dehydration at highly concentrated VS. Hence, treatment with LS was essential before dehydration of explants with VS, and the composition significantly affected their post-thaw regrowth. Owing to root's high sensitivity to both osmotic stress and biochemical cytotoxicity of VSs, cryoprotection with alternative VSs, such as 15 min exposure to B5-80% (40% glycerol and 40% sucrose, w/v) at 25 °C or 20 min exposure to A3-70% (29.2% glycerol + 11.7% DMSO + 11.7% EG + 17.4% sucrose, w/v) at 0 °C, confirmed the maximum rate of post-freezing revival. Higher freezing and rewarming rates achieved with droplet-vitrification by the use of foil strips have resulted to higher rate of post-cryopreservation regeneration (86%) compared to vitrification by using cryovials (59%). Salma et al. (2014a) exclusively studied the effect of the droplet-vitrification steps on the characteristics of cortical, pericycle and endoderm cells of apical as well as central segments of hairy roots of *Rubia akane*. In the apical root segments, plasmolysis (22–71%) was noticed shortly after the loading treatment but remained constant for the rest of the steps. In contrary, plasmolysis (39–45%) started in central segments after the sucrose pretreatment and increased to 54–68%, after the loading treatment, and then remained constant. After exposing to LN and unloading

solutions, apical segments were more rapid to deplasmolysis, whereas cortical and pericycle cells retrieved the original characteristics. However, in the central segments, only cortical cells had recovered their original characteristics, but endoderm and pericycle cells were extremely plasmolysed. Cryopreservation strongly impacted the nuclei in the central segments and displayed a very condensed nucleoplasm through the loading of treatment onwards but had not restored to the original form even after treating with unloading solution. On the contrary, very less condensed nucleoplasm was present in the apical segment cells, and it was restored to their original form after conducting the unloading treatment. The requirement of high technical skills is one of the reasons for the limited use of this cryopreservation technique. Yamamoto et al. (2011) solved this problem by designing a new cryo-plate or aluminium plate that fits within the 2 ml cryo-tubes and established a new cryopreservation technique, known as V-cryo-plate technique. There are mainly two advantages of this technique: (1) shoot tips stay stick to the cryo-plates, so the chances of injury to the explants are minimized as the plates are required to be moved as a whole; (2) due to rapid freeze/thaw, regrowth rates are quite high. Niino et al. (2013) developed the D-cryo-plate (cryo-plate dehydration) technique, on the basis of droplet-vitrification, where also the explants adhere to the aluminium plate and are exposed to each of the treatments of dehydration, cryoprotection and thawing. The importance of this method also lies with the manipulation of many explants simultaneously with the minimum chances of variation that is likely to occur (Engelmann 2014). The D-cryo-plate protocol has been tried with the polyembryonic masses of *Phoenix dactylifera* (Salma et al. 2014a, b), shoot tips of *Solanum tuberosum* (potato) (Yamamoto et al. 2012a, b), shoot tips of *Saccharum officinarum* (sugarcane) (Rafique et al. 2016) and *Petiveria alliacea* (de Almeida Pettinelli et al. 2017). Engelmann-Sylvestre and Engelmann (2015) compared the recovery percentage of shoot tips after the application of V-cryo-plate and D-cryo-plate protocol. Interestingly, after thawing, 71.0% regrowth was achieved by V-cryo-plate, but only 29.2% regrowth was noted with the D-cryo-plate method.

16.5.4 Encapsulation-Dehydration

The synthetic seed technology is the primary basis for the encapsulation-dehydration procedure to be carried out for the long-term conservation of medicinal plants. A characteristic procedure of encapsulation-dehydration cryopreservation includes encapsulation of explant within calcium alginate beads formed through 3–4% sodium and 50–100 mM calcium chloride, followed by dehydration (in sucrose solutions or under laminar airflow or else over silica gel). The encapsulating technology prevents the explant from the injury that might occur due to the severe treatments intended for dehydration (Engelmann 2009). Encapsulation-dehydration is the majorly employed procedure in the case of medicinal plants (Table 16.1). Encapsulation using higher sodium alginate dosage provides better physical safeguard to the explants. Yin et al. (2011) found sodium alginate at higher concentration

supported better efficacy of cryopreservation protocol. Besides dehydration, the addition of sucrose in the calcium alginate coating also induces rapid recovery of the explants, as recorded with *Syringa vulgaris* L. (Ozden-Tokatli et al. 2008). Even, the relative amount of the explant tissue embedded in the artificial seed is important, influencing the accomplishment of cryopreservation. Bachiri et al. (1995) demonstrated with *C. roseus* that incorporation of low amount of the explant tissue inside the bead leads to a reduced rate of regeneration. Also, addition of salicylic acid into the artificial seed improved the protocol to a great extent (Kaviani et al. 2005). Mohanty et al. (2012) reported that amalgamation of 0.4 M sucrose plus 2 M glycerol in the encapsulation matrix could also result in higher percentage of port cryopreservation regrowth. Nevertheless, the survival of the explants decreased with the increased concentration (0.5 M) of sucrose in the MS medium. This is owing to negative water potential of the high concentrations of osmoticum that reduces the needed turgor pressure for cell division (Mahajan et al. 2013). The duration for dehydration and applied concentration of sucrose after encapsulation is always a controversial subject. The existence of alginate encapsulation matrix delays the dehydration course of action. There are two ways for the execution of osmotic dehydration: (1) rapidly by incubation at concentrated sucrose solution or (2) gradually with increasing sucrose content. Dehydration over silica gel provides superior drying-pace control, but drying in a laminar airflow cabinet is practically easier. Nair and Reghunath (2009) carried out physical desiccation over silica gel and also in the laminar airflow cabinet. Around 20% moisture content on fresh weight basis confirmed to be suitable for high rate of survival (50% to 75%) in several species (Engelmann 1997; Cho et al. 2002). Different lengths of desiccation period were subjected to the preconditioned, alginate-encapsulated and precultured axillary shoot meristems in a laminar airflow cabinet. The highest rates of survival and regrowth of 56.7% and 62.2%, respectively, were achieved with 4 h of desiccation, after which the moisture content was decreased to 16%. Literatures reveal that encapsulation-dehydration may possibly provide improved safeguard than pretreatment with PVS or sucrose alone (Kaviani et al. 2008, 2010; Sopalun et al. 2010). Mandal and Dixit-Sharma (2007) reported as high as 76% post-freezing regeneration frequency using encapsulation-dehydration. Lambert et al. (2009) also confirmed that though encapsulation-dehydration is a labour intensive technique than vitrification, yet it escapes the usage of cytotoxic compounds. They encapsulated hairy roots in calcium alginate beads and precultured in the medium having rising sucrose concentration for 3 days followed by drying in the laminar airflow, which yielded the survival rates of 53% for *Medicago* and 90% for *Maesa*.

16.5.5 Encapsulation-Vitrification

Encapsulation-vitrification is an amalgamation of the two techniques, i.e. encapsulation-dehydration and vitrification. In this method, explants are encapsulated in alginate beads, followed by incubation in LS and/or PVS and then subjected

to immersion in LN (Gantait et al. 2017c) (Table 16.1). This system is gaining popularity since it is quick and physically and chemically nonhazardous and deals with even very small size of explants. The encapsulation-vitrification method is found useful over conventional cryopreservation methods and has wider applications for germplasm conservation since it effectively works with smaller shoot tips and protocorm-like bodies (PLBs) (Engelmann 1997). PLBs of medicinally important orchids are small tissues similar to somatic embryos that can potentially develop into whole plants (Gantait and Sinniah 2012). The technique was competently applied to *Dendrobium candidum* Wall. ex Lindl. (Yin and Hong 2009), *Dioscorea bulbifera* (Yin and Hong 2010), *Anemarrhena asphodeloides* (Hong and Yin 2012b), *Dioscorea opposita* (Zhao et al. 2016), etc. With an exception, Xue et al. (2008) explored and assessed the prospect of extended conservation of the germplasm of the hairy root lines of *Astragalus membranaceus*, *Eruca sativa* Mill. and *Gentiana macrophylla* Pall., following both the cold storage and cryopreservation approaches. Subsequent to 5-month cold storage, *A. membranaceus*, *E. sativa* and *G. macrophylla* hairy roots recovered growth up to 82.9, 100 and 75.7%, respectively. But, employing the encapsulation-vitrification method, the *G. macrophylla* hairy roots died, and *A. membranaceus* and *E. sativa* hairy roots survived regenerated up to 6 and 73%, respectively. Xue et al. (2008) suggested that cold storage is an appropriate substitute for the storage of hairy root lines of the three studied plant species rather than encapsulation-vitrification. Afterward, Mohanty et al. (2012) compared and made a conclusion for a competent procedure for cryopreservation of *Dendrobium nobile* PLBs, via encapsulation-vitrification or encapsulation-dehydration. In both the cryogenic approaches, PLBs were osmoprotected by the combination of 0.4 M sucrose and 2 M glycerol and encased in the alginate matrix. Between these two methods, greater survival (78.1%) and regrowth (75.9%) were obtained from encapsulation-vitrification than encapsulation-dehydration (53.3 and 50.2%, respectively). It was noted that inclusion of 0.4 M sucrose and 2 M glycerol in the alginate matrix increased the endurance rate after cryopreservation. Until now, there are only few reports on such combined techniques; however, the exploitation of these techniques will most likely turn out to be popular in the future, after the optimization of their protocols.

16.5.6 Recovery

Cryopreservation success is by large dependent upon the composition of the recovery medium. In addition, to ensure high frequency of recovery, the PVS has to be removed subsequent to thawing, by treating with dilution solution like decreasing sucrose content for 15–30 min, to avoid osmotic shock (Flachsland et al. 2006). According to Bachiri et al. (1995), rehydration commences after few hours of inoculation of LN-treated and thawed explants on the recovery medium. It was suggested to culture the explants on a semisolid full-strength MS medium to boost the penetration of nutrients for fast regrowth (Suranthran et al. 2012; Sinniah and Gantait 2013;

Gantait et al. 2015a, b, c; Zhang et al. 2015; Sharma et al. 2017). In some cases half-strength MS medium was also beneficial (Vendrame and Faria 2011; Maki et al. 2015). Following cryopreservation of *Pistacia* spp., Ozden-Tokatli et al. (2007) tested both the semisolid and liquid MS medium for recovery. They found that regeneration was better on semisolid MS medium than on liquid MS medium. A noticeable but unsettled issue of cryopreservation is the frequently observed discrepancy in rates of survival and regrowth. The parameters that help in the maximum survival usually do not facilitate the best regrowth rate. For instance, 30% survival of cryopreserved apices of *V. planifolia* shoot was achieved, but only 10% regrowth was possible (Gonzalez-Arnao et al. 2009b). This was also observed with *Atractylodes macrocephala*, which gave a 76% survival for droplet-vitrification, but regrowth frequency was lower (62%) (Zhang et al. 2015). Some unfavourable events are associated with cryopreservation such as injury to the plasma membrane and primary metabolism that can possibly have an effect on the post-thaw re-establishment of regular hormonal function. Consequently, the application of plant growth regulators could be obligatory to restore the metabolic processes that were harmed during the cryopreservation and also promotes tissue regrowth at higher rates (Touchell et al. 2002). The appropriate combination of plant growth regulators in the recovery medium such as N^6-benzyladenine (BA) alone (Sharma et al. 2017) or in combination with α-naphthalene acetic acid (NAA) (Mohanty et al. 2013) or indole acetic acid (IAA) (Ai et al. 2012) might be essential to induce the growth of the cryopreserved material (Fatima et al. 2009). Seo et al. (2007) reported regrowth of thawed shoot tips was superior in MS medium fortified with 1 mg l^{-1} of gibberellic acid (GA_3) and 0.5 mg l^{-1} of BA. Mandal and Ahuja-Ghosh (2007) followed by Nair and Reghunath (2009) also confirmed that addition of 0.2 mg l^{-1} GA_3 along with 1.5 mg l^{-1} BA and 0.2 mg l^{-1} NAA improved the regrowth of *Dioscorea floribunda* and *Clitoria ternatea* L. after LN exposure. Mandal et al. (2009) demonstrated that the post-freezing regrowth of somatic embryos was optimum when cultured in embryo conversion medium (MS medium containing 0.5 mg l^{-1} zeatin along with 400 mg l^{-1} glutamine). Contrastingly, Marco-Medina et al. (2010) noticed that hyperhydric shoots were produced in all the treatments except in the basal medium without any plant growth regulator. Increased rates of unfavourable hyperhydration up to 26.7% were observed when shoot tips were cultured in higher concentration of BA (2.22 μM) suggesting for the lower dose of BA to be employed. Likewise, shoot tips recovered in the regrowth medium containing 0.275 μM BA exhibited the highest regeneration rates (40%) with lower hyperhydration (6.7%). The incorporation of active charcoal (AC) in the culture medium was also found effective for *Miconia ligustroides*, as it protects the explant from browning and supported 70% recovery (de Oliveira Prudente et al. 2017). Furthermore, the supplementation of antioxidants such as polyvinylpyrrolidone, ascorbic acid or tocopherol to the recovery medium may be valuable because dehydration/rehydration and freezing/thawing are related with the accumulation of injurious reactive oxygen species (Cripps 2006). It is also important to note that the post-rewarming slow exposure to light raises the recovery percentage of cryopreserved plant material by eliminating the stress and inducing photo-oxidation that eventually favours tissue repair (Hong and Yin 2009). Before

fully shifting to the light, cryopreserved shoot tips of *C. rosea* were incubated in the dark for 7 days, which induced faster growth, developed healthier shoots and reduced the production of callus. Similar results were also reported for *Dioscorea alata* (Mukherjee et al. 2009) and *Passiflora suberosa* (Garcia et al. 2011), where dark incubation after rewarming and then transferring to dim light lead to successful regeneration after cryopreservation. Yet, the rate of recovery is species dependent and/or the mode of cryopreservation method used.

16.6 Problems Encountered During Cryopreservation of Medicinal Plants

The definite problem of cryopreservation that researchers have faced is the outsized plant cell and its intracellular water that takes up over 90% of the cell volume (Bachiri et al. 2000). A major proportion of the cellular water can be frozen and thus called freezable water (Crowe et al. 1990). Even in the presence of extracellular ice, a little proportion remains unfrozen and represented as 'non-freezable' water, which is surrounded by membranes and is vital to sustain the cellular structure and functionality (Hoekstra et al. 2001). It is well-established fact that development of ice inside cell interrupts the cellular structures resulting to cell death (Mazur 1984). Subsequently, withdrawing the freezable water from the tissues to be cryopreserved became the primary aim of the researchers (Engelmann 2004). The way out of this objective was obtained osmotically by introducing the cells in hypertonic solutions or by simply dehydrating the cell through air current or keeping over the silica gel. The cellular content vitrifies, i.e. solidifies into an amorphous glassy state, when the protoplast is sufficiently dehydrated (Mazur 1984). Sakai (2000) reported that the sole mechanism to avoid freezing injury was vitrification that permits cells to survive all through freezing. The minute ice crystals still are present integrated into the amorphous glass; however, no fatal injuries are caused if their recrystallization is not permissible at the time of thawing (Samygin 1974). Fujikawa and Jitsuyama (2000) also discussed about the mechanical damage of cell structures by ice crystals. The joint action of low temperature along with dehydration affecting successful cryopreservation had also been proposed by Samygin (1994). It was clear from the experiments with isolated protoplasts and liposomes that there was a shift of membrane lipid structure from liquid crystalline to gel and, with extensive dehydration, to hexagonal phases (Crowe et al. 2002). Since, the temperature for phase transition is dissimilar for an array of lipids; these alterations may result to complete loss of the cellular structures and their semipermeable properties that is caused by lateral phase separation in membrane. Protoplasts of cold-acclimated dehydration caused invasion of lipid material from plasmalemma into cell interior which leads to cell lyses during the subsequent rehydration as the membrane area was not sufficient to restore the initial volume (Uemura and steponkus 1999). Though membranes of other organelles including those of the mitochondria, tonoplast, and thylakoids are

also distressed, the plasmalemma stability is most vital for normal functioning of the cell subsequent to thawing. Additionally, superfluous dehydration can also lead to denaturation of the membrane proteins or brings about the conformational change (Samygin 1994). Dehydration also results in high cytoplasm viscosity and increases the level of toxic compounds, chiefly salts in the dehydrated protoplasts (Mazur 2004). Moreover, in the freeze-thawed mesophyll or any other plant cells, depolymerization of microtubules (Bartolo and Carter 1991) or formation of reactive oxygen species occurs (Benson and Bremner 2004).

16.7 Conclusion

Cryopreservation methods have been successful in conserving numerous plant species throughout the globe. Shoot tips are the ideal explant source for cryopreservation as they possess the highly active zone of diving cells as well as an organized structure regenerating directly into shoots avoiding the callus phase, which sometimes may incorporate mutations. Survival and regeneration rates after cryopreservation were improved by preconditioning of the explants via cold acclimation or sugar preculture, probably because of increased stability of the plasma membrane. Cryopreserved plants have been proved to be inherently steady, except in some instances where epigenetic variations were identified. Fruitful cryopreservation cannot be assured for all the medicinal plant species, since some of these show recalcitrancy towards tissue culture or cryopreservation. Cryopreservation could be progressed by efficient application of varying cooling rates and the use of accurate dosage of cryoprotectant. Further fundamental studies intended for understanding the membrane composition, membrane damage and its repair mechanism will possibly assist to clarify the cryosensitive nature of some species and improve the cryopreservation protocols for those species.

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Chapter 17
Ethnobotany: A Bridge Between Traditional Knowledge and Biotechnological Studies on Medicinal and Aromatic Plants

A. S. Vishwanathan

Abstract History is witness to plants being an integral part of human evolution, as a source of food, fuel and medicine. Traditional medicine relies extensively on plants and plant products in its holistic approach to treatment of disease, minor or major. However, many plant-based cures in traditional medicine remain undocumented secrets due to unwillingness of practitioners to divulge details for various reasons. Ethnobotanical surveys, if carried out systematically, can bring to light many of these herbal remedies which could serve as starting points for further pharmacological investigations and biotechnological studies. Micropropagation and plant tissue culture can play a key role in rapid, clonal mass propagation of medicinal and aromatic plants which are being overexploited. Secondary metabolite production by medicinal and aromatic plants can be enhanced using biotransformations and hairy-root cultures in bioreactors. A synthetic outlook to integrate traditional and modern medicine will go a long way in alleviating health-related disorders which are an impediment to progress in the medical domain.

Keywords Medicinal plants · Ethnobotany · Ayurveda · Traditional medicine · Plant biotechnology

17.1 Plants and Man: An Introduction

> "*What does interest us academically and practically is how to salvage some of the medico-botanical lore before it shall have been forever entombed with the culture that gave it birth*"
>
> – R. E. Schultes (1960)

Early land plants are known to have come into existence more than 400 million years before the first man walked the earth (Kenrick and Crane 1997). The evergreen association between man and plants is intricately woven into human

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evolution. Primitive man, as a forager, depended entirely on plants for his sustenance. Even tens of thousands of years hence, after many cultural, social, industrial and technological revolutions, plants continue to sustain man's daily needs, health and well-being. This intimate relationship with plants which has stood the test of time will continue to be the highlight of man's growth, development and evolution as a species.

The term ethnobotany has its origin in the Greek roots *ethnos* meaning people and *botane* meaning grass, put together, the study of interrelationships between humans and plants. This term has gradually expanded to include, among many other things, knowledge of plants relating to a particular region or cultural background and their applications in folk medicine. Ethnobotany can be viewed as an interdisciplinary science that unifies various conventional disciplines. Petrovska (2012) provides a concise historical account of medicinal uses of plants. In the context of this chapter, the term ethnobotany will focus specifically on medicinal and aromatic plants, and the subsequent presentation and discussion of topics, unless mentioned otherwise, will be primarily in the Indian context.

17.2 Traditional Systems of Medicine

Modern medical science relies on a fragmentary approach to treatment by making attempts to understand the physiological and pathological basis of disease and targeting specific molecules that disrupt normal functioning of the body. Although they provide instantaneous relief from discomfort, modern medicines often act by suppressing symptoms rather than eradicating the source of the problem. The use of antibiotics as therapeutic agents since the 1940s (Bush 2010) has given modern medicine an upper hand over traditional forms of medicine. In the recent past, with co-operation from advances in other disciplines, modern medical science has given way to molecular medicine where diseases can be identified and treated using interventions at a molecular and genetic level (Izraeli and Rechavi 2002). The medical model that classifies patients into various categories and tailors medical decisions, procedures and interventions to individual patients on the basis of their diagnosis or risk of disease is known as personalized or stratified or precision medicine (Collins and Varmus 2015). On account of millions of dollars being pumped into research and development of medicines across the globe annually, by the time a drug is ready for the market after extensive clinical trials, one of the major targets of the manufacturer is to obtain maximum returns on the investment. The narrower the scope of the medicine, the more the investment and, thereby, higher the cost incurred by the patient.

The skyrocketing costs and inextricably linked adverse side effects associated with modern medicines have gradually led to greater interest being shown towards traditional systems of medicine which are known to eliminate ailments and diseases from their roots. As per a report from the World Health Organization (WHO 2013), a large proportion of the population in developing countries depends on plant-based traditional medicines for healthcare. Traditional medicine adopts a more holistic

Table 17.1 Modern medicine vis-a-vis traditional medicine

	Modern medicine	Traditional medicine
Origin	Recent	Ancient
Approach	Fragmented	Holistic
Strategy	Remedial	Preventive
Basis of treatment	Treatment of pathological or physiological condition	Promotion of general well-being
Formulation	Isolated chemical molecules (purified from natural source or artificially synthesized)	Plant-based preparations (sometimes supplemented with minerals and metals in trace concentrations)
Mode of action	Suppression of symptoms	Elimination of cause
Cost	Expensive (includes cost of R&D)	Affordable
Side effects	Adverse	Negligible

approach than modern medicine, focusing more on the patient instead of the disease and on the entire body rather than merely the diseased part (Patwardhan et al. 2005). A comparative account of the key aspects of modern and traditional medicine is presented in Table 17.1.

India and China have been among the bastions of traditional herbal and folk medicine since time immemorial. Ayurveda, Unani and Siddha are three major systems of traditional medicine that are extensively practised in India.

17.2.1 Ayurveda

> "*Hitaahitam Sukham Dukhamaayustasya Hitaahitam*
> *Maanamcha Taccha Yatroktam Ayurvedah Sa Uchyate*"
>
> - Charaka Samhita (Sutrasthana, 1:41)
>
> Ayurveda (is the treatise which) describes the advantageous, disadvantageous, happy and unhappy states of life, in addition to what is good and bad for life and its measurement.

'Ayurveda' in Sanskrit literally means 'science of longevity'. The Ayurvedic school of medicine that had its roots in India more than 5000 years ago has become extremely popular across the globe. Ayurveda relies on optimizing health by balancing forces of the spirit, mind, body and the external environment (Murray 2013). The ancient tradition of Ayurveda is centred around equilibrium of seven tissue elements (*dhaatus*), three humoral elements (*doshas*), three waste products (*malas*) and the gastric fire (*agni*) (Kayne 2010). Illness is said to be a result of loss of equilibrium among these elements. Ayurveda emphasizes on preventive care by prescribing a strict dietary regimen and a specific daily routine (*dinacharya*) and seasonal routine (*ritucharya*) based on the balance of elements that can vary across individuals (Ravishankar and Shukla 2007). Diagnosis of ailments follows an extensive procedure where aspects concerned with the entire body are examined rather than only the affected part. Treatment in Ayurveda requires complete participation

of the patient and involves two aspects – medicine (*Aushadha*) and diet (*Patya*). Over 2000 different medicinal plants are said to have been described in texts related to Ayurveda (Pandey et al. 2013). The *Charaka Samhita,* a comprehensive text attributed to Charaka, one of the principal contributors to Ayurveda, provides extensive notes on therapeutics and detailed prescriptions for plant-based preparations for systematic management of different kinds of diseases (Sharma 1998).

17.2.2 Unani

The Unani system of medicine is based on Greek philosophy. Hippocrates is said to have laid the foundations of this form of medicine. Management of a disease, according to Unani medicine, depends on the diagnosis of the disease, which in turn is a function of clinical features. The body is said to be made up of four basic elements (earth, air, water, fire) having different temperaments – cold, hot, wet, dry – in various combinations (Ravishankar and Shukla 2007). If the body, comprising various tissues and organs, loses its natural ability to maintain itself, the equilibrium of the four humours – blood, phlegm, black bile and yellow bile – is disturbed and leads to pathological and physiological manifestations of the disease. Treatment involves eliminating the cause, restoring humoral balance and normalizing the tissues and organs of the body. Pharmacotherapy in Unani medicine makes extensive use of plant extracts either alone or in combinations with extracts of animal and mineral origin.

17.2.3 Siddha

Tamil Nadu in South India is the home of Siddha medicine. *Siddhars* or adepts possessing supernatural powers are said to be the pioneers of this traditional system of medicine. It is believed that ancient knowledge regarding the nuances of Siddha medicine was documented by the founding fathers in palm leaf manuscripts, some of which are in possession of families still practicing this form of medicine and administering cures. In addition to the three humoral elements – wind (*vata*), bile (*pitta*), and phlegm (*kapha*) – as in Ayurveda, diagnosis in Siddha medicine also depends on the examination of eight sites that include pulse (*nadi*), eyes (*kan*), voice (*swara*), touch (*sparsam*), colour (*varna*), tongue (*na*), faeces (*mala*) and urine (*neer*) (Ravishankar and Shukla 2007). Medicines of plant origin are supplemented by micro-quantities of mineral and metal-based preparations in Siddha medicine (Subbarayappa 1997).

17.3 Ethnomedicine: An Indian Tradition

Long before the advent of the now-popular modern Western medicine, the rural populace of India are known to have relied extensively on medicinal and aromatic plants and their products. Dependence on this system of medicine was so common in every household that even today these remedies are popularized and commercialized as 'home remedies' or, colloquially, 'Grandmother's medicine'.

Ancient practices of traditional herbal medicine are documented in religious texts and publicly available pharmacopoeias that have survived over the ages. Vaidya and Devasagayam (2007) provide a concise account of literature on medicinal plants in the form of monographs and reviews. However, on account of the rich diversity on Indian languages and dialects, caution must be exercised while translating texts from regional languages to English. However, preservation of the repository of valuable medical knowledge over the ages can also be attributed to the cultural versatility of the Indian society. Various aspects of this knowledge were associated with people from different professional backgrounds:

- Learned physicians who were well-versed in the documented forms of medical literature
- Defence personnel whose professional demands warranted practical knowledge of a handy first-aid kit comprising readily available plant resources
- Businessmen who were entrusted with the responsibility of making these natural products commercially available in the marketplace
- Most importantly, common folk who kept adding to the already available repository of knowledge by trial and error, experimenting with locally available plants and herbs

In the past, the unlettered population was in awe of local doctors who were bestowed with this wisdom about plants which seemingly had supernatural powers to revitalize a dying person and, from a more pragmatic point of view, about readily available or easy-to-prepare herbal remedies which could relieve people of minor ailments that were impediments to their daily chores and jobs. Interestingly, much of this intricate and subtle knowledge never found its way into literature. Although knowledge, as we know it today, is free to share, a considerable portion of this knowledge has remained sequestered with certain individuals or families, considered as 'custodians' of this ancient tradition, who do not divulge information because they earn their living by dispensing cures. With younger generations migrating to towns and cities in the quest for more lucrative jobs, this knowledge faces the risk of dying out due to paucity of worthy successors to whom it can be passed on. Some information regarding preparation of concoctions and poultices has remained classified, not disclosed to anyone out of the unfounded belief that the medicinal property might lose its potency if shared with others. Some healers have chosen to remain secretive out of fear of being branded as black magicians due to the extraordinary powers they exhibit using commonly available herbs.

17.4 The Way Ahead

17.4.1 Repositories of Knowledge

The Government of India upgraded the Department of AYUSH (Ayurveda, Yoga and Naturopathy, Unani, Siddha and Homoeopathy), which was part of the Ministry of Health and Family Welfare, to an independent Ministry of AYUSH in the year 2014, to facilitate studies on development and propagation of traditional systems of medicine and healthcare and to encourage exploratory and translational research on these lines. Access to the vast repositories of knowledge of traditional forms of medicine is being facilitated by information portals maintained by governmental as well as private organizations.

- The Traditional Knowledge Digital Library (TKDL) is a collaborative project involving the Council of Scientific and Industrial Research (CSIR) and the Ministry of AYUSH. The portal, accessible at http://www.tkdl.res.in, has made available close to 3,00,000 medicinal formulations from ancient texts on systems of medicines such as Ayurveda, Siddha, Unani and Yoga into five international languages with the help of information technology tools and an innovative classification system. The digital library has made information from almost 360 books on Indian systems of medicine accessible to any individual or organization at the national and international level.
- DHARA (Digital Helpline for Ayurveda Research Articles) is another such initiative implemented and maintained by AVP Research Foundation, the research wing of the Ayurvedic Trust, AVP Group of Institutions, Coimbatore, Tamil Nadu, India. This portal is a comprehensive online indexing service for research articles published in the field of Ayurveda. The initial funding for this project was received from the Central Council for Research in Ayurvedic Sciences of the erstwhile Department of AYUSH in the year 2010.

17.4.2 Ethnobotanical Studies

In this context, I am reminded of an instance some years ago when I had accompanied a colleague to a nearby village to gather some more ethnomedicinal uses of *Vitex negundo* L. (Verbenaceae). We had gone through published literature that told us that the plant was called *Vaavili* in the local language and gave us a brief idea about the medicinal applications of this plant. We did not have much difficulty in locating the house of the local expert in herbal medicine; the village folks were very co-operative. In fact, some of them were even curious about who among the two of us was unwell and what the ailment was. When we introduced ourselves as researchers from the nearby Institute and asked the venerable old gentleman about *Vaavili*, he kept staring at us with a blank gaze and curtly replied that there was no

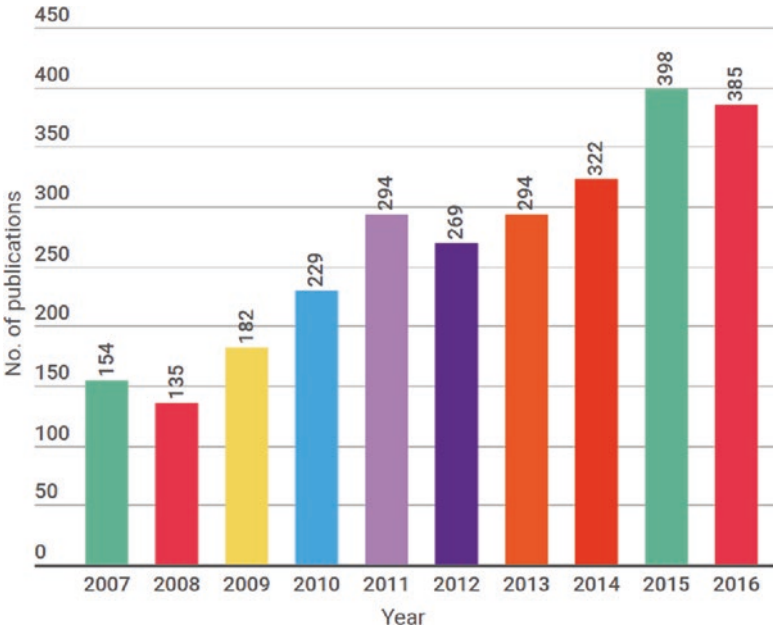

Fig. 17.1 Number of journal papers having the keywords 'ethnobotanical' or 'ethnomedicinal' during the period 2007 to 2016. (Data from Web of Science, Clarivate Analysis; accessed on 19 November 2017)

plant by that name. We were also familiar with the technical botanical description of the plant; but it was not going to help us in this situation. We tried talking about some of the applications of the plant, that we were familiar with, hoping that the gentleman could identify the plant that way. We were on the verge of giving up, when he interjected, *Vailaaku!* That was how it was referred to in the local dialect. Now that we were on the same page as him, we felt our job was half done. However, he kept giving us one reason or the other to avoid telling us about the uses of the plant, almost as if he wanted to get rid of us. It took us multiple visits and a lot of perseverance and persistence to glean some useful information from him about that one plant. As we thanked him and took leave, the broad smile on his face and the glint in his eye gave me a feeling that he had a lot more knowledge about the plant than what he had shared with us.

Reports on ethnobotanical or ethnomedicinal surveys published in peer-reviewed journals (Fig. 17.1) are an indicator of the interest evoked by this subject in the scientific community. However there are regions of the world which are yet to be explored (Fig. 17.2).

The broad spectrum of geographical and climatic conditions, ranging from sub-tropical rain-fed forests and cool mountainous regions to semi-arid barren deserts, makes the Indian subcontinent home to a bewildering diversity of medicinal and aromatic plants. Pandey et al. (2013) report that around 20,000 medicinal plants have been reported from India.

Fig. 17.2 Countries from which at least 20 journal papers have been published (having the keywords 'ethnobotanical' or 'ethnomedicinal') during the period 2007 to 2016 are shaded. (Data from Web of Science, Clarivate Analysis; accessed on 19 November 2017)

Locally available plants and plant products constitute the inventory of the self-made, local practitioners of herbal medicine. Description of plants by the illiterate and unqualified, yet knowledgeable medical experts, is often brief and non-technical. Plants possessing similar morphology could be mistaken for one another unless there is a marked distinction in the floral characteristics, phyllotaxy or composition of essential oils that lend a typical aroma to the plant. Many of the traditional methods and procedures of harnessing the medicinal potential of these plants have either got corrupted or improvised as the information was transmitted by word of mouth. It is thus critical to expedite the process of envisioning, organizing and carrying out systematic ethnobotanical studies to document this knowledge which is gradually fading away with the older generations, especially across the length and breadth of the Indian subcontinent.

17.4.3 Micropropagation and Plant Tissue Culture

Harvesting naturally growing plants is much more economical than chemical routes of synthesizing the active components. With the world showing increasing signs of inclination towards traditional herbal medicine for a more holistic cure for ailments rather the mere symptomatic treatment offered by modern medicine, medicinal plant resources run a risk of being overexploited. Clonal propagation of plants using

conventional vegetative propagation methods such as cutting, grafting and layering is common but a very slow and laborious process. In vitro methods of regeneration of plants from small pieces of somatic tissues have made it easier to produce a large number of plants from a single individual in a relatively short period of time (Bhojwani and Dantu 2013). Chaturvedi et al. (2007) provide a brief history of micropropagation of medicinal plants and outline the role of plant tissue culture techniques for in vitro cloning of medicinal plants possessing desirable traits. There is an urgent need for research institutions to take initiatives, in collaboration with government agencies, for conservation of medicinal and aromatic plants in India (Biswas et al. 2017).

17.4.4 Production of Secondary Metabolites

Plant secondary metabolites are "…*compounds that have no recognized role in the maintenance of fundamental life processes in the plants that synthesize them, but they do have an important role in the interaction of the plant with its environment.*" (Oksman-Caldentey and Inzé 2004). Pharmacological activity of plant secondary metabolites makes them the active components of traditional medicine. Roughly a quarter of the molecules used in the pharmaceutical industry are reported to have their origin in plants (Bourgaud et al. 2001). Increasing global population has had an adverse impact on the land available for cultivation of food crops necessitating optimal usage of available land for growing medicinally important plants that are valuable from the point of the pharmaceutical industry. Moreover, many plants containing high-value compounds are either not suited for large-scale cultivation on land outside their endemic habitats or are becoming endangered because of overharvesting (Rates 2001). Totipotency of plant cells has enabled their use as cell cultures for the production of the active components that confer medicinal properties on them. Production of valuable secondary metabolites employing plant cell cultures is a feasible alternative to the extraction of whole plant material. Additionally, hairy root cultures and biotransformations also have great potential for enhancing production of medicinally important secondary metabolites from plants (Paek et al. 2005). Using biotechnological methods for production of secondary metabolites ensures a continuous supply of products having uniform quality, irrespective of limitations imposed by seasonal and geographical variation (Rao and Ravishankar 2002). Advent of transgenic research has opened up possibilities of synthesizing complex molecules and compounds that are not amenable to chemical synthesis routes and are not naturally synthesized in plants (Saito et al. 1992). Efforts are required towards reduction of costs of maintaining plant cell cultures in bioreactors (Bourgaud et al. 2001) and scaling them up to commercial scale for further downstream processing.

Detailed understanding of regulatory mechanisms controlling the flux of secondary metabolite synthesis pathways and the ability to manipulate them effectively are prerequisites for commercial success of plant cell or organ cultures (Rao and Ravishankar 2002).

Fig. 17.3 Indian spices and condiments used in common home remedies

17.4.5 Synergy of Traditional and Modern Medicine

Many plants from tropical regions are directly used as therapeutic agents for curing common ailments and diseases. Some home remedies which make use of common Indian spices and condiments (Fig. 17.3) are listed below.

- Roasted and powdered cumin seeds (*Cuminum cyminum*) taken with buttermilk are effective in digestion-related disorders of the stomach.
- Black peppercorns (*Piper nigrum*) are powdered and added as seasoning to hot soups for clearing chest congestion due to cold.
- Dry ginger (*Zingiber officinale*) powder taken with a small amount of jaggery and clarified butter (*ghee*) provides relief from pain in the joints due to rheumatoid arthritis.
- A water-based decoction of black peppercorns, dry ginger, liquorice (*Glycyrrhiza glabra*), long pepper (*Piper longum*) and lesser galangal (*Alpinia officinarum*) helps to get rid of debility resulting from fever.
- Paste of dried nutmeg (*Myristica fragrans*) seeds in a few drops of water is extensively used in paediatric ailments.
- Sweet flag (*Acorus calamus*) is administered to those having a white coating on the tongue (due to bacterial infection of papillae) and is beneficial for children having speech-related disorders.
- Oak galls (*Quercus infectoria*) are very effective against ulcers in the mouth and throat.
- Essential oil of cloves (*Syzygium aromaticum*) is commonly used for relief from toothaches.
- Seeds of Turkey berry (*Solanum torvum*) and black night shade (*S. nigrum*) fried in clarified butter are used in treatment of stomach ulcers.
- Castor (*Ricinus communis*) oil is commonly prescribed as a laxative.

On a personal note, having benefited immensely from many of these home remedies during my childhood, I often resort to this form of medicine even today because the kitchen is the nearest pharmacy store! Many of the traditional Indian food preparations associated with various festivals at different times of the year and in different seasons are based on the curative properties of the ingredients. Though knowledge regarding the medicinal aspect of these food items might have been forgotten with the passage of time, mere adherence to traditional practices ensures that the health benefits are sustained over generations.

Ethnobotanical studies present modern scientists with an extensive database of remedies and cures that have been formulated and practised over centuries with varying levels of success that have been largely undocumented. From characterizing active components of plants used in treatment of specific ailments to analysing the synergistic effect of plant extracts in herbal formulations and concoctions, ethnomedicinal practices could serve as starting points for pharmacological investigations leading to clinical trials. However, caution should be exercised in cases of traditional cures associated with extensive rituals or procedures. It could be faith rather than medicine that cures the disease, in some kind of a placebo effect.

Biotechnological studies thus have a very important role in preservation, sustenance, and advancement of the rich cultural heritage of India. And ethnobotany is the bridge that possesses the potential to link our glorious past with a promising future.

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Chapter 18
In Vitro Manipulations for Value Addition in Potent Herbal Insecticidal Activities of *Chrysanthemum cinerariaefolium*

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Abstract The *Chrysanthemum* genus belongs to the genus Asteraceae which covers up to 15% of all the species of this genus. The natural insecticidal compound pyrethrin is found in *C. cinerariaefolium* and mostly found in the aerial parts such as achenes of the flowers. Chemically pyrethrin is a set of six structurally close monoterpene esters formed by esterification of two monoterpenic acids (chrysanthemic acid and pyrethric acid) with three ketone alcohols (pyrethrolone, cinerolone and jasmolone). The side effects of the chemical analogue of this molecule and eco-friendly action of pyrethrin such as rapid degradation into the environment and swift action against insects make this molecule the ultimate choice for scale-up industries thus far making this plant system of uttermost importance which needs biotechnological intervention. The in vitro research in this plant system is not completely achieved as the whole pathway-level understanding is not fully understood. Apart from it, the regeneration-level protocols in *C. cinerariaefolium* have not been established to the par, and *Agrobacterium*-based genetic transformation studies are limited which could have paved the way for better pathway-level studies, gene transfer studies and new variety development with higher pyrethrin content. The present chapter discusses the present scenario and future prospects of in vitro pyrethrin production.

Keywords *Chrysanthemum cinerariaefolium* · In vitro culture · Genetic transformation · Pyrethrin

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18.1 Introduction

The genetic improvements of the crops for their desired traits and value addition had always been the major aim in various commercial as well as medicinal value-containing plants. The in vitro techniques had been previously used to generate hybrid plants having an advantage over the non-hybrid crops in regard to their better virtues agronomically. The major emphasis of in vitro studies for crop improvements revolves around better yielding abilities of crops, their better tolerant to stress conditions and efficient resistance to crop-associated diseases. Apart from these traits, the other aspects of in vitro biotechnological intervention consider improvement in photosynthetic efficiency, enhancement of flowering abilities, fruit quality and better rooting abilities of plants (Bakry 2009).

The ever-increasing demand of secondary metabolites and their derivatives has also influenced the major medicinal and aromatic plants producing them as this leads to extractions from whole plants which results in uprooting of the plant as a whole. So there is an urgent requirement for an alternate way of production of these secondary metabolites without the constraint of damaging the whole plant species. The plant tissue culture technique has emerged as an ideal way of producing the secondary metabolites in higher amounts as it provides major quantity of relatively homogeneous, undifferentiated cells to be produced (Vanisree et al. 2004). The in vitro plant cells may be proven as a potent secondary metabolite production source as plant cells and tissue culture techniques work in a complimentary way (Fig. 18.1). In the past, various tissue culture-based techniques have been developed for genetic improvement of plants and enhancement of secondary metabolite

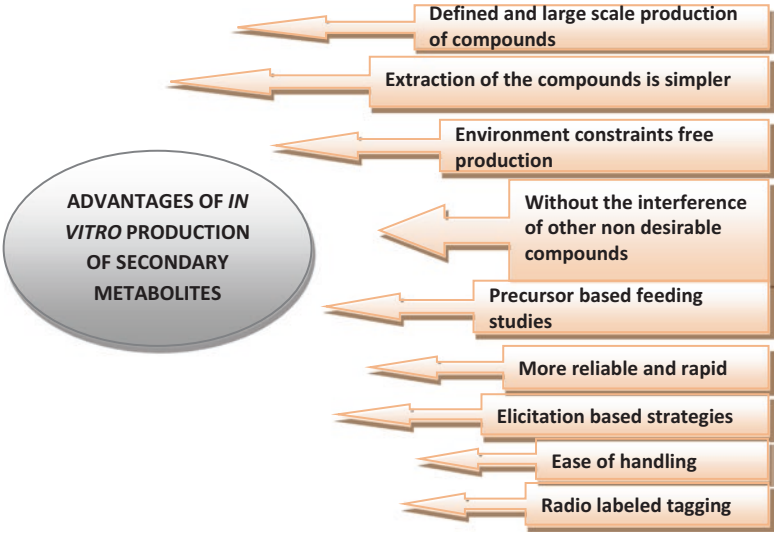

Fig. 18.1 Various tissue culture aspects and advantages of in vitro secondary metabolite production

production which includes immobilized cells, suspension culture and organ cultures (Rao and Ravishankar 2002). These tissue culture systems offer easy and efficient accumulation and extraction of secondary metabolites with low economic values. Another tissue culture-based way of efficient secondary metabolite production is through bioreactor upscaling as it is an ideal alternate for producing higher value secondary metabolite in a controlled manner (Giri and Narasu 2000). Plant tissue culture-based secondary metabolite production has proven its commercial implication also as the first pharmaceutical and dyestuff production was of "shikonin" dye which had the commercial value of $4500/kg by Mitsui Petrochemical Industry Company in Japan (Fujita 1988). Apart from it, the first industrial-level production of pharmaceutical berberine was also performed by the same company with a yield of 3.5 g per litre of culture fluid. The bioreactor upscaling of *Panax ginseng* cells for efficient production of ginsenosides was also performed in bioreactors for biomass production (Zhang et al. 2011). The bioreactor upscaling using plant cells needs a lot of modification in the bioreactor design as plant cells are more delicate and sensitive to stress as their size is larger compared to microbial cells with a rigid outer cell wall. Keeping these modifications in mind, airlift bioreactors are the preferred choice for upscaling of plant cells as compared to bioreactors having turbine impellers (Giri and Narasu 2000).

The common soil bacteria *Agrobacterium tumefaciens* and *Agrobacterium rhizogenes* are unique genetic pathogens capable of fundamentally redirecting plant metabolism in order to generate macroscopic tissue masses (crown galls and hairy roots, respectively) which support the growth of large populations of *Agrobacteria*. Central to pathogenesis is the horizontal transfer of oncogenes from the tumour-inducing (Ti) plasmids of *A. tumefaciens* and the root-inducing (Ri) plasmids of *A. rhizogenes* into the plant cell genome. These oncogenes alter the synthesis and/or transport of phytohormones *in planta*, leading to the development of the crown gall and hairy root structures from single genetically transformed plant cells (Gomez Galera et al. 2007).

The use of elicitors has shown the effects on the secondary metabolite content of the plants which may include the biotic or abiotic elicitors. The elicitation-based techniques basically target the defence response of the plant by challenging it with a physical- or a chemical-based elicitor. The elicitors are categorized into two types based on their nature: (i) biotic elicitors and (ii) abiotic elicitors. The elicitors can be defined as the substance which when used in small concentrations challenges the plant cells and triggers the defence reactions of the plants which result in induced and altered concentrations of secondary metabolites (Zhao et al. 2005). The abiotic elicitors include the substances which are of nonbiological origin which may range from ions, pH and Ca^{++} concentrations. Biotic elicitor is a term used for elicitors of biological origin which may include substances from plant cell walls (polysaccharides) and other components such as glucans and chitins as well as intracellular proteins (Kai et al. 2012). On the basis of nature and origin, the elicitors are termed as exogenous which takes into account those elicitors which have originated outside the cells such as fatty acids and polysaccharides, whereas endogenous elicitors refer to those elicitors which have originated inside the cells,

e.g. hepta-glucosides and galacturonides. Although there is a lot of work that has been done for improvement in yield of secondary metabolites through elicitation-based techniques, the mechanisms of action of elicitors are still at large and not understood to the core. Many of the assumptions were given for understanding the mechanism of action of the elicitors which majorly deals with factors affecting the membrane integrity, modulation of the pathway, calcium ions concentration variations and osmotic stress management (Zhang et al. 2004). Although elicitation-based yield improvements in plants are seen as a potent field, there is a need to optimize the conditions for better secondary metabolite yields. Among many of the parameters, the important parameters which play a major role in elicitation mechanism are age of the culture on which elicitation is to be performed, concentration and quantity of elicitors used, and exposure duration of the elicitors, among various other factors.

In future the in vitro strategies could pave the way for rapid multiplication of plants species and efficient extraction of the secondary metabolites from them as many of the aspects are nowadays being taken for consideration such as selection of high secondary metabolite-producing lines, strain improvement, optimization of the selected medium and various other parameters leading to considerable increments in secondary metabolite concentration in plants. One of the most important aspects which need to be emphasized for efficient production from plant cells via in vitro manipulations is the basic pathway understanding of the parent plants as without the proper knowledge of the key steps of the pathway, the genetic-level alteration cannot be implied successfully. Other major aspects which hinder the enhancement of secondary metabolites from plants are precursor unavailability, lack of understanding of biotransformation mechanism by the use of exogenous precursor supply and limited knowledge of metabolic engineering (Cheetham 1995).

Pyrethrins are currently the most economically important natural insecticide of plant origin and are known to be extracted from *C. cinerariaefolium***.** Pyrethrins, extracted from oil glands of achenes of flowers of *Chrysanthemum cinerariaefolium* (Asteraceae), are a set of six structurally close monoterpene esters formed by esterification of two monoterpenic acids (chrysanthemic acid and pyrethric acid) with three ketone alcohols (pyrethrolone, cinerolone and jasmolone). In recent past, the worldwide demand of pyrethrins has exceeded supply, causing a sharp augmentation in their market price. These current developments in the last decade induced an alternative way of higher production of pyrethrin with their pursuit for some novel compounds in the insecticidal field leading them to amalgamate their approach to plant tissue culture techniques. There is need to develop an alternate source for production of pyrethrins as the demand and supply ration for production of the natural insecticidal is very low.

18.2 *Chrysanthemum cinerariaefolium*: A Natural Insecticidal Source

The genus *Chrysanthemum* belongs to the genera Asteraceae and nearly margins up to 15% of the species of this genus. It also covers the related species such as *Leucanthemum*, *Dendranthema*, *Chrysanthemum* and *Argyranthemum* (Soreng and Cope 1991). The *Chrysanthemum* is one of the largest genera in Anthemideae and covers 70–150 species (Abad et al. 1995). The species of genera *Chrysanthemum* is found all over the world, but the origin of this genus is considered to be Southwest Asia and most precisely the temperate regions especially northern hemisphere up to Northern Europe, Canada, Alaska and Northern Russia (Heywood and Humphries 1977). Interestingly many of the other related species of this genus produce pyrethrin-like compounds, but no other species till now has been reported to have insecticidal activities. The overall pyrethrin yield as well as the flowering ability of the plant differs from country to country as many of the factors affect the growth and development of the plants such as climatic conditions, temperature, soil content, light exposure and various other factors (Mohandass et al. 1986). Although stem cutting and vegetative cutting are effective ways of propagation of this plant, propagation through seed is considered as the most effective mean, and aerial parts especially flowers are known to contain the maximum pyrethrin content. The temperature plays an important role in the growth of flower head and vegetative form as there are considered to be two kinds of flowers present in the plant; first of them is disc florets which are positioned at the centre and are yellow in colour, while the second is ray florets which are found on the outer rim of the flower head and are white in appearance (MacDonald 1995). Other factors which are also important are the exposure of light as long-day exposure (16 h) and short-day (8 h) conditions both help in reproductive stages of the plants (Mohandass et al. 1986).

Since ancient times, *C. cinerariaefolium* has been reported to act against many insects and showed its toxicity assessment which was later decoded as the discovery of pyrethrin occurred. The plant is assumed to be originated in Middle Eastern Europe and Persia although the "Chou" dynasty of China claims the early records which were claimed to be first century A.D., but at that time very little was known about its insecticidal properties until Staudinger and Ruzicka revealed the structure of pyrethrin. Later on the structure elucidation techniques revealed the pyrethrin contain six components from the esterification of two types of acid moieties [chrysanthemic acid (1) and pyrethric acid (2)] with three types of alcohol moieties [pyrethrolone (3), jasmolone (4) and cinerolone (5)]. Later on the other components of pyrethrin such as pyrethrin II, jasmolin and cinerin were discovered. Pyrethrins, extracted from oil glands of achenes of flowers of *Chrysanthemum cinerariaefolium* (Asteraceae), are a set of six structurally close monoterpene esters formed by esterification of two monoterpenic acids (chrysanthemic acid and pyrethric acid) with three ketone alcohols (pyrethrolone, cinerolone and jasmolone) (Ramirez et al. 2013). Although a lot of synthetic analogues as insecticidal have been used nowadays, still pyrethrin holds its position as a natural insecticidal due to its wider

spectrum of killing activities against the insects, its fast degradation in the environment and more importantly its safer use against mammals (Panda 2005). The loss of agriculture and forestry due to insects is immense as it is reported that annually δ6000 million expenditure is made for providing safety to the crops against the insects (Metcalf and Metcalf 1993). Amongst the countries which produce pyrethrin, Kenya is leading the table as it provides 70% of the pyrethrin out of the 21,000 tons pyrethrin produced worldwide followed by other countries such as Australia (Tasmania), Ecuador, Rwanda and Tanzania (Dessalgne et al. 2011). As far as India is concerned, the production of pyrethrin is very low (20 tonnes annually) as compared to leading countries in pyrethrin production (300 tonnes annually). This low supply of pyrethrin has automatically resulted in rising of the price of pyrethrin in the world market as the demand and supply ratio has been disturbed (Bisht et al. 2009).

The current scenario propels the research towards an alternative source of pyrethrin production, and in vitro plant research and production of pyrethrin under in vitro conditions could prove to be the ideal alternate production system. Apart from it, the in vitro research could also pave the way for the development of new varieties of *C. cinerariaefolium* through somaclonal variation and transgenic approach. In India, the range of pyrethrin content is noted to be between 0.9% and 1.5% DW (Pandita and Sharma 1990), whereas in other countries such as Australia, the flower yield is 140–2400 kg/ha (Bhat and Menary 1984a), and pyrethrin yield is reported to be 0.06–1.80% dry weight.

As discussed before that the flower is the only organ reported to contain pyrethrin, efforts are made to develop new varieties with better traits such as "Hypy" (Bhat and Menary 1984b), "Hansa" (Singh et al. 1988) and an Arizona variety (McDaniel 1991). The natural insecticidal pyrethrin is made up of six esters and basically belongs to the group of sesquiterpene lactone. The six esters which form the pyrethrin are cinerin I, jasmolin I, pyrethrin I, cinerin II, jasmolin II and pyrethrin II (Head 1969). Due to its rapid action and environment-friendly degradation properties, pyrethrin is fast catching up with the world market and is now one of the widely used insecticides in horticulture, agriculture, homes as well as the stores (Kennedy and Hamilton 1995). Due to this high demand and low yield of pyrethrins, a lot of effort has to be made for commercial enhancement of the yield of pyrethrum in the coming future.

18.3 Natural Insecticides: Biosafety and Market Overview

The presence of sunlight and oxygen causes degradation of pyrethrin thus narrowing their use for households use only and encouraging the use of other synthetic analogues such as pyrethroids which have shown more potential in insecticidal activities as well as their susceptibility to sunlight was also found less. Despite having the drawbacks, natural pyrethrin is still holding a lot of the promise for future as focus of the research has now been shifted towards producing these plants at the

commercial level which ultimately needs a better understanding of pyrethrin biosynthesis as well as its regulation in the plant itself.

The aerial part of the plant more precisely the inner ducts of achenes of flowers are considered to contain maximum pyrethrin content which ranges from 0.8% up to 2% of the dry wt. of the flowers (Davies 1985). Previously industry-based companies such as SANOFI Chimie (France) had adapted and used those clones of the plant which contained more than 3% of the pyrethrin content. Various factors affect the yield of pyrethrin which ranges from flower biomass yield which basically depends upon soil type, climatic conditions, genotype of the clone as well as plant diseases (Jovetic and De Gooijer 1995). Other factors that alter the yield of commercial pyrethrin production are picking interval, flower maturity, climate and drying methods applied. More importantly the ratio of the pyrethrin I and pyrethrin II decides the potency of insecticidal activity as pyrethrins II have a better kill effect and pyrethrins I are known to have a good knock-down action. A typical commercial extract can have equal amounts of pyrethrins I and II (Crombie 1980), but ratios ranging from 0.47 to 3.5 have been observed in different breeding lines (Head 1967; Bhat 1995). The use of pyrethrin is found to be safe as various toxicological studies have been done on this compound which found no side effects to human and other mammals (Shoenig 1995), as the mode of action of pyrethrin is considered to be the neuronal voltage-sensitive sodium channel (Kueh et al. 1985). Apart from its biosafety, it is easily degradable in the presence of light and air which makes it an ideal eco-friendly contender among the insecticides present in the market (Allan and Miller 1990). Although the insecticidal activity of pyrethrin is not as rapid as synthetic pyrethroids, its symbiotic use with a stabilizer (hydroquinone), synergist (piperonyl butoxide, sesamin, mysristicin) and antioxidants (tannic acid) enhances its efficiency and makes them more economically viable (Balandrin and Klocke 1988).

18.4 Biosynthesis of Pyrethrins

Pyrethrins, a group of sesquiterpene lactones is a combination of six esters is one such defense related compounds which accumulate in achenes of the flowers of the plant *Chrysanthemum cinerariaefolium* (Kikuta et al. 2011). The pyrethrins are the esters of chrysanthemic acid or pyrethric acid which are synthesized by different pathways with the help of rethrolones. The 1-deoxy-D-xylulose 5-phosphate (DXP) pathway leads to the formation of these acid moieties which contain a cyclopropane ring (Matsuda et al. 2005). The chrysanthemyl diphosphate synthase (CPPase) enzyme catalyse the formation of cyclopropane ring which gives rise to chrysanthemyl diphosphate by utilizing two molecules of dimethylallyl pyrophosphate (Rivera et al. 2001). The pyrethrolone moieties which help in the formation of chrysanthemic acid or pyrethric acid have been synthesized through linolenic acid which is derived from the oxylipin pathway (Matsuda et al. 2005).Normally one molecule of IPP and one molecule of DMAPP condense in “Head to Tail” fashion, but in case of

pyrethrin biosynthesis, two molecules of IPP and two molecules of DMAPP condensate in an unusual "Middle to Tail" (Pattenden and Storer 1973) fashion to give rise to chrysanthemyl alcohol (Godin et al. 1963) which on further oxidation leads up to the formation of chrysanthemic acid. This chrysanthemic acid is in the form of glucoside esters, and further addition of rethrolones gives rise to three esters of pyrethrin I, i.e. cinerin I, jasmolin I and pyrethrin I (Fig. 18.2). Similarly, in the presence of three molecules of rethrolone pyrethrin II, cinerin II, jasmolin II and pyrethrin II are formed from pyrethric acid (Kueh et al. 1985). Pyrethrin biosynthesis is assumed to be induced by jasmonic acid associated volatile organic compounds as jasmonic acid as well as the alcohol moieties of pyrethrins are synthesized through a common route especially considering that wounding and elicitation play a very important part in jasmonic acid biosynthesis (Wasternack 2006).

In recent developments, the seedlings of *C. cinerariaefolium* have been subjected to tagging by [1-13C] D-glucose as a precursor for studying the biosynthetic pathway of pyrethrin production. The pathway tagging study has revealed the possible role of linolenic acid in the synthesis of alcohol moiety of pyrethrin synthesis, whereas the acid moiety was found to be synthesized by D-glucose via 2-C-methyl-D-erythritol 4-phosphate (Matsuda et al. 2005).This report has given a new concept as earlier it was thought that [14C] mevalonic acid was incorporated into the flower-derived pyrethrins (Crowley et al. 1961, 1962), as it is assumed that there is a

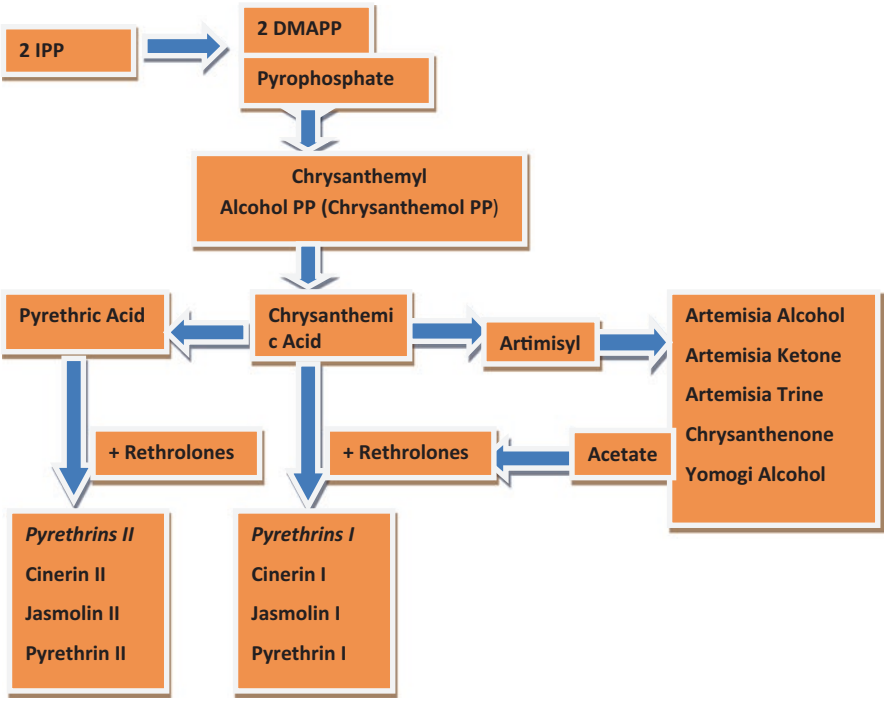

Fig. 18.2 Overview of pyrethrin biosynthesis

crosstalk between mevalonate and non-mevalonate pathway as mevalonic acid could be participating to yield acid moiety biosynthesis of pyrethrin apart from conventional biosynthesis of acid moiety from non-mevalonate pathway (Hemmerlin et al. 2003). This report has made a contradiction to the previous conventional concept as it is found that the acid moiety of the pyrethrin I is synthesized from MEP pathway in the seedlings of *C. cinerariaefolium* apart from assuming that linolenic acid was a likely contender for synthesis of alcohol moiety (pyrethrolone) of pyrethrin I. The CPPase gene or chrysanthemyl diphosphate synthase gene was first isolated by Rivera et al. (2001) which paved the way for the synthesis of chrysanthemyl pyrophosphate which contains a cyclopropane ring, and they also showed that the cyclopropanation of DMAPP is not in common head-to-tail manner. Further, the synthesis of DMAPP is from isopentenyl pyrophosphate, and an assumption was made that this IPP was made from mevalonic acid, but later works showed that 2-C-methyl-D-erythritol 4-phosphate (MEP) pathway is also involved in IPP synthesis. This finding has given a new scope to pathway elucidation in pyrethrin biosynthesis as MEP pathway occurs in plastids, but mevalonate pathway takes place in cytosols of plants as well as animals. The concept of a crosstalk between mevalonate pathway and MEP pathway was further strengthened when it was shown by the employment of [1-13C] D-glucose as a precursor showed that the MEP pathway was majorly responsible for the synthesis of acid moiety mostly in leaves (Matsuda et al. 2005). Apart from the major quantity of pyrethrin being stored in achenes of the flower (93.7%), some amount of pyrethrin is also found in receptacles (2.6%), disc florets (2.0%) as well as the ray florets (1.7%) (Head 1966). The pyrethrin compounds have been classified into two types (pyrethrin I and pyrethrin II) based on the presence of the ester linkage in them. The presence of chrysanthemoyl moiety is the characterization of type I esters of pyrethrin, whereas pyrethroyl moiety has an additional methoxycarbonyl group ester linkage. The acid moiety of pyrethrin is also characterized by its feature to contain cyclopropane ring in them. The chrysanthemyl diphosphate synthase gene has been previously isolated from *Chrysanthemum* flowers and expressed in *E. coli* which showed the catalysing ability of this gene from cyclopropane ring formation to give chrysanthemyl pyrophosphate using two molecules of dimethylallyl pyrophosphate (DMAPP) with the substrate generated by isomerization of isopentenyl pyrophosphate (IPP). The recent developments have shown that IPP is also biosynthesized via 1-deoxy-D-xylulose 5-phosphate (DXP) apart from its conventional biosynthesis from mevalonate pathway. This path-breaking finding turns the focus towards plastids as this non-mevalonate pathway or mevalonate-independent way occurs in plastid and MEP pathway takes place in the cytosol (Rohmer et al. 1996).

Matsuda (2012) assessed the biosynthesis of pyrethrum in the young seedlings with the use of 13-C labelled glucose. They considered the glucose to be the precursor for the biosynthesis of pyrethrin and found that synthesis of acid and alcohol moieties are separate events, amongst which acid biosynthesis takes place through 2-C-methyl-D-erythritol 4-phosphate (MEP), whereas the alcohol moieties are synthesized via oxylipin pathways separately. Distinctly they have also found that wound-induced volatile and nonvolatile signals affect the biosynthesis of pyrethrins

also (Matsuda 2012). The mechanical wounding of the plants results in emission of volatile organic compounds (VOCs) which help the plant to communicate at inter-plant level. The in vitro gene expression analysis of *C. cinerariaefolium* was performed by artificially damaging the seedlings and assessing its effect on pyrethrin producing genes. Placing the intact seedlings next to the wounded seedlings results in enhanced pyrethrin contents in the intact seedlings which confirms the assumption that VOCs help plant to communicate at interplanta level and intraplanta level (Kikuta et al. 2012).

For a better understanding of the pathway, Kikuta et al. (2012) were successful in purifying a protein which was claimed to play a role in the conversion of pyrethrolone to pyrethrin. Interestingly it was found that the protein that was isolated was not belonging to acyltransferase family but surprisingly it belonged to GDSL lipase family. The mutation caused in the block I domain in this protein has resulted in esterase and acyltransferase activities (Kikuta et al. 2012). It is assumed that in leaves and flowers of *C. cinerariaefolium*, the oxylipin pathway occurs, but chrysanthemic acid synthesizing enzymes are restricted to the floral parts with specific locations of glandular trichomes (Devitt- Lee et al. 2017).

In a recent study for understanding the pathway at a better level, it revealed that volatile organic compounds (VOCs) induced by wounding play an important role in pathway regulation of pyrethrin biosynthesis. The intact seedling leaves on wound-induced VOCs affected the certain set of genes, namely, chrysanthemic acid synthase (CAS), chrysanthemyl diphosphate synthase (CDS) and *Tanacetum cinerariifolium* GDSL lipase (TcGLIP), as the expression levels of these genes enhanced after the treatment with VOCs. It was suggested that for better expression of the genes, the differentiation of the tissues is important as it was found that enhanced expression of the genes was in differentiated seedlings as compared to the undifferentiated tissues (Sakamori et al. 2016).

Recently Khan et al. (2016) reported pyrethrin content from hairy root cultures as it was claimed to be the first report of genetic transformation studies mediated by *Agrobacterium rhizogenes* strain and hairy root regeneration. Amongst the different hairy root clones established, the maximum pyrethrin content was found to be in clone D3 (7.2 mg/g dw) after elicitation with biotic elicitors. Further, transcriptome-level studies in *Tanacetum cinerariaefolium* revealed that 4443 unigenes were present in the flowers and 8901 unigenes were present in the leaves. This comparative transcriptomic study between leaves and flowers delivers information regarding tissue-specific transcripts and pyrethrin's presence in the flowers (Khan et al. 2017).

18.5 Biotechnological Intervention: Improvement of Plant

Pyrethrum which is considered as one of the most important plants in the field of natural insecticidal-producing compounds needs immediate biotechnological attention as this plant suffers from low yield of pyrethrin content which falls short of the global market supply through industrial-level production. Apart from its low

pyrethrin production, the conventional cultivation methods are also not helping the cause, and industrial-level extraction processes are also not adequately optimized. These recent developments propelled the research focus shifted towards biotechnological interventions such as in vitro multiplication of plants, callus cultures, suspension and shoot and root cultures with aim to produce the varieties with better traits and desirable characters. The vegetative propagation of this plant is performed by shoot cuttings and split cuttings, but these methods are not found effective as they result in low rate of multiplication. Apart from this, the plants are also found vulnerable to attack by the nematode species which causes root knots in plants which leads to the contamination in clones by splitting of infected stock plants. The in vitro establishment and multiplication of the plants provide rapid growth with pathogen- or disease-free plants. The callus induction and regeneration of plants from callus phase in *C. cinerariaefolium* have been reported (Paul et al. 1988; Zito and Tio 1990), but the major focus has been to induce flowers under in vitro *C. cinerariaefolium* plants as flower heads are considered to contain most of the pyrethrin content (Staba and Zito 1985). Tables 18.1 and 18.2 summarize the studies conducted in vitro for *C. cinerariaefolium* establishment. The callus culture of *C. cinerariaefolium* however has shown the presence of precursors as chrysanthemic acid was found in undifferentiated mass of cells. The 14C-labelling studies have shown that glycoside rather than rethrolones esterifies the chrysanthemic acids (Zito and Tio 1990). Ahmed et al. (2011) have reported that leaf and petal explant-derived calli have the presence of pyrethrin more than the intact plants. The lack of pyrethrin synthesis in callus cultures have been majorly attributed to lack the ability to esterify monoterpene acids in pyrethrins as other steps of pyrethrin synthesis were found present in undifferentiated callus cultures (Zito and Tio 1990). Apart from it the concentration of chrysanthemic acid and pyrethric acid was found equal in plantlets and leaves, but undifferentiated cells contained almost ten times more pyrethric acid than chrysanthemic acid on a dry weight basis. The media composition and other physical factors such as pH of the medium, temperature and light intensity parameter study were also assessed on in vitro raised callus cultures of *C. cinerariaefolium.* The interesting factor which was found to affect the callus culture and pyrethrin yield was MS medium diluted two times, and pH which was found to be 5.7 for optimum growth of callus. The daylight and 23 °C temperature were found suitable for growth and multiplication of the callus culture (Hitmi et al. 2000). For rapid multiplication of shoots, the in vitro regeneration system was established from different explants such as petiolar and leaf explants.

The use of 2-4D for induction has also been studied, and it was revealed that higher concentration of 2-4D plant growth regulator affects the growth of the callus in a negative way on different genotypes of the *C. cinerariaefolium* (Obukosia et al. 2005).

The media composition played a very important role in direct regeneration from leaf explants as these explants were more responsive to adventitious shoot bud regeneration than petiolar explants. The MS medium supplemented with 4.0 mg/l BA and 0.2 mg/l 2, 4-D was found most suitable for shoot regeneration from leaf explants, whereas MS medium supplemented with 1.5 mg/l BA and 2 mg/l NAA

Table 18.1 Tissue culture studies conducted in *C. cinerariaefolium*

Explant	Hormones	Response	References
Leaves	4 mg.l^{-1}BAP 0.2 mg.l^{-1} 2,4-D	Multiple shoots formation	Hedayat et al. (2009)
Leaf/node	5.0Kn mg.l^{-1}	Axillary/multiple shoot formation	Verma et al. (2012)
Shoots	1 mg.l^{-1} NAA	Shoot proliferation	Cashyap et al. (1978)
Axillary buds	0.1 µM BAP	Multiple shoots	Wambugu and Rangan (1981)
	1.1 µM NAA		
	2 mg.l^{-1} BAP2 mg.l^{-1} IAA	Multiple shoots	Hedayat et al. (2009)
Flower buds	2 mg.l^{-1} NAA	In vitro establishment	Hitmi et al. (1999)
Capitulum	0.001 mg.l^{-1} BAP	Shoot proliferation	Roest and Bokelmann (1973)
Flower buds	2 mg.l^{-1} NAA +1 mg. l^{-1}BAP	Multiple shoots formation	Hitmi et al. (1999)
Axillary buds	1 mg.l^{-1} 2,4 D	In vitro callus induction	Zito and Tio (1990)
Shoots	0.5 mg.l^{-1} 2,4 D + 0.75 mg. l^{-1} Kn	In vitro callus induction	Cashyap et al. (1978)
Flower buds	4 mg.l^{-1} NAA + 0.4 mg.l^{-1} BAP	In vitro callus induction	Hitmi et al. (1999)
Shoots	0.05 2–4-D mg.l^{-1}	In vitro callus induction	Chumsri and Staba (1975)
Leaf/nodal parts	1.0 mg.l^{-1}-2,4-D; 0.5BAP mg.l^{-1}	In vitro callus induction	Verma et al. (2012)
Nodal explants	MS+ 40 µM BAP	Multiple shoot generation	Lindiro et al. (2013)
	MS+ 10 µM IBA	Root induction	
Shoot tips	1 mg NAA+ 1 BAP	Shoot multiplication	Grewal and Sharma (1978)
Shoot tips	0.02–2 NAA	Shoot multiplication	Wambugu and Rangan (1981)
	0.2/2 BAP 0.02		
Leaf, stem, floret	0.5 mg/l 2–4 D+ 0.5 mg/l BAP	Callus induction	Zieg et al. (1983)
Shoot tips	0.1 mg/l 2-4D+ 3 mg/l BAP		Zito et al. (1983)
Axillary buds	No PGR	Shoot multiplication	Chen et al. (1998)
Flower heads	1 mg/l NAA/ 1 mg/l BAP	Callus induction	George et al. (1999)
	2.4-D + kinetin		
Leaf explants	1 mg/l BA and 5 mg/l 2,4-D	Callus induction	Ahmed et al. (2011)
Petal explants	1 mg/l BA and 10 mg/l 2,4- D		
Nodal explants	MS+ 40 µM BAP	Multiple shoot generation	Lindiro et al. (2013)
Leaf petioles	MS+ 0.5 mg/l − 3.0 mg/l 2–4 D	Callus induction	Obukosia et al. (2005)

Table 18.2 In vitro pyrethrin detection at difference stages in *C. cinerariaefolium*

Plant's name	Source type	Pyrethrin content	References
C. cinerariaefolium	**In vitro leaves**	Py I 0.535 ± 0.013 (%DW)	Zito and Tio (1990)
		Py II 0.254 ± 0.003 (%DW)	
	Plantlets	Py I 1.266 ± 0.087(%DW)	
		Py II 0.825 ± 0.125 (%DW)	
	Callus cultures	Not detected	
T. erecta	**Callus cultures**	1.5% (%DW)	Nikam and Khan (2015)
C. cinerariaefolium	**Callus cultures**	0.023–0.113%	Ravishankar et al. (1989)
	Shoot cultures	**0.050–0.34%**	
C. cinerariaefolium	**Leaf callus**	0.22%	Rajasekaran et al. (1990)
T. erecta	**Cell suspensions**	**1.16%**	Ravishankar et al. (1989)
C. cinerariaefolium	**Callus cultures**	**35 mg %–113 mg %.**	Zieg et al. (1983)
C. cinerariaefolium	**Shoot cultures**	**50 mg %–341 mg %**	Head (1967)
	Plantlets	**2.36%**	
T. erecta	**Seedling callus cultures**	**0.9%–1.16%**	Merillon and Ramawat (2007)
C. cinerariaefolium	**Leaf derived callus**	**0.38%– 0.5%**	Dhar and Pal (1993)
C. cinerariaefolium	**Callus differentiated roots**	**ND**	Khanna and Khanna (1976), Jain (1977)
C. cinerariaefolium	**In vitro shoots**	**445.8 g 100 g^{-1} DW**	Hitmi et al. (1999)

was best suited for multiple shoot formation (Hedayat et al. 2009). The number of *Chrysanthemum* cultivars and their mutants was in vitro established with the emphasis on optimization of the explants best suited for regeneration and multiplication. Interestingly the plants established from adventitious shoot buds showed altered inflorescence and colours when shifted to glasshouse conditions. However, the plants raised through shoot-tip explants or adventitious buds from leaf explants showed noticeable phenotypic change (Zalewska et al. 2011). The rapid in vitro multiplication using nodal explants of *C. cinerariaefolium* has also been established by Lindiro et al. (2013) and they stated that the difference in the level of cytokinin and auxin for generation of microshoots and roots as far as their number and length were concerned. In the latest in vitro developmental studies, germacratrien-12-oic acid was assumed to be the candidate as a precursor for the synthesis of all the sesquiterpene lactones present *in C. cinerariaefolium* which depends upon the regio (C6 or C8)- and stereoselective (a or b) hydroxylation of germacratrien-12-oic acid (Ramirez et al. 2013). The in vitro GUS expression analysis on different cultivars showed that *Agrobacterium* strains EHA105 and MOG101, containing pMOG410, gave more numbers of blue-stained units per explants, and the in vitro explants source was proven more efficient in uptaking of GUS gene compared to other

explants sources (Boase et al. 1988). The *Agrobacterium*-based genetic transformation studies in *C. cinerariaefolium* are restricted as there are few reports in this area of research. The hairy root cultures of the plants were reported by Khan et al. (2016) which states that high pyrethrin yielding clones of the plants were established using *A. rhizogenes* strain A4. Among the selected hairy root clones, two of the highest pyrethrin yielding clones were elicited with bacterial and fungal elicitors, and their phytochemical analysis resulted in pyrethrin yield compared to "Avadh" variety of *C. cinerariaefolium.*

The direct shoot bud regeneration from hairy root clones of *Chrysanthemum cinerariaefolium* for transgenic plant production has previously never been reported, so the experiment has been designed by the authors at their own lab to check the regeneration ability of hairy root clones for its spontaneous shoot proliferation from the different hairy root clones. For the regeneration experiment, the 20-day-old hairy root clones were initially shifted to different combinations of MS media supplemented with BAP and NAA growth hormones to check the regeneration response (Table 18.3). Most of the hairy root clone showed initiation of callusing response on 15th day observation as these media combination yielded nodule like structure

Table 18.3 Different media combination for shoot bud regeneration from hairy root cultures of *C. cinerariaefolium*

Media composition	Response		
	15th day	25th day	35th day
MS + 1 mg/l NAA + 1 mg/l BAP	Signs of callus formation on tips	No further differentiation	No further differentiation
MS + 2 mg/l NAA + 1 mg/l BAP	Small callus-like growth on the root tip	Green compact structure	No further differentiation
MS + 3 mg/l NAA + 1 mg/l BAP	Signs of callus formation on tips	Initiation of callusing	Callus was green compact structure with nodules
MS + 1 mg/l NAA + 2 mg/l BAP	Swelling of the roots with intermittent nodule formation	Loose fragile callusing covering the explant	Fragile, loose callusing shifted to low BAP combinations
MS + 1 mg/l NAA + 3 mg/l BAP	Swelling of the roots with intermittent nodule formation	Nodule growth turns to callus	Loose fragile callusing covering the explant shifted to low BAP combinations
MS + 1 mg/l TDZ (synthetic seeds)	Germination of seeds with hairy root multiplication	Multiplication of HR without callusing phase	HR growth covered complete surface of petri plate with callusing
MS + 2 mg/l TDZ (synthetic seeds)	Germination of seeds with hairy root multiplication	Fast multiplication of HR without callusing phase	HR growth covered complete surface of petri plate with callusing
MS + 3 mg/lTDZ (synthetic seeds)	Immediate callusing response from hairy root clones	Intermittent swelling of roots with callusing	Swelling converted to callusing which covered whole HR with regeneration

intermittently. On the 35th day of their subculturing, an interesting observation was made that all high BAP-containing medium showed the morphologically different appearance of callus which was fragile and loose in nature which was assumed to give rise to somatic embryos. Further incubation of this hairy root clone on the same media combinations till the 35th day showed complete callusing of the explants. No further differentiation was observed; therefore those calli were shifted to low BAP-containing medium (MS + 0.1 mg/l BAP) on which these fragile loose calli showed no further differentiation (Fig. 18.3) and were not able to generate to somatic

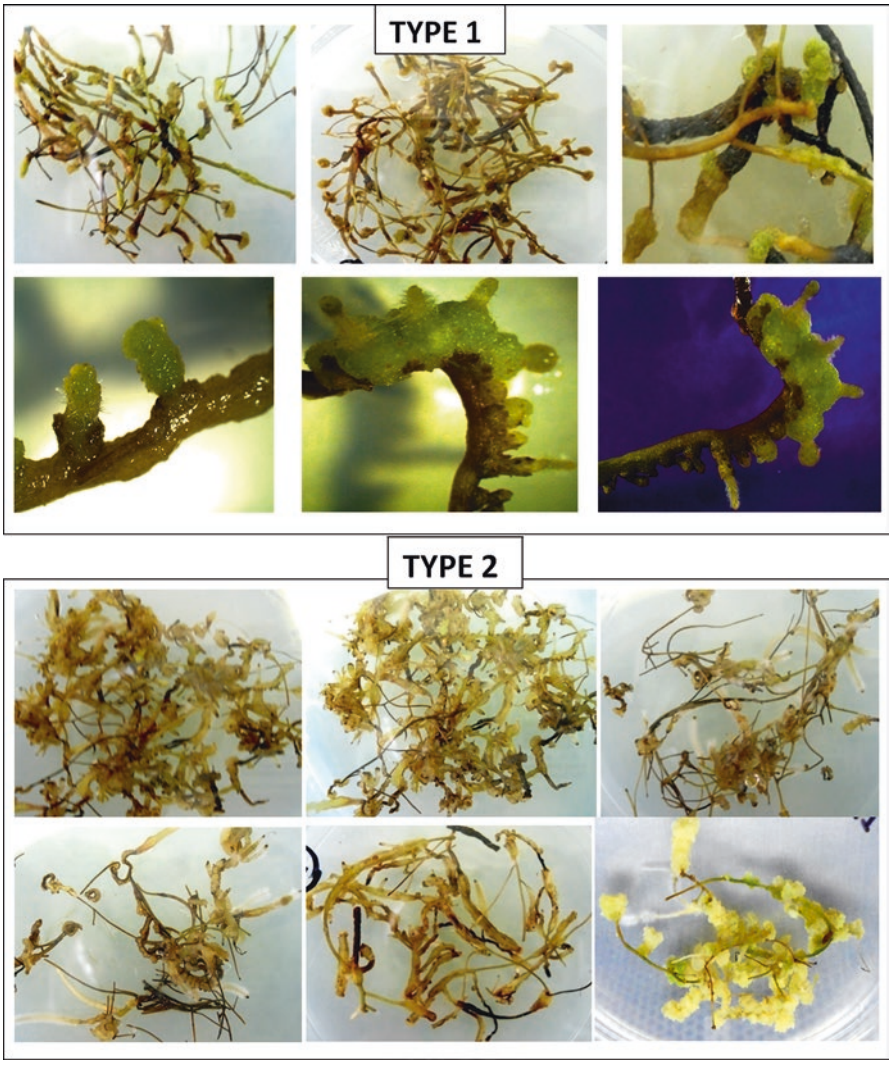

Fig. 18.3 Different types of callus observed on MS medium supplemented with NAA and BAP. Type 1, green compact nodular structures; type 2, fragile loose callus

embryos even after 3 months of their shifting**.** On the other hand, the MS medium supplemented with high NAA plant growth hormone showed a different appearance of the callus which was green compact structure with nodules and may give rise to shoot buds directly. These different appearance calli were further shifted to low BAP and NAA concentration containing MS medium for further regeneration into somatic embryo and shoot development, respectively.

Green compact nodular structures which were observed on high NAA supplemented (Fig. 18.3) were shifted to low NAA containing MS media combinations, but no shoot bud initiation was observed even after 2 months of their transfer. Further subculturing of these explants showed no improvement and no shoot bud initiation signs.

One experiment was also set with MS medium containing high TDZ plant growth regulator with synthetic seed-mediated approach. To check the response of hairy root clones for regeneration, different concentrations of sodium alginate, i.e. 1, 3 and 5% (w/v), on MS medium supplemented with high TDZ plant growth regulators were tested. Amongst the three tested concentrations of Na-alginate which were tested, beads of ideal morphology, in terms of isodiametric shape, texture and rigidity, could be obtained with the 3% concentration (Fig. 18.4). After optimization of the sodium alginate concentration, the actively growing tips of hairy root clones were shifted to the MS medium with different concentration of TDZ-enhanced growth regulator (MS + 1.0–3.0 mg/l TDZ). The synthetic seed showed hairy root multiplication response on 15th day response on MS medium supplemented with 1 mg/l TDZ and 2 mg/l TDZ which further continued till 25th day which covered the whole petri plates. The synthetic seeds showed proper initiation of the seeds without any callus formation in hairy root clones. Till the 35th day, the multiplication of hairy roots continued, but there were no signs of callus formation and regeneration response. On the contrary the third combination of MS medium with TDZ (MS + 3 mg/l TDZ) showed the immediate callusing response from hairy root clone on the 15th day observation as the synthetic seed started to germinate. On the 25th day response check, the germination of synthetic seed was proper with intermittent swelling of roots which continued till the 35th day as the swelling converted to callusing which was further converted to proper callus phase. These calli were further shifted to low TDZ-containing medium, but even after 30 days of their transfer, the callus showed no signs of regeneration.

The direct regeneration protocol from leaf explants has also been developed in *C. cinerariaefolium*, and using this direct regeneration protocol, the *A. tumefaciens*-mediated genetic transformation has been carried out using gus gene under the control of the chrysanthemum RbcS promoter. Successful transformation studies resulted in having stable expression of *gus* gene in the transgenic *C. cinerariaefolium* plants (Mao et al. 2013). Other pyrethrin yielding plant such as *Tagetes erecta* has also been studied for their genetic transformation studies using *A. tumefaciens* strainLBA 4404 containing p *CAMBIA* 121 having *gus* reporter gene. The genetic transformation studies resulted in gus-containing transgenic plants with best results obtained using hypocotyls as explants (Gupta and Rahman 2015).

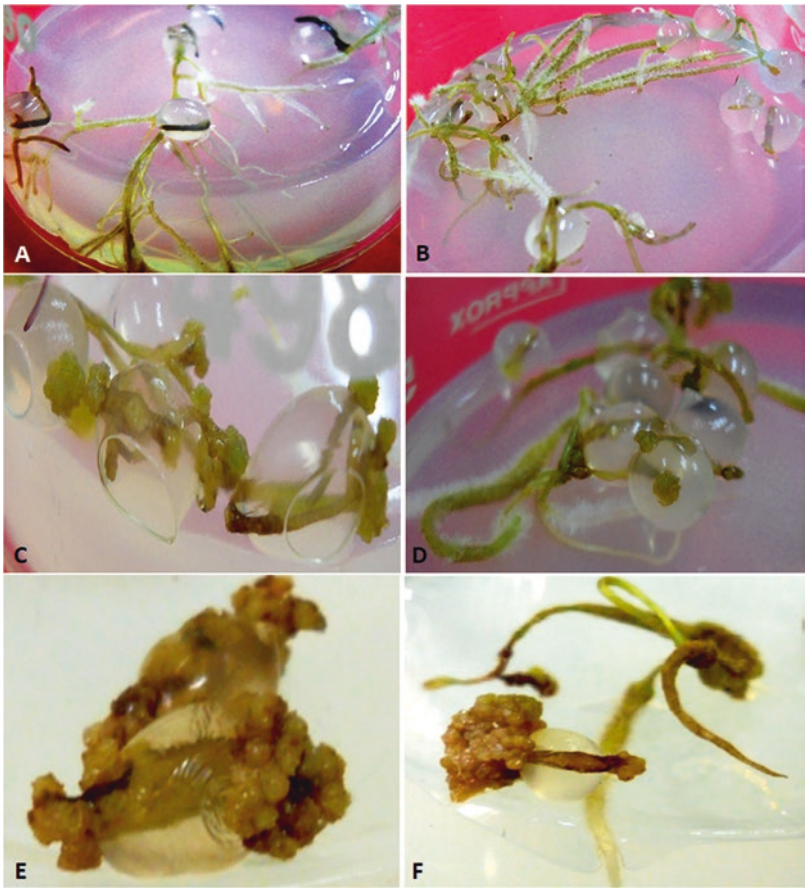

Fig. 18.4 Synseed-mediated regeneration response of D3 hairy root clone supplemented with TDZ. (**a**)–(**b**) proliferation response on MS + 1.0 mg/l TDZ and MS + 2.0 mg/l TDZ; (**c**)–(**f**) callusing response on MS + 3.0 mg/l TDZ

18.6 Future Prospects

The in vitro research in *C. cinerariaefolium* has been an area of interest as production of pyrethrin from an alternate source in a prerequisite in pyrethrin research. Although attempts have been made to produce pyrethrin under in vitro undifferentiated cultures such as callus and suspension, still a more elaborate approach towards this direction is required as the presence of pyrethrin in these undifferentiated tissues is in limited amounts. For an approach towards improved pyrethrin production, each step of biosynthesis needs to be characterized fully which in turn will help in diverting the flux in the direction of pyrethrin production. More elaborate studies of pathways will also help in blocking the other unnecessary pathways which are hindering the synthesis of pyrethrin production *in planta*. As far as the gene

characterization and overexpression studies are concerned, the gene transfer methods need to be explored in this plant system as reports of transgenic plant production in *C. cinerariaefolium* are quite limited. Apart from biolistic methods for gene transfer studies, *Agrobacterium tumefaciens-/rhizogenes*-mediated approaches need to be explored more for more efficient gene transfer. Future outcomes in improved pyrethrin production will also depend upon the protocol establishment for effective direct and indirect regeneration from different explants and somaclone variant generation which could help in better variety development under in vitro condition followed by their transfer to glasshouse- and field-level trials.

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Chapter 19
Biotechnological Approaches for Genetic Improvement of Fenugreek (*Trigonella foenum-graceum* L.)

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Abstract Fenugreek (*Trigonella foenum-graecum* L.) is one of the important medicinal plants of ancient medicinal systems due to its high nutraceutical and pharmaceutical properties. Seeds and leaves of Fenugreek contain phytochemicals like diosgenin and trigonelline. It is a cultivated plant of the modern world for medicinal uses, an edible vegetable, and a forage plant. Advancement in industrial and biotechnological techniques for the isolation of phytochemicals increase the demand of Fenugreek, and its breeding programs are based on improving the secondary metabolites compared to other uses. Recent advancement in modern biotechnological approaches enables researchers to develop elite cultivars of desired traits in a short time. Application of modern techniques like artificial mutations under in vitro conditions, characterization using molecular markers, and development of successful plant tissue culture techniques, genetic transformation techniques, and functional genomics studies have significant potential to improve Fenugreek traits. The study highlights the application of biotechnological approaches used for the development of elite Fenugreek traits for the researchers for future breeding programs. Furthermore, the research gap and areas to improve research have been highlighted in this present study.

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Abbreviation

2,4-D	2,4-dichlorophenoxyacetic acid
B5	Gamborg medium
EMS	Ethyl-methanesulfonate
IAA	Indole acetic acid
IBA	Indole-3-butyric acid
IPA	Indole-3-propionic acid
MAS	Marker-assisted selection
MMS	Methyl-methanesulfonate
MS	Murashige and Skoog medium
NAA	α-Naphthaleneacetic acid
NaN_3/SA	Sodium azide
OD	Optical density
PCR	Polymerase chain reaction
PEG	Polyethylene glycol
PGRs	Plant growth regulators
QTL	Quantitative trait locus
TDZ	Thidiazuron
UV	Ultravoilet
WP	Woody plant
ϒ-rays	Gamma rays

19.1 Introduction

Plants have a key role in human diets due to providing nutrition and biochemicals that are used as medicines; humans know the use of these herbs to cure acute or chronic diseases since ancient times. Fenugreek (*Trigonella foenum-graecum* L.) is one of the oldest plants used by different human civilizations due to its high medicinal, nutraceutical, and pharmaceutical properties and also used as an edible or spice herb. It is considered as one of the most important ingredients in old medicinal systems like Indian Ayurvedic medicines and Chinese/Tibetan medications (Zandi et al. 2017).

Fenugreek is an annual dicotyledonous herb of the Fabaceae family (subfamily – Papilionoideae). The genotypes of Fenugreek possess a wide variability in morphology growth and development, seed size and color, plant biomass, and seed production. The plant has short stature with 40–60 cm high glabrous stem, trifoliate leaves with ovate leaflets (Acharya et al. 2006a), and twin flowers. There are two types of plants; the early mature plants take 80–90 days, while late-maturing plants

Table 19.1 Major bioactive compounds of Fenugreek seeds

Compound	Chemical name	References
Carbohydrates	Galactomannans	Meghwal and Goswami (2012)
Proteins	Lecithin, albumin	Naidu et al. (2011)
Lipids	Unsaturated lipids, phospholipids, glycolipids	Chatterjee et al. (2010)
Free amino acids	4-Hydroxyisoleucine, lysine, histidine, arginine	Isikli and Karababa (2005)
Vitamins	A, B1, B2, C, nicotinic acid, niacin	Leela and Shafeekh (2008)
Minerals	Zn, P, Mn, Fe, Ca	Al-Jasass and Al Jasser (2012)
Coumarins	Trimethyl coumarin, trigocoumarin, methyl coumarin	Raju et al. (2001)
Flavonoids	Vitexin, vecenin-1, quercetin, orientin, tricin, saponaretin, naringenin, luteolin, lilyn, kaempferol, isoorientin, apigenin, isovitexin, 7-O-D glucopyranoside	Blumenthal et al. (2000)
		Suavare et al. (2000)
		Naidu et al. (2011)
		Meghwal and Goswami (2012)
Phenolics	Vanillic acid, gentisic acid, gallic acid	Rababah et al. (2011)
Saponins	neotigogenin, diosgenin, fenugrin, hederagin, glycoside, gitogenin, foenugracin, tigogenin, smilagenin, yamogenin, yuccagenin, trigonoesides, sarsasapogenin	Raju et al. (2004)
Volatile oils	N-alkanes, sesquiterpenes	Meghwal and Goswami (2012)

take 100–115 days from germination to maturity (Petropoulos 2002). Seeds are available in different color range of olive green, brown, cinnamon, or lighter green in color with seed size of 3.5–6 mm long and 2.5–4 mm wide (Petropoulos 2003).

Fenugreek seed is the most attractive part of the plant due to different bioactive compounds (Taylor et al. 2000; Acharya et al. 2006b). However, the amount of these chemicals is associated with genotypes or ecological factors which consequently affect the minor- or polygenes which in turn affect the phytochemical production (Fehr 1998; Acharya et al. 2008). Major bioactive compounds and chemicals contained in Fenugreek seeds are given in Table 19.1.

Fenugreek has high nutritional values due to presence of macro- and microelements and dietary food fiber in leaves and seed (Thomas et al. 2011). Fresh leaves are used as vegetable (Balch 2003), and dried leaves are utilized as flavoring agent (Olaiya and Soetan 2014). Fenugreek seeds are the major constituent of oily pickles for special flavor in the Indian subcontinent (Najma et al. 2011) and for bread making in Egypt as a staple food (Mehrafarin et al. 2011). Seeds are rich in proteins

Table 19.2 Pharmacological and therapeutic uses of Fenugreek

Disease/disorders	References
Aging	Kaviarasan et al. (2004)
Anemia	Mahmoud et al. (2012)
Antibacterial	Premnath et al. (2011)
Antifertility	Aswar et al. (2010)
Antimicrobial	Jasim et al. (2017)
Anti-obesity	Kumar and Bhandari (2015)
Antioxidant	Ktari et al. (2017)
Antiulcer	Al-Meshal et al. (1995)
Cancer/anticarcinogenic	Mohamed et al. (2015)
Diabetes	Jiang et al. (2017)
Hypercholesterolemia/antilipidemic	Sharma and Choudhary (2016)
Immunodeficiency/immunomodulatory	Moradi Kor and Moradi (2013)
Indigestion and flatulence	Sauvare et al. (2000)
Inflammation	Piao et al. (2017)
Kidney disorders	Belguith-Hadriche et al. (2013)
Myocardial infarction	Panda et al. (2013)
Skin irritation	Meghwal and Goswami (2012)

(Naidu et al. 2011), carbohydrates (Najma et al. 2011), minerals (Hegazy and Ibrahim 2009; Jani et al. 2009), and vitamins (Sharma 1986). However, the use of Fenugreek leaves after boiling, frying, or steaming resulted in depletion of certain vitamins (Leela and Shafeekh 2008). Fenugreek is an important constituent in food processing as food adhesive, stabilizer and emulsifier (Jani et al. 2009), food gum (Sowmya and Rajyalakshmi 1999), alcoholic beverages (Jani et al. 2009), bread making (Raju et al. 2001, Meghwal and Goswami 2012), and food preservative (Betty 2008).

Fenugreek seeds are rich in phytochemicals having certain pharmacological properties and are highly effective against diseases. The major use of Fenugreek as medicinal plant includes lactation, stimulant, and condiment (Betty 2008) in India, treating labor pain by Romans, leg weakness and edema by Chinese (Yoshikawa et al. 2000), and diabetes by Africans and Asians (Miraldi et al. 2001). Other folkloric uses for Fenugreek include anemia (Kaviarasan et al. 2004), kidney disorders (Xue et al. 2011; Belguith-Hadriche et al. 2013), skin irritation (Suavare et al. 2000; Meghwal and Goswami 2012), and other diseases like arthritis, chronic cough, dropsy, epilepsy, gout, liver disorders, paralysis, piles, and respiratory disorders (Ahmadiani et al. 2001; Tayyaba et al. 2001; Kaviarasan et al. 2004; Amin et al. 2005), whereas the use of modern technologies enables humans to use these bioactive compounds more efficiently against different diseases and disorders (Table 19.2).

19.2 Genetic Resources of Fenugreek

Fenugreek or *Trigonella* has a large number of species throughout the world, and around 260 species have been reported (Petropoulos 2002; Basu 2006). Some of these *Trigonella* species are cultivated like *T. balansae*, *T. calliceras*, *T. spicata*, *T. occulta*, *T. lilacina*, *T. corniculata*, *T. spinosa*, *T. caerulea*, *T. radiata*, *T. maritima*, *T. cretica*, *T. polycerata*, and *T. foenum-graecum.* The most cultivated among these species is *T. foenum-graecum* due to its high demand for its bioactive compounds used for medicinal purposes (Petropoulos 2002). These *Trigonella* species and landraces show their distribution in Australia, Asia, Africa, and Europe. The major Fenugreek-producing countries are India, China, Turkey, Pakistan, Spain, India, France, Morocco, Egypt, and Ethiopia (Petropoulos 2002; Acharya et al. 2008), whereas the crop is still cultivated as a minor crop in North America (Canada and the United States) (Fig. 19.1).

The word *Trigonella* means "little triangle" due to triangular-type resemblance of leaves, and *foenum-graecum* means "Greek hay" due to its early introgression from Greece (Basu 2006). However, there are different theories about the exact ancestors and origin of *T. foenum-graecum* besides of its cultivation since 4000 BC (Acharya et al. 2008). The probable ancestors of *T. foenum-graecum* are *T. gladiata* (Petropoulos 1973) and *T. gladiate* (Petropoulos 2002). Regarding center of origin of Fenugreek, different geographical regions have been reported by different researchers. Duke et al. (1981) reported the Mediterranean region as the center of origin for Fenugreek. Earlier, De Candolle (1964) and Fazli and Hardman (1968) reported Asian regions (Mesopotamia, Persia, Punjab, and Kashmir) and European

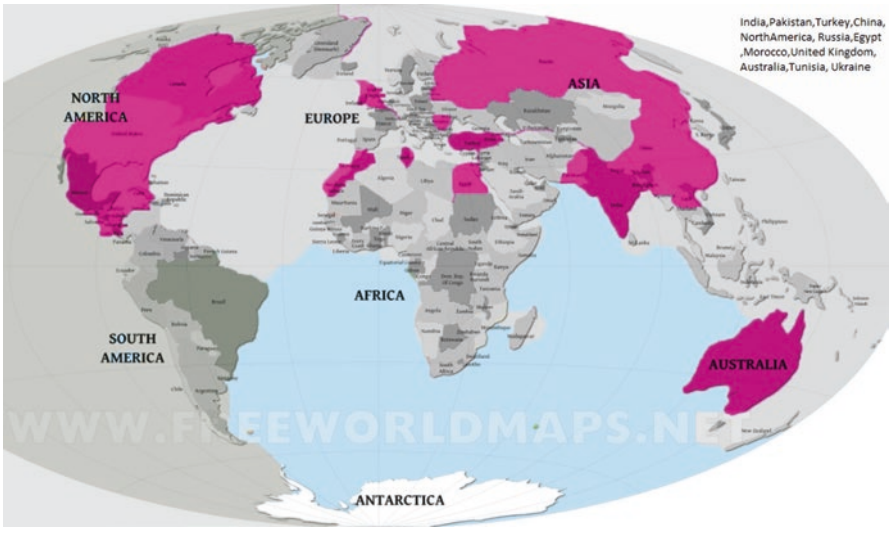

Fig. 19.1 Geographical distribution of Fenugreek (*T. foenum-graecum*) – pink color shows the presence of Fenugreek

regions (Greece, Italy, and Spain) as an origin of Fenugreek, whereas Turkey was reported as a center of origin for Fenugreek (Dangi et al. 2004) due to the presence of a number of Fenugreek genotypes. Currently, 51 species of Fenugreek have been recorded in Turkey having endemic *Trigonella* species with distribution in the Black Sea and Mediterranean region.

Germplasm collection of any plant/crop has significant impact on its improvement through conventional breeding or application of modern biotechnological tools. The germplasm collection of Fenugreek is available at the Plant Gene Resources of Canada, Saskatoon in Canada (Acharya et al. 2006b); National Bureau of Plant Genetic Resources, India (Basu et al. 2014); Longerenong Agricultural College and University of Melbourne, Australia (McCormick et al. 2009); and USDA ARS Plant Introduction Station, Washington.

Genetic improvement of any crop can be accomplished through the application of traditional or modern biotechnological approaches. The commercial use of Fenugreek all over the world is mainly based on its bioactive compounds like diosgenin, and there is dire need to improve Fenugreek germplasm for steroidal industry and for food industry (Petropoulos 2002; Basu 2006; Acharya et al. 2008) with more yield. Currently, breeding programs for Fenugreek improvement include selection (Prajapati et al. 2010, Basu et al. 2014), hybridization (Acharya et al. 2008), and mutation (Rajoriya et al. 2016) used singly or in combination of more than one method (Mehrafarin et al. 2011). It is difficult to develop new cultivars by hybridization under field conditions due to self-pollinated nature of Fenugreek (Petropolous 2002; Acharya et al. 2008).

Application of modern biotechnological approaches enables researchers to develop elite cultivars of desired traits in a short time. Therefore, modern techniques like induced mutations under in vitro conditions (Petropolous 2002), characterization using molecular markers, development of successful plant tissue culture techniques (Aasim et al. 2009), and genetic transformation techniques have significant potential to improve Fenugreek traits. In recent years, researchers are focusing on the isolation and utilization of secondary metabolites of Fenugreek due to advancement in industrial and biotechnological techniques. On the other hand, work related to its genetic or biotechnological improvement is also confined to secondary metabolites rather than its improvement for human consumption, food processing, or as forage plant. The study highlights the application of biotechnological approaches used for the development of elite Fenugreek traits with main focus on its phytochemicals and molecular genetic diversity.

19.3 Mutation Breeding of Fenugreek

The importance of medicinal metabolites of Fenugreek along with its nutritional values opens a new window for its cultivation worldwide. Furthermore, low agronomic practices, soil fertility, and adaptation to wide climatic regions are the major factors for its distribution (Petropolous 2002; Acharya et al. 2006a, b; Montgomery

et al. 2006). However, there is still need to breed/develop new varieties which can cope with local climate and can produce more yield with higher concentrations of nutraceutical compounds in order to increase its economic value. Mutation breeding is one of the most commonly practiced techniques for generating genetic variation in existing gene pool of economic plants (Toker et al. 2007) up to a certain extent (Fehr 1993) and can facilitate selection in the local environment (Yadav et al. 2007). Furthermore, a large number of alleles can be manipulated for a particular trait (Chopra 2005). The final result of this mutation breeding is recessive and segregated as 3:1 ratio in diploid plants like Fenugreek, and there is need to check the results up to the second generation (Micke and Donini 1993).

Mutagens (physical or chemical) are playing a vital role for the development of new traits with more yield and quality by developing resistance to biotic or abiotic stresses. In Fenugreek breeding program, mutagens have been applied for the development of genetic variability for desirable traits. Mutation breeding in Fenugreek is carried out through spontaneous mutation or induced mutation (Petropolous 1973; Singh and Singh 1976; Laxmi and Datta 1987) using chemicals or physical mutagens like radioactive rays. There are few examples of development of Fenugreek varieties through spontaneous mutation (Petropolous 1973; Singh and Singh 1976; Laxmi et al. 1980; Laxmi and Datta 1987) like RH 3129 variety that was developed from spontaneous mutation of Moroccan cultivar having twin pods and high diosgenin contents (Laxmi et al. 1980; Petropolous 2002).

The first study reported for Fenugreek mutants by using chemical mutagens was reported by Auerbach (1961), and the seeds were obtained successfully, whereas colchicine treatment to shoot apex resulted in tetraploid plants (Roy and Singh 1968). Further studies using physical mutagens (UV irradiation, ϒ-rays) or chemical mutagens (ethyl-methanesulfonate (EMS), methyl-methanesulfonate (MMS), and sodium azide (NaN_3 or SA) have been successfully reported by different researchers. Anis and Wani (1997) reported the meiotic abnormalities (bridges, cytomixis, laggards, nondisjunction, precocious movement of chromosomes stickiness, univalents, etc.) due to caffeine treatment. Similar types of effects were also observed on the morphological characters of Fenugreek which was directly proportional to caffeine dosage.

Sodium azide (SA/NaN_3) is one of the most commonly used chemical mutagen for developing Fenugreek mutants. Siddiqui et al. (2007) applied 0.1, 0.2, 0.3, 0.4, and 0.5% NaN_3 at 24 ± 1 °C for 72 h on root tip cells of *T. foenum-graecum* for cytogenetic changes. Application of NaN_3 resulted in decreased seed germination (%), radicle length, and mitotic index, whereas increased chromosome stickiness, bridge formation, precocious separation, and lagging chromosomes were also observed. Prabha et al. (2010) checked 1.5 mM, 3.0 mM, and 4.5 mM of sodium azide for 4, 6, and 8 h and recommended the 4.5 mM sodium azide applied for 4 h for selecting new genotypes with higher seed yield of Fenugreek. Kapoor and Srivastav (2010) treated seeds with sodium azide and obtained as tetraploid and mixoploid in M_2 generation with meiotic abnormalities. Further study of M_3 generation revealed the decreased RAR in tetraploid and reduced chiasma per chromosome for both types of mutants.

Ethyl-methanesulfonate (EMS) is another mutagen that has been used for seed quality and production (Basu 2006). Earlier, Chaudhary and Singh (2001) obtained determinate mutant by treating Rmt 1 (indeterminate variety) with 0.10% EMS for 4 h and rose up to M3 generation. Their results revealed the significant difference on plant growth and insect infestation compared to Rmt 1 and UM 305 (spontaneous mutant). Basu et al. (2008) successfully developed Fenugreek mutants with early maturity, determinate growth, and high seed yield by employing EMS.

Besides using single mutagen, a number of studies reflected the use of more than one chemical mutagen or comparison of physical and chemical mutagens. The first report on comparison of multiple chemical mutagens was reported by Jain and Agarwal (1987). They obtained two- to fourfold steroidal sapogenins (diosgenin and tigogenin) in seeds and plants of *T. foenum-graecum* when treated with chemical mutagens (EMS, MMS, and NaN3) at low concentrations. Contrarily, treated with higher concentrations resulted in decreased sapogenins contents, whereas Agarwal and Jain (2015) treated *T. foenum-graecum* and *T. corniculata* seeds with different concentrations of EMS, MMS, and NaN_3 for steroidal sapogenins production. Both steroidal sapogenins were enhanced with the application of all three mutagens with maximum augmentation at 0.1 M EMS.

The studies related to comparison of physical and chemical mutagens by researchers reported the variable effects of these mutagens on developing Fenugreek mutagens. Gadge et al. (2012) treated the Fenugreek seeds with UV radiation and ethidium bromide at different doses and reported ethidium bromide as better mutagen compared to UV or UV+ ethidium bromide. Bashir et al. (2013a) applied ϒ-rays, EMS, and SA at different dose/concentration on M_1 and M_2 generations. Their conclusion was decreased mutagenic effectiveness with the increased mutagen dose/concentration, and mutagenic efficiency of mutagens was EMS > SA > ϒ-rays. In another study, Bashir et al. (2013b) reported decreased germination percentage, seedling height, percent survival, and pollen fertility with increased dose/concentration of the mutagens. They also concluded that EMS treatments were more superior to ϒ-rays and SA in inducing pollen sterility (EMS > ϒ-rays>SA). Recently, Rajoriya et al. (2016) investigated the mutagenic effect of ϒ-rays, EMS, and SA and obtained M_1 and M_2 generations. Their results revealed decreased germination, seedling height, and plant survival with increased doses/concentrations of mutagens. Comparing mutagens, ϒ-ray treatment was more detrimental than other mutagens on plant growth and survival. Application of physical and chemical mutagens in these studies revealed the variable effects of mutagens on newly developed mutants. The difference in results might be due to difference in genotypes, mutagens, concentration, and mode of application. However, it is important to note that multiple mutagens have been employed for the development of new superior mutants in recent years.

19.4 Molecular Genetic Diversity of Fenugreek

In the modern era of the twenty-first century, exploiting the natural biodiversity for novel alleles in order to improve the production, quality, nutritional value, and adaptation to different geographical regions has immense importance in modern breeding programs. Increasing human population and demand for nutrition can be coped with the application of modern plant breeding for elite crops with high yield. However, scarcity of local genetic material and use of elite cultivars resulted in erosion of genetic material and brought the crops/plants to an endangered level. Therefore, there is a need to save these endangered landraces by using biotechnological techniques for conserving the elite genes which control the yield and quality for the coming future.

Exploitation of phenotype and genotype variations in order to characterize and managing genetic diversity and germplasm collection of different plant species have been done during the last few decades. Advancement in genome mapping and sequencing methods provide a toolbox for researchers/scientists to explore the structure and function of the genome of desired organism (Baloch et al. 2017). Molecular markers enable to measure direct genetic diversity and allow to proceed further beyond indirect diversity measures, based mainly on morphological traits or geographical origin of that species. Currently, different marker systems are available for the monitoring of genetic diversity, and these molecular markers have been employed for the determination of genetic diversity of Fenugreek (Table 19.3).

Table 19.3 An overview of molecular markers used for genetic diversity of Fenugreek

Molecular markers	No of accessions/genotypes/varieties etc	References
RAPD	17 varieties	Sundaram and Purwar (2011)
	61 accessions	Choudhary et al. (2013)
	7 accessions	Haliem and Al-Huqail (2014)
	5 cultivars	Modi et al. (2016)
	48 genotypes	Mamatha et al. (2017)
AFLP	20 landraces	Ahari et al. (2014)
	24 accessions	Al-Maamari et al. (2014)
ISSR	49 accessions	Randhawa et al. (2012)
RAPD/ISSR	17 accessions	Dangi et al. (2004)
	30 genotypes	Tomar et al. (2014)
	8 varieties and 6 populations	Hora et al. (2016)
RAPD/AFLP	5 varieties	Kumar et al. (2012)

19.4.1 Random Amplified Polymorphic DNA (RAPD) Markers for Fenugreek Genetic Diversity

Studies on Fenugreek regarding the application of molecular markers revealed the use of RAPD markers more than other markers like AFLP, ISSR or comparison of two markers like ISSR/RAPD or RAPD/AFLP markers. RAPD primers (18) for assessing the genetic diversity and species relation of two taxonomically *Trigonella* species and 61 accessions were reported by Sundaram and Purwar (2011). They recorded a total of 141 bands, and 74 were polymorphic with 66–100% polymorphic band range with an average of 52.85%. Genetic similarity values of 0.66–0.90 showed the moderate to high genetic variability, whereas these populations were divided into two main clusters with two separate subgroups. Choudhary et al. (2013) evaluated the genetic variability of 17 varieties using morphological and 17 RAPD markers and recorded 57.66% polymorphism. They also divided these 17 varieties into two major clusters with 12 varieties in cluster-I and 5 varieties in cluster-II. Similarly, these varieties were also distributed into two major clusters on the basis of morphological dendrogram. It was interesting to note that morphological analysis of some varieties was not accordingly to RAPD analysis due to environmental factors.

Haliem and Al-Huqail (2014) analyzed the correlation between biochemical characteristics and genetic variation of seven wild accessions of Fenugreek collected from different ecogeographical regions by using RAPD markers. The results of molecular analysis revealed high polymorphism (94.12%), whereas 90.00 and 93.75% total polymorphism values were recorded for acid phosphatase and glutamate-oxaloacetate transaminase. Modi et al. (2016) analyzed the 5 *Trigonella* cultivars to assess the genetic diversity by using 11 RAPD primers. They reported a total of 80 bands of 200–3060 bp size, of which 66 were polymorphic with 82.50% polymorphism. They also reported Jaccard's similarity coefficient of 0.266–0.615 and constructed a dendrogram which revealed two clusters. Mamatha et al. (2017) analyzed the genetic diversity of 48 *Trigonella* genotypes by using 30 RAPD markers which yielded 119 bands of 50.00–91.66% polymorphism with 79.21% polymorphism, whereas polymorphism information content (PIC) value was ranged 0.66–0.90, and these genotypes were clustered into 10 groups at 0.75 similarity coefficient.

19.4.2 Amplified Fragment Length Polymorphism (AFLP) Markers for Fenugreek Genetic Diversity

There are only two reports which revealed the use of AFLP markers for Fenugreek. Twenty Fenugreek accessions collected from different parts of Oman with 4 accessions from Iraq and Pakistan were compared by Al-Maamari et al. (2014). They employed 6 AFLP markers and attained 1852 polymorphic loci from 24 accessions.

The highest genetic diversity (H) of 0.2146 was recorded for Omani populations as compared to 0.0844 (Pakistan) and 0.1620 (Iraq). Their results proved the cultivation of Fenugreek for long time with frequent exchange of genetic material among Fenugreek accessions cultivated in Oman. Another study by Ahari et al. (2014) revealed the use of 20 landraces of Iranian Fenugreek genetic diversity with the help of AFLP markers. They obtained 147 bands with 50–500 bp size and 87% polymorphism by using 5 AFLP primers. The results of PIC were scored 0.79 (Kashan), 0.93 (Broojerd), and 0.93 (Kashan), whereas genetic similarity coefficient was scored 44–94% among landraces.

19.4.3 Inter Simple Sequence Repeat (ISSR) Markers for Fenugreek Genetic Diversity

There is only a single report regarding use of ISSR markers for assessing the genetic diversity of Fenugreek. Randhawa et al. (2012) analyzed the 49 accessions of Fenugreek collected from different locations using 19 morphometric and 186 ISSR markers. The morphometric data classified the accessions into two clusters with ~65% similarity. Initial screening with 100 ISSR primers resulted in 21 polymorphic primers, and these 21 primers generated 186 amplicons with 92.4% polymorphism, whereas 47 accessions were classified as single group with ~65% similarity on the basis of cluster analysis.

19.4.4 ISSR/RAPD Markers for Fenugreek Genetic Diversity

Most of the studies on molecular genetic diversity of Fenugreek have the use of two markers for assessing and comparing the genetic diversity. Dangi et al. (2004) studied genetic diversity of 17 accessions of *T. foenum-graecum* and 9 accessions of *T. caerulea* collected from different parts of the world by using ISSR, RAPD, and ISSR+RAPD markers. Their results revealed the distribution of accessions from different geographical regions of both species into different groups. They also reported higher genetic similarity indices of *T. caerulea* compared to *T. foenum-graecum.* Similarly, molecular and biochemical characterization of ten Fenugreek accessions was reported by Harish et al. (2011) using ISSR and RAPD markers.

A study by Tomar et al. (2014) using 30 RAPD and 20 ISSR markers and 30 Fenugreek genotypes yielded 250–1300 bp products, whereas a relatively higher proportion of polymorphic bands were recorded for RAPD (76.78%) compared to ISSR (68.08%). The dendrogram constructed for RAPD and ISSR revealed the classification of genotypes into two main groups. Recently, Hora et al. (2016) checked the genetic diversity of 8 varieties and 6 populations of Fenugreek collected from Northern India by using 100 ISSR and 400 RAPD markers. The polymorphism

among different Fenugreek varieties and populations was recorded 42.91% for RAPD and 55.66% for ISSR markers. They also reported the effective use of cluster analysis for unraveling the genetic variation within the accessions and use of RAPD and ISSR markers for assessing the genetic diversity and genetic relationship.

19.4.5 RAPD/AFLP Markers for Fenugreek Genetic Diversity

Nine RAPD and 17 fluorescently labeled AFLP primers for assessing the genetic diversity of 5 varieties are cultivated in India by Kumar et al. (2012). They reported 47 bands with 200–5000 bp size and average polymorphism of 62.4% for RAPD markers, whereas 669 bands with 50–538 bp size were amplified for AFLP primer combinations (PCs). The mean genetic diversity (Nei's 1973) of 23.83% (RAPD) and 2.1% (AFLP) was recorded across all loci. Results also revealed more polymorphism for RAPD markers compared to AFLP markers, whereas reproducibility and authentication of AFLP markers were more compared to RAPD markers.

The studies on molecular markers reflected the use of these markers for optimization of genetic diversity by using a single marker or comparison of two markers for same number of accessions, genotypes, varieties, etc. In all these studies, researchers used variable number of accessions/genotypes/varieties collected from their own region or other regions of the world. In general, there is need to use more detailed work with more focus on using a number of accessions/genotypes/varieties for future studies to select target-specific superior traits on the basis of molecular markers for specific geographical region with more yield and quality.

19.5 In Vitro Cell/Tissue Culture of Fenugreek

Fenugreek is an important medicinal plant that contained bioactive compounds like alkaloid, saponins, choline, steroidal sapogenins trigonelline, trigocoumarin, and trimethyl (Aasim et al. 2014). Although, fenugreek varieties developed all over the world have better morphological characteristics, wide geographical adaptation, and more yield, the primary objective of these efforts made to date to improve Fenugreek is based on these bioactive compounds. It is very important to understand the variations that occur in metabolites production or medicinal pathway (Al-Habori and Raman 2002) for genetic improvement of Fenugreek.

Plant cell and tissue culture techniques provide direct production of elite plants or induction of callus, cell suspension cultures, somatic embryogenesis, or genetic transformation (Aasim et al. 2014) for the production of economically important diosgenin and trigonelline (Oncina et al. 2000; Ramesh et al. 2010). The results of different researches show the advantage of isolation of secondary metabolites through in vitro cell culture compared to whole plant or seeds taken from field conditions. Furthermore, the cells or plants taken from in vitro culture are consistent

and elite in nature due to being grown under controlled environment. Furthermore, application of different chemicals/enzymes/organic compounds or controlled change in culture conditions more efficiently makes it possible to change the metabolite concentration. There are a number of reports available which highlight the use of different plant tissue culture techniques like callus culture, cell suspension culture, protoplast culture, and organogenesis under in vitro production which have been employed for genetic improvement and phytochemical production.

19.5.1 In Vitro Cell Suspension Culture of Fenugreek

Cell suspension culture is the most common technique used for the synthesis of secondary metabolites. Furthermore, it also allows the researchers to check the efficacy of different chemicals or organic compounds on cell growth and subsequently secondary metabolites production of economic plants. This technique was first employed by Cerdon et al. (1945) in Fenugreek, and they reported 20% decreased cell growth when culture medium was provided with 125 μM diniconazole compared to control after 21 days of culture. The reduction in plant growth due to diniconazole treatment resulted in 50% decreased total sterol contents. Later on, Khanna et al. (1975) gained more sapogenin contents by adding cholesterol in the suspension culture medium. Positive bearings of mevalonic acid on steroidal sapogenin synthesis during cell suspension cultures of Fenugreek tissues were reported by Trisonthi et al. (1980). Similarly, application of cholesterol in cell suspension culture also resulted in enhanced sapogenin contents (Brain and Williams 1983). A clear correlation between copper and de novo synthesis of medicarpin (isoflavonoid pterocarpans) using cell suspension culture has been reported by Tsiri et al. (2009), whereas 37% more trigonelline contents have been reported by adding nicotinic acid in the cell suspension culture of Fenugreek (Ramesh et al. 2010).

19.5.2 In Vitro Protoplast Culture of Fenugreek

The studies about protoplast culture of Fenugreek are limited and used for both in vitro isolation of secondary metabolites and shoot regeneration. The first study on protoplast culture was reported by Shekhawat and Galston (1983), and they successfully gained green calli and leafy shoots. They used mesophyll protoplasts taken from leaf explant followed by culture on medium enriched with 0.1 mg/l 6-Benzylaminopurine (BAP) and Zeatin. Christen (2002) successfully developed protoplast culture using root apices explant, but they failed to convert it into shoots. However, successful shoots induction from protoplast taken from root apices were reported by Petropoulos (2002) and Mehrafarin et al. (2010). They also achieved more trigonelle contents from callus that were 3–4-folds more than seeds and 12- to 13-folds more than roots and shoots.

19.5.3 In Vitro Callus Culture of Fenugreek

Callus culture is an important technique used for plant proliferation, somatic embryogenesis, cell suspension culture, protoplast culture, and isolation of secondary metabolites in Fenugreek. Most of these studies on callus culture of Fenugreek were used or developed for secondary metabolites isolation rather than shoot/plant proliferation. Callus induction using different explants, plant growth regulators, and culture conditions proved to be more economic and efficient for secondary metabolites production compared to seeds. Joshi and Handler (1960) reported the importance of nicotinic acid and s-adenosylmethionine for trigonelline production enriched with additional adenosine triphosphate (ATP) and $MgCl_2$ in the culture medium. Their results revealed three- to fourfold more trigonelline contents compared to seeds. They also reported 12- to 13-fold more trigonelline contents from callus culture than roots or shoots culture. Khanna and Jain (1973) reported higher steroidal contents (diosgenin, gitogenin, tigogenin) and spirostane derivatives from callus culture using 1 mg/l 2,4-D on agar solidified MS medium. The best culture time for the production of these metabolites were optimized as 6-week-old callus cultures. Radwan and Kokate (1980) attained more trigonelline contents (15.6 mg/g of dry wt) after 4 weeks of culture which were 3- to 4-folds more than seed and 12- to 18-folds more than roots/shoots culture, whereas increased trigonelline contents were also recorded on medium supplemented with 10 mg/l 2,4-D, IAA, IPA, and NAA.

Higher trigonelline contents from calli compared to in vivo culture using different explants were presented by Ahmed et al. (2000). The trigonelline contents under in vivo conditions were recorded as 0.45 mg/g (leaves), 0.21 mg/g (stems), and 0.29 mg/g (roots), whereas trigonelline contents from calli were recorded as 0.61 mg/g (leaves), 0.30 mg/g (stems), and 0.40 mg/g (roots). Oncina et al. (2000) also used calli of different explants for diosgenin production and obtained 2.2 mg/g of dry wt. (leaf), 0.74 mg/g (stem), and 0.60 mg/g (root) from 45-day-old calli. Rezaeian (2011) reported increased callus induction with increase in 2,4-D and achieved maximum callus induction from shoot apical meristem explant after 45 days of culture, whereas diosgenin contents were high in leaf calli compared to shoot or root callus. Variable effects of mannitol and sodium chloride on calli growth and secondary metabolites levels were reported by Hussein and Aqlan (2011). The highest total chlorophyll and protein contents from callus culture (2.727 mg/g) compared to 0.789 mg/g from in vitro regenerated shoots and 0.421 mg/g from fresh callus were recorded (Prabakaran and Ravimycin 2012). Recently, importance of harvesting time, type of media, and plant organ on the concentration of diosgenin of Fenugreek was highlighted by Ciura et al. (2015). The highest content of diosgenin was recorded from leaves compared to stems, roots, and callus culture. They also reported the highest content of diosgenin between the 21st and 38th day of growth. Alalwani and Alrubaie (2016) checked the effects of PEG and combination of PEG+magnetic water (0% PEG+1000G, 3% PEG +1000G, 6% PEG +1000G, 9% PEG +1000G) on the production of trigonelline from callus of *T. foenum-graecum* L. Provision of 1 mg/L BA +1 mg/L 2,4-D was optimized for callus induction, whereas

9% PEG and 9% PEG +1000G magnetic water resulted in maximum trigonelline contents from callus

Besides use of callus for secondary metabolites production, a number of studies revealed the successful use of different explants and culture conditions for callus induction. Shekhawat and Galston (1983) reported 0.1 mg/l of BAP, zeatin, glutamine, and asparaginase in the culture medium as best for callus induction and differentiation, rapid cell division, and growth, whereas Azam and Biswas (1989) reported MS medium enriched with NAA, 2,4-D, kinetin, and coconut water for callus induction and growth of Fenugreek. El-Bahr (1989) reported MS medium enriched with 3% sucrose and 2 mg 2,4-D for optimum callus induction of Fenugreek. Seyedardalan and Mahmood (2013) reported direct somatic embryogenesis using hypocotyls. MS medium containing 3 mg/l picloram+0.5 mg/l BAP was optimal for globular embryos induction followed by 2 more weeks for maturation. Abd Elaleem et al. (2014) successfully developed callus from cotyledons and hypocotyls explants. MS and B5 media augmented with 2,4-D and NAA resulted in 100% callus induction.

In recent years, number of studies highlighted the successful callus induction using different explants and culture conditions but failed to obtain shoot induction from induced callus. Aasim et al. (2010) achieved callus induction from hypocotyl explant but failed to get shoots from induced calli. El-Nour et al. (2013) induced calli by using 8- to 20-day-old cotyledonary node and hypocotyl explants cultured on MS and B5 media containing different PGRs. They achieved maximum callusing index value (2.8) from MS medium enriched with 1.5 mg/l, 2,4-D using hypocotyls and cotyledons explants. In another study, El-Nour et al. (2015) successfully achieved callus induction of Fenugreek using cotyledons and hypocotyl explants cultured on MS medium containing 0.5 mg/l Kin with different concentrations of 2,4-D and NAA. Among explants, hypocotyl explant was more responsive than cotyledon for callus induction. The highest mean callus index for hypocotyl (3.50 ± 0.15) and cotyledon (2.41 ± 0.18) was recorded on medium enriched with 4.0 mg/l NAA+ 0.5 mg/l Kin and 1.0 mg/l 2, 4-D, respectively, after 6 weeks of culture. In both studies, they failed to induce shoots from calli.

19.5.4 In Vitro Organogenesis/Regeneration of Fenugreek

In vitro organogenesis of Fenugreek is one of the greatest challenges for researchers to develop reliable and reproducible protocol, although a number of studies on in vitro regeneration through direct or indirect organogenesis or direct or indirect somatic embryogenesis have been reported for Fenugreek. But these studies have major drawbacks like difficulties in propagation, rooting, and adaptation which make this plant recalcitrant in nature. Therefore, callus induction or somatic embryogenesis employing different techniques like cell suspension culture, callus induction, or protoplast culture for secondary metabolites production are more preferable compared to organogenesis. Although, reports are available which reflect the

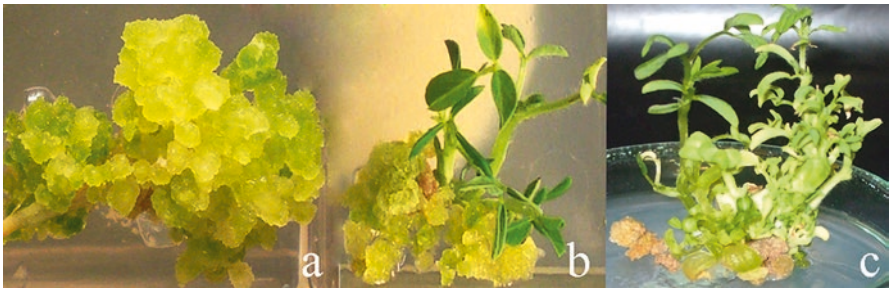

Fig. 19.2 Callus induction and shoot regeneration from hypocotyl and cotyledon node explant. (**a**) Callus induction on hypocotyl explant; (**b**) shoot regeneration using BAP-NAA; (**c**) and kinetin (Aasim et al. 2010)

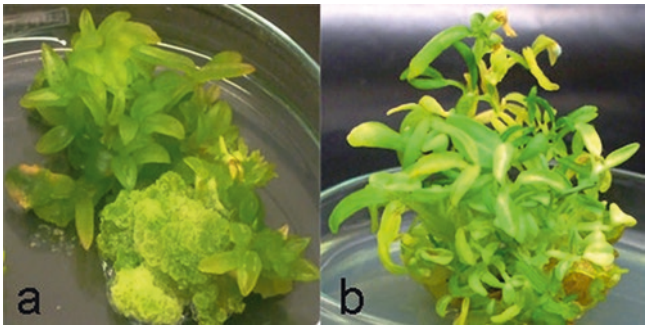

Fig. 19.3 Shoot regeneration from cotyledon node explant. (**a**) Hyperhydric shoots on MS medium supplemented with TDZ and (**b**) normal shoots on MS medium supplemented with TDZ-IBA (Aasim et al. 2010)

development of protocol in order to gain plants/plantlets under in vitro for further studies like genetic transformations. Khawar et al. (2004) successfully obtained in vitro regenerated shoots induction from apical meristem but failed to get rooted plantlets.

Different explants (cotyledonary nodes, leaves, and hypocotyl) of Fenugreek cultured on different PGRs like TDZ-IBA, BAP-NAA, and kinetin were tested by Aasim et al. (2010). There was no shoot regeneration from hypocotyl explants on any medium, but cotyledonary node explants responded well to BAP-NAA, kinetin (Fig. 19.2), and TDZ-IBA (Fig. 19.3) to induce multiple shoots. Among these PGRs, TDZ-IBA induced more number of shoots compared to others. However, they did not achieve rooted plantlets, and no acclimatization was performed.

Afsharie et al. (2011) checked the efficacy of different basal medium salts, PGRs, and explants (stem segments, embryos, and hypocotyls) for in vitro regeneration potential of Fenugreek. Their results revealed that both B5 or MS medium with 2.5 mg/l BAP + 0.5 mg/l NAA were optimum for somatic embryogenesis and 1.5 mg/l BAP + 0.5 mg/l NAA for shoot regeneration. Prabakaran and Ravimycin

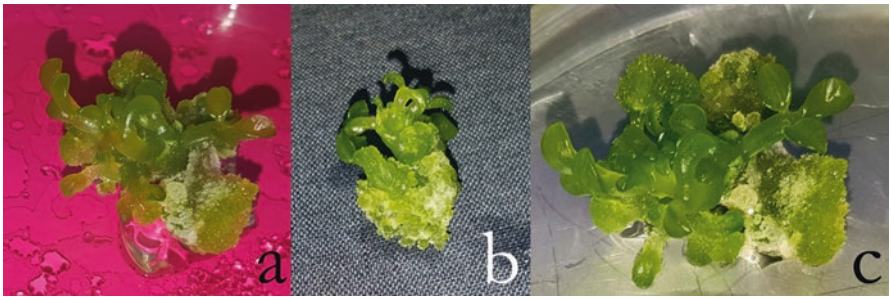

Fig. 19.4 Multiple shoot regeneration of Fenugreek (*Trigonella foenum-graecum* L.) using cotyledonary node explants, (**a**) callus induction, (**b**) shoot induction, and (**c**) multiple elongated shoots (Taşbaşi et al. 2017)

(2012) reported successful use of shoot tip explants for multiple shoot induction of Fenugreek. They achieved a maximum of two and four shoots from medium supplemented with 1.0 mg/l BA and 0.5 mg/l Kin, respectively, after 30 days of culture. However, no information about rooting and acclimatization of in vitro regenerated shoots was provided.

Indirect organogenesis through somatic embryogenesis was reported by Al-Mahdawe et al. (2013) using cotyledonary node explants. The process involved callus induction>somatic embryogenesis>secondary somatic embryos and embryoids>rooting> plantlets. Although they achieved plantlets, no information was given about plantlets transferred to soil. Pant et al. (2013) used different explants (leaf, stem, root, cotyledonary node, and hypocotyl) of Fenugreek on media supplemented with different PGRs. They achieved maximum shoot induction from leaf and stem explant cultured on medium containing 0.5 ppm BAP, whereas maximum shoots from cotyledonary node were achieved from medium supplemented with 0.1 ppm TDZ. Vaezi et al. (2015) cultured hypocotyl and cotyledon explants on MS medium provided with 2,4-D and Kin for callus induction followed by subculture to medium containing BAP and NAA for shoot induction. 5.0 mg/l BAP + 5.0 mg/l NAA was found best for maximum number of shoots per explant from hypocotyl explant.

Recently, two studies on in vitro regeneration of Fenugreek have been reported about the efficacy of sucrose concentration, explants age, and explant type (Taşbaşi et al. 2017; Kavci et al. 2017). Cotyledonary nodes and leaf explants taken from 18- to 20-day-old c seedlings were cultured on Phytagel-solidified MS medium with different sucrose concentrations (1.5, 3.0, 4.5, and 6.0%) and TDZ (0.40, 0.80, and 1.20 mg/l) + 0.20 mg/l NAA. Both explants induced 100% callus but no shoot induction from leaf explants, whereas a maximum of 18.75 shoots/shoot buds were achieved from MS medium enriched with 0.40 mg/l TDZ + 0.20 mg/l NAA and 1.5% sucrose concentration (Fig. 19.4-Taşbaşi et al. 2017).

In another study, Kavci et al. (2017) used 10- and 20-day-old cotyledonary node explants and cultured on Gelrite-solidified MS medium containing TDZ (0.40, 0.80, and 1.20 mg/l + 0.20 mg/l NAA) and different sucrose concentrations (1.5, 3.0, 4.5,

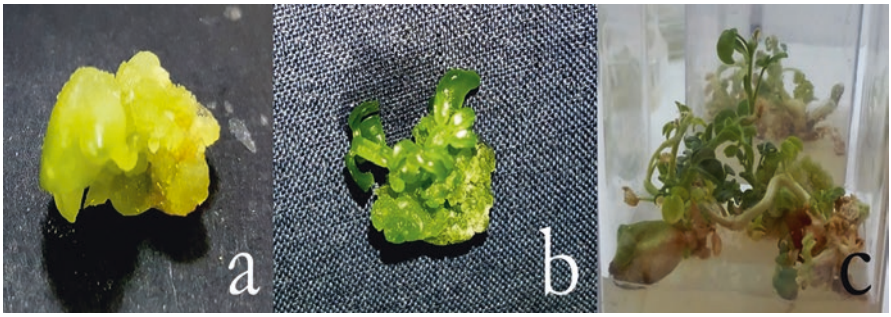

Fig. 19.5 Multiple shoot regeneration of Fenugreek (*Trigonella foenum-graecum* L.) using cotyledonary node explants, (**a**) callus induction and (**b, c**) multiple shoot induction (Kavci et al. 2017)

and 6.0%). They reported callus induction followed by somatic embryogenesis (100%) after 4 weeks of culture followed by development of shoot buds and shoots. Twenty-day-old explants were more effective than 10-day-old explants. A maximum number of shoots/shoot buds were recorded on medium containing 0.80 mg/l TDZ + 0.20 mg/l NAA + 4.5% sucrose. Burdak et al. (2017) inoculated shoot apex explant of different genotypes using different growth variants. Maximum callus induction frequency was recorded on MS medium supplemented with 0.5 mg/l BAP + 0.5 mg/l 2,4-D, whereas de novo shoot regeneration was achieved after subculturing of calli to 0.5 mg/l BAP-containing medium followed by rooting on medium supplemented with 0.2 mg/l IAA (Fig. 19.5).

Application of plant cell and tissue culture techniques in Fenugreek revealed the significance and superiority of this biotechnological tool. Different techniques like in vitro cell suspension culture, protoplast culture, and callus induction have been reported more advantageous for metabolites compared to seed and plant. On the other hand, few reports also reflected the successful use of callus for somatic embryogenesis and shoot induction. The study also reveals the successful in vitro organogenesis from different explants and culture conditions. However, information about rooting and adaptation is very rare or not provided which shows the recalcitrant nature of *Trigonella* plant and challenge for researchers to develop reproducible and complete plant tissue culture protocol for the application of other biotechnological techniques for its improvement. Development of in vitro regeneration of Fenugreek plantlets will allow researchers to incorporate genes of interest through genetic transformation studies.

19.6 Genetic Transformation Studies in Fenugreek

Genetic transformation of desired trait or gene to medicinal plant in order to obtain economically and medicinally important bioactive molecules or compounds has been common in the past years. However, there are few studies which successfully

report the use of *Agrobacterium tumefacien*s or *Agrobacterium rhizogenes* in Fenugreek. *A. rhizogenes* has been used for the production of hairy roots of Fenugreek in order to produce important secondary metabolites like diosgenin. Although the number of these studies are very low, they revealed the successful use of different *A. rhizogenes* strains for hairy root production followed by production of diosgenin (Merkli et al. 1997) or trigonellin (Raheleh et al. 2011) contents.

Merkli et al. (1997) established successful hairy root culture of *Trigonella foenum-graecum* L. using *Agrobacterium rhizogenes* strain A4 by infecting 2-week-old stems of sterile plantlets. They checked the root growth and diosgenin contents of hairy roots cultured on different mediums like WP, MS, and B5 for 35 days. The maximum growth (606 mg) with maximum growth index (80) was recorded from WP medium containing 3% sucrose, whereas maximum diosgenin content (0.040% dry weight) was achieved from on-half WP liquid medium with 1% sucrose compared to control (0.024% dry weight). Raheleh et al. (2011) used different *A. rhizogenes* (A4, 9126 and 15,834) and two different techniques for infection (cocultivation and injection) for checking the transformation efficacy and trigonellin production of two Iranian masses of *T. foenum-graecum.* (Zanjan and Borazjan). They achieved 100% hairy root production from all strains, whereas 26% transformation efficiency was recorded by injection method. They also achieved the highest trigonelline amounts of 14.89 (Borazjan – 28 days) and 14.03 mM g-1 DW (Zanjan – after 7 days).

Besides using *A. rhizogenes* for hairy root and secondary metabolites production, it has been used for gene function or expression. Shahabzadeh et al. (2013) evaluated the transformation frequency using *A. rhizogenes* strain K599 harboring a *GFP* gene. They inoculated the leaf and stem explants taken from two different ecotypes (Karaj and Bushehr) with three different OD_{600} concentrations (0.8, 1.2, and 1.6). Stem explant induced more hairy roots (8.09) with 81.3% transformation frequency compared to leaf explant, whereas a maximum of 8.76 transgenic hairy roots, 79.76% transformation frequency, and 0.77 d^{-1} growths rate of transgenic roots were recorded at OD_{600} of 1.2 for K599 strain. Their results reflected the importance of genotype, type of strain, explant, and inoculation condition for successful production of transgenic hairy roots for subsequent secondary metabolites production in Fenugreek.

Besides the use of *A. rhizogenes*, there is single report available on the use of *A. tumefaciens* for genetic transformation in Fenugreek by Khawar et al. (2004). They inoculated 1-week-old cotyledon, root, and hypocotyl explants with oncogenic *A. tumefaciens* strain A281 harboring β-glucuronidase (GUS) gene. Tumors induced with GUS gene were expressed by histochemical analysis, and presence of *uidA* gene was successfully confirmed by PCR amplification. There is no report available which highlights the use of economically important gene like insect or herbicide resistance genes in Fenugreek. Similarly, use of other technologies for genetic transformation like biolistic or protoplast is not available. This might be due to lack of proper tissue culture protocol, rooting problems, and transformation efficiency.

19.7 Genomic Studies of Fenugreek

A limited number of functional genomic studies of Fenugreek have been reported to date irrespective of large number of studies about isolation, characterization, and clinical studies of diosgenin and other bioactive compounds of Fenugreek. However, studies related to genes responsible for the biosynthesis of these phytochemicals are very rare. Similarly, a limited number of studies about genome sequencing are available to date. The first study about de novo transcriptome analysis, diosgenin pathway, and genes responsible for diosgenin biosynthesis in *T. foenum-graecum* was reported by Vaidya et al. (2012). They used sequencing messenger ribonucleic acid (RNA) aided with a SOLiD 4 Genome Sequencing Analyzer for transcriptome analysis. They obtained a total of 42 million high-quality reads, and de novo assembly was performed using Velvet at different *k*-mer, Oases, and CLC Genomics Workbench, which yielded 20,561 transcript contigs, and 18,333 transcript contigs were annotated functionally. About 6775 transcripts were found related to plant biochemical pathways including the diosgenin biosynthesis pathway according to Kyoto Encyclopedia of Genes and Genomes pathway mapping.

Chaudhary et al. (2015) investigated the effects of methyl jasmonate (MeJA) on diosgenin biosynthesis and gene expression of six Fenugreek varieties. Application of 0.01% MeJA significantly increased diosgenin levels from 0.5%–0.9% to 1.1%–1.8% within 12-day-old seedlings, whereas MeJA also upregulated the expression of two pivotal genes of the mevalonate pathway, the metabolic route leading to diosgenin: 3-hydroxy-3-methylglutaryl-CoA reductase (*HMG*) and sterol-3-β-glucosyl transferase (*STRL*). Increased gene expression of *HMG* and *STRL* genes was recorded for Gujarat Methi-2 and Kasuri-2 variety. They concluded the use of MeJA as a promising elicitor for diosgenin production by Fenugreek plants.

Ciura et al. (2017) reported the first report on the next-generation sequencing of cDNA-RDA products of Fenugreek. They used methyl jasmonate for elicitation and cholesterol and squalene as precursor feeding for enhancement of sterols and steroidal sapogenins of in vitro grown plants for representational difference analysis of cDNA (cDNA-RDA). Differential, factor-specific libraries were subjected to the next-generation sequencing for identifying genes responsible for diosgenin biosynthesis. Approximately 9.9 million reads were obtained, trimmed, and assembled into 31,491 unigenes with an average length of 291 bp. Functional annotation and gene ontogeny enrichment analysis was achieved by aligning all unigenes with public databases. They identified the novel candidate genes responsible for diosgenin biosynthesis and validated their expression by using quantitative RT-PCR analysis. Their results revealed the biosynthesis of diosgenin from cycloartenol via cholesterol. These results open the new window for the breeders and researchers to understand the biosynthesis pathway, genes responsible for biosynthesis, and genome sequence to find more functional genes responsible for plant growth and production of bioactive compounds of Fenugreek.

19.8 Conclusion

Fenugreek is an underutilized plant all over the world where it is used for various purposes based on the demand of the community that ranged from its use as vegetable to spice and medicinal plant. The wide distribution of plants in different geographic regions has wide genetic variability, but studies related to its genetic variability are very limited. Although extensive work related to artificial mutation using physical and chemical mutagens have been reported under in vitro conditions for its bioactive compounds, there is also need to do more work on its agronomic characterization and acclimatization to different environmental conditions. Similarly, different plant tissue culture protocols have been employed successfully with aim to improve the major bioactive compounds contents. But success about the development of in vitro grown plantlets is still the challenge for the researchers for the application of modern biotechnological tools like genetic transformation studies to incorporate genes of interests. The main drawback of Fenugreek is the availability of limited work related to its functional genomics, gene expression studies, genome sequencing, and other plant omics. There is also a need to explore the potential of plant by applying biological tools like QTL or MAS in order to identify the potential genes for future conventional or modern breeding programs for developing elite cultivars against biotic or abiotic stresses to improve yield and nutraceutical values. The potential of Fenugreek as medicinal plant has been exploited well compared to its other uses. There is also a need to exploit the potential of Fenugreek as forage crop and edible uses by developing new cultivars with the aid of biotechnology.

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Chapter 20
Biotechnological Advancement in an Important Medicinal Plant, *Withania coagulans*: An Overview and Recent Updates

Mangal S. Rathore, Kusum Khatri, Jasminkumar Kheni, and Narpat S. Shekhawat

Abstract *Withania coagulans* (Stocks) Dunal is an important and high-value medicinal plant of *Solanaceae*. It is popularly called Indian cheese maker or vegetable rennet. Plant possesses multiple medicinal properties, and the reproductive failure and overexploitation from wild habitat forced this species towards the verge of complete extinction. Plant tissue culture and recent advancements in the field of biotechnology like genomics, proteomics and metabolomics have enormous potential for the genetic improvement of plant species and facilitate the development of new methods for plant germplasm conservation, evaluation and improvement. Though *W. coagulans* is known for multiple medicinal properties, however, it has not been given considerable attention for genetic improvement. The present chapter will focus on the development and recent contribution of advanced biotechnological interventions in genetic improvement of *W. coagulans*. This chapter will provide a comprehensive information on the development of in vitro methods for conservation of germplasm, mass-scale multiplication and their employment in genetic transformation and secondary metabolite production. Further genomics, proteomics and metabolomics updates on *W. coagulans* have been discussed, and these will facilitate researchers a ready-to-use source of information.

Keywords In vitro clonal propagation · Encapsulation · Withanolides · Ashwagandha · Germplasm conservation · Genomics and metabolomics

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20.1 Introduction

Withania is a member of *Solanaceae*. *Withania* genus comprises of a large number of species with global distributions. In literature the genus is reported to comprise of about 23 species. *W. somnifera* (L.) Dunal popularly called 'ashwagandha' and *W. coagulans* (Stocks) Dunal popularly called 'vegetable rennet' are predominating medicinal plants of Indian and other traditional systems of medicine (Tuli and Sangwan 2010). These species are distributed in east of the Mediterranean region extending to South Asia and found in many parts of Pakistan and India (Chadha 1976). *W. coagulans* is an important medicinal plant found in western arid regions of India. *W. coagulans* rank second after *W. somnifera* to be used for different medicinal purpose in India. *W. coagulans* is named as 'Akri' or 'Puni-ke-bij' or 'Paneer-Bandh' in Hindi, 'Vegetable rennet' in English, 'Khamjira' in Panjabi and 'Punirband' or 'Punir-ja-fota' in Sindhi. *W. coagulans* is a small shrub of 110–140 cm height having rigid aerial system covered with grey-whitish hairs. The leaves are long/lanceolate oblong or sometimes ovate, narrow at the base and have a short stalk. The flowers range 10–12 mm in size and found in axillary cymose clusters. These are yellowish, dioecious and polygamous in nature. The fruits are called berries and ranges 10–12 mm in diameter. The berries are red, smooth and covered by leathery calyx. The seeds are glabrous, ear shaped and of dark brown colour. Seeds possess fruity smell.

Solanaceae is a family of plants which are naturally rich in medicinally important alkaloids; however, alkaloids/withanaloids are not restricted to this family. *W. coagulans* has a rich history of medicinal attributes including milk coagulating properties. The plant species has been named as Indian cheese maker or vegetable rennet due to coagulant properties of its fruit and leaves. The Withanin is the major enzyme responsible for coagulation properties. The berries of *W. coagulans* are used to clot the milk and prepare the paneer. As per an estimate, a spoon of decoction (prepared by mixing the berry power with boiling water) can coagulate a gallon of milk in an hour. As compared to *W. somnifera*, *W. coagulans* is phyto-chemically unique, and the following unique characteristics were discussed by Mishra et al. (2013).

1. The neuroactive metabolite withanolide A is produced predominantly in aerial parts of *W. coagulans* and in underground parts, i.e. roots of *W. somnifera*. This makes commercially viable and easier harvest of withanolide from *W. coagulans*.
2. *W. coagulans* produces sterols and withanolides of potential biogenetic significance, for example, ergosta 5,25-diene 3β,24-diol, withacoagin, and withacoagulins.
3. It produces withanolides containing either bridge between C-14 and C-20.
4. It also produces a few unique hydoxylated withanolide like coagunolide which includes 20β-hydroxy-1-oxo-(22R)-witha-2, 5, 24-trienolide, withacoagulin, and 17β-hydroxy-14α,20βepoxy-1-oxo-(22R)- witha-3,5,24-trienolide.

Despite enormous and important qualities and limitation to the production or availability of plant material, plant species has not received considerable attention for biotechnological studies including its regeneration, transformation, cultivation aspect, uses as sources of key genes for metabolite pathways and genetic improvement. Among different species of *Withania*, cultivation of *W. somnifera* and *W. coagulans* has been reported (Mirjalili et al. 2009); however, in India to the best of our knowledge, there are no reports on the systemic cultivation of *W. coagulans*. The plant is commercially important because of the ability of its fruits (Paneerdodhi) to coagulate the milk and the presence of various metabolites of medicinal importance. Due to multiple medicinal applications, the plant species is harvested from the wild which harms the natural biodiversity. Natural propagation of this plant species are through seeds, and it is very slow and unreliable due to environmental constrains. Also, the polygamodioecious nature of flowers and self-incompatibility (Gilani et al. 2009) limits the chances of seed set. Due to these reasons, the plant species are found only in the vegetative state under arid conditions. *W. coagulans* cannot reproduce fast enough in the wild to keep up with the exploitation rate; hence, the rate of regeneration and exploitation is not balanced. Further due to ruinous harvesting practices, the genetic diversity of *W. coagulans* in it natural habitat got endangered, and plant species have been declared critically endangered. Hostile environment, habitat disturbances, reproductive failure and overexploitation threatened the survival of *W. coagulans* and rendered the plant species vulnerable to complete extinction.

Numerous studies on various aspects are available on *W. somnifera*. Limited studies are available on *W. coagulan*s, and no systemic attempts were made to develop it as a commercial crop and for its genetic improvement to exploit for commercial purpose. With NCBI search only 87 research publications could be seen on *W. coagulans*. In genomics and proteomics, no any cutting edge research has been carried out, and only 39 and 26 sequences of nucleotide and proteins, respectively, have been submitted to public domain https://www.ncbi.nlm.nih.gov/gquery/?term=withania+coagulans). There is need to carry out systemic investigations on this plant species to develop a sustainable way or strategies to get benefits from this plant species without affecting it in its natural habitat. From plant genetic resource point of view, special attentions are needs for it's in situ and ex situ conservation, and the development of in vitro multiplication methods could help large-scale restoration programs of *W. coagulans* through mass-scale multiplication of elite germplasm. From pharmaceutical point of view, the understanding of secondary metabolism and different biosynthetic pathways producing commercially important metabolites could facilitate the pathway engineering to produce metabolite of interest through phytofarming. In the present chapter, we focused on the biotechnological developments in *W. coagulans*, and attempts were made to present an overview and recent updates in this plant species (Figs. 20.1, 20.2 and 20.3).

Fig. 20.1 A plant of *W. coagulans* growing in natural habitat ((**a**), Photograph taken by Dr. Kheni from Amreli district in Gujarat, INDIA) and fruits of *W. coagulans* (**b**)

20.2 Medicinal Properties of *W. coagulans*

The plant species has not been considerably explored because of its restricted distribution in terms of habitat, scarcity of plant material in wild and little scientific efforts for its genetic improvement for commercial cultivation. Each part of the plant have commercially and medicinally importance. The plant is commercially important because of the ability of its fruits (Paneer-dodhi) to coagulate the milk and the presence of coagulin H, an immunosuppressive drug. The Withanin enzyme in sweet berry fruits is responsible for milk coagulating properties in this plant species. The twigs are used to clean the teeth, and the inhalation of the plant smoke provides relief in toothache (Dymock et al. 1972; Krishnamurthi 1969). The fruits popularly called berries are known to possess sedative, emetic, alterative and diuretic properties and used to treat liver complaints, asthma and biliousness. Flowers are useful in the treatment of diabetes (Bown 1995). The other parts of plant species are known for treating the digestive disorders, flatulent colic, nervous exhaustion, disability, insomnia, wasting diseases, failure to thrive in children, impotence, intestinal infections and blood impurity. These multiple medicinal properties have been reported to be associated with withanolide contents produced (Glotter 1991; Chen et al. 2011). Plant species is used to treat ulcers, rheumatism, dropsy and senile debility. Plant species also possesses antimicrobial, anti-inflammatory, antitumor, hepatoprotective, antihyperglycemic, cardiovascular, immunosuppressive, immunomodulatory (rheumatism, nephronia, degenerative diseases), free radical scavenging and central nervous system anti-depressant activities (Maurya et al. 2010). A few metabolites like coagulin H, coagunolide, coagulin

Fig. 20.2 Multiple shoot induction after repeated transfer of nodal explants in *W. coagulans* (**a**), in vitro cultured leaf of *W. coagulans* showing multiple shoot bud differentiation (**b**), multiple shoot cultures of *W. coagulans* generated using nodal shoot segment from mature plant (**c**), in vitro rooted plantlets of *W. coagulans* (**d**), ex vitro rooting and coupled acclimatization of rooted plantlet of *W. coagulans* (**e–f**), ex vitro rooted plantlet of *W. coagulans* (**g**) and acclimatized plantlets of *W. coagulans* under nursery conditions (**h–i**)

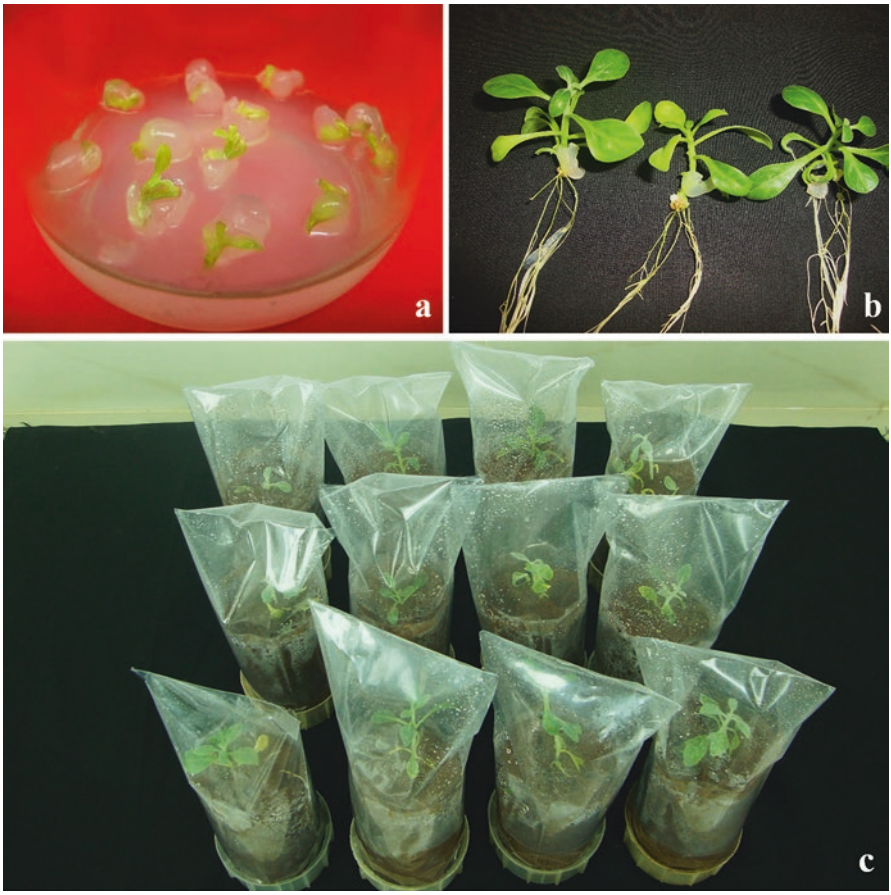

Fig. 20.3 Shoot bud induction from encapsulated micro-shoots of *W. coagulans* under in vitro conditions (**a**), encapsulated bead germinated plantlets of *W. coagulans* (**b**) and plantlets produced through encapsulation technology directly on sterile soil (**c**) under controlled conditions

C, coagulin L, withanolide F and 17-hydroxywithanolide K are antidiabetic metabolites and are known to occur in this plant species in minute quantity. The withanolides, a class of C28 triterpenes derived compounds, are known to possess healing properties. The aqueous extract of *W. coagulans* exhibited free radical scavenging activity (Budhiraja et al. 1986; Hemalatha et al. 2004). The hepatoprotective property of 3F-hydroxy-2, 3 dihydro-withanolide F from fruit of *W. coagulans* was studied, and Budhiraja et al. (1986) reported withanolide F more active than hydrocortisone for hepatoprotective effect. Withaferin A has tumour inhibitory property against human carcinoma of nasopharynx and mitotic poison arresting property and possesses antiarthritic and anti-inflammatory effect. Withaferin A suppresses arthritic syndrome without any toxic effect and inhibits angiogenesis (Mohan et al. 2004).

20.3 In Vitro Cultures of *W. coagulans* and Germplasm Conservation

W. coagulans is an important medicinal plant and exposed to different kinds of threats in its natural habitat for its own survival. The plant species have been reported critically endangered in its natural habitat. Therefore, sustainable harvest of this plant species from the wild is not a viable option as this will result in loss of germplasm. There is need to devise some other strategies to meet the requirement of the plant species for traditional medicine and to propagate to prevent the erosion and encourage the amplification of germplasm. The biotechnological studies focused mainly on the dominating species of *Withania*, i.e. *W. somnifera*, and numerous reports on different aspects of this plant species are available. The biotechnological studies on *W. coagulans* initiated in the last 10 years, and we felt that it is still in a nascent phase of development; however, considerable progress has been made predominantly in the field of tissue culture, and a few good reports are available in literature. In vitro propagation in *W. coagulans* has been standardized and reported by Kaur (1992), Rathore (2005) Rathore et al. (2012, 2016), Rathore and Kheni (2015), Jain et al. (2009, 2011, 2016), Valizadeh and Valizadeh (2009, 2011) and Joshi et al. (2016). The in vitro propagation is a vegetative propagation without involvement of meiotic division. In vitro clonal propagation, direct organogenesis/plantlet regeneration and callus cultures are the main approaches in tissue culture propagation. Propagation of plantlet using seed as explant is also one of the approaches; however, the progeny may differ from mother plant as the seed itself is a product of sexual process. In vitro propagation serves rapid and large-scale production method for commercial crops, genetic improvement and conservation of germplasm, and supply of plant material for different purpose including genetic transformation.

Leaf and internode explants were cultured to develop callus culture and plantlet regeneration in *W. coagulans* (Valizadeh and Valizadeh 2009). Callus induction was reported on Murashige and Skoog's medium (Murashige and Skoog 1962) containing supplemented combination of with 2,4-dichlorophenoxy acetic acid (2,4-D) and cytokinins, namely, 6-benzylaminopurine (BAP) and kinetin (Kin). Valizadeh and Valizadeh (2009) reported shoot regeneration from internode-derived callus and 75% survival of tissue-cultured plants; however, the regeneration frequency was claimed unsatisfactory.

Jain et al. (2009) reported efficient micropropagation using nodal segments from adult plant of *W. coagulans*. Multiplications of shoots were achieved on medium containing BAP and Kin along with phloroglucinol. Phloroglucinol has been reported to improve the multiplication of shoots and in vitro root regeneration in cultures. Shoots were rooted in vitro, and pulse treatment of shoots with phloroglucinol and choline chloride improved the rooting on medium supplemented indole-3-butyric acid (IBA), phenylacetic acid and choline chloride (Jain et al. 2009). The true-to-type nature of regenerated shoots was established using RAPD fingerprinting which demonstrated suitability of axillary shoot culture for large-scale multiplication of selected genotypes. Valizadeh and Valizadeh (2011) reported

micropropagation protocol for *W. coagulans* using nodal explants. Multiple shoots were achieved on media containing combination of BAP and IBA. Shoots were rooted in vitro with IBA, and 75% survival of soil transplanted plants has been reported. Rathore et al. (2012) reported a simple, rapid and cost-effective micropropagation system for *W. coagulans* for mass-scale production of true-to-type plantlets using nodal shoot segments from mature plant. BAP, IAA and IBA were used in the protocol. Use of ascorbic acid, citric acid, adenine sulphate and L-arginine in culture medium was reported to exhibit beneficial effects during different stages of tissue culture. Further repeated transfer of proliferated shoot buds, i.e. transfer of newly differentiated shoot buds along with mother explant, has been claimed to improve multiplication of shoot in culture. Both in vitro and *ex vitro* rooting of cloned shoots were reported, and it was first reported on *ex vitro* root regeneration in *W. coagulans*. The roots differentiated under *ex vitro* conditions have been reported to better adapt to the anchoring medium, and these plantlets acclimatized faster compared to the in vitro ones. Also *ex vitro* rooting minimizes the root damage during transplantation to soil (McClelland et al. 1991) and provides greater resistance to stress as the rooting quality is better in *ex vitro* rooted plantlets (Bonga and Aderkas 1992). Further single step of plantlet generation from cloned shoots through coupled *ex vitro* rooting of shoots and hardening of plantlets minimizes the time for plantlet production and thus several times cheaper (Singh et al. 2009). The *ex vitro* rooted plantlet survived more than 90% after soil transplantation. The protocol was claimed cheaper and commercially superior due to the use of commercial grade sugar instead of pure and laboratory grade sucrose, use of less expensive commercial grade agar-agar in culture medium instead of branded agar-agar, higher rate of shoot proliferation and multiplication rate, single-step *ex vitro* rooting and hardening of plantlets in the greenhouse. These features, higher rate of multiplication and easier technique for direct rooting and hardening in single step, made this protocol superior to previously reported methods on micropropagation of *W. coagulans*. The protocol was reported reproducible and easy to follow for large-scale restoration programs through true-to-type mass-scale multiplication of *W. coagulans*. Joshi et al. (2016) cultured nodal segments from field-grown plants and observed verification in cultures on medium supplemented TDZ and naphthoxyacetic acid (NAA). Further, Joshi et al. (2016) reported use of meta-topolin (*m*T) along with NAA for induction of health shoots in *W. coagulans*.

Among different in vitro approaches, encapsulation technology is an exciting and rapidly growing area with considerable impact on conservation and delivery of tissue-cultured plants in a more economical and convenient way (Rai et al. 2009). Rathore et al. (2016) employed tissue culture technique for conservation of germplasm of *W. coagulans*. The authors reported alginate encapsulation of in vitro derived micro-cuttings of *W. coagulans* and subsequent in vitro plantlet regeneration for rapid multiplication, short-term storage and germplasm distribution. Use of 3.0% sodium alginate and 100 mM calcium chloride was claimed as the most suitable matrix for encapsulation, and these synseeds exhibited 95% regeneration potential. IBA pulse treatment at the base of micro-cutting before encapsulation was shown to produce complete plantlets during regeneration. The encapsulated

micro-cutting storage at 4 °C under sterile conditions for 60 days exhibited 72% plantlet regeneration and thus reported to facilitate genetic restoration programs, short-term storage and germplasm distribution (Rathore et al. 2016). Similar to this alginate encapsulation and plantlet regeneration has been developed in various medicinal plants including *W. somnifera*. Jain et al. (2016) describe method for mass multiplication of *W. coagulans*, assessment of fidelity of regenerated by RAPD fingerprinting and estimation of bioactive compounds (withanolides) in tissue culture-produced plantlets with TLC and reverse phase HPLC.

20.4 Genetic Transformation in *W. coagulans*

Genetic engineering in plants is sophisticated technology, and this makes possible manipulations that are outside the repertoire of breeding or cell fusion techniques. With genetic engineering, genes can be accessed from exotic sources and introduced into a crop. Genetic transformation allows researcher to express the important gene/s from a source in to prokaryotic and eukaryotic systems. In *W. coagulans* genetic transformation would facilitate genetic improvement, functional genomic, understanding of withanolide metabolism and metabolic engineering for improved phytopharming of the targeted metabolites. Different genes (*WcTDS* and *WcTR*-1) were cloned from *W. coagulans* have been expressed heterologously in *E. coli* to study the kinetic properties of recombinant proteins to understand the secondary metabolism (Kushwaha et al. 2013; Jadaun et al. 2017). Genetic transformation protocols for higher plant species are needed for genetic improvement of plant species for different agronomic traits, understanding of functional genomics, biosynthesis of metabolites and metabolic/pathway engineering to improve the targeted metabolites or bioactive compounds. Gene transfer methods in plants involve both direct and indirect transformation systems; however, the main focus remains to achieve the maximum number of stably transformed plants. The widely used methods include *Agrobacterium*-mediated gene transfer and microprojectile bombardment with DNA or biolistics approach. Genetic transformation protocols have also been reported in *W. coagulans*.

Mirjalili et al. (2009) produced hairy root cultures after transforming *W. coagulans* with C58C1 strain of *Agrobacterium tumefaciens* harbouring pRiA4 vector. The transformed roots produced important bioactive compounds, i.e. withanolide A and withaferin A. Further accumulation of withanolide A during early phase of cultures and of withaferin A during late phase of culture was demonstrated. This work clearly showed possibility of development of potential bioreactor system for production of withanolide A with promising cell lines. Further, Mirjalili et al. (2011) reported transformation of *W. coagulans* with *squalene synthase* (*AtSS*1) gene from *Arabidopsis thaliana* using *Agrobacterium rhizogenes* A4. The transformed root tissues were hairy and found to exhibit increased capacity for biosynthesizing phytosterols and withanolides, which was positively correlated with expression level of the transgene under control of overexpressing CaMV35S promoter. Mishra et al. (2013)

reported an efficient and reproducible *Agrobacterium*-mediated genetic transformation protocol in *W. coagulans* using in vitro derived leaf explants. The protocol was developed using LBA4404 *Agrobacterium* strain harboring the pIG121Hm binary vector with β-glucuronidase gene (gusA), reporter gene under the control of CaMV35S promoter. Further, Mishra et al. (2013) discussed the optimal conditions to achieve higher frequency of transformation and achieved 100% frequency of transient GUS expression with 5% stable transformation efficiency. Further, a comparison was done for types of withanolides in transgenic and non-transgenic plants.

20.5 Proteomic Aspect of *W. coagulans*

Proteins play the vital roles in living organisms and different reactions. These are known to have the most important functions for a living organism. Proteomics deals with the experimental analysis of proteins and specifically involves purification and characterization. Not much work has been done, and it is not wrong to say there has not been any systematic investigation on proteome of *W. coagulans* to unveil the hidden potential of this plant. A few important enzymes involved in biosynthesis of metabolites were expressed in heterologous system and purified to understand the kinetic behaviour. Kushwaha et al. (2013) expressed *WcTR*-1 encoding tropine-forming tropinone reductase in *E. coli* and produced recombinant protein. The recombinant protein was purified, and kinetic properties were investigated to understand the secondary metabolism in *W. coagulans*. The tryptophan decarboxylase, a key enzyme for synthesis of metabolites possessing indolyl moiety in *W. coagulans*, was studied. By expressing *WcTDC* in *Escherichia coli*, the recombinant enzyme was produced and subsequently studied to understand kinetics of catalysis (Jadaun et al. 2017). The results revealed adaptability of the plant species to hot arid regions and also provided insights in understanding of withanamide biosynthesis.

Naz et al. (2009) purified and characterized partially a milk coagulating protease from *W. coagulans*, and with SDS-PAGE a 66 kDa molecular weight protein was reported to have milk coagulating activity. The coagulation activity was shown to increase with $CaCl_2$ concentration and decreases with increasing temperature. Pezeshki et al. (2011) reported extraction of protease from fruits of *W. coagulans* and assessed its proteolytic potential on Iranian UF white cheese as compared with pure chymosin and fungi rennet. Pezeshki et al. (2011) reported comparable properties of cheeses made using *W. coagulans* with cheeses produced using different rennet preparations except pH which was lower in cheeses made with *W. coagulans*. The enzyme-induced gelation is an important biochemical steps in cheese preparation. Beigomi et al. (2014) characterized a protease from *W. coagulans* fruits for milk-clotting activity. The protease was shown to have excellent thermal stability, and a fraction of 66 kDa molecular weight having the highest milk-clotting activity was reported in SDS-PAGE. Gel formation was monitored using low-amplitude oscillatory rheology at different temperatures. With Arrhenius plot the temperature dependence of gelation was discussed, and it was shown that an increase in tempera-

ture decreases the gelation onset time, gel formation rate and the final gel strength (Beigomi et al. 2014). The enzyme stability against a wide range of temperatures makes this protease suitable for cheese manufacturing industries. Kazemipour et al. (2016) carried out a proteomic and zymographic analysis of berries in *W. coagulans* and demonstrated scientific basis of milk coagulation properties by showing presence of effective protease. Sodium chloride and enzyme concentrations have been shown to influence rennet coagulation time, and calcium chloride was reported to improve the clotting activity. Complete inhibition of milk coagulation by a protease inhibitor (pepstatin-A) indicates the proteomic nature of enzyme responsible for coagulation. Kazemipour et al. (2016) show presence of aspartic proteases and lower concentration of metalloproteases in fruit extract and further projected use of fruits extracts in dairy industry for milk clotting as an alternative of calf rennet. The traditional and medicinal use of *W. coagulans* confirms the safety of this plant for different industrial applications, and wide range of adaptability of milk coagulating enzyme make the plant system suitable for commercial exploitation by cheese-making industries.

20.6 Metabolic Aspect of *W. coagulans*

W. coagulans possess for multiple medicinal properties. *W. coagulans* is rich in steroidal lactones, also known as withanolides. Withanolides are reported from each part of this plant including roots. These properties are known to be attributed to withanolides that are present in the plant (Atta-ur-Rahman et al. 1998b, 1999; Atta-ur-Rahman et al. 2003). Withanolides are polyhydroxy C28 steroidal lactones occurring naturally and contain ergostane nucleus and a lactone-containing side chain. In withanolides a six- or five-membered lactone or lactol ring is attached to an ergostane skeleton. These Dragendorff's test positive compounds, however, are not N-containing. Withaferin A is the first member of this group, and it was isolated from *W. somnifera* (Lavie et al. 1965). In *W. coagulans* most of the work was carried out on its metabolic constitution, and this section will provide a comprehensive summary on metabolic composition in this plant species. The twigs and leaves of the plant are known to contain withanolides A and B, withacoagin, coagulin, etc. The leaves comprise of chlorogenic acid, and the berries contain free amino acids, esterases, essential oils and alkaloids (Bandyopadhyay and Jha 2003). The fruits or berries contain milk coagulating enzymes, esterases, free amino acids, fatty oil, essential oil and alkaloids. Proline, hydroxyproline, valine, tyrosine, aspartic acid, glycine-asparagine, cysteine and glutamic acid are the main amino acids. Fourteen different alkaloidal fractions have been reported from the alcoholic extract of fruits. The oleic, linoleic, palmitic, stearic and arachidonic acids are main fatty acid contents. The seeds are reported to contain 12–14% fatty oil. The oil has a high content of linoleic acid and b-sitosterol which in combination are responsible for the hypocholesterolaemic effect (Atal and Sethi 1963). The defatted seed meal contains 17.8% sugars having D-galactose and D-arabinose in 1:1 proportion and

maltose in traces amount (Salam and Wahid 1969). In case of *W. coagulans*, suitable public databases are not available for details of its metabolites and other functions. The PhytoChemical Interactions Database (PCIDB) provides a very brief detail about *W. coagulans* genes, proteins, metabolic pathways and their interactions http://www.genome.jp/db/pcidb/kna_species/10338). PCIDB uses KNApSAcK database, and this database showed taxonomic details, 5 entries for metabolites, 27 entries for ChEMBL Protein interactions and 7 for genes in CTD interactions. A summary of important metabolites in different parts of *W. coagulans* has been given in Table 20.1 (adopted from Maurya et al. 2010; Khodaei et al. 2012).

Table 20.1 Summary of important metabolites in different parts of *W. coagulans*

S. No.	Plant part	Compound	Remark
1.	**Whole plant**	(22R), 20β-hydroxy- 1-oxowitha-2,5,24-trienolide	Naz (2002)
		(22R)-14,20-epoxy-17ß-hydroxy-1-oxowitha-3,5,25-trienolide	
		[14α, 20 β, 27- trihydroxy- 1-oxo-(22R)- with a-3,5,24-trienolide]	Dur- E- Shahwar (1999)
		Δ^3isowithanolide F	Velde et al. (1983)
		14, 15β- epoxywithanolide I: [(20S, 22R) 17β, 20β-dihyroxy -14β, 15β- epoxy- 1- oxo- witha-3,5,24-trienolide]	Choudhary et al. (1995)
		17β- hydroxywithanolide K: [(20S, 22R) 14α, 17β, 20β-trihydroxy 1- oxo- with a-2, 5, 24- trienolide]	
		17β, 27 dihydroxy-14, 20- epoxy -1- oxo- 22R- witha-3, 5, 24-trienolide	Atta-ur-Rahman et al. (1993)
		17β,20β- dihydroxy- 1- oxo- witha-2,5,24- trienolide	Choudhary et al. (1995)
		17β-hydroxy-14α,20α-epoxy-1-oxo-(22R)-witha-3,5,24-trienolide	Atta-ur-Rahman et al. (2003)
		Amyrin	Naz (2002)
		Bispicropodophyllin glucoside	Nur-E-Alam et al. (2003)
		Coagulansins A and B	Jahan et al. (2010)
		Coagulin and Coagulin A	Dur- E- Shahwar (1999)
		Coagulin F: [27-hydroxy-14,20-epoxy-1-oxo-(22R)-witha-3,5,24-trienolide]	Atta-ur-Rahman et al. (1998a)
		Coagulin G: [17â,27-dihydroxy-14,20-epoxy-1-oxo-(22R)- witha-2,5,24-trienolide]	

(continued)

Table 20.1 (continued)

S. No.	Plant part	Compound	Remark
		Coagulin H: 5α, 6β, 14α, 15α, 17, 20- hexahydroxy- 1- oxo- witha – 2, 24 – dienolide	Atta-ur-Rahman et al. (1998d)
		Coagulin I: [(14R,17S,20£,22R)-5α,6β,17-trihydroxy-14,20-epoxy-1-oxo-witha-2,24-dienolide]	
		Coagulin J: 3β, 27 dihydroxy- 14, 20 epoxy-1-oxowithania-5, 24-dienolide	
		Coagulin K: 14,20- Epoxy- 3ß) -1- oxowit-(O- ß-D-glucopyranosyl ha- 5,24-dienolide	
		Coagulin L: (14R, 17S, 20S, 22R)-14,17,20- trihydroxy- 3β-(O-β-D-glucopyranosyl)-1-oxowitha-5, 24- dienolide	
		Coagulin M: 5α, 6β, 27-trihydroxy- 14, 20- epoxy- 1- oxo-witha- 24 enolide	Atta-ur-Rahman et al. (1998b)
		Coagulin N: 15α, 17-dihydroxy- 14, 20- epoxy- 3β- (O- β-D-glucopyranosyl)- 1- oxo- witha – 5, 24- dienolide	
		Coagulin O: 14, 20- dihydroxy- 3β-(O- β- D-glucopyranosyl)- 1- oxo-with a- 5, 24- dienolide	
		Coagulin P: 20,27-dihydroxy-3β-(O-β-D-glucopyranosyl)-1-oxo-(20S,22R)-witha-5,14,24-trienolide	Atta-ur-Rahman et al. (1999)
		Coagulin Q: (20S,22R)-1β,3β,20-Trihydroxy-witha-5,24-dienolide 3-O-β-D-glucopyranoside	
		Coagulin R: 3β,17β-dihydroxy-14,20-epoxy-1-oxo-(22R)-witha-5,24-dienolide	
		Coagulin S: (20S, 22R) – 5α, 6β, 14α, 15α, 17β, 20, 27-heptahydroxy- 1- oxo- witha –24- eno-lide	Nur-E-Alam et al. (2003)
		Coagulin U	Naz (2002)
		Methyl-4 – benzoate	
		ß-sitosterol	
		ß-sitosterol glycoside	
		Withanolide G	Atta-ur-Rahman (1998)

(continued)

Table 20.1 (continued)

S. No.	Plant part	Compound	Remark
		Withanolide I	
		Withanolide J	
		Withanolide K	
		Withapakistanin: [17β, 20 β- dihydroxy- 14, 15β- epoxy-1-oxo-(22R)- with a-3,5,24 trienolide]	Dur- E-Shahwar (1999)
		Withasomniferine-A: [17β, hydroxyl- 6α, 7α -epoxide-1-oxo-(22R)-witha-4,24-dienolide]	
2.	**Aerial parts (stem and leaves)**	(20R,22R)-14,20a,27-trihydroxy-1-oxowitha-3,5,24-trienolide	Huang et al. (2009)
		(22R)-14a,15a,17b,20b-tetrahydroxy-1-oxowitha-2,5,24-trien-26,22-olide	
		5,20α (R)-dihydroxy-6α,7α-epoxy-1-oxo-(5α) witha-2,24-dienolide	Subramanian et al. (1971)
		Ajugin E	Nawaz et al. (1999)
		Chlorogenic acid	Anonymous (1966)
		Coagulin B	Atta-ur-Rahman et al. (1998a)
		Coagulin C	Atta-ur-Rahman et al. (1998 c)
		Coagulin D	
		Coagulin E	
		Withacoagulin A: (¼(20S,22R)-17β,20β -Dihydroxy-1-oxowitha-3,5,14,24-tetraenolide	Huang et al. (2009)
		Withacoagulin B: (¼(20R,22R)-20β,27-Dihydroxy-1-oxowitha-3,5,14,24-tetraenolide	
		Withacoagulin C: (¼(20S,22R)-14a,15a,17β,20β -Tetrahydroxy-1-oxowitha-3,5,24-trienolide	
		Withacoagulin D: (¼(20S,22R)-14a,17β,20β,27-Tetrahydroxy-1-oxowitha-3,5,24-trienolide	
		Withacoagulin E: (¼(20R,22R)-14β,20β -Dihydroxy-1-oxowitha-2,5,24-trienolide	
		Withacoagulin F: (¼(20R,22R)-14β,20β -Dihydroxy-1-oxowitha-3,5,24-trienolide	

(continued)

Table 20.1 (continued)

S. No.	Plant part	Compound	Remark
		Withacoagulin G	Youn et al. (2013)
		Withacoagulin H	
		Withacoagulin I	
		Withanolide F	Huang et al. (2009)
		Withanolide L	
3.	**Fruits**	(17S,20S,22R)-14α,15α,17β,20β-tetrahydroxy-1-oxowitha-2,5,24-trienolide (a coagulanolide)	Maurya et al. (2008)
		20β, hydroxy -1- oxo- (22R) – witha – 2, 5. 24- trienolide	Atta-ur-Rahman et al. (2003)
		3β- hydroxy-2,3-dihydrowithanolide F	Budhiraja et al. (1983)
		3β,14α,20α_F,27-tetrahydroxy-1-oxo-20R,22R-witha-5,24-dienolide	Ramaiah et al. (1984)
		3β-hydroxy- 2,3-dihydrowithanolide Hk	
		5α, 27- dihydroxy- 6α, 7α- epoxy-1-oxowitha- 2, 24- dienolide	Anonymous (1966)
		5α, 17α- dihydroxy- 1- oxo- 6α,7α-epoxy- 22 R- with a- 2, 24-dienolide	
		5α, 20α (R) dihydroxy- 6á, 7á-epoxy-1- oxowitha- 2, 24- dienolide	
		Capryloyl hexaglucoside	Ali et al (2014)
		Ergosta-5,25-diene-3β,24 ε -diol	Velde et al. (1983)
		Geranilan-10-olyl dihydrocinnamoate	Ali et al. (2015)
		Geranilan-8-oic acid-10-olyl salicyloxy- 2-O-b-D-glucofuranosyl-(6”→1”’)-O-b-D-glucofuranosyl-6”’-n-octadec-9””,11””- dienoate	
		Geranilanolyl salicylic glycoside	
		Menthyl tetraglucoside	Ali et al. (2014)
		n-dotriacont-21-enoic acid	Ali et al. (2015)
		n-heptacosanyl linolenate	Ali et al. (2014)
		n-nonacosanyl linolenate	
		n-octacosanyl linolenate	
		n-octatriacont-17-enoic acid	Ali et al. (2015)
		n-tetratriacontanoic acid	
		Sitosterol-β-D-glucoside	Ramaiah et al. (1984)
		Withacoagulin: 20β,27-Dihydroxy-1-oxo-(22R)-witha-2,5,24-tetraenolide	Atta-ur-Rahman et al. (2003)
		Withacoagulinyl tetraglucoside	Ali et al. (2014)
		Withaferin	Neogi et al. (1988)
		Withanolide D	Budhiraja et al. (1983)
		Withanolide H: 14α, 20α_F, 27-trihydroxy-1-oxo-20R, 22R-with a-2,5,24- trienolide	Ramaiah et al. (1984)

(continued)

Table 20.1 (continued)

S. No.	Plant part	Compound	Remark
4.	**Roots**	(20R, 22R) 6α, 7α- epoxy- 5α, 20-dihydroxy- 1- oxo- witha-2, 24- dienolide	Neogi et al. (1988)
		(20S, 22R) 6α, 7α- epoxy- 5α-hydroxy- 1- oxo-witha-2,24- dienolide	
		5,27-Dihydroxy-6α,7α-epoxy-1-oxo-(5α)-witha-2,24-dienolide	Sethi and Subramanian (1976)
		Withacoagin	Neogi et al. (1988)
		Withaferin A	Subramanian and Sethi (1969)
5.	**Seed**	D- Arabinose	Anonymous (1966)
		D- Galactose	
		Linoleic acid	

20.7 Molecular Biology/Genomics Aspect of *W. coagulans*

The study of genomes or genomics involves investigations and analysis of the genomes of an organism. It helps in the comparison of one genome with other and to understand the structure, function, diversity and evolution of genomes. The larger size and complexity of genome in higher plants are the major bottlenecks in whole genome sequencing and, however, with technological advancement genome sequencing, are progressing day by day. Till date scare information is available on genome sequencing in *Withania* genus, and a few reports on database are available (Afendi et al. 2012; Gupta et al. 2013). Most of works on *Withania* focused on the dominating species of this genus, i.e. *W. somnifera*. Gupta et al. (2013) reported *de novo* assembly and functional annotation in genome of *W. somnifera*; however, no information is available on *W. coagulans*. In this plant species, it is important to know the phytochemical genomics under biosynthesis, function and regulation of metabolites in *W. coagulans*. Khan et al. (2009) reported *MPF*-1- and 2-like gene clones in *W. coagulans*. In literature a few reports on gene cloning and characterization are available. Tropinone reductases (TRs) are a class of enzymes converting tropinone into tropane alcohols and which are important intermediary steps for biosynthesis of tropane esters of medicinal importance, namely, hyoscyamine/scopolamine and calystegins. The tropane alkaloids biosynthesis has been reported to be limited in roots, and these are stored in aerial parts. Kushwaha et al. (2013) reported cloning of a tropine-forming tropinone reductase (*WcTR*-I) from leaf tissues of *W. coagulans*. Subsequently the gene was heterologously expressed in *E. coli*. Jadaun et al. (2017) characterized tryptophan decarboxylase (TDC) from *W. coagulans*. The *WcTDC* was expressed heterologously in *Escherichia coli*, and kinetic properties were studied for recombinant TDC enzyme under withanamide biosynthesis.

20.8 Future Prospects

There are 23 species reported in *Withania*. The high-end molecular biology technique could be employed to generate the molecular signature for proper identification or differentiation *W. coagulans*. Trait-associated molecular marker can be developed. Further, these can be used to characterize the elite germplasm to be used in hybridization programs to develop commercially suitable cultivars. Among different species of *Withania*, cultivation of *W. somnifera* and *W. coagulans* has been reported (Mirjalili et al. 2009); however, in India to the best of our knowledge, there is no report on systemic cultivation of this species. From an agricultural point of view, development of agrotechnology is the need of the hour, and this could facilitate the large-scale cultivation to fulfil the commercial requirements for this plant. The in vitro propagation facilitates mass multiplication and germplasm conservation of rare, endangered and threatened medicinal plants. In *W. coagulans* tissue culture technique could be employed to develop elite cultivar through genetic engineering. Further cell culture technique could be employed to produce commercially important metabolites through phytopharming. The genomics, proteomics and metabolomics data can be generated, and key regulators of metabolite biosynthetic pathways can be identified. Subsequently the generated knowledge could be employed in phytopharming of commercially important metabolites through metabolic engineering.

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Chapter 21
Tissue Culture in Mulberry (*Morus* spp.) Intending Genetic Improvement, Micropropagation and Secondary Metabolite Production: A Review on Current Status and Future Prospects

Tanmoy Sarkar, Thallapally Mogili, S. Gandhi Doss, and Vankadara Sivaprasad

Abstract Mulberry (*Morus* spp.) is a woody, perennial, highly heterozygous, fast-growing plant and grown mainly for its foliage worldwide under various agroclimatic zones (tropical, subtropical and temperate) of Asia, Africa and the Americas. Mulberry leaves are the sole food source for monophagous and domesticated mulberry silkworm, *Bombyx mori* L. Moreover, mulberry fruits are fleshy, succulent and delicious berries. The fruits are low in calories and contain health-promoting phytonutrients such as polyphenols, minerals and vitamins having medicinal importance for its antioxidant, antitumor, neuroprotective activities and hypo-lipidemic/macrophage activating effects. Genetic improvement of mulberry is mainly aimed for improving productivity and quality of leaf for silk production. Conventional plant breeding techniques including tissue culture and molecular biology methods are employed in mulberry genetic improvement programmes to develop varieties for improved leaf productivity and biotic/abiotic stress tolerance. This review focuses on various tissue culture approaches such as in vitro regeneration, micropropagation, genetic transformation, somaclonal variation, in vitro selection, suspension culture, and much more which often supplement the traditional breeding methods. Further, characterization and production of secondary metabolites from mulberry tissues through suspension culture which are becoming a blooming option for commercial exploration of bioactive compounds have been discussed.

Keywords Organogenesis · Somatic embryogenesis · Somaclonal variation · Double haploid · Somatic hybrid · Transgenic mulberry · Secondary metabolite

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21.1 Introduction

Mulberry (*Morus* spp.; family *Moraceae*) is a typical deciduous, cross-pollinated tree or shrub with deep root system. Mulberry leaf is alternate, stipulate, petiolate, entire or lobed in nature. Its catkin possesses pendent or drooping peduncle bearing unisexual flower. The fruit (sorosis) consists of collection of individual achenes. Mulberry grows worldwide under varied climatic conditions between latitudes of 50^0N and 10^0S and from a sea level to as high as 4000 m which includes China, India, Thailand, Brazil, Uzbekistan and other countries. Mulberry is said to be originated in the northern hemisphere, especially in Himalayan foothills and subsequently spread to the tropics of southern hemisphere (Tikader and Dandin 2005; Vijayan 2010). In most of the mulberry growing countries especially in China and India, its improvement is mainly focused on enhancing the foliage yield and quality. Mulberry leaves are the sole food source for the monophagous mulberry silkworm, *Bombyx mori* L. (Lepidoptera). Mulberry leaf is rich in proteins, carbohydrates and moisture, which are of prime importance for silkworm rearing. Diploid (2n = 28) and triploid (3n = 42) mulberry varieties are cultivated for silkworm rearing in around 2.20 lakhs ha of land with assured irrigation in India. The sustainability of silk industry across the globe is directly related to the production and continuous supply of high-quality mulberry leaves. China stands first in mulberry silk production, while India occupies the second place with highest consumption in the world.

Approximately 68 mulberry species have been reported across the world, and majority of them occur in Asia with China (24 spp.), Japan (19 spp.) and India (4 spp.). Only few mulberry species such as *M. alba*, *M. indica*, *M. bombycis*, *M. latifolia* and *M. multicaulis* are widely cultivated for foliage to feed silkworms, while *M. laevigata*, *M. rubra*, *M. alba* and *M. nigra* are grown for edible fruits and *M. serrata* for timber (Datta 2000). Approximately 4800 mulberry germplasm resources are being maintained in China, Japan, South Korea, France, Italy and Bulgaria, while 1269 accessions are being conserved in India. *Morus* species shows extensive variation at ploidy level, ranging from diploid to decosoploid ($2n$ = 28 to 308) including a haploid, *M. notabilis* (Yile and Oshigane 1998).

Mulberry leaf is also used as fodder for livestock in various countries. Different parts of mulberry are also exploited for medicinal properties in various countries such as Korea, Japan and China to treat diabetes, paralysis, beriberi, etc. (Kim et al. 2003). Mulberry leaf is rich in several antioxidants, amino acids, minerals, vitamins, etc., which are of pharma- and neutra-ceutical value to the human beings and also consumed as dried leaf powder or tea. Few mulberry species are also cultivated for edible fruits and for medicinal purposes in Japan, China, Korea, Thailand, India and other countries. Further, mulberry tree is also being utilized for urban green cover and landscaping (Tipton 1994).

Mulberry shows extensive genotypic and phenotypic variations for disease resistance, abiotic stress tolerance, leaf yield and quality, phytochemicals, etc. Mulberry genetic improvement programmes are mainly focussed on improving stress toler-

ance and enhancement of leaf productivity and quality, while less attention has been paid until recently to improve fruit yield (Zhang et al. 2016). Various abiotic stresses (drought, low temperature, high salinity and alkalinity) and biotic factors (infestation of insect pests, snails, nematodes and pathogenic infections by virus, fungi and bacteria) are experienced in widespread areas across the globe adversely affecting leaf yield and quality. Traditional breeding approaches relying on morphological- and physiological-based phenotyping contributed greatly towards the development of stress-tolerant, high leaf-yielding and high leaf-quality mulberry varieties. However, since the last two decades, several modern approaches are being employed for accelerating mulberry genetic improvement programmes towards enhancement of potential foliage yield which includes physiological trait-based breeding (Mamrutha et al. 2010; Mishra 2014; Naik et al. 2014), genetic engineering (Jianzhong et al. 2001; Sajeevan et al. 2017), molecular breeding (Mishra 2014; Naik et al. 2014; Thumilan et al. 2013, 2016), omics (Khurana and Checker 2011; Dhanyalakshmi et al. 2016; Saeed et al. 2016) and tissue culture techniques (Narayan et al. 1989; Lakshmi Sita and Ravindran 1991; Susheelamma et al. 1996; Thomas et al. 1999). Various aspects and applications of plant tissue culture techniques towards propagation, genetic improvement, conservation and value addition of mulberry are being discussed in this review.

21.2 Application of Tissue Culture in Mulberry Genetic Improvement

Plant tissue culture is a technique for growing, multiplying and maintenance of plant cells, tissues or organs isolated from the mother plant, under nutritionally and environmentally supportive environment (in vitro), and sterile condition (Thorpe 2007). It is essentially based on the principle of cellular totipotency and concept of the cell theory proposed by Matthias Jakob Schleiden and Theodor Schwann (Vasil 2008). Plant tissue culture has found its applications not only in basic and applied research but also in commerce and trade. Various types of explants such as cell, anther, ovule, embryo, protoplast-derived somatic hybrid and meristem/buds are utilized for in vitro regeneration of whole plants. Tissue culture techniques were employed in mulberry for numerous applications such as micropropagation, callus culture, organogenesis, somatic embryogenesis, somatic hybridization, development of transgenics, in vitro screening of genotypes for stress tolerance and yield, production of haploids and triploids, and secondary metabolites (Figs. 21.1 and 21.2). Further, cryopreservation of mulberry tissues at ultra-low temperature (Niino 1995; Rao et al. 2007, 2009) and production of synthetic seeds by encapsulation of apical/axillary buds or somatic embryos with sodium alginate and calcium chloride (Pattnaik et al. 1995; Kamareddi et al. 2013) are the other important applications of tissue culture for mulberry germplasm conservation and propagation.

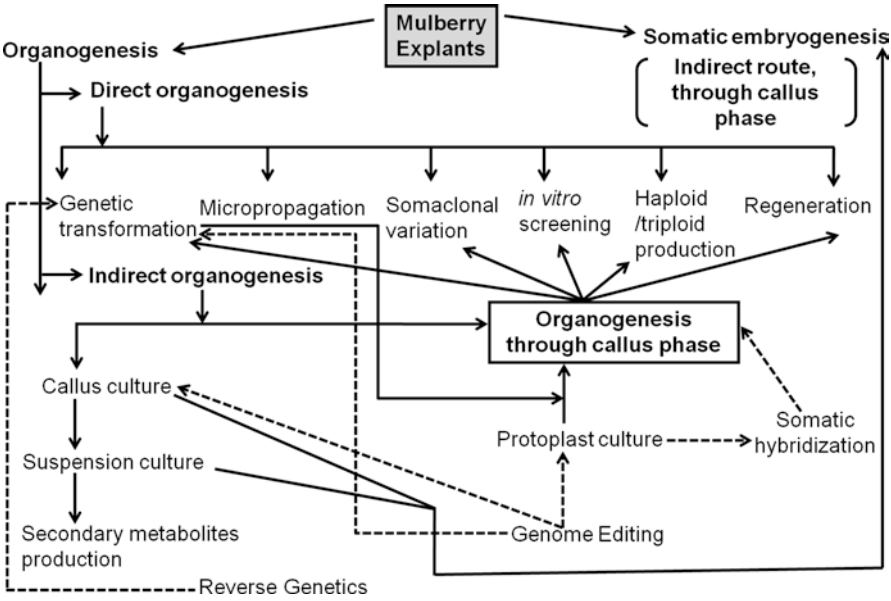

Fig. 21.1 Integration of tissue culture and molecular biology techniques in mulberry (*Morus* spp.) for genetic improvement

21.3 Micropropagation

Mulberry being highly heterozygous, cross-pollinated, dioecious, perennial and woody tree is usually propagated through stem cuttings and bud grafting, but not via seed propagation. However, the traditional vegetative propagation method is also influenced by various factors such as genetic makeup of mother plant, its origin and physiological condition, age of the stem, rooting ability, loss of vigour, environmental conditions, seasonal variations and many more. Hence, tissue culture-based micropropagation (clonal propagation) has emerged as a viable option to address the constraints of vegetative propagation. Further, micropropagation emerged as viable option for multiplication of a specific mulberry genotype/cultivar with its full genetic potential within a short period of time under controlled conditions. Micropropagation of mulberry was demonstrated for the first time by Ohyama (1970) in developing whole plants from axillary buds of *M. alba* cultured in Murashige and Skoog (MS) medium supplemented with growth regulators. The review on micropropagation by Vijayan et al. (2011a) enlists the different media, hormonal combinations, carbon sources and gelling agent concentration employed by various researchers to develop micropropagated mulberry plants, based on genotype/species specificity. Surface sterilization of explants and maintenance of aseptic conditions for culturing are the most important aspect in any tissue culture technique. The explants, collected from field-grown mulberry plants for tissue culture, are thoroughly washed in running tap water for 1 h followed by immersion in a

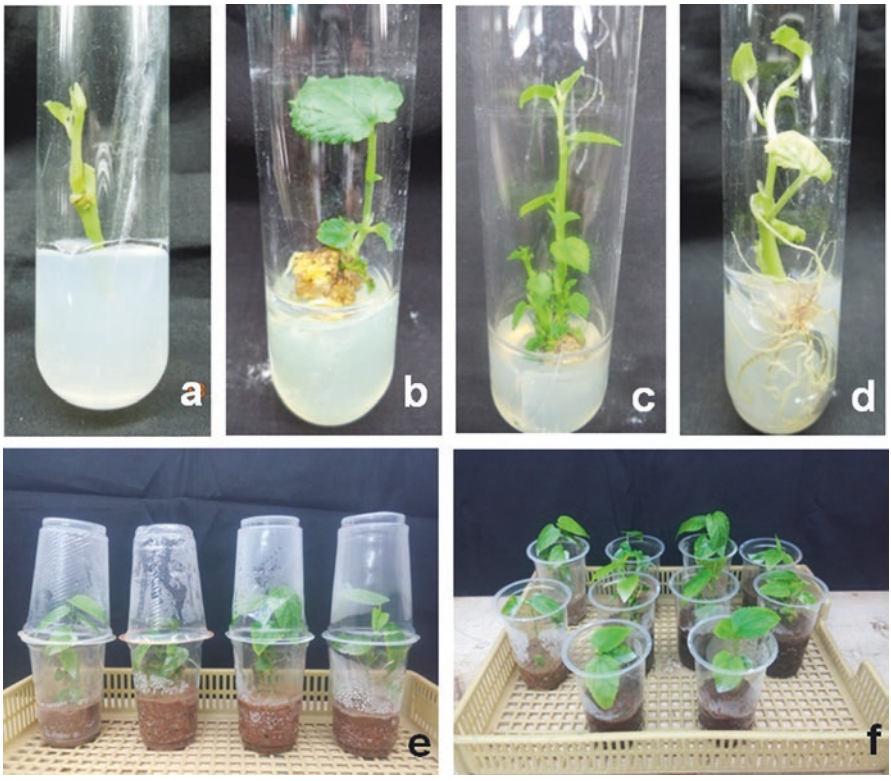

Fig. 21.2 Development of tissue culture raised mulberry (*Morus* spp.) through in vitro regeneration: In vitro shoot regeneration from axillary bud (**a**), callus tissue (**b**) and multiple shoot induction (**c**) through subculturing of in vitro regenerated shoots, in vitro root induction (**d**), hardening (**e**) and acclimation (**f**) of mulberry plants

liquid detergent (1%; v/v) such as Labolene/Tween 20 for 4–5 min or 0.2% Cetavlon (v/v) for 10 min and again washed thoroughly in running tap water for removing the traces of detergent/disinfectant (Sajeevan et al. 2011; Chattopadhyay et al. 2011; Raghunath et al. 2013). Further, the explants were treated with 0.5% systemic fungicides like Bavistin (0.5%; w/v) for 30 min and rinsed 2–3 times with sterile distilled water. Surface sterilization is usually undertaken by treating the explants with ethyl alcohol (70%; v/v) for 1–3 min followed by rinsing twice with sterilized distilled water (Sajeevan et al. 2011; Gogoi et al. 2017). Subsequently, the explants were soaked in $HgCl_2$ (0.1%; v/v) prepared in distilled water for 3–8 min with gentle shaking and rinsed 5–6 times in autoclaved distilled water for removing the traces of $HgCl_2$ (Bhatnagar and Khurana 2003; Raghunath et al. 2013; Sajeevan et al. 2017). The shoot tips (1 cm long) and nodal explants (1–2 cm long) of juvenile or adult shoot of current year with dormant auxiliary buds are most suitable for micropropagation in mulberry (Bhau and Wakhlu 2003).

The shoot initiation parameters such as media composition, hormonal composition, carbon source, pH of the media, type of gelling agent and its concentration are very crucial. The MS medium supplemented with 0.5 mg L^{-1} 6-benzylaminopurine (BAP), 3% sucrose, and pH 5.6–5.8, 0.7–0.8% agar was found optimum for mulberry micropropagation (Lalitha et al. 2013; Saha et al. 2016; Bhau and Wakhlu 2003; Enomoto 1987; Pattnaik and Chand 1997; Thomas 2003). However, half-strength MS medium showed better rooting as compared to full strength with the hormonal combination of 0.5 mg L^{-1} NAA (α-naphthaleneacetic acid) for *M. alba*, *M. indica*, *M. multicaulis* and *M. latifolia* (Vijayan et al. 2003). Further, higher concentration of BAP (>2 mg L^{-1}) and auxins such as NAA and IBA (indole-3-butyric acid; >1.0 mg L^{-1}) is inhibitory for shoot initiation, multiplication and rooting (Vijayan et al. 1998; Bhau and Wakhlu 2003). Thidiazuron (TDZ) is one of the most active cytokinin-like substances employed for multiple shoot induction in mulberry (cv. S1635). However, TDZ at a concentration (>2.27 μM), instead of direct organogenesis, induced callus tissues and produced rosette of shootlets from nodal explants (Lalitha et al. 2013). Addition of gibberellic acid (GA_3) in TDZ-free MS medium amended with BAP promoted multiple shoot proliferation and elongation (Lalitha et al. 2013; Saeed et al. 2015). The above studies demonstrated that the success of micropropagation in mulberry is dependent on growth regulator combinations, explant type and genotype, indicating that a single protocol might not be applicable to all the genotypes. Tissue culture technique itself may induce genetic variations; hence genetic fidelity of the micropropagated plants needs to be analysed using molecular markers (Saha et al. 2016). Various explants, media and combination of growth regulators such as auxin, cytokinin and gibberellic acid were used for obtaining different phases of growth in the micropropagation of mulberry genotypes/cultivars belonging to different *Morus* spp., and rooted plantlets are hardened and transferred to soil (Gogoi et al. 2017; Choudhary et al. 2015; Vijayan et al. 2011a).

21.4 Regeneration

Whole mulberry plant can be regenerated through organogenesis or somatic embryogenesis (Fig. 21.1). Organogenesis is a complex phenomenon involving *de novo* formation of organs from explants either through direct route or intermediate callus phase. However, the success of organogenesis and callus induction depends on various factors such as selection of explants, age of explant, type and composition of media, specific growth regulators, genotype, sources of carbohydrate and gelling agent. A variety of explants have been used for direct organogenesis and indirect organogenesis via callus phase in mulberry (reviewed by Sarkar et al. 2017). Explants from cambial regions (Narasimhan et al. 1970), hypocotyl segments (Shajahan et al. 1997), cotyledons (Thomas 2003), internodal stem segments (Vijayan et al. 1998) and young leaves (Chitra and Padmaja 2005) were found best for callus induction in MS medium supplemented with 2,4-dichlorophenoxyacetic acid (2,4-D). Plantlets of the mulberry (*M. alba*) were also regenerated from leaf

through callus phase. Callus formation was obtained on MS medium containing a combination of 1 mg L^{-1} 2,4-D and 0.5 mg L^{-1}BAP. Shoots were regenerated from the callus on MS medium supplemented with 1 mg L^{-1} BAP. Subsequently, the regenerated shoots rooted on MS medium containing either 0.5 mg L^{-1} IBA or NAA (Bhau and Wakhlu 2001). Augmentation of medium with coconut water (150 ml L^{-1}) and casein acid hydrolysate (100 mg L^{-1}) as nitrogen sources could enhance callus induction (Susheelamma et al. 1996). Higher concentration of cytokinin especially BAP and less auxin induces regeneration of shoot buds from the callus.

However, development of plant regeneration protocols using somatic tissues paved the way for the genetic improvement of woody tree like mulberry for the development of transgenics, somaclonal variants, somatic hybrids and multiplication of desirable genotypes (Fig. 21.2). Direct organogenesis has the advantages of inducing lesser genetic variations helping to maintain the genetic fidelity of regenerated plants. Indirect organogenesis (through callus phase) might induce genetic variations resulting in the development of somaclonal variants. As mulberry is highly heterozygous and outbreeding in nature, it is better to utilize tissues such as leaf, petiole as explants instead of cotyledons/hypocotyls to maintain the genetic makeup of a particular genotype via regenerated plants. Plants developed through cotyledons/hypocotyls, a product of cross-pollination and sexual reproduction might not generate true-to-type plants. However, regeneration of mulberry plant using leaf and petiole explants is difficult to achieve, and it is also genotype dependent as in other tree species. Hence, various regeneration (through direct organogenesis) protocols have been developed since a long time in mulberry using petiole and leaf explants (Kim et al. 1985; Machii 1990; Vijayan et al. 2000; Bhau and Wakhlu 2001; Chitra and Padmaja 2005; Raghunath et al. 2013). In most of the cases, shoot buds regeneration from leaf explant mostly occurred from the midrib or cut end of leaf lamina-petiole region (Vijayan et al. 2011b). However, regeneration of mulberry plants from petiole explants through organogenesis is yet to be achieved; hence more research in this area is required.

Silver nitrate ($AgNO_3$) is used in plant tissue culture medium as silver ions in the form of nitrate play a crucial role in influencing somatic embryogenesis and morphogenesis such as shoot regeneration and root formation, which are prerequisites for successful micropropagation, whole plant regeneration and genetic transformation (Kumar et al. 2009). Raghunath et al. (2013) reported shoot bud induction in mulberry (cv. V1) from leaf explants cultured in MS medium amended with TDZ (1.0 mg L^{-1}), IAA (2.0 mg L^{-1}) and $AgNO_3$ (2.0 mg L^{-1}). Further, whole mulberry plantlets were successfully regenerated through direct and indirect organogenesis from various explants (leaf, hypocotyl, cotyledon, intermodal segment, nodal explant and apical bud) of various genotypes/cultivars (S1, K2, DD, S36, V1, Chinese white, Kokuso27, etc.) belonging to different species like *M. alba*, *M. indica*, *M. latifolia*, *M. multicaulis*, *M. macroura*, *M. nigra*, etc. (Vijayan et al. 2000*;* Bhau and Wakhlu 2001; Bhatnagar et al. 2001; Lu 2002; Bhau and Wakhlu 2003; Kavyashree 2007, Rao et al. 2010; Zaki et al. 2011; Akram and Aftab 2012; Chitra et al. 2014).

Somatic embryogenesis is an important approach for plant propagation, regeneration and genetic manipulation in tissue culture. It is based on the ability of competent cells of somatic tissue to change their differentiation pathway to become somatic embryos, analogus to zygotic embryo, either directly or via callus phases, which subsequently develop into whole plants through tissue culture (Santos et al. 2005). Several attempts have been made to develop somatic embryos in mulberry with limited success. Shajahan et al. (1995) demonstrated development of embryo-like structures through suspension culture of hypocotyls derived callus from *M. alba*. Subsequently, globular and heart-shaped embryos were developed from callus derived from zygotic embryos cultured on MS medium supplemented with 2 mg L^{-1} 2,4-D and 0.5 mg L^{-1} BAP (Agarwal 2002). Secondary embryogenesis lead to the development of well-matured cotyledonary embryos from small embryogenic clumps comprising roughly 20–30 globular and heart-shaped embryos cultured on MS medium supplemented with 0.05 mg L^{-1} 2,4-D, 0.1 mg L^{-1} BAP and 6% sucrose (Agarwal et al. 2004). However, adventitious shoot and root formation from somatic embryo and subsequent regeneration of whole plant in mulberry are not reported so far.

21.5 In Vitro Screening and Somaclonal Variation

Abiotic stress (drought, cold, salinity and alkalinity) tolerance is a complex phenomenon involving interaction of several genes through signal transduction pathway. However, to withstand the ill-effects of abiotic stresses, plants devise different adaptive mechanisms such as morphological and developmental changes as well as alteration in physiological, biochemical and molecular processes (Sarkar et al. 2014, 2016; Sarkar 2014). Screening of mulberry genotypes for abiotic stress tolerance under real field conditions is not an easy approach as salinity, drought and alkalinity levels in the field vary depending on season, soil depth, soil type and agroclimatic zone. This approach also demands for land preparation, allocation of land under particular agroclimatic area or specific soil type, manpower, resources, time, adequate funds and technical expertise. Further, plant-environment interactions influenced by other factors interfere with the expression of abiotic stress tolerance at whole plant level. Hence, in vitro regeneration-based screening of mulberry open up a viable option to select plants under controlled environmental and simulated stress conditions, which may facilitate expression of the plant's innate ability to tolerate various abiotic stresses and help to select plants at initial stage for subsequent screening under real field condition. In vitro screening of axillary buds and shoot tips under salt stress paved the way to identify salinity tolerant mulberry genotype with efficiency, rapidity and cost-effectiveness resulting in the identification of salinity (NaCl), alkalinity (Na_2CO_3 + $NaHCO_3$, pH 8.5–10) and drought (polyethylene glycol, PEG) tolerant genotypes (Tewary et al. 2000). Fourteen MS media combinations supplemented with various concentrations of cytokinins (kinetin and BAP) have been evaluated in three mulberry genotypes (G2, G3 and G4) to determine the optimum responses of nodal explants towards sprouting of shoot buds and length of regenerating shoots under in vitro stress conditions. In vitro screening method demonstrated that

G4 genotype can tolerate up to 1.0% NaCl and pH (9.5) in MS medium with appropriate hormonal combinations. Moisture stress-tolerant genotype (S13) exhibited higher bud sprouting and shoot growth under 4% PEG in MS medium (Tewary et al. 2000). Similarly, attempts were also made to identify mulberry genotypes tolerant to salinity and alkalinity (Vijayan et al. 2004; Ahmad et al. 2007).

Plants regenerated from in vitro cultured explants through organogenesis and callus phases may possess an array of genetic and epigenetic changes referred as somaclonal variations. Hence, continuous cell line culture under appropriate selection pressure were used to develop salinity tolerant mulberry plants. This approach led to development of a somaclonal variant in mulberry (*M. alba* cv. S1) exhibiting higher leaf yield and more number of branches than the mother plant (Narayan et al. 1993). Similarly, Susheelamma et al. (1996) also isolated somaclonal variant with shorter intermodal distance, thicker leaves, higher chlorophyll and moisture content and better moisture retention capacity than mother plant from plantlets developed through leaf-derived callus culture of mulberry cv. S14.

21.6 Genetic Engineering in Mulberry

Genetic engineering provides plant biotechnologists with a unique opportunity to transfer gene(s) from alien sources across higher taxonomic unit, when traditional or molecular breeding approaches may not work well due to the unavailability of specific gene(s)/QTLs (quantitative trait loci) governing particular trait of interest in natural plant populations. In vitro regeneration potential or cellular totipotency in mulberry is highly genotype dependent (Raghunath et al. 2013), and the success of genetic transformation is dependent on various factors such as regeneration potential of genotype, choice of explant (Sarkar et al. 2016), *Agrobacterium* strains, recombinant plasmids (Bhatnagar and Khurana 2003) and many more. Genetic transformation in mulberry with heterologous genes has been attempted since long through particle bombardment, *Agrobacterium* rhizogenes-mediated, electroporation and *in planta* techniques (Sugimura et al. 1999; Oka and Tewary 2000; Machii 1990; Machii et al. 1996; Bhatnagar et al. 2002). However, these attempts were not successful as regeneration of transformed plants could not be achieved. Over the time, attempts have been made to develop efficient regeneration and genetic transformation protocols by various research groups using different explants, mulberry genotypes and heterologous genes including methods of transformation (Sarkar et al. 2017). Subsequently, an efficient and reproducible protocol for the genetic transformation using *Agrobacterium tumefaciens* was reported in mulberry (*M. indica* cv. K2) by utilizing explants such as hypocotyl, cotyledon, leaf and leaf-derived callus. The explants were precultured for 5 days on regeneration medium (MS) supplemented with TDZ (0.1 or 1.1 mg L^{-1}). Afterwards, the explants were used for mulberry genetic transformation (cocultivation with *A. tumefaciens*) in the presence of 200-250 μM acetosyringone in liquid MS medium supplemented with 1.1 mg L^{-1} TDZ (Bhatnagar and Khurana 2003; Sajeevan et al. 2017).

Agrobacterium tumefaciens-mediated genetic transformation in mulberry (*M. indica*) leads to the development of transgenic plants with tolerance to various abiotic and biotic stresses (Table 21.1). Most of the times, cotyledon, hypocotyl and leaf-derived callus have been used as explants for genetic transformation (Bhatnagar and Khurana 2003; Lal et al. 2008; Saeed et al. 2015). Addition of appropriate concentration of acetosyringone in cocultivation medium enhanced transformation efficiency in mulberry with other key factors being pre-culture, cocultivation and immersion time, and bacterial concentration (Yong et al. 2010). Hypocotyl and cotyledon explants excised from 10- to 15-day-old in vitro raised seedlings were used in genetic transformation (Bhatnagar and Khurana 2003; Sajeevan et al. 2017). While, the leaf of the field-grown plant was cultured on MS medium containing IAA (2 mg L^{-1}), TDZ (1.1 mg L^{-1}) and $AgNO_3$ (2 mg L^{-1}) and kept initially in dark and thereafter in light condition for over 2 months for the production of leaf-derived callus. Further, the regenerating callus was used for genetic transformation (Bhatnagar and Khurana 2003; Das et al. 2011). Leaf explants from in vitro grown axillary bud cultured on MS medium supplemented with TDZ (0.1 mg L^{-1}) have also been used for genetic transformation. The explants were immersed for 30 min in *A. tumefaciens* suspension prepared in liquid MS medium containing 1.1 mg L^{-1} TDZ and 200 μM or 250 μM acetosyringone followed by coculturing of explants on solidified MS medium amended with 1.1 mg L^{-1} TDZ for 3 days in dark. Subsequently, the cocultured explants were washed in liquid MS medium containing TDZ (0.1 or 1.1 mg L^{-1}) and cefotaxime (250 mg L^{-1}) to kill *Agrobacterium* cells (Bhatnagar and Khurana 2003; Sajeevan et al. 2017). Thereafter, the explants were cultured on shoot induction/selection; shoot elongation and rooting medium supplemented with appropriate hormonal combinations to generate transgenic plants for stress tolerance (Table 21.1).

21.7 Development of Haploid and Polyploid Mulberry

Development of inbreed lines in mulberry through traditional breeding methods is very difficult task due to inbreeding depression, long juvenile period, high heterozygosity, dioecious and outbreeding nature. Hence, production of haploid plants through in vitro androgenesis and gynogenesis, and subsequent development of double haploid lines through colchicine treatment are the easiest and most rapid methods for generating complete homozygous (inbreed) lines (Dunwell 2010; Germaná 2011). Shoukang et al. (1987) first attempted the anther culture for development of haploid mulberry and only observed division of cultured pollen. Subsequently, Katagiri (1989a), Katagiri and Modala (1991) and Katagiri and Modala (1993) reported division of in vitro cultured pollen, formation of callus from binucleate cultured pollen and induction of organ-like structures from pollen-derived callus. Sethi et al. (1992) reported embryo differentiation from mulberry anther cultures maintained in the dark at 26 ± 1 °C for a period of 15 days. Development of rooted embryoids of mulberry (*M. indica*; cv. RFS135)

Table 21.1 Development of transgenic mulberry for stress tolerance through *Agrobacterium tumefaciens*-mediated genetic transformation

Transgene	Genotype	Explants	Media composition			Performance of transgenic plant	References
			Shoot induction	Shoot elongation	Rooting		
Hva1	*M. indica* (cv. K2)	Hypocotyl, cotyledon	MS medium + TDZ (1.1 mg L^{-1}) + kanamycin (50 mg L^{-1}) + cefotaxime (250 mg L^{-1})	MS medium + BA(0.5 mg L^{-1}) + GA_3 (0.5 mg L^{-1}) + $AgNO_3$ (2 mg L^{-1}) + kanamycin (50 mg L^{-1})	MS medium + NAA (1 mg L^{-1}) + activated charcoal (0.1%)	Tolerance to drought and salinity stress	Lal et al. (2008)
Osmotin	*M. indica* (cv. K2)	Leaf-derived callus, Hypocotyl, Cotyledon	MS medium + IAA (2 mg L^{-1}) + TDZ (1.1 mg L^{-1}) + $AgNO_3$ (2 mg L^{-1}) + kanamycin (50 µg/ml) + cefotaxime (250 mg L^{-1})	MS medium+ BAP (0.5 mg L^{-1}) + GA_3 (0.5 mg L^{-1}) + $AgNO_3$ (2 mg L^{-1}); MS medium + BAP (1 mg L^{-1})	Half-strength MS medium + IBA (1 mg L^{-1})	Tolerance to drought and salinity stress; resistance to fungi	Das et al. (2011)
Hva1	*M. indica* (cv. K2)	Hypocotyl, cotyledon	MS medium + TDZ (1.1 mg L^{-1}) + kanamycin (50 mg L^{-1}) + cefotaxime (250 mg L^{-1})	MS medium + BA(0.5 mg L^{-1}) + GA_3 (0.5 mg L^{-1}) + kanamycin (50 mg L^{-1}) + $AgNO_3$ (2 mg L^{-1})	MS medium + NAA (1 mg L^{-1}) + activated charcoal (0.1%)	Tolerance to drought, salinity and cold stress	Checker et al. (2012)
bch1	*M. indica* (cv. K2)	Leaf-derived callus, Hypocotyl, Cotyledon	MS medium + TDZ (1.1 mg L^{-1}) + kanamycin (50 mg L^{-1}) + cefotaxime (250 mg L^{-1})	MS medium +BA(0.5 mg L^{-1}) + GA_3 (0.5 mg L^{-1}) + $AgNO_3$ (2 mg L^{-1}) + kanamycin (50 mg L^{-1})	MS medium + NAA (1 mg L^{-1}) + activated charcoal (0.1%)	Tolerance to UV, high temperature and irradiance stress	Saeed et al. (2015)
SHN1	*M. indica* (cv. M5)	Hypocotyl, Cotyledon	MS medium + TDZ (0.1 mg L^{-1}) + cefotaxime (200 mg L^{-1}) + kanamycin (50 mg L^{-1})	MS medium + TDZ (0.1 mg L^{1}) + cefotaxime (200 mg L^{-1}) + kanamycin (50 mg L^{-1})	MS medium + IBA (0.5 & 1.0 mg L^{-1}) with or without activated charcoal (0.1%)	Enhanced leaf moisture retention capacity	Sajeevan et al. (2017)

from uninucleate microspores through callus phase has also been reported (Jain and Datta 1992) but with negligible reproducibility. Successful attempts have been made to produce haploid mulberry plant through in vitro gynogenesis. Lakshmi Sita and Ravindran (1991) reported development of gynogenic plants from ovary cultures, without callus phase in *M. indica*. Further, in vitro grown unpollinated ovary was cultured on MS medium supplemented with 2,4-D (4.5 μM), glycine (6660 μM) and proline (1738 μM), and the regenerated gynogenic haploid mulberry plants (2n = x = 14) were subsequently transferred to the soil (Thomas 1999; Thomas et al. 1999). However, production of haploid plants and homozygous lines based on double haploidy in mulberry are not reported in the recent past.

The triploidy nature is of significant importance in tree species that are economically important for biomass and leaf yield production, because they promote vegetative growth by preserving huge amounts of photosynthetic energy and also by not being channelled to seed and fruit production. The endosperm of diploid angiosperm is a triploid tissue having three sets of chromosomes, a result of double fertilization occurred in higher plants (Hoshino et al. 2011; Thomas 2002). However, various natural and in vivo induced triploids have been reported by several research groups in mulberry (Das et al. 1970; Katagiri et al. 1982; Dwivedi et al. 1989; Thomas et al. 2000). In nature, triploid plants are sterile and cannot set seeds and fruits. Many of the triploid mulberry plants are superior to the diploids, especially in terms of leaf yield and nutritive qualities (Seki and Oshikane 1959). Desired traits of triploid mulberry could be maintained through clonal propagation without any loss of foliage yield (Vijayan et al. 2011a). The production of triploid through conventional breeding is lengthy and tedious approach, and hence, endosperm culture offers an excellent system for demonstration of morphogenesis and plant regeneration (Bhojwani and Razdan 1996). Thomas et al. (2000) reported regeneration of Indian mulberry (cv. S36) from endosperm tissue via callus phase. The endosperm tissue was isolated from the seeds of young fruits obtained from open pollination and cultured for callus induction on MS medium supplemented with 5 μM 2,4-D and different concentrations of auxin, cytokinin and gibberellic acid along with various nitrogen sources such as tomato juice, yeast extract, casein hydrolysate and coconut milk. Subsequently, shoot buds were regenerated by subculturing callus on medium containing cytokinin alone or in combination with NAA. Maximum callus induction (70–72%) was obtained on MS medium supplemented with BAP (5 μM), NAA (1 μM) and coconut milk (15%) or yeast extract (1000 mg L^{-1}). The MS medium containing TDZ (1 μM), or BAP (5 μM) and NAA (1 μM) was found to be the best one for shoot induction. The plantlets were transferred to the soil after cytological confirmation of triploid nature of regenerated plants (Thomas et al. 2000; Thomas 2002). Further, in vitro production of tetraploid mulberry from apical buds in MS medium supplemented with 0.1% colchicine and BAP (2 mg L^{-1}) has also been reported (Chakraborti et al. 1998). However, only diploid and triploid mulberry genotypes are of great importance in terms of commercial cultivation due to their high leaf palatability.

21.8 Protoplast Culture and Somatic Hybridization

Isolation of protoplasts from plant cells using enzymes was reported for the first time by E.C. Cocking (1960). Subsequently, Takebe et al. (1971) reported regeneration of whole plant from tobacco leaf protoplasts. Protoplasts can also take up foreign DNA through exposed cell membrane with the help of physical (electoporation) and chemical (polyethylene glycol) treatments, thus paving the way to develop transformed cells, tissue and whole plant. Isolated protoplasts also act as model systems for demonstrating various biochemical and molecular processes. Somatic hybridization through protoplast fusion paved the way for the development of interspecific hybrid with traits of both the donor plants, which is not possible through conventional breeding approaches due to sexual incompatibility (Johnson and Veilleuz 2010). Further, tropical mulberry flowers at the end of winter and the beginning of spring, while temperate mulberry does the same in the middle of spring to the first half of summer (Doss et al. 1998). Non-synchronized flowering pattern forms the constraint for initiating hybridization experiments between tropical and temperate mulberry for genetic enhancement and amalgamation of traits. Hence, somatic hybridization in mulberry could be a viable option to address the issue, and several efforts have been made to isolate protoplast from mesophyll cells, primary and secondary callus culture of mulberry (Wei et al. 1994; Umate et al. 2000a, 2000b; Mallick et al. 2016). A combination of 2% cellulase, 1% macerozyme and 0.5% macerase (Katagiri 1989b; Umate et al. 2005) in the presence of 0.5 M mannitol (osmoticum), macro- and micronutrients of B5 medium, and additional vitamin supplements was found to be optimal for higher protoplast isolation (Umate et al. 2005; Umate 2010). Further, presence of zeatin (2.3 l μM), 13.5 μM dicamba (2-methoxy-3, 6-dichlorobenzoic acid) and 2,4-D (2.3 μM)/NAA (2.7 μM) in protoplast culture medium supported the development of microcalli from protoplasts. Subsequently, shoots were regenerated from microcalli cultured on MS medium containing TDZ (4.5 μM) and IAA (17.1 μM). The regenerated shoots have been rooted on MS medium supplemented with 4.9 μM IBA (Umate et al. 2005) or half-strength MS medium supplemented with IBA (0.5 mg L^{-1}) and 0.1 mg L^{-1} BAP (Wei et al. 1994). Interestingly, fusion product of mulberry protoplasts known as somatic hybrid was successfully achieved using chemical fusogen (Ohnishi and Kiyama 1987) and electrofusion (Ohnishi and Tanabe 1989). However, regeneration of whole plant from somatic hybrid is yet to be reported in mulberry necessitating further research in this direction to realize full potential of somatic hybridization in mulberry.

21.9 Secondary Metabolite Production

Secondary metabolites including flavonoids are valuable compounds which are used as medicines, spices, dyes, insecticides, cosmetics and foods (Zhong 2001). The production of secondary metabolites employing plant tissue culture methods

mainly depends on cell proliferation and differentiation (George 2008). Diverse external factors such as temperature, light, pH of media, growth regulators especially auxin and salt concentration influence the production of secondary metabolites in cultured cells (Zenk et al. 1977; Smetanska 2008). Secondary metabolites are often produced in cultured cells in suspension (liquid medium), following callus culture. The concentration of auxin and cytokinins, and the ratio between them are crucial factors for the callus production, growth of callus (Zenk 1978) and production of secondary compounds. Medicinally and economically important secondary metabolites of mulberry include rutin, mulberroside A, morusin, cyclomorusin, quercetin, etc. and have been produced by suspension cultures of leaf-derived callus and callus-derived adventitious roots (Lee et al. 2011; Inyai et al. 2015; El-Mawla et al. 2011). Rutin, a flavone with anticancer and anti-ageing activity, and dietary effects have been produced in the presence of 5 mg L^{-1} IAA, but rutin secretion into liquid medium enhanced in the absence of IAA. Addition of auxins such as IAA, 2, 4-D and NAA not only enhanced the development of callus and callus-derived adventitious roots but also the protein and rutin content in the cultured cells. Supplementation of medium with cytokinin (BAP and kinetin) reversed the phenomenon (Lee et al. 2011). Inyai et al. (2015) showed that immobilization of callus-derived free cells suspended in liquid medium with calcium alginate and subsequent elicitation with yeast extract and methyl jasmonate enhanced the production of mulberroside A in suspension cultures. Elicitors induce signal transduction pathways triggering the transcription of biosynthetic enzymes involved in the formation of defence compounds like secondary metabolites in plants (El-Mawla et al. 2011; Saeed et al. 2015).

21.10 Conclusions and Future Prospects

Considerable achievements have been made employing tissue culture techniques for in vitro propagation; generation of transgenics, somaclonal variants and haploids; regeneration of whole plant; and production of secondary metabolites in mulberry. The transgenic mulberry so far developed need to be evaluated under confined field trials to demonstrate tolerance level under stress conditions (Sarkar et al. 2017). Regeneration of mulberry is genotype dependent and requires continuous efforts to develop genotype-independent regeneration and genetic transformation protocols using leaf explants retaining the genetic fidelity of the mother plant except for the heterologous gene(s). Further, the endosperms, hypocotyls and cotyledons derived from the crosses between two elite parents could be used as explants to generate promising diploids, triploids and polyploids including transgenics retaining the elite genetic background of parental lines in mulberry. Somaclonal variants already developed need further validation for possible commercial exploitation. In spite of development of haploid mulberry plant, there are no reports on the generation of double haploidy-based homozygous lines, and future research towards this approach need to be initiated for the faster mulberry genetic improvement. Somatic embryogenesis

could be an important route for regenerating mulberry transgenics; further, till date no report is available on regeneration of whole mulberry plant from somatic hybrids. Hence, further research in these directions need to be initiated.

Several secondary metabolites with ample medicinal value are reported from mulberry; commercial production of these phytochemicals through suspension culture could add value to the sericulture industry. Further, integrated approach of genomic tools such as metabolomics, proteomics and transcriptomics could aid in identifying novel secondary metabolites in mulberry and their production for commercial exploitation. Genetic engineering and reverse genetic approaches such as virus-induced gene silencing, RNA interference and genome editing tools in tandem with suitable genotype-independent regeneration protocols can provide valuable insights in mulberry functional genomics for the identification of trait-specific novel genes. Further, integration of genetic engineering and molecular biology techniques along with appropriate whole plant regeneration schemes coupled with traditional breeding methods, adequate fund flow and concerted efforts could witness breakthroughs in genetic improvement of mulberry especially for biotic/abiotic stress tolerance and higher productivity/quality of foliage and fruits.

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Chapter 22
In Vitro Conservation Strategies for *Gloriosa superba* L.: An Endangered Medicinal Plant

Ritu Mahajan, Pallavi Billowaria, and Nisha Kapoor

Abstract The immense value of plants remains in their incredible potential, a bit of which has been discovered till now and ample still remains to be discovered. The capacity of plants to produce a diverse array of unique chemical compounds attracts the attention of researchers and pharma industry towards the advantage of procuring these biological compounds as medicines for human health. *Gloriosa superba* L., the plant of enormous medicinal importance, has been listed among the endangered plants a few decades back. The plant is exploited for the colchicine which adds a characteristic feature to the medicinal value of this seasonal herb. Biotechnological approaches involve several efficient and cost-effective techniques which further resulted in the manipulation of this endangered plant to enhance its yield. Even the use of callus culture, cell suspension and hairy roots recommends coherent and productive formula for conservation and production of colchicine so as to fulfil the increasing demands of the pharma industry. The chapter reviews the in vitro propagation and conservation efforts made by several workers to increase and maintain the germplasm and isolation of colchicine from in vitro grown cultures.

Keywords *Gloriosa superba* · Micropropagation · Endangered · Conservation · Secondary metabolites

22.1 Introduction

The wonderful potential that plants carry inside them had made the life possible on earth, millions of years ago (Mergeay and Santamaria 2012). Plants once changed the scenario of earth's atmosphere from a reducing to the present oxidizing one (Piombino 2016). Since early civilizations man has remained obligate over the rich diversity of plants. Exploiting plants for enormous needs of his livelihood, man has realized the medicinal importance of plants also. Secondary metabolites, the low

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molecular weight compounds produced by plants, have attracted the interests of man since ages.

According to a report by the World Health Organization (WHO), about 75% of the world's population relies on plant-derived medicines for their health concern, and from them around 21,000 plants are known to possess medicinal potential (Pan et al. 2014). The collaboration of natural products chemistry and synthetic chemistry has aided in inventing new molecules by manipulating the natural products of the plants. But due to the risk of side effects and other associated safety unease, still products of natural origin are much favoured by the developed nations (Ekor 2013). Plants have a remained major source of medicine for about 3.4 billion population of the developing world according to a WHO report (Khan et al. 2007).

In spite of the tremendous positive effects in fulfilling medicinal demand, many of these plants have been badly affected in accomplishing the increasing demands of society. Over-exploitation due to different human activities like lack of cultivation methods, excessive collection by pharmaceutical companies, poor growth, distribution in specific areas and overharvesting has led to the destruction of real wealth of plants which have resulted in blacklisting many important genera into threatened, endangered and extinct category (Chen et al. 2016).

There is an urgent need for the protection of these valuable plants by new techniques of propagation, multiplication and conservation. Then only the pharmaceutical demand can be accomplished concurrently by using alternative strategies rather than destroying the whole plant in its natural habitat. Biotechnology approaches of mass propagation, synthetic seed production, cryopreservation, genetic transformation, in vitro secondary metabolites isolation and elicitation strategies have been proved useful for conservation of plant as well as meeting the demands of society (Cruz-Cruz et al. 2013).

In vitro propagation techniques since the last few years have made a strong effort for increasing the number and production of quality plants. The technique along with cryopreservation makes conservation of germplasm of better quality stock plants possible. Even the synthetic seed formation increased the efficiency of plant tissue culture manifolds by increasing the chances for a long-time stable storage of elite varieties (Lata et al. 2011). Isolation of high-value secondary metabolites from in vitro cultures opened new opportunities for enhancing their production by using the natural principle of stress or elicitation (Giri and Zaheer 2016).

Gloriosa superba L., a herbaceous climber belonging to family Liliaceae, grows in the tropical and subtropical environment of Africa and Asia including India, Sri Lanka, Bangladesh, Burma and Malaysia (Mahajan 2015). In India, the climber grows in the states of Tamil Nadu, Maharashtra, Madhya Pradesh and many parts of the Himalayas and Northern India. The herbaceous climber grows well in the rainy season and is well adapted to sandy loam soils. The stunning flowers and medicinal value of the herb have drawn a great attention towards mass cultivation of plant. The plant has been enlisted as an endangered species in the IUCN Red List of endangered species (Wable and Kharde 2009). The plant has medicinal value as it is a rich source of an important alkaloid, colchicine that plays role in plant breeding studies and cytogenetics where it induces polyploidy by causing mitotic arrest (12). The

plant has potent role for anti-abortive, antimicrobial and anticancer activity (Nikhila et al. 2014; Mahajan 2015).

22.2 Conservation Strategies

22.2.1 In Vitro *Propagation*

Sprouting of tubers in *G. superba* is irregular. Due to poor seed germination and continued asexual propagation through tubers, genetic variability of *G. superba* has become low. It resulted in reduced vigour, less tolerance to biotic and abiotic stress, low yield and decreased population (Ade and Rai 2012). A number of pathogens associated with the plant also reduce the yield of crop to a considerable limit.

Being an important medicinal plant, various workers have tried different conservation strategies for its propagation and preservation. Anandhi and Rajamani (2017) proposed mutagenic treatments for generation of variability in *Gloriosa* for its improvement. Plant culture system provides an advanced and alternative protocol to meet the importance of this valuable plant. It has several advantages over the conventional method of propagation. It makes plant available throughout the year, and also plants free from any microbial contamination are produced. The time required for growth of plants is reduced and is seasonal independent. In *Gloriosa*, tubers are not available throughout the year, and also the seed viability is poor. Thus in vitro propagation methods can result in increased plant number, colchicine extraction from in vitro cultures, germplasm conservation and genetic improvement of the plant.

The production of superior plant material also depends on many factors, and one of the important factors is the tuber quality. Muruganandam and Mohideen (2007) observed that the tubers weighing 51–70 g were ideal as they resulted in high sprouting rate, increased plant height, number of flowers, good fruit set, high seed yield and suitable pod. However, tubers less than 30 g in weight were comparatively low yielding. Even, significant correlation exists between seed yield per plant and some phenotypic and genotypic characters of the plant. Chitra and Rajamani (2009) calculated positive or negative correlation of quality characters among 18 genotypes using 13 different physiological and biochemical traits. Krause (1986) proposed that high yield of seeds can be obtained using cross-pollination rather than self-pollination in tuber-raised plants.

Poor seed germination, susceptibility towards pests and excessive collection in habitats for medicines are the main casual factors provoking the need of plant tissue culture. Seeds are highly priced in the world market, and enough plant material is not sufficient from tubers (Mahajan et al. 2016). Even, multiplication using vegetative propagation is not possible in *G. superba* because due to strong apical dominance in plant, any damage to the plant part may cause death of the whole plant. Somani et al. (1989) made first attempt for in vitro propagation of *G. superba* using

apical buds which still remain the favourable explant for successful propagation (Kumar et al. 2015).

22.2.1.1 Seed Germination

Various reports in literature propose poor seed germination and low viability of seeds in *G. superba*. Singh et al. (2015a) obtained seed germination using 1.0 mg/l BAP with 0.5 mg/l GA_3.They observed that a minimum period of 49 days is required for germination. Anandhi et al. (2013, 2016) proposed different treatments for effective germination of seeds and tubers. Seeds soaked in hot water for 1 h promoted germination up to 32.75% and sprouted earlier in 48.35 days. Even the ethanol treatment at 550 ppm was effective as it resulted in maximum sprouting in 6.33 days with improved plant vigour. However, chilling temperature treatment before inoculation does not result in any positive effects on regeneration of the plants (Finnie and Staden 1989), whereas Mahajan et al. (2016) observed 10–15% seed germination after 3 weeks following cold treatment.

22.2.1.2 Shoot and Root Induction

Yadav et al. (2012) tried different sterilization treatments for in vitro propagation of *G. superba*. They observed that sterilization of tubers with 0.1% $HgCl_2$ for 5 min eradicated maximum contamination. Also, MS medium supplemented with 2.0 mg/l 6-BA and 0.5 mg/l NAA along with 3% sucrose proved good for *in vitro* tuber formation and shoot regeneration. They also observed that increase in the concentration of sucrose up to 4% resulted in formation of large tubers. Maximum roots were induced on half MS medium containing 1.0 mg/l indole-3-butyric acid (IBA) and 0.5 mg/L naphthalene acetic acid (NAA). Hassan and Roy (2005) obtained four shoots per culture using apical and axillary buds as explants on MS containing 1.5 mg/l BA and 0.5 mg/l NAA. Repeated subculturing on 15% coconut water along with 2 g/l activated charcoal resulted in increased number of increased shoots. Roots formed well on MS containing 1.0 mg/l IBA and 0.5 mg/l IAA (Hassan and Roy 2005). Change in concentration of growth regulators effects time for shoot initiation and multiplication.

BAP remains a good hormone for shoot initiation even alone and proved to be the best when combined with NAA. Khandel et al. (2011) performed in vitro propagation by shoot induction in *G. superba* from apical shoot buds and meristems on MS medium. 2.0 mg/l BAP + 0.5 mg/l NAA proved the best medium for shoot initiation. 90 ± 7.0% plants formed shoots from meristems and 88 ± 6.2% from apical shoot buds. Kinetin otherwise was not effective either alone or with BAP. However, 0.5 mg/l kinetin proved to be good for the highest shoot initiation and proliferation from tuber explants when used with 2.0 mg/l BAP. Sprouted tubers always remained good explants for in vitro propagation of *Gloriosa*. Custers and Bergervoet (1994) observed that low level of 6-benzyladenine (up to 1 mg/l) improves plant growth

and while increasing the concentration of BA up to 10 mg/l results in proliferation of multiple shoots.

22.2.2 In Vitro *Tuberization*

G. superba being an endangered plant and because of poor seed germination and slow tuber multiplication needs a well-developed protocol for in vitro tuberization for its rapid multiplication. Many workers have revealed that in vitro tuber formation is controlled by several factors, including the hormonal combinations (Hannapel et al. 2017).

In vitro corms were raised under in vitro conditions from 30-day-old multiple shoots which resulted in production of dormant and nondormant corm buds (Sivakumar et al. 2003). Best response from dormant corm buds was recorded on MS containing B5 vitamins along with growth regulators 2iP and adenine sulphate and 6% sucrose, while in case of nondormant corm buds, MS containing growth regulator kinetin and adenine sulphate gave a good response. A number of efforts have been made to increase the population of *G. superba*, but the ratio of explants to generated plants remained 1:1. Since, *G. superba* tuber has only two apical buds, and it was observed that the plant dies on the removal of these apical buds. So, a protocol was standardized in which nondormant tubers of *G. superba* L. were grown on MS containing 4.0 mg/l BAP and 1.0 mg/L NAA by Anandhi and Rajamani (2012). A 100% primary and secondary tuber formation was observed, while Rishi (2011) reported B5 medium supplemented with 3% of sucrose best for the microtuberization in *G. superba* resulting in the production of 180–240 tubers within 6 months.

Shimasaki et al. (2009) observed the efficient method of tuber formation in *G. superba* is by using longitudinal section (LS) of stem branches. The number of tuber formation increased in the presence of higher concentrations of sucrose and showed maximum number of tubers when sucrose at concentration of 80 g/l along with TDZ (3 μM) was used. The number of tuber formation from decapitated stem explants was more than the double of the apical stem explants. When treated with trehalose (40 g/l) and TDZ (3 μM), the number of tuber formation was maximum (9.0), while an increase in trehalose concentration to 80 g/l decreased the tuber formation. Thus, the LS have higher ability for tuber formation. Further, combining TDZ at concentration of 3 μM along with 80 g/l sucrose or 40 g/l trehalose are highly effective for multiple tuber formation in the stem cultures of *G. superba*.

Kolar and Basha (2014) proposed a reliable method of producing in vitro tubers from seed explants and estimation of colchicine from plants of Pachaimalai Hills in Tamil Nadu. A high rate of in vitro seed germination (72.5%) was observed on MS basal supplemented with 0.5 mg/l GA_3 and 1.0 mg/l BA along with 1% sucrose, after an overnight treatment of soaking the seeds in 1% GA_3. The germinated seeds

induced 90% in vitro tubers after 6 weeks of culture when transferred to MS basal medium containing 1.0 mg/l BAP + 0.05 mg/l GA_3 + 9.5 mg/l NAA and 6% sucrose, after 6 weeks of culture. They observed that colchicine content was found to be maximum in in vitro grown tubers (0.14%) as compared to naturally growing seeds, tubers and leaves. But, Kumar et al. (2015) observed that in vitro formed tubers have less colchicine content as compared to in vivo grown tubers. However, they recorded that the colchicine content of these in vitro grown microtubers increased as they grow old.

Kumar et al. (2015) studied that MS supplemented with 6% sucrose and 35.5 μM BA along with citric acid and polyvinyl pyrrolidone-40 resulted in microtuber formation, while individual microtuber subcultured at 8.88 μM BA resulted in individual shoot formation. They also recorded 90% survival rate of *in vitro* formed tubers. Ghosh et al. (2007) produced in vitro tubers in *G. superba* without any growth regulators added to the medium. They tried three different basal medium for the production of in vitro tubers from nondormant tubers of *G. superb*a. They observed that each explant resulted in the production of three tubers from every explant after 12 weeks of culturing. Healthy green shoots originated from these tubers were able to produce good roots on half MS basal with one-fifth nitrates. Thus, this method of high rate of in vitro tuber production without any requirement for growth regulator can increase the number of plants and reduce the burden over wild plant material, further leading to a major contribution in conservation of *G. superba*.

22.2.2.1 Callus Induction

Callus culture is a good substitute source for production of in vitro plants. Sivakumar and Krishnamurthy (2000) tried to regenerate in vitro plants in *G. Superba* using six different explants including dormant and nondormant corm buds. Ninety-eight percent callus induction was observed in MS supplemented with different concentration and combinations of auxins and cytokinins. Samarajeewa et al. (1993) reported the callus and multiple shoots on Gamborg B5 medium supplemented with kinetin at a concentration of 0.46 μM along with BAP and IAA 0.04–0.28 μM/L using nondormant corm buds as explants.

Ade and Rai (2012) tried Gamborg B5 along with BAP and IBA. Primarily they could not observe any response at the lower concentration of BAP, but on increasing the concentration of BAP, a better response was observed. Morphology of in vitro grown shoots, roots, buds and leaves was found to be very similar to in vivo grown plants, after histo-morphogenetic studies.

Rishi (2011) succeeded in getting 98% callus from nondormant corm bud explants on 2 ppm NAA and 0.5 ppm kinetin in B5 medium using different explants, while Arumugam and Gopinath (2012) also used corm buds as explants on MS medium supplemented with growth regulators. The callus derived from corms was highly efficient to generate multiple shoots even up to 43 shoots per explant. The observations of Ade and Rai (2012) also supported Rishi (2011) regarding the

efficiency of MS medium and Gamborg B5 medium. Rishi (2011) observed the maximum number of shoots on MS containing 4.52 μM2,4-D + 13.30 μM BAP as compared to IBA and IAA, while Gamborg B5 medium along with BAP, kinetin and IBA also presented good results. MS and Gamborg B5 medium were found to be more supportive for the growth of *G. superba* as compared to Nitsch media, N6 and White's medium. Micropropagation protocol of high efficiency was developed by Ozdemir et al. (2011) using MS and Gamborg B5 medium along with a number of growth hormones. Thus attaining multiple shoots induced from nondormant corm buds, callus induction and root formation confirm the significance of in vitro conservation.

Arumugam and Gopinath (2012) formulated protocol for in vitro propagation using corm buds as explants. Among the different hormones tested, MS + 1.0 mg/l 2,4 D + 0.5 mg/l IAA for 4 weeks generated yellow callus, while the callus transferred to half MS + 1.0 mg/l kinetin +1.5 mg/l BAP along with 20% coconut water generated maximum multiple shoots. Root explants of *G. superba* resulted in 94.40% yellow callus. This callus when transferred to MS + 2.0 mg/L 2 4-D + 1.0 mg/l IAA + 0.75 mg/L NAA resulted in multiple shoots, while MS medium containing 8.0 mg/L GA_3 + 4.0 mg/l IAA + 2.0 mg/l BAP resulted in 93.80% roots (Gopinath et al. 2014).) Regenerated plantlets showed 80% survival rate when transferred to field conditions.

Mahajan et al. (2016) observed creamy white and friable callus from in vitro grown leaves as explants on MS medium supplemented with 3.0 mg/l 2,4-D (Fig. 22.1) and recorded that a high concentration of 2,4-D (5.0 mg/l) turned explants brown, while Nikhila (2014) observed that growth regulators 0.15 mg/l NAA and 0.25 mg/l BAP were best for callus induction. They further observed that a high shoot regeneration (92.60%), with an average number of (4.2 ± 0.22) shoots per explant, was recorded on transferring the callus on to the MS medium containing BAP (3.0 mg/l), 2,4-D (1.0 mg/l) and Kn (0.5 mg/l). Also, 91.80% root regeneration was obtained on MS medium supplemented with 2.5 mg/l IBA after 4 weeks of subculturing with an average of 3.4 ± 0.21 roots per explant. The roots were healthy with root hairs.

22.2.2.2 Somatic Embryogenesis

Development of small embryos from the tissues of different plants called as somatic embryos has been reported by many workers in *G. superba* (Jadhav and Hegde 2001; Madhavan and Joseph 2008; Mahajan et al. 2016). Jadhav and Hegde (2001) reported the formation of somatic embryos in MS medium supplemented with 2,4-D, casein hydrolysate and coconut water which were further confirmed using histological studies. Addition of BAP resulted in shoot formation, while BAP and IBA resulted in root formation. Madhavan and Joseph (2008) observed somatic embryos directly from leaf explants which in addition of 2 mg/l NAA and 0.05–0.15 mg/l TDZ resulted in initiation of microcorms. The microcorms on transfer to MS medium supplemented with 2 mg/l NAA and 1 mg/l BAP resulted in somatic

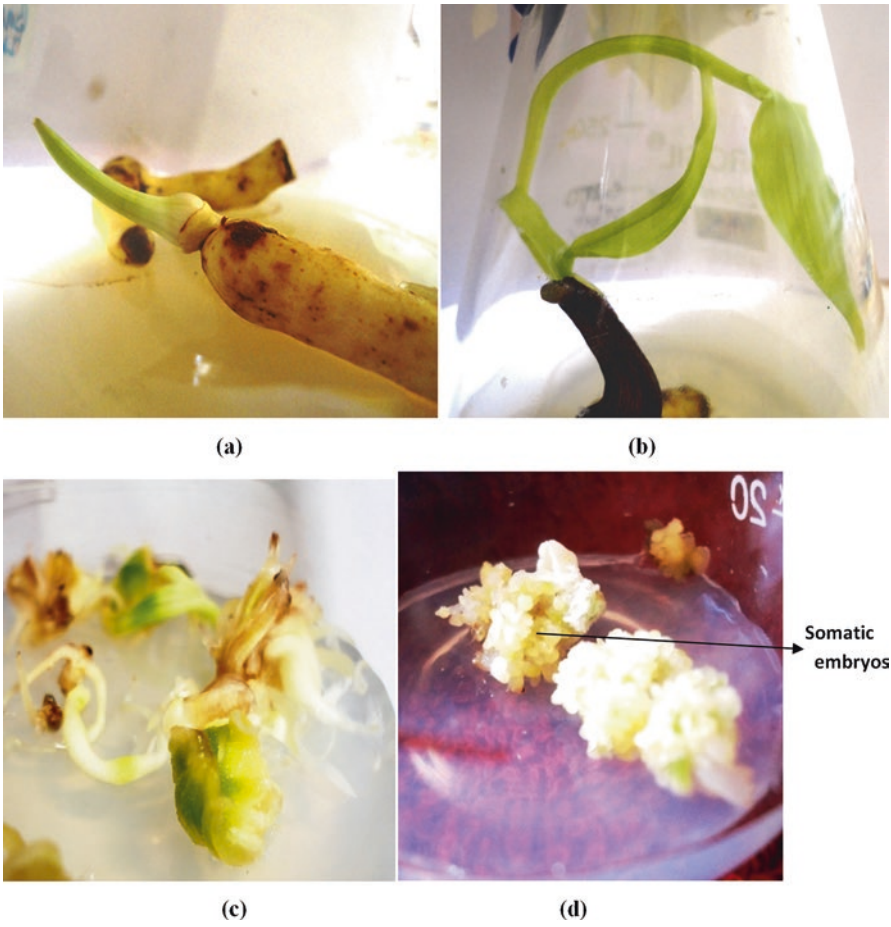

Fig. 22.1 (**a**) Initiation of shoot from the tuber; (**b**) multiplication of shoots in MS medium supplemented with BAP, kinetin and GA_3; (**c**) induction of shoots and roots from the callus; and (**d**) formation of somatic embryos from the callus

embryos. The mature embryos were recovered on their transfer to MS medium containing abscisic acid at the concentration of 0.5–1 mg/1.

Nikhila et al. (2015) found 0.5 mg/l 2, 4-D + 0.25 mg/l kinetin hormone to be best for somatic embryogenesis in *G. superba*, while Mahajan et al. (2016) observed that the subculturing of embryogenic callus on MS medium containing 2,4-D, BAP and kinetin resulted in globular structures, while heart-shaped somatic embryos were formed in addition of growth regulator BAP only to MS medium (Fig. 22.1).

22.2.2.3 Hardening of In Vitro Grown Plants

Hardening is one of the most critical steps of plant tissue culture where in vitro raised plants get acclimatized to the outside environment. Venkatachalam et al. (2012) transferred the in vitro rooted plantlets with expanded leaves into plastic cups containing sand and soil in the ratio of 1:2, while Chatterjee and Ghosh (2015) placed micropropagated plants at room temperature for 7–10 days after washing prior to their transfer to a to earthen pots containing soilrite before covering them with polythene bags. They observed that the regenerated plants grew well and were phenotypically similar to the parental stock once exposed to field conditions. Yadav et al. (2013) used biological hardening by inoculating the in vitro grown *Gloriosa* plantlets with *Acaulospora laevis*, *Glomus mosseae* and a mixed AMF (arbuscular mycorrhizal fungi) strain and observed that the survival of plantlets was significantly improved. Even, the colchicine content was high in the tubers of plants inoculated with *A. laevis* and *G. Mosseae* (Yadav et al. 2013).

22.3 Genetic Transformation Through Hairy Root Induction

Singh et al. (2015b) observed that the climbers, such as *G. superba*, have many problems associated with their propagation and breeding. Thus, strategies using plant transformations including *Agrobacterium*-mediated technologies are routinely being used for the improvement of such plants. This can help in the production of desired transgenics and also in introducing various types of biotic and abiotic resistance in plants. Leela and Agastian (2013) made an attempt to induce hairy roots in *G. superba* callus using *Agrobacterium rhizogenes* (MTCC strain 2364) by cocultivation on medium supplemented with 20 mg/l acetosyringone. The content of colchicine in hairy roots was determined by HPLC technique. They also recorded that the nitrogen, amount of inoculum and ferric ions are the important factors that affect growth of hairy roots and colchicine production.

22.3.1 Production of Colchicine from In Vitro Cultures

Production of colchicine from callus cultures of *G. superba* is effected by number of factors such as growth hormones, nutritional factors and precursors of colchicine. Sucrose and ammonium nitrate in the medium promote biomass formation and colchicine content. Elevation in colchicine concentration was observed by Pandurangan and Philomina (2010) when tyrosine at concentration 40 μM was used in the medium. Maximum biomass (0.31 g) was obtained in 22–27 days of culture, while the colchicine (1.02 mM) production was maximum on the 25th day.

Sivakumar et al. (2004) suggested maximum phenylalanine ammonia-lyase and tyrosine ammonia-lyase activity during the early growth period, while late growth

phase suppresses production of secondary metabolites and results in poor elicitation. High sucrose concentration is inhibitory for growth of cells, while 3% sucrose increased biomass and colchicine content up to 1.51 mM (Hayashi et al. 1988), while colchicine production decreases in addition of 2,4-D. Also, the nitrogen source also effects the production of colchicine. He observed an increase in colchicine production with 20 mM ammonium and 40 mM nitrate, while cations such as Fe^{2+}, Ca^{2+} and PO_4^{3-} inhibit the formation of colchicine.

Certain biotic and abiotic elicitors enhance the secondary metabolite production in in vitro cultures (Naik and Al-Khayri 2016). Ghosh et al. (2002) recorded increased colchicine content up to 1.9 mg/g cell dry wt. in root cultures treated with a combination of 20 mg/l *p*-coumaric acid and tyramine. Even Yoshida et al. (1988) recommended *p*-coumaric acid and tyramine as important precursors that trigger the formation of colchicine. Ghosh et al. (2006) induced root cultures of *G. superba* and treated them with various biotic and abiotic elicitors like methyl jasmonate, silver nitrate, calcium chloride, cadmium chloride, aluminium chloride, yeast extract and casein hydrolysate. They observed that the colchicine content was increased by 50-fold with methyl jasmonate and 63-fold increase with aluminium chloride, while calcium chloride provided a good medium for biomass increase. Colchicine was released maximum into the medium when treated with 10 mM cadmium chloride. However, yeast extract and casein hydrolysate did not improve colchicine accumulation.

22.4 Conclusions and Future Prospects

The plant *G. superba* has high medicinal potential, and at the same time, limited supply has made the situation critical because of the high demands from herbal industries. Due to the lack of awareness and interest of the farmers, the cultivation approach for conservation is not cost-effective. Thus, in vitro biotechnological approaches can lead to mass multiplication of plants and can be supportive in reducing pressure on the natural plant germplasm. Till date, many workers have made progress regarding development of rapid and reliable protocol for in vitro plant propagation systems for *G. superba*. Further, these tissue culture techniques in *G. superba* could be employed as an alternative and continuous method for producing secondary metabolites at a large scale that can play a vital role in various pharmaceutical industries, and the in vitro production of these secondary metabolites can be enhanced by following a systematic approach. The use of gene transfer technology in *G. superba* can be used as a powerful tool for its genetic improvement as it considerably overcomes some of the agronomic and environmental barriers, which otherwise would not be achievable through conventional propagation methods. Further, the use of molecular markers will help in screening the plants for various useful characteristics and selection of elite propagules/clones.

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Chapter 23
Somaclonal Variations and Their Applications in Medicinal Plant Improvement

Frédéric Ngezahayo

Abstract Plant tissue culture is an important tool for various investigations in many plants including medicinal plants. Different techniques are used to in vitro cultivate medicinal plants for mass propagation, conservation, and secondary metabolites production. They include micropropagation, axillary bud, shoot culture, root, and callus culture, organogenesis, somatic embryogenesis, and cell suspension culture. For the production of phytochemicals, cell suspension and callus cultures are most preferred followed by root and shoot cultures and somatic embryogenesis. However, plant tissue culture may generate somaclonal variations as a result of gene mutation and/or changes in epigenetic marks, particularly with highly differentiated explants and callus stage passage. On one hand, the occurrence of somaclonal variation may be an obstacle for both in vitro propagation and germplasm conservation, while it is exploited in many crop plant improvements on the other hand. In the present chapter, possible somaclonal variation following medicinal plant tissue culture and their consequent implication in the regulatory network of secondary metabolites production are presented.

Keywords Medicinal plant · Tissue culture · Somaclonal variation · Secondary metabolites

23.1 Introduction

Plant in vitro culture is the process by which an explant (plant cell, tissue, or organ) is cultivated under aseptic conditions. This implies a prepared medium containing macro- and micronutrients with sources of carbon and addition of plant growth regulators. Exploiting the plant cell totipotency, Haberlandt was the first to predict that one could successfully cultivate artificial embryos from vegetative cells (reviewed by Trevor 2007). From the beginning of plant tissue culture, different media of various compositions have been established, but the one developed by

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Murashige and Skoog (1962) is the most used in plant propagation in combination with auxins and cytokinins as growth regulators. From this period, different in vitro techniques were established with the main goal of plant improvement. These techniques include callus culture, meristem and shoot tip culture, bud culture, organogenesis and embryogenesis, and cell suspension culture (Chawla 2002). In vitro culture has then been enlarged to a wide range of plants such as crop plants, horticultural plants, forest trees, and medicinal plants. These applications can be divided conveniently into many areas such as plant breeding (Poehlman 1987), pathogen-free plants (Taskin et al. 2013), and production of phytochemicals in medicinal plants (Pant 2014). However, plant in vitro culture is often accompanied with genetic and or epigenetic alterations termed as somaclonal variations (reviewed in Kaeppler et al. 2000; Miguel and Marum 2011; Smulders and de Klerk 2011; Us-Camas et al. 2014). It was recently shown that somaclonal variations are important in the improvement of horticultural plants (Krishna et al. 2016). Though medicinal plants have been widely cultivated in vitro, the possible somaclonal variations following their cultivation and their potential implications in their improvement are not well documented. We hope to highlight different techniques used in in vitro culture of medicinal plants, subsequent somaclonal variations, and their possible implication in the production of secondary metabolites through the present chapter.

23.2 *In Vitro* Culture of Medicinal Plants

Several medicinal plant species have been in vitro cultivated for vegetative propagation. They include *Ocimum basilicum* L. (Daniel et al. 2010), *Anoectochilus formosanus* H AYATA (Zhang et al. 2010), *Swertia chirayita* (Roxb. ex Fleming) H. Karst (Joshi and Dhawan 2007), *Curcuma longa* L. (Panda et al. 2007), *Alpinia galanga* L. (Parida et al. 2011), *Rauwolfia serpentina* Benth. (Ilahi et al. 2007), *Talinum portulacifolium* L. (Thangavel et al. 2008), *Aegle marmelos* (L.) corr. (Puhan and Rath 2012), *Cocculus hirsutus* (L.) Diels (Meena et al. 2012), *Baliospermum montanum* (Wild.) Muell. Arg. (Sasikumar et al. 2009), *Pluchea lanceolata* (Oliver and Hiern) (Kher et al. 2014), *Ajuga bracteosa* (Kaul et al. 2013), *Moringa oleifera* Lam. (Saini et al. 2012), *Asclepias curassavica* L. (Reddy et al. 2012), *Hybanthus enneaspermus* (L.) F. Muell (Sivanandhan et al. 2015), *Dendrobium longicornu* (Dohling et al. 2012), *Gentiana kurroo* Royle (Kaushal et al. 2014), *Lychnophora pinaster* Mart. (De Souza et al. 2007), *Aristolochia bracteolata* Lam. (Sebastinraj and Sidique 2011), *Cleome rutidosperma* DC. (Deventhiran et al. 2017), *Lantana camara* L. (Srivasta et al. 2011), *Saintpaulia ionantha* Wendl. (Al-Sane et al. 2005), *Hypericum perforatum* (Wang et al. 2015), *Swertia lawii* Burkill (Kshirsagar et al. 2015), *Taxus baccata* L. (Amini et al. 2014), *Psoralea corylifolia* L. (Ahmed and Baig. 2014), *Spilanthes acmella* Murr. (Abyari et al. 2016 L), *Rauvolfia serpentina* (Singh, et al. 2009), *Curculigo orchioides* Gaertn. (Nagesh et al. 2010), *Centella asiatica* L. (Joshee et al. 2007), *Oplopanax elatus* (Moon et al. 2013), *Wedelia*

calendulacea Less. (Sharmin et al. 2014), *Hypoxis hemerocallidea* Fisch., C.A. Mey. & Avé-Lall. (Kumar et al. 2017), *Plumbago rosea* L. (Borpuzari and Borthakur 2016), *Tylophora indica* (Burm.f.) Merrill (Chandrasekhar et al. 2006), *Hovenia dulcis* Thunb (Yang et al. 2013a), *Euphorbia fusiformis* (Srinivas and Reddy 2017), *Myristica malabarica* Lam. (Iyer et al. 2009), *Tylophora indica* (Burm.f.) Merr. (Sahai et al. 2010), *Boerhaavia diffusa* Linn. (Sudarshana et al. 2008), *Leucojum aestivum* L. (Ptak et al. 2013), *Pimpinella tirupatiensis* Bal. and Subr. (Prakash et al. 2001), *Tetrapleura tetraptera* (Schmm. And Thonn.) Taub. (Opabode et al. 2011), *Eleutherococcus senticosus* Maxim. (Chen-Guang et al. 2011), *Stevia rebaudiana* Bertoni (Lopez-Arellano et al. 2015), *Wattakaka volubilis* (L.f.) Stapf (Chakradhar and Pullaiah 2014), *Ceropegia juncea* Roxb. (Nikam and Savant 2009), and *Hyoscyamus aureus* (Besher et al. 2014). It is supposed that tissue culture is an alternative potential for their conservation (Pant 2013). Indeed, over exploitation of medicinal plants may negatively impact on plant population in its natural habitat. Thus, depending on the technique used, tissue culture can be used as an alternative method for plant regeneration as well as for in vitro secondary metabolite production (Iriawati et al. 2014).

In addition, plant tissue, cell, or organ culture systems represent a potential source of valuable and renewable phytochemicals which are not produced by other means (reviewed by Mulabagal Vanisree and Tsay Hsin-Sheng 2004). Medicinal plant biotechnology is therefore very advantageous in providing continuous and reliable source of plant pharmaceuticals and could be used for the large-scale culture of plant biotechnology from which these metabolites can be isolated (Debnath et al. 2006).There are several methods of plant tissue culture used in medicinal plants such as shoot culture, meristem culture, organogenesis, callus culture, micropropagation, axillary bud culture, cell suspension culture, and somatic embryogenesis. From the above scientific literature, it is documented that highly differentiated tissues including leaves, petiole, and seeds are utilized as well as preexisting meristems, such as axillary buds and shoot tips. Moreover, it is evident that cultures that go through a callus phase (somatic embryogenesis, cell suspension culture, and callus culture) are predominant than the production of plants via axillary branching for example (Table 23.1).

23.3 *In Vitro* Culture and Secondary Metabolites Production in Medicinal Plants

More than 100 medicinal plant species have been in vitro cultivated in order to increase the production of secondary metabolites by means of cell suspension culture (Khanpour-Ardestani et al. 2015; reviewed by Mulabagal Vanisree and Tsay Hsin-Sheng (2004); Srivastava et al. 2011; Al-Sane et al. 2005; Wang et al. 2015; Amini et al. 2014), callus culture (reviewed by Mulabagal Vanisree and Tsay Hsin-Sheng 2004; Nakka and Devendra 2012; Mittal and Sharma 2017;

Table 23.1 List of some medicinal plants in which somaclonal variation has yet analyzed

Plant name	Culture method	Media composition	Molecular marker used	Genetic variation	References
Viola pilosa Blume	Axillary bud culture	MS + 1 mg/l BA+0.25 mg/l Kn (shoot induction)	RAPD, ISSR	No	Soni and Kaur (2014)
		MS + 1 mg/l IBA (root induction)			
Dendrobium nobile Lindl.	Pseudostem segments culture	MS + 1.5 mg/l TDZ (Protocorm like bodies induction)	RAPD, SCoT	No	Bhattacharyya et al. (2014)
		MS + 1.5 mg/l TDZ and 0.25% activated charcoal (root induction)			
Spilanthes acmella (L.) Murr.	Axillary bud culture	MS + 1.0 mg/l BAP (shoot induction)	RAPD, ISSR	No	Yadav et al. (2014)
Zingiber officinale Rosc.	Axillary bud culture	MS+ 1 mg/l BA, 1 mg/l IAA and 100 mg/l adenine sulfate	Cytophotometric estimation of 4C nuclear DNA content, RAPD	No	Mohanty et al. (2008)
Plantago major	Callus culture	MS + different concentrations 2,4-D and KIN (callus induction)	ISSR	Yes	Esmaeili et al. (2014)
Rauvolfia serpentina (L.)	Indirect organogenesis	MS+ BAP + NAA (callus induction)	ISSR	Yes	Saravanan et al. (2011)
		MS+ 22.19 μM BAP+ 86,864 μM ADS (shoot induction)			
		MS+ 17.74 μM BAP+ 32.57 μM ADS (direct regeneration)	ISSR	No	
Guadua angustifolia Kunth	Axillary bud culture	MS+ 2 mg/L BAP+ 10 mg/L adenine sulfate (shoot induction)	RAPD, ISSR	No	Nadha et al. (2011)
Hibiscus sabdariffa L.	Meristem culture	MS+ 0.1–2.0 mg/L BAP and kinetin (soot induction)	RAPD	Yes	Govinden-Soulange et al. (2010)
		MS+ 1.5–2.5 mg/L IBA (root induction)			

Boerhaavia diffusa L.	Shoot culture	MS+ 2 μ M BAP (shoot induction)	RAPD	No	Patil and Bhalsing (2015)
		MS+ 1 μ M-10 μ M IAA and 1 μ M-10 μ M IBA (root induction)			
Celastrus paniculatus Willd.	Shoot culture	MS+ 0.5 mg/L BAP and 0.1 mg/L NAA (shoot induction)	RAPD, ISSR	No	Senapati et al. (2013)
		½ MS+ 0.5 mg/L IAA (root induction)			
Dendrocalamus strictus (Roxb.) nees	Nodal culture	MS+ 4 mg/l BAP (shoot induction)	RAPD, ISSR	No	Goyal et al. (2015)
		MS+ 3 mg/l NAA (root induction)			
Tylophora indica Burm F. Merrill.	Indirect organogenesis	MS+ 2.0 mg/l BAP + 0.5 mg/l IBA (callus induction)	ISSR	No	Sharma et al. (2014)
		MS + 0.1 mg/l TDZ (shoot induction)			
		½ MS + 0.5 mg/l IBA			
Amorphophallus rivieri Durieu	Indirect organogenesis	MS + 1.0 mg/L NAA and 1.0 mg/L BAP (callus induction)	ISSR	Low	Hu et al. (2011)
		MS + 0.5 mg/L NAA + 2.0 mg/L BAP (shoot and root induction)			
Echinacea purpurea (L.) Moench	Organogenesis	½ MS+ 0.1 mg/l NAA + 1 mg/l 6-BA (shoot initiation)	AFLP	Yes	Chuang et al. (2009)
Artemisia absinthium L.	Organogenesis	MS+ 0.5 mg/l 2,4-D+ 0.5 mg/l KIN (callus induction)	ISSR, SSAP	Yes for callus regenerated plants. No for regenerated plants from nodal explant	Kour et al. (2014)
		MS+ 4.5 mg/l 6-BAP+ 0.5 mg/l NAA (shoot induction)			
		MS+ 0.5 mg/l IBA (root induction)			
Amorphophallus albus Liu and Wei	Morphogenesis from callus	MS+ 5.37 μM NAA+ 4.44 μM 6-BA (callus induction)	RAPD, ISSR	Yes	Hu et al. (2008)

(continued)

Table 23.1 (continued)

Plant name	Culture method	Media composition	Molecular marker used	Genetic variation	References
Maesa spp.	Axillary bud culture	MS+ 4.4, 8.8, 13.2 and 22.2 lM BA or MS+ 4.4, 8.8, 13.2 and 22.2 lM BA +5, 10.7 and 13.5 lM NAA	Flow cytometry	No	Faizal et al. (2011)
Aloe barbadensis mill.	Indirect organogenesis	MS+ 0.25–1.0 mg/l NAA + 2% sucrose	RAPD, ISSR	No	Sahoo and Rout (2014)
Aloe vera (L.) Burm.f.	Stem culture	MS+ 2.5 mg/L 6-BA	Mitotic karyotype study, RAPD	No	Haque and Gosh (2013)
Smallanthus sonchifolius (Poepp. and Endl.) H. Robinson	Indirect somatic embryogenesis	MS + 1 mg/L thiamine+100 mg/ Lmyo-inositol+1 mg/L 2,4-D+ 0.01, 0.05, or 0.1 mg/L BA	Fow cytometry, ISSR	Yes	Viehmannova et al. (2014)
Justicia betonica Linn.	Indirect organogenesis	MS+ 1.5 mg/L NAA and 0.5 mg/L BAP	Mitotic index and nuclear size	No	Yaacob et al. (2013)
Ducrosia anethifolia	Organogenesis	MS + 2,4-D + NAA BA+ KIN (various concentrations)	AFLP	Yes	Shooshtari et al. (2013)
Mandevilla velutina	Callus culture	MS + 2 mg/l 2,4-D + 2 mg/l + 6-BA+3 mg/l 6-furfuryl-aminopurine	Morphogenetic and biochemical analysis	Yes	Maraschin et al. (2002)
Curcuma longa L.	Callus culture	LS + 3 mg/L 2,4-D	Biochemical analysis	Yes	Roopadarshini and Gayatri (2012)
Curcuma longa L.	Axillary bud culture	MS + 1–5 mg/L 6-BA+1–2 mg/L IAA, NAA, KIN	Cytophotometric, RAPD	No	Panda et al. (2007)
Anoectochilus formosanus HAYATA	Axillary bud culture	MS + 1 mg/L NAA + 2 mg/L 6-BA	ISSR	No	Zhang et al. (2009)

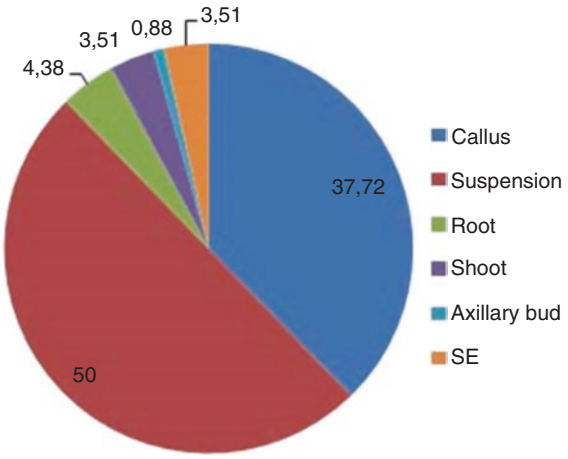

Fig. 23.1 Frequency (%) representation of utilization of plant tissue culture techniques in plant secondary metabolites production

Khanpour-Ardestani et al. 2015; Arya et al. 2007; Besher et al. 2014; Ataei-Azimi et al. 2008; Obae et al. 2011; Nikam and Savant 2009; Roopadarshini and Gayatri 2012), root culture (reviewed by Mulabagal Vanisree and Tsay Hsin-Sheng 2004; Mahdieh et al. 2015), shoot culture (reviewed by Mulabagal Vanisree and Tsay Hsin-Sheng 2004; Nandhini et al. 2015; Chen et al. 2014), somatic embryogenesis (Bhattacharyya et al. 2014; Iriawati et al. 2014; Pathak et al. 2012; Ptak et al. 2013), and axillary bud culture (Faizal et al. 2011). This shows the importance of callus and cell suspension cultures in the production of secondary metabolites (Fig. 23.1). Depending on the medicinal plant species used, compounds such as alkaloids, flavonoids, saponins, and many more others, are frequently produced by tissue culture techniques, principally by cell suspension and callus cultures. For example, the production of flavonoids is enhanced by callus culture, suspension cultures, transformation, and other techniques (reviewed in Bansal and Bharati 2014). Mulabagal Vanisree and Tsay Hsin-Sheng (2004) showed also the importance of callus culture (38.54%) and suspension culture (61.46%) in the production of the plant pharmaceuticals. Investigations have proved that the quantity of secondary metabolites from in vitro propagated plants is higher than that from in vivo grown ones (Ataei-Azimi et al. 2008; Nakka and Devendra 2012; Chen et al. 2014; Sivanandhan et al. 2015) with more compounds in leaves than stem and roots (Sivanandhan et al. 2015). However, secondary metabolites production depends on the medium composition and the phytochemicals category (Karalija and Paric 2011; Besher et al. 2014; Mahdieh et al. 2015). For example, the highest content of hyoscyamine was in a medium containing varied categories of macronutrients, three carbon sources (thiamine-HCL, pyridoxine, and nicotinic acid), kinetin and naphthalene acetic acid as growth regulators, and sucrose (50 g) than in a medium with less ingredients, different growth regulators, and sucrose (30 g) in which the lowest content of tropane alkaloids was in callus, while the highest content of scopolamine was in the wild plants (Besher et al. 2014). In addition, phytochemical compounds respond differently to the same medium (Karalija and Paric 2011). The in vitro culture

method also influences the quality and quantity of produced secondary metabolites. In *Echinacea angustifolia* DC., caffeic acid derivatives are significantly produced in shoots from axillary bud culture, while alkamides are mainly accumulated in callus- and leaf-regenerated shoots (Lucchesini et al. 2009). The production of phytochemical compounds depends also on preexisting secondary metabolites which influence the tissue culture technique, and it is genotype-dependent (Obae et al. 2011). In *Maesa* species, for example, there is no difference in secondary metabolites between regenerated plants and their controls (Faizal et al. 2011). In the same culture method, phytochemical compounds quantities are differentially synthesized, non-embryogenic callus producing more secondary metabolites, such as alkaloids, terpenoids, and phenolics than embryogenic callus (Iriawati et al. 2014).

23.4 Somaclonal Variation in Medicinal Plants

The major consequence of plant in vitro culture is the occurrence of somaclonal variation as a result of gene mutation or epigenetic alterations (Larkin and Scrowcoft 1981; Gould 1986; reviewed by Kaeppler et al. 2000; reviewed by Krishna et al. 2016). Explant type, explant source, mode of regeneration, culture length period, and the number of subculture cycles, culture environment, genotype, and ploidy are some of the sources of variations detected in plant tissue culture (reviewed in Krishna et al. 2016). Though somaclonal variations can also be generated by preexisting somatic mutations present in the mother plant (Karp 1994), highly differentiated tissues (roots, leaves, and stems) generally produce more somaclonal variations than axillary buds and shoot tips (Duncan 1997). Data from scientific literature showed that leaves, petiole, and seeds are more frequently used as explants from medicinal plants. Moreover, it is generally believed that somaclonal variations predominantly occur at the callus stage, which represents a highly stressful conditions to the plant genome (Larkin and Scowcroft 1981), whereas micropropagation achieved by enhanced branching from preexisting primordial organs such as shoots or axillary buds should largely preserve fidelity of the donor plants (Hao and Deng 2003; reviewed by Ngezahayo and Liu 2014). In medicinal plants, instead of analyzing somaclonal variations, many efforts are concentrated on large-scale multiplication and phytopharmaceutical compounds for commercial and medical uses. On a few plants studied, there is no genetic somaclonal variation from in vitro culture without passage to callus stage. Despite this, it is possible to speculate that the main in vitro techniques used in the production of secondary metabolites, i.e., cell suspension and callus cultures and to a lesser degree somatic embryogenesis, are possibly accompanied by somaclonal variation. In addition, it was observed that cultures that go through a callus phase (cell suspension and callus cultures, and somatic embryogenesis) are predominant which are also supposed to generate somaclonal variation. In crop plants (*Arabidopsis thaliana, Fragaria×ananassa*, *Triticum aestivum* L., *Oryza sativa* L., *Gossypium hirsutum* YZ1, *Zea mays* L., *Citrus sinensis* L. Osb.), it has been observed that callus, shoot tip cultures, and

somatic embryogenesis are accompanied by microRNA gene expression in which microRNAs accomplish different roles such as regulation of target genes, response to stress, rejuvenation in micropropagated plants, embryogenic callus formation and somatic embryogenesis, embryogenesis and postembryonic development, down-regulation of target genes, response to hormone depletion, and light photoperiod (Luo et al. 2006; Chu et al. 2016; Qiao and Xiang 2013; Yang et al. 2013b; Chavez-Hernández et al. 2015; Szyrajew et al. 2017). MicroRNA expression and histone modifications have also been observed in cell suspension cultures of *Arabidopsis thaliana* (Tanurdzic et al. 2008; erdasco et al. 2008), *Solanum tuberosum* (Law and Suttle 2005), and in callus culture of *Zea mays* (Alatzas and Foundouli 2006). Thus, although the similar results are not yet clarified in medicinal plant tissue culture with a passage to callus stage, microRNAs expression was observed in callus culture of *Taxus* trees (Zhang et al. 2015). Other epigenetic variations (cytosine DNA methylation alterations and histone modifications) are also supposed to occur.

23.5 Roles of Somaclonal Variation in Regulating Secondary Metabolites Biosynthesis

Secondary metabolites production is often observed in medicinal plant tissue culture process, especially in cell suspension and callus cultures compared to untreated plants. Callus stage is accompanied by somaclonal variations which are probably among the potential triggers of the above phytochemicals (Fig. 23.2). Somaclonal variation is the result of genomic mutations or epigenetic alterations from plant tissue culture process (Larkin and Scrowcoft 1981; Gould 1986; reviewed by Kaeppler et al. 2000; reviewed by Krishna et al. 2016). Though somaclonal variation in in vitro medicinal plant cultured is not well documented, one shall speculate that it is presumably involved in the regulating network of secondary metabolites production. Indeed, in *Curcuma longa* L., significantly high curcumin, oleoresin, and volatile oil contents (%) were observed in somaclonal variants when compared to the normal regenerants and also control plant (Roopadarshini and Gayatri 2012). This may be particularly by epigenetic alterations, i.e., heritable covalent modifications of the chromatin which are not due to DNA sequence change (reviewed by Us-Camas et al. 2014). For example, heterochromatin (tightly packed) due to DNA methylation and/or histone modifications silences genes that are in the heterochromatin zone because of non-availability for the transcription machinery (reviewed by Us-Camas et al. 2014). In turn, DNA methylation is thought to be mediated by a microRNA pathway (Wu et al. 2010). Three main epigenetic mechanisms are widely studied in plant tissue culture, i.e., DNA methylation, histone modifications, and microRNA (Smulders and de Klerk 2011; reviewed by Us-Camas et al. 2014). Among these, DNA methylation has been shown to involved *Medicago truncatula* somatic embryogenesis by regulating gene expression of somatic embryos (Kurdyukov et al. 2014). Secondary metabolites whose biosynthesis pathways have been frequently studied are flavonoids and alkaloids. For example, flavonoid

Fig. 23.2 Probable mechanism of secondary metabolites production in medicinal plant in vitro culture as a result of somaclonal variations

biosynthesis is a multistage process involving a range of enzymes catalyzing each stage (Ferreyra et al. 2012). In stressful conditions such as salt stress exposure, it has been observed that there is an epigenetic regulation of flavonoid biosynthetic and antioxidant pathways (Barthi et al. 2015). In this case, one should speculate that epigenetic regulation is related to the genes implicated in the biosynthetic pathway of those compounds in tissue culture. By analyzing alkaloid content in in vitro callus and field grown plants, it was concluded that there exists a regulation at transcriptional level of alkaloid biosynthesis pathway (Pathak et al. 2012). Moreover, the development of medicinal plant cell culture techniques has led to the identification of more than 80 enzymes of alkaloid biosynthesis (reviewed in Kutchin 1998), showing also the possible involvement of epigenetic marks in this process. In *Taxus* species, the overexpression of the miRNAs increased the genes of secondary metabolites such as taxol, phenylpropanoid, and flavonoid biosynthesis, thereby suggesting their function as crucial factors that regulate the entire metabolic network during tissue culture process (Zhang et al. 2015). However, only a few genes are related to secondary metabolites, indicating that other factors than miRNAs are also present in

the regulatory process (Zhang et al. 2015). It may be probable that DNA alterations and histone modifications are implicated, particularly when they occur at gene promoters.

23.6 Conclusions

Several strategies have been followed to in vitro cultivate cells, organs, and tissues from medicinal plants for two main benefits, mass propagation and secondary metabolites production. Plant biotechnology has used a wide range of those techniques, i.e., shoot culture, meristem culture, organogenesis, callus culture, micropropagation, axillary bud culture, cell suspension culture, and somatic embryogenesis. Micropropagation without passage to callus phase (axillary bud culture for example) is a promising method for mass propagation and conservation concerns due to its ability to produce true-to-types regenerated plantlets. Tissue culture methods such as cell suspension and callus cultures are devoted to produce secondary metabolites. Nonetheless, they present a potential risk of generating somaclonal variations which are not well documented in the case of medicinal plants, particularly in the epigenetic aspects as potential factors in gene regulatory network. Thus, their possible implication in the triggering of secondary metabolites production needs to be elucidated.

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Part III
Conventional and Molecular Approach

Chapter 24
Genetic Improvement of Medicinal and Aromatic Plants Through Haploid and Double Haploid Development

Sweta Sharma, Kshitij Vasant Satardekar, and Siddhivinayak S. Barve

Abstract Medicinal and aromatic plants (MAPs) produce secondary metabolites that are pharmacologically and economically important. These compounds are distributed/limited in a particular species, genus, or family and are reported to play an important ecological role like pollinator attractants, adaptations to environmental and biological stresses (chemical defenses). The concentration of these secondary metabolites is very low and highly variable, thus making them high-value low-volume products. Advances in biotechnology, particularly haploid and double haploid (DH) production, have opened new avenues for breeding, genetics, transformation, and mapping studies in these MAPs. This will allow means for the commercial exploitation of such rare plants and the chemicals they produce in medicines, aromatic industries, and plant growth and for insect and weed control. This chapter details the different methods of producing haploids and DHs, factors influencing their generation, and their use in genetic improvement of these MAPs. Few MAP species in which haploid and DHs have been studied are also briefly discussed.

Keywords Medicinal and aromatic plants · Haploids · Double haploids · Androgenesis

24.1 Introduction

Medicinal and aromatic plants (MAPs) have a long history of being used by humans for therapeutic and culinary purposes. Medicinal plants have helped generations as traditional therapeutic aid for cure or prevention of any ailments and boost the human health and well-being, whereas the aromatic plants, as the name suggests, produce essential oils that are taken in use for their aroma and flavor. These medicinal and aromatic properties are due to secondary metabolites found in a plant species or related group of species. These secondary metabolites show wide chemical diversity

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which in turn gives them diverse activity usually helping plants for protection against varied microbial pathogen and pests.

Due to high costs of development, time consuming developmental process and side effects of synthetic drugs, there has been shift in interest to develop alternative plant derived drugs from plants showing antimicrobial activities. These are relatively safer and dependable. Thus MAP species are of considerable importance in the pharmaceutical industry. They play a significant role for the discovery of new drugs. The initial experiments on antimicrobial properties in plants date back to the nineteenth century. Since then many species have been identified and brought into use in medicine, e.g., *Papaver somniferum*, *Rauvolfia serpentina*, and *Digitalis* sp. Other well-known drugs of plant origin are antibiotics (e.g., penicillin, tetracycline, erythromycin), antiparasitics (e.g., avermectin), antimalarials (e.g., quinine, artemisinin), lipid control agents (e.g., lovastatin and analogs), immunosuppressants for organ transplants (e.g., cyclosporine, rapamycins), and anticancer drugs (e.g., paclitaxel, irinotecan) (Harvey 2008).

The potential MAPs can be selected based on several criteria like:

1. Randomly screening the local flora for selected bioassays and for the potential drug molecules
2. Screening of the plants having traditional and ethno-medicinal usage history
3. Grazing habits of the animals

As the MAP species are not cultivated on large scale in farms and their collection is restricted in the wild, these species present the agriculturist a bright potential. However, there is high species diversity prevalent among these MAPs in the wild. Thus their economic value and sustainable use become a function to the genotype of the planting material, making it necessary that the available genotypes are screened and identified from the natural population for breeding. Breeding plays a pivotal role in the production of improved superior agricultural variety. Despite its huge potential, MAP breeding has not been fully explored. Conventional breeding methods are highly time consuming and make use of conservative tools for manipulating the plant genotype. To shorten this time and avoid this "*n*" number of generation in getting the desired genetic improvement, various biotechnological techniques were worked upon. With the discovery of natural sporophytic haploid in *Datura* (Blakeslee et al. 1922), and later production of haploids from anther culture in *Datura* (Guha and Maheshwari 1964, 1966), haploids in higher plants led to the use of double haploid (DH) technology in plant breeding. Soon Kasha and Kao (1970) achieved another milestone by making an important discovery of induction of haploids through interspecific crosses of haploids in barley (*Hordeum vulgare* L.). This discovery revolutionized and accelerated the plant breeding process worldwide using DH technology allowing breeder to achieve homozygosity at all loci in a single generation after genome duplication.

Haploids are plants (sporophytes) that contain a gametic chromosome number (*n*) and are smaller, sterile, and with reduced vigor due to the nonavailability of one set of chromosome during meiotic pairing. On inducing chromosome doubling, the

fertility can be restored in the obtained DHs which are completely homozygous. These haploids serve as a new variety in self-pollinated crops and as homozygous line for cross-pollinated species. In addition to breeding, haploids and double haploids are also of great use in genetic studies, such as gene mapping, marker/trait association studies, location of QTLs, and genomics and as targets for transformations. The haploid technique in combination with other molecular biological technique opens the path for improved novel breeding techniques like mutation breeding, backcrossing, hybrid breeding, and genetic transformation.

24.2 Methods of Development of Haploids

Haploids generated from a heterozygous individual and converted to diploid create instant homozygous lines, bypassing generations of inbreeding. Never ending efforts of biotechnologist and plant breeders in search of new techniques for producing haploids in plant species have led to the development of various methods. Different species respond differentially to these methods. Generally two methods are used to produce haploids. First, haploids can be induced from rare interspecific or intergeneric crosses, in which one parental genome is eliminated after fertilization. Second, cultured gametophyte cells may be regenerated into haploid plants, but many species and genotypes are recalcitrant to this process.

24.2.1 In Vitro Induction of Maternal Haploids (Gynogenesis)

In vitro induction of maternal haploids is called gynogenesis. In gynogenesis, haploid cells of the female gametophyte, i.e., unfertilized egg cell, are stimulated to develop into an embryo. Gynogenic haploids are known to be more genetically stable than androgenic haploids and have been reported in many several species, such as onion, sugar beet, cucumber, squash, gerbera, sunflower, wheat, barley, carrot, etc. (Bohanec 2009; Chen et al. 2011; Kiełkowska et al. 2014). The immature female gametophyte when cultured in vitro on the medium leads to the formation of mature embryo sac (Musial et al. 2005). Under optimal conditions the egg cells undergo sporophytic development (haploid parthenogenesis) (Bohanec 2009) and can develop into haploid plant directly or take intermittent callus route and subsequently form haploid embryos or plants. Several biotic and abiotic factors significantly influence/determine the success and efficiency of the technique in the given species. Factors like the genotype of donor plants, growth conditions, media components, type and concentration of carbohydrates, and plant growth regulators are crucial factor. In onion efficiency of up to 51.7% was achieved depending on the donor genotype which was stable for two consecutive years (Bohanec and Jakše 1999). Induction rates were even higher in identified onion genotypes, achieving

frequencies of 196.5% embryos from a double haploid line (Javornik et al. 1998) or 82.2% for an inbred line (Bohanec 2003). Developmental stage of gametes, the pretreatment of flower buds prior to inoculation, in vitro culture media, and culture conditions are other factors affecting the embryogenic response of female gametes in culture.

Gynogenesis is commercially used in onion (*Allium cepa*) and sugar beet (*Beta vulgaris*) and some trees. In onion (*A. cepa*), growth temperature of the donor plant before flowering plays a vital role in gynogenesis. The use of DHs in onion breeding produces recombinant inbreds allowing the breeder to save cost and time. In sugar beet (*B. vulgaris*), cold treatment of inflorescences (8.8 °C for 1 week) combined with high temperatures (30.8 °C) during the induction phase improves the response (Weich and Levall 2003).

Gynogenesis can also be induced by using irradiated pollen (Sauton 1989; Kurtar et al. 2002). This method is highly laborious and is also limited by irradiation type and dose (Dal et al. 2016). Suboptimal dose leaves the generative nucleus with partial damage which can still fertilize the egg cell. At increased dose the haploid regeneration frequency is reduced. This technique, despite its low efficiency, has been used in species that do not respond to other proven methods.

24.2.2 Wide Hybridization

Wide hybridization is another method of developing haploids by crossing of distantly related species. It can be a cross of two individuals belonging to different species of same genus (also called interspecific hybridization) or different genera (intergeneric hybridization). This technique is of great importance as it allows plant breeder to transfer desirable traits between species. The process has been mostly reported in cereals (Chen et al. 2013; Zhang et al. 2008). Cell division after normal double fertilization results in the formation of hybrid zygote and endosperm. Subsequently the zygote loses a set of paternal chromosomes, forming haploid embryo. Even the endosperm development is aborted due to this chromosome elimination. All the methods used in this technique are fully known to breeders like emasculation, pollination, and embryo culture, circumventing the need of any specialization. For survival of haploid embryo, they need to be rescued and cultured in vitro. Barley presents the best example of this technique called "bulbosum method" (Kasha and Kao 1970; Sanei et al. 2011). However while choosing species for the wide hybridization, one has to be cautious about the flowering time of both the species. Wide hybridization is also effective in double haploid production in wheat. Piosik et al. (2016) proved the applicability of this technique in *L. sativa*. He crossed the lettuce with *H. annuus* or *H. tuberosus*. The resulting embryo was rescued and cultured in vitro. Haploids obtained were however infertile. The application of wide hybridization for haploid generation in MAPs is still not realized commercially.

24.2.3 Centromere-Mediated Genome Elimination

Development of plants having chromosome only from one parent can also be achieved by the help of molecular biological techniques. While analyzing molecular basis for the genome elimination in interspecific crosses, a theory of unequal behavior of the centromeres from the two parent species at mitotic spindle was proposed which can cause genome elimination of one of the parent (Henikoff and Dalal 2005; Forster and Thomas 2005; Jin et al. 2004). Ravi and Chan (2010) developed haploid *Arabidopsis thaliana* plants by changing centromere protein CENH3, the centromere-specific histone, in one of the parent's genome. Chromosomal position of CENH3 provides for docking of the kinetochore complex. Thus if any changes are induced in this protein, they are unable to assemble at centromere correctly (Allshire and Karpen 2008). When a parent with altered CENH3 is crossed to a parent with wild-type genome, chromosomes from the mutant parents are lost generating the haploid plant with wild-type half genome. Spontaneous generation of DHs is achieved from haploid during meiotic nonreduction. Maternal and paternal haploids can be generated through reciprocal crosses. This technique has been successfully used in breeding programs of *Arabidopsis* by converting tetraploid *Arabidopsis* into diploid. Screening of such haploid can be eased by adding seed-specific fluorescent reporter by Ravi et al. (2014). CENH3 being present in all eukaryote allows this method to be applicable to haploids in any plant species to produce. Successfully demonstrated in *Arabidopsis*, applicability of this type of genome elimination in economic crops is yet to be explored. This approach has been successfully employed in maize (Kelliher et al. 2016). However, its use in MAPs has not yet been reported.

24.2.4 Induction of Paternal Haploids (Androgenesis)

Among all the available methods to obtain haploids and DHs, in vitro anther or isolated microspore culture is the most widely used method. Gametic embryogenesis results in haploid plants and is a perfect example of cellular totipotency (Reynolds 1997). After the discovery of *Datura innoxia* by Guha and Maheshwari in 1964, several reviews on androgenesis have been published giving chronological advancement of this technique by Magoon and Khanna (1963), Kasha (1974), Zhang et al. (1990), Jain et al. (1996–1997), Smykal (2000), Maluszynski et al. (2003a, b), Andersen (2005), Palmer et al. (2005), Xu et al. (2007), Germanà (1997, 2006, 2007, 2009), Seguí-Simarro and Nuez (2008), Seguí-Simarro (2010), Touraev et al. (2009), and Dunwell (2010). This method has been successful in many economical crop especially Solanaceae, Cruciferae, and Gramineae families (Dunwell 1986; Hu and Yang 1986). Woody trees are characterized by high heterozygosity, and conventional breeding is restricted in them due to long-generation cycle with a long juvenile

period. Gamete embryogenesis is the only way for breeding and obtaining homozygosity in woody plants (Germanà 2006, 2009). Almost all the MAPs in which haploid generation has been successful have made use of this method.

Embryogenesis in pollen can be induced by anther culture or by microspore culture. Anther culture comes handy as it is simple and does not require skill. It is also a good system to study cellular, physiological, biochemical, and molecular processes involved in pollen embryogenesis, whereas, microspore culture requires modern equipment and skilled researcher as microspore isolation needs removal of all the somatic tissue. Haploid generation via anther culture may produce diploid, somatic callus, and subsequently embryos. The pathway selected is influenced by the number of factors like physiological state of the donor plant pretreatment and stress applied. Isolated microspores, when given the optimal combination of culture conditions and stresses, are diverted from the normal gametophytic developmental pathway to a sporophytic pathway. It produces embryos and haploid plants which subsequently on treating with colchicine lead to the development of DHs. Though haploid and DHs have proved to be a potent breeding tool, breeders often face problem like low frequencies of embryo induction, albinism, plant regeneration, plant survival, and the genotype- and season-dependent response genotypes (Maluszynski et al. 2003a). There is no universal protocol that will result in microspore embryogenesis in all species.

Upon stress induction the microspore undergoes several morphological changes like cellular enlargement, vacuole regression, and nuclear migration (Touraev et al. 2001 and Maraschin et al. 2005). However, not much literature is available on molecular basis of microspore embryogenesis. Many researchers have focused on studying transcriptional changes during the microspore embryogenesis process (Kyo et al. 2003; Maraschin et al. 2006; Amatriaín et al. 2006; Hosp et al. 2007; Joosen et al. 2007; Malik et al. 2007; Tsuwamoto et al. 2007). In barley, transcriptome analysis was done in different lines after stress treatment, and based on their expression, three clusters of genes were identified by (Amatriaín et al. 2009). Genes related to structural and functional changes in membrane were found to be affecting the ability of the microspore to divide and form embryos. Genes related to stress response, transcription and translation regulation, and degradation of pollen-specific proteins were associated with green plant production, while expression of genes related to plastid development was associated with albino plant regeneration.

Generation of albino plants is one of the major problems during microspore embryogenesis. Stress-treated microspore has showed differentiation of plastids to amyloplast accumulating starch. This occurrence has been associated with the expression of albino phenotype (Caredda et al. 2000). In wheat studies revealed that microspore-derived albino plants were lacking plastid ribosomes (Hofinger et al. 2000; Zubko and Day 2002). The differential expression of plastid-related genes after stress treatment suggested that although albinism is manifested at the time of plant regeneration, it is determined earlier in microspore embryogenesis or even at the time of sampling (Caredda et al. 2000, 2004). It was also hypothesized that there are different mechanisms governing plastid disappearance during pollen maturation in albino genotypes and during microspore dedifferentiation (Amatriaín et al. 2009).

24.3 Factors Affecting Haploid Generation

Switching of developmental process from normal gametophytic to sporophytic forms is the basis of the microspore embryogenesis. This divergence from regular gametogenesis to sporogenesis usually requires a trigger like cold shock, heat shock, and carbohydrate and nitrogen starvation. Stress treatment allows primary ontogenetic route of microspore to be compromised, rerouting it to androgenesis for the induction of microspore embryogenesis.

24.3.1 Genotypes for Double Haploid Production

The donor genotype is known to be one of the most important factors in various tissue culture systems. Different cultivars of the same species often exhibit diverse responses. Thus in haploid breeding programs, it becomes necessary to identify and select responsive genotypes. Genotypic dependency of haploid production from unfertilized ovule culture has also been reported in several species (Bohanec 2009; Chen et al. 2011). Doi et al. studied 43 genotypes of *G. triflora*, *G. scabra*, *G. triflora* var. *japonica* f. *montana*, and their interspecific hybrids. Their research revealed that despite the genotypic variations in the frequency of embryo-like structure (ELS), gynogenesis can be utilized for production of haploids and DHs among a wide range of gentian genotypes. In one of their comparative experiments, they also reported that genotypic dependency in unfertilized ovule culture was less as compared with that of anther culture (Doi et al. 2011). Genotype-independent gynogenesis protocol is also reported in plants such as onion, sugar beet, and carrot (Bohanec and Jakše 1999; Geoffriaeu et al. 1997; Gurel et al. 2000, Kiełkowska and Adamus 2010). The effects of season, genotype, and their interaction on haploid production in gerbera were evaluated on the four genotypes responsive to gynogenesis (Alberto Tosca et al. 1999). Naked mature unfertilized ovules were collected from the four genotypes and analyzed. Of the four genotypes tested, two gave more calli in the spring and one in the autumn, and the fourth was hardly affected by seasonal variations, whereas shoot recovery depended on both the season of ovule collection and the genotype. Studies carried out on *Solanum tuberosum* showed that the ability to undergo microspore embryogenesis is a heritable recessive polygenic trait (Chupeau et al. 1998; Rudolf et al. 1999; Smykal 2000).

The developmental stage of the microspores used for haploid culture is crucial for success and varies depending on the species. Buds or tillers are typically harvested when the microspores are at the uninucleate to early binucleate stage. Acetocarmine and DAPI (4,6-diamidino-2-phenylindole) are the stains most commonly used for determining the developmental stage of the microspore (Fan et al. 1998). For many species the plant material is collected and used immediately, while for most cereals tillers are selected; placed in nutrient solution, media, water, or inducer chemicals; and kept for up to several weeks prior to microspore isolation. Most temperature

pretreatment is at 4–10 °C, but short heat shock condition of 33 °C for 48–72 h can also be used (Liu et al. 2002). The tillers and buds are surface sterilized prior to microspore isolation to eliminate bacterial or fungal contaminants. For surface sterilization usually almost the same protocols are used involving brief (1–2 min) immersion of the plant material in ethanol (70%), followed by immersion in sodium hypochlorite (6% or less) with a drop of Tween for several minutes (up to 15 min), followed by several washes with sterile distilled water. Mercuric chloride has also been used but should be avoided because of its toxic effects. In order to avoid deleterious effects from surface sterilization, treatments should be kept to the minimum which will provide contaminant-free plant material.

24.3.2 Climatic Condition of Donor Plants

A prerequisite for successful and consistent microspore culture response is healthy, pest-free donor plants. Seeds are planted with adequate spacing to allow for vigorous growth; plants are fertilized and watered regularly, screened, and treated as required to minimize disease and insect infestations. Donor plants can be grown in the field, in the greenhouse, or in environmentally controlled growth chambers. Growth chambers allow for the control of temperature, humidity, photoperiod, and light intensity and provide an enclosed space where the incidence of disease or insect infestation can be minimized and effectively treated when necessary. In field-grown donor material, contamination rate can be an issue, and embryogenic potential can be adversely affected. A combined approach is also useful, e.g., in asparagus seeds are planted in the greenhouse, and the seedlings are then transplanted to the field. At the end of the growing season, the crowns are taken, vernalized at 4 °C for up to 6 months, and then planted in a growth chamber (Wolyn and Nichols 2003). Donor plant conditions not only play a role in microspore culture response, i.e., the production of embryos, but also in regeneration of these embryos to plants. It has been reported that donor plants of barley grown under growth chamber conditions produced more DH green plants than donor plants grown under greenhouse conditions (Dahleen 1999). This is particularly important in cereal crops where albinism is a major problem. The temperature at which the donor plants are grown also plays a critical role in microspore culture response. For *Brassica* species, the donor plants are initially grown at 20/15 °C, and just prior to bolting, the temperatures are reduced to 10/5 °C (Ferrie and Keller 1995). This slows the growth of the plant and allows for a longer time period during which buds at the appropriate developmental stage can be selected. The cold temperature stress of the donor plants results in a higher frequency of microspore embryogenesis, and while embryos can still be obtained from greenhouse-grown plants, the response is decreased. Cooler than normal temperatures are also beneficial for barley, with winter barley (15/12 °C) requiring lower temperatures than spring barley (18/15 °C) (Kasha et al. 2003). However a cold temperature stress is not a requirement for other species such as asparagus (Wolyn and Nichols, 2003), pepper (Lantos et al. 2009), or *Saponaria vaccaria* L. (Kernan and Ferrie 2006).

24.3.3 Cold Treatment

A cold pretreatment (0–10 °C, 0.5–7 days) has been shown to be beneficial for many medicinal plant species (Ferrie 2013). Cold pretreatments applied to the microspores are supposed to induce cytoskeletal and nuclear rearrangements, to increase intracellular ABA levels, to slow down degradation processes in the anther tissues, and to assure survival of a greater proportion of microspores (Maraschin et al. 2005; Shariatpanahi et al. 2006). In borage, the application of a cold pretreatment at 4 °C for 4 days to the excised anthers significantly increased the frequency of embryogenic calli and embryos produced. It is important that this pretreatment must be applied to anthers and not to flower buds. Cold pretreatments applied to the flower bud are ineffective (Vagera and Havranek 1985; Tipirdamaz and Ellialtioğlu 1998; Ozkum and Tipirdamaz 2002; Irikova et al. 2011).

Cold pretreatment was found to be beneficial for production of ELSs from unfertilized ovule culture of gentians, *Beta vulgaris* (Gurel et al. 2000; Lux et al. 1990) and *Triticum durum* (Sibi et al. 2001). Cold pretreatment for 1–2 weeks is beneficial for effective induction of ELSs in gentians. On the contrary no positive influences of cold pretreatment on gynogenic response were reported in *Cucurbita pepo* (Metwally et al. 1998) and *Guizotia abyssinica* (Bhat and Murthy 2007). In *G. triflora*, Pathirana et al. (2011) also recommended cold pretreatment (4 °C for 48 h) on anther and ovary culture.

24.3.4 Heat Shock

Stress induction in plant has become an integral component for microspore embryogenesis. The application of a mild heat shock to cultured anthers is one of the most used stresses to induce microspore embryogenesis (Shariatpanahi et al. 2006). Such treatments have been found to augment the embryo formation. Heat shock treatment alone is sufficient to deviate the microspore toward embryogenesis in a number of species such as rapeseed and pepper (Custers et al. 1994; Abdollahi et al. 2004; Parra-Vega et al. 2013), among others. Heat shock influences microtubule distribution and blocks further gametophytic development, during which acentric nucleus migrates to more central position and mitosis ultimately results in a symmetrical division with two daughter cells, similar in size and organelle distribution (Shariatpanahi et al. 2006; Liu et al. 1995; Fan et al. 1998). Exposure of isolated microspores to high temperature is considered to be a key factor for embryogenesis induction. Ahmadi et al. (2011) reported that in *B. napus* microspore culture, elevated temperature (30 °C) not only efficiently induced microspore embryogenesis but also accelerated the process of embryogenesis. It was found in triticale that a cold pretreatment could be useful to induce embryogenesis with efficiency lower than with heat shock, but without compromising cell viability, which eventually prevailed in the final embryo yield (Zur et al. 2009). Detailed studies were performed on

Brassica oleracea, and results showed that heat shock proteins accumulated in anthers as in leaves of plant. However a temperature as high as 40 °C was inhibitory to embryo formation (Fabijanski et al. 1991). Contrary to the many studies conducted, Binarova et al. (1997) showed late-bicellular stage pollen can be induced to undergo embryogenesis on the application of severe heat shock of 41 °C. Embryogenic induction was linked to the synthesis and nuclear localization of HSP70. Detailed cellular changes during heat shock induction and embryo development of cultured microspores of *Brassica napus* were done by Telmer et al. (1995). Heat shock has been reported to increase the embryogenic route in several plants like wheat (Touraev et al. 1996c), tobacco (Touraev et al. 1996a, b), and *Brassica* (Telmer et al. 1995; Binarova et al. 1997; Custers et al. 1994), while the cold shock and starvation induced microspore embryogenesis in maize, wheat, barley, and rice (Indrianto et al. 1999). The developmental stage of microspore at the time of culture initiation is of crucial importance, with late-vacuolated microspore to early-bicellular pollen grain being the responsive stages to heat stress (Pechan and Keller 1988).

24.3.5 Type of Sugar

Sugars are the source of carbon and energy and also act as an osmotic regulator in the induction medium (Ferrie et al. 2005). The type and concentration of sugars in the induction medium have been found to influence androgenesis (Ferrie et al. 2005). Sucrose, maltose, glucose, and fructose are the main carbohydrates used in culture media for androgenesis, with sucrose predominating (Ferrie et al. 2005). Androgenesis in *Cumis sativas* is influenced by the type and concentration of sugars used (Ashok Kumar and Murthy 2004). For *Triticum aestivum* (Indrianto et al. 1999) and *Secale cereale* (Immonen and Anttila 1998), maltose was used as carbon source, while for strawberry (Owen and Miller 1996) and *T. aestivum* (Chu et al. 1990), glucose and fructose, respectively, are proved to be the best carbon sources. Further, sugar concentration has also been shown to influence embryogenesis in many species (Ferrie et al. 2005): 0.09 M sucrose for *Quercus suber* (Bueno et al. 1997) and 0.18 M sucrose for *Oryza sativa*.

24.3.6 Amino Acid

The importance of amino acids for the haploid production was realized long back in 1973 by Nitsch and Norreel. They obtained pollen plants on medium containing extract from embryogenic *Datura* anther. When analyzed, this extract was found to be rich in amino acid serine (Nitsch 1974). However experiments for haploid response in *Nicotiana tabacum* using serine were not successful (Horner and Street 1978). Further studies revealed that in *Nicotiana tabacum* it was glutamine which has a significantly higher amount in androgenic anther (Horner and Pratt 1979). Ashok Kumar and Murthy (2004) reported that in *Cucumis sativus* embryos developing on B5 medium

fortified with combination of glutamine, glycine, arginine, asparagine, and cysteine amino acids resulted in maximum plantlet regeneration.

24.4 Use of Haploids and Double Haploids in Genetic Improvement

With the recognition of biosafety and sustainability of plant-based medicine, there has been a surge of interest in expanding DH for F1 hybrid production to high-value crops such as medicinal and aromatic plants. However breeding has been highly neglected among these species. Development of double haploid technology in MAPS will not only allow providing high, stable, and predictive yields of the raw biochemicals processed by pharmaceutical and nutraceutical industries but also has the potential to make significant advancements in genetic and biochemical studies toward better understanding of the secondary metabolite synthesis.

24.4.1 Breeding

Breeding is one of the most important factors for commercially viable crop in phytopharmaceutical field. The basic purpose of breeding is getting homogeneity, high content of important constituents, factors influencing the success of postharvest processing, and appropriate morphological properties. For this all the natural variations available in the species are screened. As biochemical and metabolic processes are under genetic control, even small spontaneous change at the gene level can manifest itself at the molecular level resulting in difference in proteins, i.e., enzymes. This leads to dissimilarities in the metabolism among plants of the same species providing a wide base of natural variation for identifying and selecting of traits of interest and considering it in future improvement programs. Haploids are the best material to screen these traits and validate the phenotype because they allow expression of even the recessive genes allowing better selection methods to be implemented. The deleterious genes that are recessive are not expressed in heterozygous state and get fixed causing inbreeding depression. Haploids, on the other hand, fix these genes in one generation which thereby get eliminated from genetic pool of the species allowing more viable combination of gene to sustain. However, monoploid haploids are usually smaller with reduced vigor and are sterile. To restore the fertility, it is a must that their chromosomes are doubled forming the DHs.

Haploid system is very useful when screening plant variations for breeding. Application of haploids technology relies on three main plant factors:

1. Embryogenic potential of male or female gametophytes
2. Chromosome doubling with or without colchicine treatment
3. Regeneration of plants from embryo

This technique speeds up the plant breeding by allowing the development of homozygous line in one generation rather than several generations of conventional breeding. In self-pollinated crop, these double haploids represent a new cultivar. They allow the expression of recessive alleles which thus becomes the phenotype of the variety or cultivar which after several cycles of crossing and selection leads to the gradual improvement of the species. In cross-pollinated crops, these DHs can be used as parental lines for hybrid production or as homozygous lines for breeding. A new breeding technique, reverse breeding, makes use of both DHs and backcrossing (Driks et al. 2009). In this technology, DHs are developed from microspores of plants in which meiotic recombination has been suppressed by gene knockout of key meiotic genes. The resulting recombinant inbred populations can be screened via molecular markers to identify those with complementary combinations of chromosomes to allow an original heterozygous parent of the DH to be reconstructed by hybridizing the two individuals. Consequently, different parents with different chromosome constitutions can be identified to reconstruct existing F1 hybrids. The technique however is limited to crops with a haploid chromosome number of 12 or less and in which spores can be regenerated into DHs. However DH development is genotype dependent and thus is not applicable to all genotypes and species of plant. Another example of molecular breeding was developed using reversible male sterility and DH production, F1 hybrid breeding. This technique uses molecular tools causing single- or double-point mutations in tobacco GS1 and fusing it with tapetum-specific TA29 or the microspore-specific NTM19 promoter. This construct when transformed in tobacco causes male sterility. Homozygous DH male-sterile plants were generated through microspore embryogenesis from these transformed plants. Fertility restoration was achieved by spraying plants with glutamine or by pollination with pollen matured in vitro in glutamine-containing medium.

24.4.2 Mutation Breeding

Conventional mutagenesis like conventional breeding is a time-consuming process. In seed the mutagenesis is limited by production of chimera and loss of desired traits. Double haploid technique has proved to be an important tool when fixing mutation in mutation breeding. Haploids also provide a valuable genetic system for mutagenic studies (Christianson and Chiscon 1978). Higher plants have large number of genetic markers and thus become suitable for genotoxicity and cytotoxicity studies. Besides, knowledge of in vitro selection studies also needs to be developed to screen the mutated haploids. Haploid microspore can be isolated and given mutagenic treatment. They can be then cultured on petri plate and regenerated into plants. Seeds can also be treated with mutagenic agent. Gametes formed after germination of treated seeds can be used for haploid development. All mutations, whether dominant or recessive, are expressed in haploids, thus giving phenotypic validation. After diploidization, the regenerants in which mutation has been fixed are screened for traits of interest. This technique has been successful in the development of new

cultivars. *Brassica* is the most extensively studied system for mutational breeding and thus also serves as ideal candidate for mutagenic studies. The effectiveness of mutagenic treatment depends on both dosage and stage of microspore (He et al. 2000). Various mutagenic agents used are EMS (ethyl methanesulfonate), ENU (ethyl nitrosourea), NaN3 (sodium azide), MNU (*N*-methyl-*N*-nitrosourea), gamma rays, X-rays, and UV (ultraviolet) (Szarejko and Forster 2007).

24.4.3 Somatic Hybridization (Protoplast Fusion)

In somatic hybridization the protoplasts, i.e., cell devoid of the cell wall, from two genetically different species are experimentally fused. The resulting parasexual hybrid protoplasts have heteroplasmic cytoplasm and nuclei from two parents. This technique allows production of interspecific or intergeneric hybrids. The technique takes the advantage of enzymatic digestion of the cell wall. For higher plants cellulase and pectinase or macerozyme are used. Bacterial cell walls are degraded by the action of lysozyme. Fungal wall is degraded by Novozyme 234 which includes glucanase and chitinase. Lysozyme and achromopeptidase are used for cell wall degradation in *Streptomyces* (Narayanswamy 1994; Jogdand 2001). Isolated protoplast is either spontaneously fused together forming plasmodesmatal contact. However protoplast isolated from different sources does not fuse spontaneously due to the negative charge on the plasma membrane and requires induction for fusion. This induced fusion can be mechanical, chemical (Pasha et al. 2007; Jogdand 2001; Srinivas and Panda (1997), or electrofusion (Ushijima et al. 1991; Dimitrova and Christov 1992). Somatic hybrids have been generated by protoplast fusion in many plant species. One of the finest examples of somatic hybrid is pomato fusion product of *Lycopersicon esculentum* (tomato) and *Solanum tuberosum* (potato) (Melchers 1978). This technique has been successful for introducing various traits in plants like disease resistance gene, genes responsible for the tolerance of cold, frost, and salt. These somatic hybrids also provide the only mean of genetic recombination in asexual or sterile plants. Tu et al. (2008) produced intertribal somatic hybrids of *Raphanus sativus* and *Brassica rapa* with medicinal plant *Isatis indigotica*.

In gentians somatic hybridization was attempted in order to broaden the genetic diversity in secondary metabolites between cell suspension-derived protoplasts of diploid *Gentiana kurroo* Royle with leaf mesophyll-derived protoplasts of tetraploid *G. cruciata* L. (Tomiczak et al. 2017). Protoplast fusion was accompanied by polyploidization and spontaneous elimination of genome parts of either fusion. It was observed that most of the hybrids were genetically closer to *G. cruciate* and all inherited chloroplast from mesophyll of *G. cruciate.* Genes responsible for various useful traits such as disease resistance, nitrogen fixation, frost hardiness, drought resistance, herbicide resistance, and heat and cold resistance can be transferred from one species to another by protoplast fusion. Somatic hybridization has been successful for many important citrus rootstocks and scion cultivars (Grosser 1994) and citrus-related species (Jumin and Nito 1996). Somatic hybridization between two

diploids results in the formation of an amphidiploid which can be avoided by using haploid protoplasts. Chuong et al. (1988) produced cytoplasmic hybrid in *Brassica napus* wherein haploid protoplast from cytoplasmic atrazine resistant (CATR) and cytoplasmic male sterile (CMS) *B. napus* was fused.

24.4.4 Molecular Gene Transfer

Microspore and microspore-derived haploid embryos or plants are useful tools for transformation studies. They are excellent starting material because of the single cell, haploid nature of the microspore. After chromosome doubling the regenerating embryos or plants produce a homozygous true-breeding plant carrying the gene of interest. A highly efficient double haploid protocol is required for any transformation protocols. Various methods of transformation have been studied in various plant species *Agrobacterium tumefaciens* (Pechan 1989; Dormann et al. 2001), particle bombardment (Fukuoka et al. 1998; Nehlin et al. 2000), microinjection (Jones-Villeneuve et al. 1995), and electroporation (Guerche et al. 1987; Jardinaud et al. 1993), combined particle bombardment, and *Agrobacterium*-mediated transformation (Abdollahi et al. 2009a). A novel transformation system in *triticale* involved the use of cell-penetrating peptides (Chugh et al. 2009), which have the capability to translocate across cell membranes, and has been used in mammalian systems to move molecules into the cell. Microspores are different from other cells due to the thick microspore exine that creates a barrier for uptake of the DNA. In order to enhance microspore transformation, some researchers have used enzymes or other methods to reduce or puncture the exine. Microspore-derived embryos (MDE) can be used as the starting material for transformation using microinjection (Neuhaus et al. 1987; Swanson and Erickson 1989; Huang 1992) or a combination of microprojectile bombardment and DNA imbibition (Chen and Beversdorf 1994). Transformation and DH technology have also been used to create homozygous lines with enhanced tolerance to clubroot (Reiss et al. 2009) or pollen beetle (Ahman et al. 2006). Once transformed these haploids are used to form transgenic lines which on chromosome doubling form DHs, lines homozygous for the trait of interest, thereby speeding up the breeding process. Combining microspore culture technology with the newer techniques such as the use of zinc fingers for precise gene targeting may also speed up the breeding process. Haploid transformation allows a novel method for studying secondary metabolism in medicinal plants which can be altered to overproduce certain phytochemicals of interest, to reduce the content of toxic compounds, or even to produce novel chemicals. Pathway elucidation and metabolic engineering have been useful to get enhanced yield of the metabolite of interest or for producing novel metabolites. Heterologous expression of putative plant secondary metabolite biosynthesis genes in a microbe is useful to validate their functions and in some cases, also, to produce plant metabolites in microbes.

Though the protocols to transform (embryogenic) calli using *Agrobacterium*, particle bombardment, and electroporation established many species, the regeneration

efficiency is very low. Further even more challenging is to fix a transgene in population for further evaluation of self-incompatible species. These limitations can be overcome via developing microspore transformation methods like *Agrobacterium*, particle bombardment or cell-penetrating peptides (CPPs), and subsequent DH induction and regeneration (Eudes et al. 2014). *Agrobacterium*-mediated transformation was successfully used in rice (Otani et al. 2005), wheat (Chauhan and Khurana 2011; Liu 2009), and maize (Aulinger et al. 2003), and even transcription activator-like effector nuclease (TALEN)-mediated gene knockout DH transformants have been developed (Gurushidze et al. 2014). Protein molecule, designed endonucleases (i.e., zinc-finger nucleases, transcription activator-like effector nucleases, CRISPR/Cas9, and meganucleases) in the form of protein, and enzymes can be delivered in cell for targeted mutation allowing transforming and mutating plant without transgene introduction (Bilichak et al. 2015, Chang et al. 2014) and wheat (Chugh et al. 2009) microspores by CPPs. These types of peptides can thus be used for both transformation and transgene-free genome editing. Combining mutagenesis and transformation studies with DH techniques significantly reduces the time, space, and costs required to obtain modified homozygotes for genotype-phenotype validation. The use of DHs allows such studies to be accomplished with relatively smaller number of population as required in conventional self-fertilizing one.

24.4.5 Genetic Mapping

QTL mapping is one of the major applications of DHs. As the DHs are homozygous true-breeding lines, they are now considered ideal for production of mapping populations in developing trait-linked markers. In potato, DHs derived from heterozygous tetraploid varieties offer segregating plant material for finding trait-linked markers with bulked segregant analysis. DHs have been extensively used for chromosome mapping and mapped genetic markers in a number of species, such as rapeseed (Delourme et al. 2013), wheat (Cabral et al. 2014), and barley (Sannemann et al. 2015). Segregating DH populations provides excellent opportunities to find marker-trait associations through linkage mapping. Tuvesson et al. (2007) developed DH mapping population for marker-trait associations in rye. Rye shows significant inbreeding depression. To generate mapping population, two DHs are crossed to produce F1 progeny. The individuals are again used to form DHs. Parental DHs and the F1-derived DHs are crossed to a tester in order to keep them alive. The recombination among gametes allows determination of genome organization, agricultural traits, and map markers. From a single heterogeneous genotype, many DHs can be induced where each microspore-derived plant is a unique product of recombination between the chromosomal pairs of the donor which simultaneously allows phenotyping and analysis of the inheritance of markers and genes. Large number of DHs that can be developed from a single plant has one more advantage. It allows fine mapping and map-based cloning approach (Gao et al. 2013). In out-pollinating species, the study can be started with only one DH parent in the initial cross to

produce a segregating population. DHs are also ideal in establishing marker-trait associations in bulked segregant analysis (BSA). BSA and DH analysis have been successful in establishing marker-assisted selection for several breeding traits, mainly disease and pest resistance but also quality traits (Michelmore et al. 1991). DHs currently play an important role in genomics. A popular method of identifying genes controlling a trait is to trawl through expressed sequence tags (ESTs) and to map their chromosome position relative to the trait in question. DHs play a vital role in integrating genetic and physical maps, thereby providing precision in targeting candidate genes. Mutant populations derived from homozygous DH line are another way of linking genes to phenotypes. A single plant allows fine mapping or even map-based cloning approaches.

24.5 Case Study

*24.5.1 Haploid Development in Neem (*Azadirachta indica*)*

Neem (*Azadirachta indica* A. Juss.), an evergreen tropical forest tree, is a renewable source of various useful products. Almost every part of this tree – seeds, leaves, roots, bark, trunk, and branches – has multiple uses. Though many plants produce insecticidal and insect-repellent agents, neem offers far more effective and environment-friendly measure for pest control and elimination in agriculture. Of the many bioactive ingredients isolated from this tree, the most notable are azadirachtin and salanin, extracted from the seeds. Neem-based products such as 'Azatin', 'Turplex', 'Align', and 'Margosan' have been introduced as insecticides in US markets. Another compound obtained from leaves and flowers of the neem tree (*Azadirachta indica*) is nimbolide. Cytotoxic effects of limonoid have been studied on human choriocarcinoma (BeWo) cells. The results revealed that it has immense potential in cancer prevention and therapy based on its antiproliferative and apoptosis-inducing effects (Kumar et al. 2009).

Despite such huge potential as herbal drug, its improvement is hampered by highly heterozygous nature, long reproductive cycle, and recalcitrant seed. The in vitro production of haploids is extremely valuable in plant breeding and genetics of such highly heterozygous, long-generation tree species. However a very negligible work has been done in this direction. Chaturvedi et al. (2003) studied the feasibility of haploid production in neem using anthers at the early- to late-uninucleate stage of pollen. Androgenic haploids of the neem tree were produced by anther culture at the early- to late-uninucleate stage of pollen. Haploid formation occurred via callusing. Histological analysis revealed that in 4-week-old cultures, the anther wall cells had started dividing, while the microspores appeared to be unchanged. However, in 8-week-old cultures, the anther locules were filled with the callus.

24.5.2 *Haploid Development in* Peltophorum pterocarpum

Increasing antibiotic drug resistance has warranted the scientists to search for new antimicrobial substances from medicinal plants. New and potent antimicrobial agents, particularly antifungal and antibacterial, are being actively investigated. *P. pterocarpum* commonly known as copper pod belongs to the family Caesalpiniaceae. This plant is known for the medicinal value of bark and leaves among various regional tribes. Few attempts were made on inhibitory activity against certain pathogenic bacteria and fungi. Studies confirmed that the plant bark and leaves have antimicrobial, antioxidant, antifungal, apoptotic, and hematological activity (Sukumaran et al. 2011). Biswas et al. (2010) investigated into the hepatoprotective activity of the ethanolic extract on rats and found that they have the potential to treat paracetamol-induced hepatic damage and some liver diseases. This activity was attributed to the antioxidants present in it. Not only bark and leaves, even flower extracts are potential candidate for medicinal value (Sukumaran et al. 2011). The compound named terrestribisamide isolated from *P. pterocarpum* flowers showed moderate antibacterial and antifungal activities against tested strains. Besides it also showed good anticancer activity. This anticancer potential of the compound can further be tapped upon (Raj et al. 2012). Raj et al. isolated bergenin (C-glycosyl benzoic acid) from *P. pterocarpum* flowers and tested its antimicrobial activity against bacteria and fungi. It showed antifungal activity against *Trichophyton mentagrophytes*, *Epidermophyton floccosum*, *Trichophyton rubrum*, *Aspergillus niger*, and *Botrytis cinerea*. Another research group studied the antioxidant and antiglycemic potential of different plant parts from *P. pterocarpum* (Manaharana et al. 2011). Broad-range activity of plant extracts was said to be due to the presence of many antimicrobial compounds or their synergic effect. Thus active fractions should be isolated, analyzed, and studied for in vivo efficacy for identification and development of antimicrobial drugs (Nathana et al. 2012)

The development of haploid callus, embryos, and plantlets from cultured anthers and the various factors affecting androgenesis in *Peltophorum pterocarpum* (copper pod) was studied long back in 1987 (Rao and De 1987). Unfortunately no further attempts were made for haploid generation in the species. Pretreatment of flower buds at moderate temperature of 14 °C for 8 days was most effective for callus production. The color of the anther was found to be a reliable and efficient indicator for identification of suitable stage of anther for culture. The frequency of anthers which produced callus and shoots was highest when anthers were cultured at mid- or late-uninucleate stage. A high sucrose concentration of 10% is a specific media requirement for androgenesis. The haploid nature of the embryos, callus, and regenerated plants ($n = 14$) was confirmed by chromosome count.

24.5.3 Haploid Development in Echinacea purpurea

Purple coneflower (*Echinacea purpur*ea L.) is a popular medicinal herbs used in Europe and America for the treatment of infections, inflammations, and insect bites (Hobbs 1998). This plant is known to have 216 medicinally active compounds (Murch et al. 2006). Various classes of biologically active components obtained from different species of *Echinacea* are phenolic acids, alkamides, polyacetylenes, glycoproteins, and polysaccharides (Bauer and Wagner 1991). The main phenolic compounds in *Echinacea* are caffeic acid derivatives such as cichoric acid in *E. purpurea* and *E. pallida* and echinacoside in *E. angustifolia* (Harborne and Williams 2004). Another compound echinacoside, found in *E. angustifolia*, is a broad-spectrum antibiotic, inhibiting a broad range of viruses, protozoa, bacteria, and fungi. Echinacein shows activity against hyaluronidase, an enzyme that microbes produce to penetrate tissues and cause infection (Pons 1992). Its immunomodulatory activities like stimulating T-cell production, phagocytosis, lymphocytic activity, and cellular respiration (anti-oxidation) activity against tumor cells have been established by various researchers (Barrett 2003).

However *Echinacea* yield has shown considerable variability in their composition mainly because of the use of variable plant material and extraction methods (Abbasi et al. 2007). Haploid plants can serve to overcome this problem by marker-linked trait studies and also making possible to generate completely homozygous diploid through diploidization of haploids. These DH lines can be used as crossing parents for the production of hybrid seeds with high growth vigor. In purple coneflower, anther culture has been attempted but plant regeneration failed (Bhatti et al. 2001). Zhao et al. (2006) reported for the first time haploid plant regeneration by culture of anthers in purple coneflower. Combination of BA with NAA in the callus induction medium was observed to be favorable in the formation of embryogenic calluses. The use of 2,4-D resulted in soft and watery calli, and few plants could regenerate. Higher BA concentrations caused severe vitrification of the regenerated shoots. Roots of haploid plants were much thinner and stomata were also small. Seeds with hybrid vigor acquired by crossing between homozygous lines have high application potential in agriculture production. Purple coneflower is generally cross-pollinated (Li 1998), but the mechanism for protection against self-pollination is by protandry and is not self-sterile (Sejdler and Dabrowska 1996). Regeneration of haploid plants by anther culture enables us to apply breeding program for the production of hybrid vigor seeds for this important medicinal plant. Chen et al. (2016) in an attempt to develop double haploid developed tetraploid plants.

The future research priorities of *Echinacea* include the biochemical aspect and research on the development of large-scale bioreactors. This has tremendous potential in the discovery of new compounds that are synthesized in low quantities. The use of bioreactors for somatic embryo and artificial seed production minimizes the variability in *Echinacea* and thus any chemical variability. Both organogenesis and somatic embryogenesis are important in *Echinacea* as the secondary metabolites produced could be a source of new drugs for pharmaceutical industry. Thus the development of DHs offers an opportunity for selection of elite germplasm lines,

biochemical and molecular characterization of biosynthetic pathways of the compounds of interest, and enhanced phytochemical production. In addition haploids provide best system for studying natural and induced genetic variations which form potential source for production of novel compounds. DHs also speed up researches on mapping, isolation, and cloning of the genes controlling the production of medicinally important compounds and more efficient and robust transformation systems.

24.5.4 Haploid Development in Borage

Borago officinalis commonly known as borage is cultivated along the Mediterranean Basin, Western Asia, and certain regions of North Africa, South America, and Continental Europe. Traditionally, it is used for culinary purposes in some regions of Spain, Italy, France, and Germany. Borage seeds are also good commercial sources of gamma-linolenic acid (GLA). GLA has beneficial effects on brain aging and improves both memory and N-methyl-D-aspartic acid receptor function (Biessels et al. 2001). Wettasinghe and Shahidi (1999) studied antioxidant and free radical-scavenging properties of ethanolic extracts of defatted borage (*Borago officinalis* L.) seeds and concluded that borage extract can be supplemented in oils and meat products replacing the synthetic antioxidants for retarding lipid oxidation.

It also exhibits antispasmodic, bronchodilator, vasodilator, and cardio-depressant activities. The activities suggest the spasmolytic effects of borage crude extract are probably via Ca^{++} antagonist mechanism (Gilania et al. 2007). Borage also has immune-modulator properties (Harbige et al. 2000). Borage oil is effective for the treatment of ailment like diabetic neuropathy and rheumatoid arthritis (Horrobin et al. 1993, Kast 2001). Phytochemical studies reveal that borage contains tannins, resine, ascorbic acid, beta carotene, niacin, riboflavin, rosmarinic acid, and flavonoids (Bandoniene and Murkovic 2002; Duke 2001).

Breeding programs in this plant are restricted due to its multifactorial self-incompatibility. Such systems limit the production of pure (homozygous) lines by self-pollination (Leach et al. 1990). To overcome this limitation, Eshaghi et al. (2015) resorted to the androgenesis. They studied various factors influencing the production of haploid embryos. Results showed that the developmental stage of the microspore is the most critical factor of all and same is correlated with the bud size. Various other factors were also evaluated. Stress played an important role in the androgenic response of borage. Heat shock was effective to induce embryogenesis. The best embryogenic calli and embryogenic response were obtained at 32 °C during 3 days. Treatment at 30 °C for 14 days produced calli which was not derived from microspores and instead was most likely to be derived from anther wall tissues. Similar stimulating effect of prolonged exposure to heat on anther wall tissues has been observed in other species also (Parra-Vega et al. 2013). The use of maltose instead of sucrose improved the efficiency of embryo induction probably due to osmotic stress (Bohanec et al. 1993; Raquin 1983; Ferrie and Caswell 2011; Calleberg and Johansson 1996).

Further extending the study, Hoveida et al. (2017) investigated the effect of chemical and physical stress on the development of double haploids. They explained the effect of different mediums, colchicine treatment, n-butanol, centrifugation, and electroporation treatments. The best response for callus was obtained using AT3 medium, and B5-NLN medium produced the maximum number of embryo-like structures (ELSs). Frequency of ELS formation and plant regeneration was significantly enhanced by the colchicine treatment. Treatment of borage anthers with 0.2% n-butanol enhanced the viability of induced calli. Centrifugation and electrical current were effective individuals for callogenesis and for ELSs, callus viability, and plant regeneration, respectively.

24.5.5 Haploid Development in Gentians

Gentiana triflora is a flowering plant of the genus *Gentiana* and is native to China, Mongolia, Eastern Russia, Korea, and Japan. Gentian petals are predominantly blue due to the presence of blue and stable anthocyanin gentiodelphin (delphinidin3-*O*-glucosyl-5-*O*-(6-*O*-caffeoyl-glucosyl)-3′-*O*-(6-*O*-caffeoyl-glucoside). They have been used in local medicine and are reported to have antimicrobial (Nat et al. 1982), antifungal (Sluis and Labadie 1981), hepatoprotective (Lian et al. 2010), and antilipidemic activities (Vaidya et al. 2009). Yamada et al. (2014) initiated the studies to decipher the molecular mechanism of anti-inflammatory property of root extract of gentian. They concluded that inhibition of TNF-a, iNOS, and Cox-2 expression by gentiolactone might be a probable model explaining the anti-inflammatory property. Gentians show intense inbreeding depression, thus making homozygous line development a challenging job. Homozygous parental lines are crucial for F1 hybrid breeding. Thus the researchers have resorted to the development of homozygous lines via haploid and double haploid generation. Doi et al. (2010) studied gentian plant (*Gentiana triflora*) for their response to haploid generation via anther culture system. It was observed that half-strength modified Lichter (NLN) medium along with high concentration of sucrose and heat shock treatment (130 g/l) was beneficial for embryogenesis from anther. He also concluded that developmental stage and genotype of the anthers used were crucial factors determining the efficiency of the haploid generation. However, due to low embryogenic efficiency of anther culture and genotypic dependency, Doi et al. (2011) resorted to gynogenesis, an alternative process for haploid and/or DH induction. Unfertilized ovule culture has more advantages than anther culture as it tends to overcome the abovementioned problems. Further extending the study, Doi et al. (2013) analyzed the effect of various factors and reproducibility of their protocol in different genotypes and spatially different labs on embryo-like structure (ELS) production from unfertilized ovule culture. Cold pretreatment promoted ELS generation. To check the reproducibility of their protocol, they also tested their developed method in two different labs and concluded that gynogenesis is a stable and reproducible method for developing haploids and DHs in gentians. Such studies will lay a path for not only breeding but also genetic and developmental studies of gynogenesis.

24.5.6 Haploids in Stevia

There is an increasing demand of products that are herbal and promote health. *Stevia rebaudiana* is one such promising herb belonging to Asteraceae family. It is distributed in the United States through Mexico and Central America. It is also found in non-Amazonian South America, southward to Central Argentina (King and Robinson 1987).

The worldwide researches in stevia have mainly focused on the sweet-tasting diterpenoid steviol glycosides (SGs), which are used as a non-sucrose and non-caloric sweetener in a wide range of food products. In stevia, the SGs are mainly accumulated within its leaves, followed by stems, seeds, and roots. In addition to its sweetening property, it has medicinal values and uses. Eight sweet steviol glycosides have been isolated and identified from *S. rebaudiana* leaves (Kinghorn and Soejarto 1985). Among the known SGs, the most abundant glycoside in stevia leaf is stevioside. Rebaudioside-A (Reb-A), the second most abundant compound, is better suited than stevioside for use in foods and beverages due to its pleasant taste. Thus there is a big challenge for agronomists and plant breeder to maintain the desirable level of Reb-A/stevioside ratio in stevia leaves.

To achieve the consistency in stevioside/rebaudioside-A yield, a superior planting material is required. Low seed germination rate and self-incompatibility present in the species result in plants of varied genotypes and phenotypic traits, which does not allow generation of homogenous population in terms of important traits such as desired stevioside ratio and yield. This limits the breeders for the production of superior homozygous line prerequisite for breeding. The major research programs have focused on the tissue culture of stevia to raise homogenous quality material in a short time span. However no empirical report is available on successful generation of haploids and subsequent double haploids. Flachsland et al. (1996) regenerated plants from anthers cultured in vitro under defined conditions which were later identified as diploid indicating their somatic origin.

24.5.7 Haploids in Carrot

Daucus carota is an important root vegetable being used from ancient times for rich bioactive compounds like carotenoids, anthocyanins, chlorogenic acid, essential oils, and dietary fibers (Sun et al. 2009; Sharma et al. 2012). Traditionally carrot has been used in medicine for its antimicrobial activity (Tavares et al. 2008) and to treat hypertension (Gilani et al. 2000). *D. carota* has also been reported to have hepatoprotective activity (Bishayee et al. 1995). Not only the roots but aerial parts of plant are reported to produce essential oil having antimicrobial against the human enteropathogen *Campylobacter jejuni* (Rossi et al. 2007). Root extracts can also be used for preparation of pharmaceutically important molecules (Yadav et al. 2002).

This species shows high inbreeding depression which is a major drawback for its breeding programs. Conventionally developed inbred lines on the other hand take

much longer and are not completely homozygous; thus complete potential of hybrid generation cannot be tapped upon. With the discovery of genetic-cytoplasmic male sterility (CMS), the use of F1 hybrid along with CMS has speeded up the production of hybrid in carrot (Simon et al. 2008). However after several generations, that also results in inbreeding depression. To overcome this limitation, several researchers worked on haploid and DH generation (Matsubara et al. 1995; Adamus and Michalik 2003; Staniaszek and Habdas 2006; Li et al. 2013). Ovule or ovary cultures are an alternative method for haploid production (Kiełkowska et al. 2014). Haploids can also be developed by the application of irradiated pollen or pollen of other species or genera (Foroughi-Wehr and Wenzel 1993). Kiełkowska and Adamus (2010) studies with different species as a pollen source narrowed down to parsley. It was observed that parsley pollens germinate on carrot stigma and induces ovule to develop but does not cross with it.

Heat shock had an adverse effect on carrot ovules. The genotype of the donor plant is one of the most important factors affecting haploid plant induction regardless of the methodology used (Phippen and Ockendon 1990; Martinez et al. 2000; Chen et al. 2011). Li et al. (2013) conducted haploid study on 47 carrot accessions over 4 years of time span. Cold and heat pretreatment generally have a negative impact on the induction of microspore embryogenesis, but a short pretreatment showed a positive influence on some accessions.

In carrot effect of various components on androgenetic response has been studied in detail by many researchers. Different media have been used for androgenic plant regeneration (Andersen et al. 1990; Matsubara et al. 1995; Tyukavin et al. 1999). In androgenetic studies usually it is observed that the regeneration medium is different from the medium used for induction of embryogenesis. Stress or component applied to induce embryogenesis if not removed or reduced in concentration hinders the regeneration ability of the embryos (Bajaj 1990; Chauvin et al. 1993; Takahata and Keller 1991). The ability to regenerate plants from androgenetic embryos depends mostly on the applied cultivar.

Copper-tolerant androgenic haploids were obtained by Kowalska et al. (2008). Kiszczak et al. (2017) conducted a comparison study between androgenesis via anther culture and microspore culture and concluded that keeping endogenous and exogenous factors uniform, the anther culture technique is more efficient during the plant regeneration stage for immediate short-term results (4–8 weeks), and survival rates were also higher. However with microspore culture, it takes longer (12–24 weeks), but numbers of regenerated plants are higher due to secondary embryogenesis.

24.6 Conclusion

MAPs continue to play an important role in general wellness of the people both rurally and as home-based remedies for cure of illness. These natures' reservoirs should be tapped upon for their potential pharmacological and therapeutic properties. Any advancement toward the discovery, identification, and isolation of novel molecule of medicinal importance from such plants will have promising future both

in pharma and agricultural fields. Most of these plants are not cultivated but harvested from the wild. This leads to variable concentrations of the desired compound. Though much neglected, haploid and DH production approach is the need of time for generating superior identified plants for homogeneous harvest for drug discovery and screening programs. As homozygous genotypes actually represent phenotype, they can be used for phenotypic validation, genetic, biochemical, transformation, and agricultural studies. They have been very instrumental in accelerating crop improvement programs of maize, rice, wheat, barley, brassica, and several others. Double haploid generation allows breeders to surpass several generations of selfing and crossing in convention breeding programs. Gradually with the invention of new technologies and improvement in older ones, more effective methods of developing DH line are coming up. This will help scaling up of medicinal and aromatic products from plants, which will contribute to countries' economy and generate employment in agricultural and pharma sector.

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Chapter 25
Role of Molecular Marker in the Genetic Improvement of the Medicinal and Aromatic Plants

Anubha Sharma, Nitish Kumar, and Iti Gontia Mishra

Abstract Several molecular markers have been developed for breeding major crops owing to their significance, ease, and suitability. Out of these DNA markers are frequently used ones; therefore, in this chapter, we describe the DNA markers to map major genes with regard to their principle, applicability, and methods. The two major classes of DNA markers are based on (i) DNA hybridization, e.g., restriction fragment polymorphism, DNA chips, etc.,. and (ii) polymerase chain reaction (PCR), e.g., SSR, RAPD, AFLP, and SNP. Developing trait-linked markers involves the segregation of populations demonstrating target traits followed by reliable phenotyping methods. With the help of these techniques, trait-linked markers may be used in two situations: (i) in the absence of any biological information and (ii) with available information about the trait.

Keywords Aromatic plants · Medicinal plants · Isozyme · Breeding · Alleles · Fingerprinting

Abbreviations

AFLP	Amplified fragment length polymorphism
EST	Expressed sequence tag
GBS	Genotyping by sequencing

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ISSR	Inter-simple sequence repeats
PCR	Polymerase chain reaction
QTL	Quantitative trait loci
RAPD	Random amplified polymorphic DNA
RFLP	Restriction fragment length polymorphism
SNP	Single nucleotide polymorphism
SSR	Simple sequence repeats
STR	Short tandem repeat
STSs	Sequence-tagged sites

25.1 Introduction

Medicinal and aromatic plants are the most exclusive source of lifesaving drugs used worldwide for health-related issues. Herbal drugs contain the secondary metabolites of the medicinal plants. Phytochemical compounds or secondary metabolite affects the physiological processes of a living organism. Hence major focus of the researchers is to improve their specific content (metabolite) which is useful because of its medicinal or aromatic property. Since these are the important aspects in the medicinal field of science, we should find the methods for its improvement and more effective breeding methods. The initial step to improve their quality and efficacy is correct identification and DNA fingerprinting analysis. DNA fingerprinting analysis deals with the detection and exploitation of naturally occurring DNA sequence polymorphisms. It is a powerful tool in the field of medicinal plants for accurate identification. Molecular markers play an important role in deducing the plant's genotype. Morphological, biochemical, and DNA-based molecular markers are generally used to find the genetic diversity for plant species.

The classical meaning of molecular "markers" refers to locating similarities or dissimilarities among individual, cultivars, or breeding lines using phenotypic characters or closely linked genes. Molecular markers may be used to select the desirable phenotype at the seedling stage to avoid false breeding. In the case of plant breeding, these markers rely upon genetic variations observed among individuals within alleles at particular gene/loci. A molecular linkage map can be constructed by analyzing the marker genotypes of each plant and then calculating the genetic distances between marker pairs based on their recombinant frequency. The use of molecular markers in breeding is a widespread technique for commodity crops such as the cereals, but for medicinal plants, there are only a few reports. DNA markers can be utilized for validating the medicinal plant specimen used for making pharmaceutical products and also in case of marker-assisted breeding.

25.2 Types of Molecular Marker

25.2.1 Protein Markers

Prior to the development of DNA markers, markers based on morphological and biochemical characteristics were universally used. Under biochemical markers, isozyme (multiple forms of enzyme) markers are most commonly used, which are protein enzymes encoded by one or more loci/alleles, and may be distinguished on the basis of gel mobility during electrophoresis followed by specific staining (Winter and Kahl 1995; Jinek et al. 2012; Manzo-Sanchez et al. 2015). Isozymes having the same enzymatic activity may be differentiated according to the charge or size. Isozymes are, however, limited in number and unevenly distributed on the chromosome, and enzyme activity often depends upon the age or type of plant tissue (Yang et al. 2015). Even so, the ease and economy of isozyme analysis make it suitable for various studies, e.g., in groundnut, chickpea, and barley (Javid et al. 2004; Iqbal et al. 2005). Masoumi et al. (2012) calculated variations in seed protein in different medicinally important plants such as Iranian cumin, fennel, and longleaf for grouping them in accordance to their seed protein (biochemical marker).

In another medicinal plant *O. sanctum*, unique banding profiles of enzymes, i.e., esterase, peroxidase, acid phosphatase, and alkaline phosphatase, represented its fingerprint (Johnson 2012; Hammad 2009). Hence fingerprinting via use of isozymes as marker represents genetic variability within and among plant populations and is widely and efficiently revealed by isozyme electrophoresis (Hamrick and Godt 1990).

25.2.2 DNA Markers

It is well known that climatic conditions, age of an individual, and other physiological conditions do not affect DNA markers, so these are more reliable than morphological or chemical traits (Balasubramani et al. 2010; Manokar et al. 2017). DNA marker techniques are based on the use of sequence differences among species or each individual within a species. Recently nuclear ribosomal internal transcribed spacer (ITS) region is used for species identification of Gambhari. Gambhari (*Gmelina arborea* Roxb.) is a medicinally important plant wildly distributed in Southeast Asia. In India it is distributed in Northwestern Himalayas (Dhakulkar et al. 2005). Its extract has been used for the treatment of various health problems like

anthrax, asthma, bronchitis, cholera, epilepsy, etc. (Manokar et al. 2017). Another plant *Baliospermum montanum* which is commonly found in India and nearby countries has medicinal value because of the presence of a certain pharmaceutical compound. The secondary metabolite such as codeine, L-dopa, reserpine, and digitalis is useful for the treatment of various diseases. Like several other plants, molecular marker RAPD is used for the genetic diversity in various plant species of *Baliospermum montanum* (Muazu et al. 2016). Unusual pairing of sister chromosomes or recombinations causing rearrangement in the chromosomes result in genetic variability in individuals of a group, e.g., insertions, inversions, translocations, deletions, or reduplications. Such rearrangements in the genetic material vary in lengths from one to a million base pairs. DNA mutations also exist in the form of nucleotide substitution events (Graham et al. 2010). Hence by using these characteristic variations of the genetic material, tools like DNA-DNA hybridization or PCR are commonly used. In the former technique, a probe, i.e., a short but precise sequence of DNA homologous to the target site, is tagged with a radioisotope and hybridized with the DNA specimen. DNA variations may be detected depending on the probe's specificity or on its length as exemplified by restriction fragment length polymorphism (RFLP). For PCR the requirement of template DNA is very small, and PCR-based tools are relatively simple and inexpensive. They involve loci such as minisatellites or microsatellites; site-specific primers, e.g., sequence-tagged sites (STSs) or expressed sequence tags (ESTs); and random primers, e.g., random amplified polymorphic DNA (RAPD) or amplified fragment length polymorphism (AFLP).

It has been proved that nuclear ribosomal ITS region shows high level of divergence between species, but it is highly conserved within species. Therefore they are the most preferred genetic markers for species-level identification as shown in so many reports (Qiao et al. 2009; Balasubramani et al. 2010; Selvaraj et al. 2012; Rai et al. 2012; Cheng et al. 2016).

25.3 Molecular Markers Using Hybridization Methods

25.3.1 RFLP (Restriction Fragment Length Polymerase)

RFLP is known as a first-generation technique, and the basis of many DNA marker methods (Jones et al. 2009) targets mutations (additions/deletions/alterations) in recognition sites of restriction enzymes, leading to distant shift in fragment size (Tanksley et al. 1989). The main advantages of RFLP markers are due to its codominant nature, reproducibility, and specificity, and prior sequence information is not warranted. Abd EI-Twab and Zahran (2010) have shown phylogenetic relationship between *Matricaria recutita*, *Achillea fragrantissima*, and *Artemisia arborescens* using RAPD, ISSR, and RFLP technique. These plants are mainly found in Egypt and are disease resistant. Similarly, *Bulbus fritillariae* (*BF*), commonly used as antitussive herb in China, includes four species that are more competent as herbs compared to others and is identified using PCR-RFLP analysis (Wang et al. 2007).

Similarly, identification, detection, and quantification of aromatic plants have been accomplished using RFLP. Recently, comparison of 5S *rRNA*-NTS gene spacer region has served as the basis for identifying medicinal and aromatic plants at both interspecific and intraspecific levels. The plant species *Picea glauca* and *Pseudotsuga menziesii* (known for their essential oils) has shown variations in the NTS region ranging from 101 bases to 880 bases in *P. glauca* and *P. menziesii*, respectively (Liu et al., 2003).

25.4 Molecular Markers Using Polymerase Chain Reaction (PCR)

25.4.1 Minisatellite/Microsatellite Markers

Minisatellites are DNA sequences ranging from 9 to 100 bp and may be found repeatedly along the genome (Rogstad 1993). The number and size of repeats differ and, however, is maximally under 1000 bp. Variability in the number of repeats is another kind of polymorphism called variable number of tandem repeats (VNTRs). They are measured by either hybridization using probes or PCR using specific primers. Variations in length of fragments that hybridize with a probe targeted to minisatellites indicate the presence of VNTRs. Primers flanking the repeat regions can alternately be used via PCR; the size of the amplicon varies to indicate the presence of VNTRs.

Martins et al. (2013) developed a new set of microsatellite (SSR) markers for *Smilax brasiliensis* (sarsaparilla) which is used in traditional medicine as a tonic, antirheumatic, and antisyphilitic. This plant has been sold in Brazilian pharma without any quality control, any herbal drug should have a quality check first before making it available in the market for medicinal use. *Rhodiola rosea* L. is also a famous plant and used as a medicine because of its adaptogen properties. It has been reported that it has a positive effect on cardiovascular and central nervous system (Kelly, 2001). The extract of *R. rosea* is used to treat various illnesses including tiredness, depression, anemia, impotence, and neural disorders (Brown et al., 2002). Veress et al. (2015) have developed new primer pairs to test *R. rosea* populations, and they have found two novel variable microsatellite loci in the genome of *R. rosea*.

RAPD

Random amplified polymorphic DNA (RAPD) markers are used to characterize the genetic variability of medicinally important plants (Satovic et al. 2002; Vieira et al. 2003; Singh et al. 2004; De Masi et al. 2006). The advantage of using RAPD for genetic diversity is that it does not require any prior sequence information (Palumbi 1996). Recently Tiwari et al. (2016) analyzed genetic variations among *Cassia tora* from Central India using RAPD Markers. *Cassia* is a medicinal plant containing medicinally significant components such as anthraquinones and sennosides (used for formulating Ayurvedic cough syrups/expectorants, healing skin diseases, e.g., ringworm, eczema, and scabies). *Aegle marmelos* is another example which wildly

grows in India and is well known for its medicinal uses. This tree contains furocoumarins, flavonoids, and various other essential oils. All parts of the bael tree have great medicinal importance. The PCR-based RAPD markers have been widely used to study biodiversity in various medicinal plants including *Aegle marmelos* (Martínez et al. 2005; Govarthanan et al. 2011). Recently, many genetic diversity studies have been done in various plants like parasite *Cuscuta* (Khan et al. 2010), *Convolvulus pluricaulis* (Ganie et al. 2015), and *Evolvulus alsinoides* Ganie and Sharma 2014) using RAPD as a molecular marker.

25.4.2 Amplified Fragment Length Polymorphism (AFLP)

It is a type of RAPD which is commonly used to detect restriction site polymorphisms without prior sequence information through PCR (Vos et al. 1995). AFLP analysis is one of the robust multiple locus fingerprinting techniques among genetic marker techniques that have been evaluated for genotypic characterization (Ghosh et al. 2011; Misra et al. 2010; Percifield et al. 2007; Saunders et al. 2001). Technique-wise this method is similar to RFLP analysis; however, a small subset of these fragments are exhibited, and the primers determine the number of fragments obtained. AFLP is advantageous over other techniques due to the presence of multiple bands obtained from different parts of the genome, thereby preventing overinterpretation or misinterpretations owing to point mutations or single-locus recombinations that may impact other genotyping methods. However, the alleles are not determined easily, and this is the main disadvantage of AFLP markers.

Hyoscyamus sp. (Solanaceae family) is a famous source of tropan alkaloids, i.e., hyoscyamine, scopolamine, and atropine, and is cultivated for its medicinal importance (Suzuki et al. 1991; Kartle et al. 2003). Etminan et al. (2012) analyzed the genetic diversity within a set of 45 Iranian *Hyoscyamus* sp. using AFLP and retro/AFLP markers and generated significant data for future training and breeding programs to manage germplasm resources.

Swertia chirayita is known for treating asthma and liver disorders (Brahmachari et al. 2004). In India it is mainly found at high altitude and Western Ghats. AFLP is a method of choice for discriminating closely related species and authentication of herbs. It was earlier reported in *Plectranthus* genus by Passinho-Soares et al. (2006) and *Swertia* sp. by Misra et al. (2010). They proved that AFLP provides a validating tool for detecting adulterants in crude drug formulations of *Swertia* and for maintaining quality standards of herbal drug industry. *Zanthoxylum acanthopodium* and *Zanthoxylum oxyphyllum* leaves used for essential oils extraction purpose. This oil is used in cosmetics and perfume industries. Apart from its essential oil, it is also used as ethnomedicine in Northeast India. Scientist used AFLP for the authentication of two *Zanthoxylum* species to determine adulteration-related problems faced by pharmaceutical industries to supplement conventional drug assessment protocols (Gupta and Mandi 2013). Similarly, molecular markers, AFLP-2_31 and SAMPL-3_60, may be used in marker-assisted programmer to improve breeding efficiency of pharmaceutical properties for the spice plant, oregano (Azizi et al. 2016).

Uses of molecular markers are a rapid method as it can help to take out the relevant information from any part of the plant tissue at every developmental stage. With the help of linked DNA markers, breeder can take out reliable information before pollination. Phenotypic evaluation of genetic traits is often complicated by environmental factors. However, environmental conditions do not show its effect on DNA marker. The breeder can evaluate their material independently of the environmental conditions (environmental conditions can be favorable or unfavorable for morphologic and/or biochemical marker expression).

Molecular marker can also be used for diagnostic analysis to show the presence of traits for disease resistance. It can be performed by the tightly linked DNA markers with the target gene without resorting to pathogen inoculation in the field. Furthermore, molecular markers facilitate introgression of genes into selective cultivars in advance of the occurrence of a certain type of diseases or biotypes of insects.

With the help of molecular marker, one can select complex traits in a very precise manner. As of now, all of us know that polygenic traits are often difficult to select for using conventional breeding approaches. DNA markers linked to QTL allow them to be treated as single Mendelian factors. Besides analyzing and selecting the interesting characters, molecular markers allow the researchers also to analyze the wild species with potential interest for the breeding program. Numerous articles that consist of DNA markers (Reiter 2001; Avise 2004; Mohler and Schwarz 2005; Falque and Santoni 2007) are available.

So far desirable genetic markers should have the following features:

(a) Show high level of genetic polymorphism.
(b) Be codominant (heterozygous individuals can be distinguished from homozygous ones).
(c) Allelic features should be clearly distinguished in them (so, the different alleles can be easily detected).
(d) Have appropriate distribution throughout the genome.
(e) Have neutral selection.
(f) Have an easy tracking (the entire process can be automated easily),
(g) Low-cost genotyping.
(h) Have a high repeatability (the data can be stored and shared between laboratories).

Suitable DNA markers should be polymorphic in the DNA level and can be expressed in all tissues, organs, and various developmental stages (Dudley 1993).

25.5 Conclusion and Future Directions

It is clear that the molecular markers are extremely useful source in medicinal plant breeding and enhancement of secondary metabolite of aromatic plants. Marker-assisted selection can benefit in improving the traits as they directly deal with the genes accountable for expression of some important traits. Molecular markers are

helpful to get the insides of genetic mechanism of secondary metabolites as well as provide tools to recognize QTLs for determining quantitative variation for secondary metabolites. These markers often exhibit low level of polymorphism, but it is definitely balanced by their higher interspecific transferability. They are frequently used to deduce the functional diversity of the germplasm. These attributes of molecular marker make them more suitable for genetic diversity study of medicinal and aromatic plants. In actual fact, we will be shifting to the whole genome-based selection strategies as the specific recombination events are hunted and changes are evaluated on a genome-wide scale. SSR- and SNP-based GMMs are the choicest marker for studying genetics and breeding of crop plants. The utilization of allele-specific, functional markers (FMs) for the genes controlling agronomic traits is critical advert for plant breeding. Hence, SSR and SNP markers along with other types of markers which mainly focus on functional polymorphisms within genes are to be developed in the coming years.

In the last few years, emphasis has been given on the generation of transgenic plants for the betterment of yield and quality of some vital medicinal plants and plants having secondary metabolites. However, the use of biotechnological tools for the improvement of medicinal plant species has to confront many restrictions such as gene silencing and multitrait genes and no significant improvement in the enviable secondary metabolites or medicinal drug so that one can use it for profitable business venture. With the time lapse, there are many newer approaches for genetic exploitation of metabolic pathways that have been acknowledged. DNA-based marker studies have been applied to crops and also for authentication of medicinal plants. The application of such tool needs further research for broader employment and characterization of medicinal and aromatic plants. Since the use of transgenic crops is gaining popularity but has to be used with some ethical and biosafety issues, hence genomics can also be used by creating "cis-genic crops," i.e., transfer of the cis-genes from wild relatives to cultivated species. The selection of the most suitable marker system, however, needs to be decided on the basis of particular plant and will highly rely on many concerns including the availability of technology.

Conflict of Interest It is declared that the authors have no competing interests.

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Chapter 26
Genetic Engineering Potential of Hairy Roots of Poppy (*Papaver* spp.) for Production of Secondary Metabolites, Phytochemistry, and In Silico Approaches

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Abstract Opium poppy is one of the most important medicinal plants, because of its secondary metabolites (alkaloids). Opium as such is an important product, which has many uses and abuses. Its alkaloids are widely used in modern pharmacopeia. *Agrobacterium rhizogenes* (hairy roots), mediated hairy root culture, is also used for secondary metabolite production under in vitro conditions. Hairy roots are able to grow fast without phytohormones and to produce the metabolites of the mother plant. India is the only country where UN has given license to produce opium from latex. The application of opiate alkaloids, mainly in hydrochloride, sulfate, and phosphate forms, is restricted in some well-defined therapeutic fields. A major component among alkaloids is morphine, having analgesic in nature and used mainly to control severe pain and sedative effects. Poppy seeds have been described as tonic and aphrodisiac, promote luster of the body, enhance capacity to muscular work, and allay nervous excitement. Plant of such economic importance is affected by various biotic and abiotic factors leading to yield loss. Biotic factors include fungi, bacteria, viruses, nematodes, and birds too. This important plant has huge prospects in pharma industry, and on other hand, it is facing lots of challenges in the form of illicit trade, drug abuse, and biotic and abiotic stresses.

Keywords Opium poppy · Alkaloids · Hairy root · Secondary metabolite

26.1 Introduction

The opium poppy (*Papaver somniferum* L.), member of family Papaveraceae, is an important plant known from ages for its medicinal value (Neligan 1927). Its active components are being used in acute pain. Besides India, it is grown in European

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countries only for seeds and oil; however, in India it is grown under strict vigilance of the Government of India and International Narcotics Control Board (INCB), Vienna, for extraction of opium too.

Opium poppy has been known as useful plant since Neolithic era (8000 years ago). The history of opium poppy as an early companion to human is dated back to archeological excavation of last 100 years (cf. Tetanyi 1997). The sleep-inducing property of this plant was known to Greeks in the sixth century BC (cf. Veslovskaya 1976). In India, opium poppy was brought by Arabs in the seventh century AD, and the plant itself was introduced as a crop only in the thirteenth century. The opium poppy is historically most important medicinal plant and also a very unique source for producing opium alkaloids. The earliest description of poppy in Indian literature is available in *Dhanwantri Nighantu* C.a. 1000 AD (Sharma 1973). In 1757, after the battle of Plassey, the opium monopoly became direct legacy of the Britishers, and Bihar was the province where the best quality of opium was produced in large quantities. Major poppy-cultivating states of India are Uttar Pradesh, Madhya Pradesh, and Rajasthan.

The opium poppy is a dicotyledonous plant belonging to group polypetalae, series Thalamiflorae, and family Papaveraceae. The family is further subdivided into Papaveroideae, which is characterized by plants containing latex tissues (Trease and Evans 1972). Papaveraceae is among the families under the natural order Rhoedales (Papaverales) which was later incorporated into order Ranunculales (Gottlieb et al. 1993). The genus comprises nearly 100 species (Fedde 1909) and is affiliated to the section Mecones comprising five species, (i) *P. somniferum*, (ii) *P. setigerum*, (iii) *P. glaucum*, (iv) *P. glacile*, and (v) *P. dicaisnei*, among which *Papaver setigerum* (2n = 44) is a close relative and probably the ancestor of opium poppy (Hammer and Fritsch 1977). However, *P. setigerum* grows wild in the Southern Mediterranean and Canary Islands and is still very similar to the cultivated opium poppy species, *Papaver somniferum* (2n = 22). Hrishi and Hrishi (1960) observed a fairly good genomic affinity between the two species. However, the chromosomal differences might have been responsible for restricted or no gene flows leading to reproductive isolation, hence speciation. *Papaver bracteatum* also known as *P. orientale* is basically an ornamental poppy. *P bracteatum* lack morphine but contains thebaine as major alkaloid.

The opium poppy plant is an erect growing herb, 100–120 cm in height and with nearly 2.0 cm. thick pithy stem. The radical leaves are elongated, thick, and soft. Leaves are 30–40 cm long and 15–30 cm broad. The leaves are sessile, oval-ovate or ovate-oblong, irregularly lobed with cordate-amplexicaul base enveloping the stem. The lower leaves on stem have short petioles. These may be glabrous above but glaucous below and sharply toothed on the margin with larger teeth alternating by smaller ones. In young leaves, the trichomes are small and simple, growing in size along with the growth of the leaves and then getting branched. The reproductive stage is characterized by drooping bud, having 10–15 cm long peduncle, which may sometimes be hairy. Flowers are solitary on long peduncle, bisexual, regular with two caducous sepals. Petals are four, free, and generally white; however, in "Malwa" forms they are large, rose, lilac, or purple colored with deeply fringed margins. The

stamens are hypogynous, indefinite, arranged in several whorls. Anthers are yellow, linear, oblong, or dilated. Pollen grains are 3-zonocolpate with spiratexine surface (Sharma 1980). There are many united carpels. Stigma is capitate with 8–14 lobes. Style is absent. Ovules are many and placentation parietal. Fruit is a capsule. There are 2–15 capsules per plant. The immature capsule is covered with waxy coating, which imparts grayish-blue tint to the capsule. The rind of the capsule gets hard and woody before the capsule is fully grown. The mature capsule is pale brownish in color. It has unique swollen ring below where it joins the stalk. Openings by pores are beneath the persistent stigma. The number of stigmatic rays on the capsule indicates the numbers of septa in the capsule. The stigmatic disc may be flat, concave, or convex. Morphine, codeine, thebaine, narcotine, and papaverine are the most important alkaloids, which are present in the latex of immature fruits. Cost of opium is quite high. However depending on the grade of opium, its cost ranges from INR 600 to INR 2500 per kg, while the open-market price of opium is between INR 25000 and INR 30,000 per kg in 2013. Its cost in international market is approx. 5000 to 31, 000 US$ per kg. *Agrobacterium rhizogenes*-mediated genetic transformation for the development of hairy roots exhibited extensive branching. Hairy root culture results in the production of major alkaloids found in the mother plant and also sometimes de novo compounds (Nader et al. 2006). These transgenic roots or "hairy roots" are a good source of in vitro secondary metabolite production (Hamill et al. 1987) such as tropane alkaloids (Flores and Filner 1985; Oksman-Caldentey and Arroo 2000) and many other metabolites (Giri and Narasu 2000).

26.2 Phytochemistry of *Papaver somniferum* Alkaloids

Poppy juice is the latex containing many important alkaloids, obtained from poppy. This is called opium and contains more than 40 isoquinoline alkaloids including morphine (Preininger 1985). These are colorless, odorless, insoluble, and extremely bitter and unpleasant in taste (Meijerink et al. 1999). In addition to the phenanthrene alkaloids, which include the analgesics morphine and codeine, other important classes of tetrahydroisoquinoline alkaloids found in opium poppy include the benzylisoquinolines, such as the vasodilator papaverine and the antispasmodic noscapine, and the benzophenanthridines, such as the antibiotic sanguinarine (Fig. 26.1; Phillipson 1983; Preininger 1985). Table 26.1 represents the major alkaloids present in poppy straw or latex of *P. somniferum.*

Alkaloids are produced by plants as a defensive agent against predation of animals, insects, and pathogens. They are secondary plant metabolites (Zenk and Juenger 2007). Alkaloids are classified on the basis of their amino acid precursor; they also contain nitrogenous bases (Dewick 2002). Opium poppy contains a group of alkaloids called benzylisoquinoline (BIA) that have tyrosine. Several plants contain benzylisoquinoline (BIA) alkaloids. Till date approximately 2500 BIA alkaloids are reported across several plant families. However, morphine and codeine are produced in opium poppy only (Hagel et al. 2007; Ziegler and Facchini 2008).

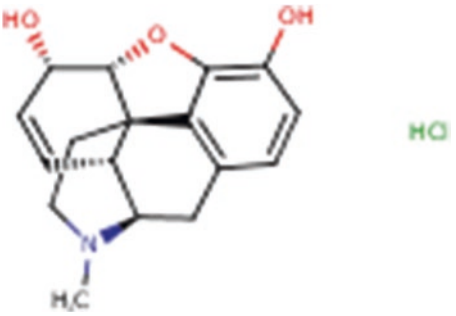
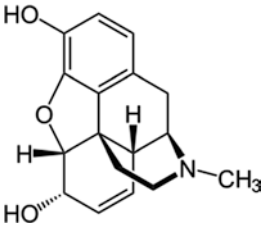
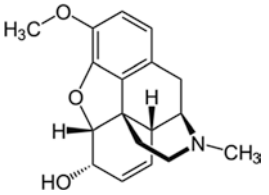
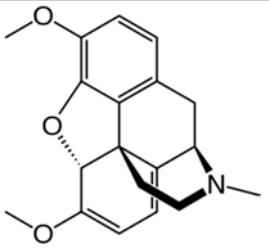
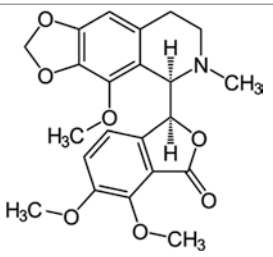


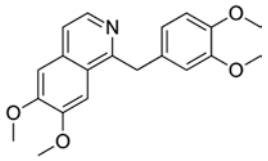
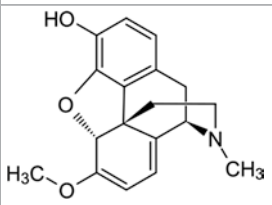
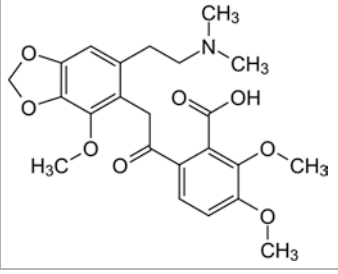
Fig. 26.1 Morphine hydrochloride

Table 26.1 Natural alkaloids contained in opium or poppy straw

	Alkaloid	Chemical formula	Chemical structure	% in Dried latex	Effect on humans
1.	Morphine	$C_{17}H_{19}O_3N$		9–17%	Analgesic and narcotic
2	Codeine	$C_{18}H_{21}O_3N$		0.8–2.5%	Analgesic and narcotic
3	Thebaine	$C_{19}H_{21}O_3N$		0.3–1.5%	Mild analgesic and sedative
4	Narcotine/ noscapine	$C_{22}H_{23}O_7N$		2–9%	Antitussive and apoptosis inducer

(continued)

Table 26.1 (continued)

	Alkaloid	Chemical formula	Chemical structure	% in Dried latex	Effect on humans
5	Papaverine	$C_{22}H_{21}O_4N$			Vasodilator
6	Oripavine	$C_{18}H_{19}O_3N$			Toxic and low therapeutic index
7	Narceine	$C_{22}H_{22}O_8N$			Mild analgesic

26.2.1 Morphine

Morphine is the principal alkaloid in opium with 9–17% of total alkaloids present in dried latex. It belongs to the class of naturally occurring alkaloids, with tetrahydroisoquinoline nucleus. Merck started its commercial marketing in 1827 (Courtwright 2009). First isolated by Friedrich Sertürner, morphine is though to be the first isolated compound from plants. He named the substance after the Greek god of dreams as *Morphium*, because of its sleep-inducing nature (Fisher 2009). Morphine is popular as analgesic and narcotic because of its effects on the central nervous system and on smooth muscle. It is widely used to relieve pain. Morphine has been approved for the treatment of chronic pain since January 2017. Morphine is sold under many names and is on the World Health Organization's list of essential medicines, the most effective and safe medicines needed in a health system (WHO Model List of Essential Medicines 2015).

Manufacture of morphine has risen over the past two decades, reaching a record level of 523 tons in 2013. Apart from therapy, morphine, like codeine, is also being used for conversion into other opioids. Its consumption for the treatment of moderate-to-severe to chronic pain has raised almost fourfold globally over the past two decades. Major consumption of morphine still remains in high-income countries, while consumption levels in most other countries remains very low (*Narcotic Drugs* 2014).

Traditionally, morphine has been used in a wide variety of clinical situations. It is used in acute and chronic pain and to sedate, in cardiac disease, pulmonary disease, gastrointestinal disease, and spasticity. Morphine helps in patient's tolerance for pain and to reduce discomfort. Besides that it helps in mood alteration, euphoria and dysphoria, and drowsiness. The World Health Organization recommends the use of morphine in their three-step analgesic ladder for moderate-to-severe pain resulting from cancer and should be given orally in fixed intervals (WHO 1996). It is a very effective analgesic given orally for cancer chronic pain, but dosage must be individualized (Walsh 1984). Continuous intravenous infusion of morphine is suggested for relief of pain in intensive care patients (Barre 1984). It is also very commonly used for pain control in the perioperative period. Morphine and gabapentin were found effective in an optimized mouse model of multiple sclerosis-induced neuropathic pain (Khan et al. 2014). Nebulization of morphine has been reported to improve chronic cough (Rutherfold et al. 2002). Administration of morphine in analgesic doses significantly reduced tumor metastasis following surgery (Page et al. 1993). Another frequent use of morphine is in alleviating pain during myocardial infarction and during labor pains. Morphine is used in addition to the treatment of both acute coronary syndrome and decompensated congestive heart failure (Breall et al. 2005).

Morphine acts on both innate and cell-mediated immunity, and its treatment suppresses a variety of immune responses, involving major cell types of immune system, viz., NK cells, T cells, B cells, macrophages, and polymorphonuclear leukocytes (McCarthy et al. 2001). In addition to analgesic effects, morphine and other opioids have many other immunomodulatory effects, which have therapeutic implications. The potential usefulness of these drugs may range from conditions associated with inflammation to malignancy (Dinda et al. 2005).

Morphine has significant side effects; continuous use will cause breathing problems. When ingested, morphine produces intense excitement or euphoria and a state of well-being. Regular use can result in the rapid development of tolerance to these effects, thereby having a high potential of addiction and intense physical dependence. Thus development of withdrawal symptoms upon cessation is generally observed. Potentially serious side effects include a severe respiratory distress and low blood pressure.

Heroin (black tar heroin) is usually white or brown powder sometimes a black sticky substance synthesized from morphine. This drug could be administered either by inhalation, by sniffing or smoking, and thirdly by injecting. All these routes of administration deliver the drug to the brain very rapidly. When it enters the brain, heroin is converted back into morphine. Intravenous injection of this drug leads to dry mouth, flushing of skin, heaviness in extremities, and feeling of euphoria accompanied by mental dysfunctioning. It has high risk for addiction, due to its extreme effects in the brain.

The pharmacokinetics of morphine is well established, and three types of opioid receptors have been identified, viz., μ, κ, and δ receptors. Of these three receptors, morphine interacts predominantly with the opioid mu (μ)-receptor, the binding sites for which are widely distributed in the human brain. The density of receptors is high

in the posterior amygdala, hypothalamus, thalamus, nucleus caudatus, putamen, and certain cortical areas. Activation of the μ1 receptor leads to pain relief, while activation of the μ2 receptor can cause effects such as respiratory depression and addiction. Sedation or sleepiness is caused by morphine's activation of the κ receptor subtype (Dinda et al. 2005). Since morphine has a marked immunosuppressive effect, the opioid receptors have been demonstrated in immunoinflammatory cells as well; thus, a possible mechanism for the direct action of opiate on these cells is possible (McCarthy et al. 2001). Genetic association has also been strongly suggested to influence the response to opioids, and a number of works have suggested that this variability may be caused by several genes. Readily absorbed from all mucous membranes, morphine can be administered intravenously, intramuscularly, subcutaneously, intrathecally, orally, or transdermally. After epidural administration the majority of morphine is absorbed into the systemic circulation, whereas only 5% of the administered dose crosses the dura. Following absorption, it is rapidly distributed and crosses the blood-brain barrier. It undergoes extensive first-pass metabolism in the liver resulting in much lower peak concentration after oral administration than after parenteral administration (Glare and Walsh 1991). The liver is the primary site of morphine metabolism through the process of glucuronidation. Morphine-3-glucuronide is the principal metabolite and is biologically inactive. Another active metabolite, normorphine, is formed mainly after oral administration but is rarely found in the plasma. The kidney excretes morphine and its metabolites, but in patients with renal insufficiency, the metabolites accumulate (Hasselstrom and Sawe 1993; Christrup 1997).

26.2.2 Codeine

Second important alkaloid of opium poppy is codeine. It's a phenanthrene opioid related to morphine but is mild analgesic and sedative. It also acts centrally to suppress cough (Schroeder and Fahey 2004). It also shows affinity for mu (μ) receptor, however, with comparatively less affinity than morphine. Its main therapeutic action is analgesia. Codeine's analgesic activity is, most likely, due to its conversion to morphine. The minimum effective concentration is highly variable and influenced by numerous factors, including age, previous opioid use, and general medical condition. However, the effective dose for patients that have developed tolerance is significantly higher than the opioid-naive patients. Presently almost all of the manufactured codeine is acquired from morphine through a semisynthetic process. Manufacture of codeine stood at more than 350 tons in 2013. Codeine is the most commonly consumed opiate in the world, particularly as one of the component of the cough syrups, in terms of the number of countries in which it is consumed.

Codeine is generally administered orally because it is absorbed very well. After demethylation by the live enzymes, codeine gets converted to morphine. This is further metabolized and undergoes glucuronidation. More than 70 percent of the codeine undergoes glucuronidation and form codeine-6-glucuronide, and the remaining

percent undergoes demethylation to form norcodeine. The glucuronide metabolites of morphine are morphine-3-glucuronide (M3G) and morphine-6-glucuronide (M6G). Morphine and morphine-6-glucuronide are both active and have analgesic activity, whereas norcodeine and M3G do not have any analgesic properties.

26.2.3 Thebaine

Thebaine or paramorphine is a white, crystalline, slightly water-soluble alkaloid present in opium in small quantities. It is the most poisonous opium alkaloid and is, therefore, not used for therapeutic or recreational purposes. Thebaine is the main alkaloid found in *P. bracteatum* Lindl; it is later converted to codeine by synthetic processes. Almost all of the produced thebaine is converted industrially into a variety of compounds including oxycodone, oxymorphone, nalbuphine, naloxone, naltrexone, buprenorphine, and etorphine. The alkaloid is metabolized via O-demethylation. Thebaine has a sharp astringent taste, and its consumption can give rise to symptoms similar to pesticide strychnine, which are severe muscular convulsions and death through asphyxia. Long believed to have no morphine-like agonistic properties, thebaine is ten times more toxic than morphine. Also it has now been established to have physical and psychological effects, when large doses are ingested over a certain period, like consuming poppy seed tea. Thebaine has significant dependence potential, and for this reason it is controlled in Schedule II of the Controlled Substances Act as well as under international law.

Manufacture of thebaine increased sharply after the late 1990s and reached an all-time high of more than 150 tons in 2012. This seems to continue, due to the high demand for thebaine alkaloid (Narcotic Drugs Report 2014).

26.2.4 Noscapine

Noscapine is benzyl isoquinoline alkaloid, also obtained from poppy plant. It does not have pain-killing properties and thus is used as antitussive, against coughing, related to inflammation. Studies have suggested anticancer effect of noscapine in melanoma, ovarian cancer, gliomas, breast cancer, lung cancer, and colon cancer. The anticancer property of noscapine is due to its tubulin-binding, anti-angiogenic property that causes cell cycle arrest and induces apoptosis in cancer cells both in vitro as well as in vivo (Ming et al. 2016). Major drawbacks of this drug are its short (less than 2 h) biological half-life, poor absorption, low aqueous solubility, and widespread first-pass metabolism, thereby requiring large doses for effective treatment (Singh et al. 2013).

Noscapine can increase the effects of centrally sedating substances such as alcohol and hypnotics. It has been shown to increase the effects of anticoagulant, warfarin (Ohlsson et al. 2008). The antitussive effects of noscapine opioids are mediated

predominantly by mu- and kappa-opioid receptors. Also mu 2- rather than mu 1-opioid receptors are involved in mu-opioid receptor-induced antitussive affects (Kamei 1996).

26.2.5 Papaverine

Papaverine is the antispasmodic alkaloid. The name has originated from Latin word *papaver* meaning poppy. It was first described and isolated by George Merck (Merck 1848). Papaverine differs from morphine both structurally as well as functionally. It is used primarily in the treatment of visceral spasm, involving the intestines, heart, or brain, and also in the treatment of erectile dysfunction. It is a potent vasodilator particularly of the proximal, intermediate, and distal cerebral arteries. Therefore for this reason it is used as cerebral and coronary vasodilator during balloon angioplasty (Liu and Couldwell 2005) and coronary artery bypass surgery (Takeuchi et al. 2004). Papaverine may also be used as a smooth muscle relaxant in microsurgery where it is applied directly to blood vessels.

Papaverine is not a first-line drug for migraines but has also been found to be effective in prophylaxis or preventative measure of migraine headaches (Sillanpää and Koponen 1978; Vijayan 1977). Frequent side effects of papaverine treatment include tachycardia, constipation, retention test (used to determine hepatic function), increased transaminase levels, increased alkaline phosphatase levels, and vertigo.

26.2.6 Oripavine

Oripavine is the chief metabolite of thebaine. Oripavine is also a potential analgesic same as morphine but has low therapeutic indexes due to its high toxicity. Toxic signs were recorded in animal models included epileptic type convulsions followed by death. Oripavine got much attention with the development of its semisynthetic derivatives named orivinols. All these semisynthetic molecules, known as Bentley compounds, represent the first series of "super-potent" μ-opioid agonists, with some of them being more than 10,000 times the potency of morphine as an analgesic (Bentley et al. 1965, 1967).

26.2.7 Narceine

Narceine is the crystalline opium alkaloid with bitter taste and little solubility in water. The compound has narcotic effects and can be used as a substitute for morphine. The word is derived from Greek word *nárkē*, meaning numbness. Narceine

belongs to the family of stilbenes; that means they are organic compounds containing a 1,2-diphenylethylene moiety, which are derived from the common phenylpropene (C6-C3) skeleton building block. Narceine is used as mild relaxant for smooth muscles. Recently it has been studied to cause genotoxicity in the HT29, T47D, and HT1080 cancer cell lines (Afzali et al. 2006d).

26.3 Medicinal Importance of *Papaver* Alkaloids

The application of opium is known and practiced by pharmacists nowadays. Its powder (*Pulvis opii*), tincture (*Opii siccum*), etc. are legal in many pharmacopeias. However, modern applications require more processed forms and products, which adhere strict industrial standards. The application of opiate alkaloids, mainly in hydrochloride, sulfate, and phosphate forms, is restricted in some well-defined therapeutic fields. Morphine has both analgesic and sedative action and is used to relieve extreme cases of pain, in case of myocardial infraction, in inoperable cancer, typhoid fever, internal hemorrhages, and trauma. Derivatives of morphine-like apomorphine hydrochloride and morphine hydrochloride are used to avoid side effects associated with morphine. Codeine is used both as analgesic as well as antitussive to relieve persistent coughs. Papaverine is used as a smooth muscle relaxant in asthmatic, gastric, intestinal spasms, and coronary ailments.

Drugs derived from opium are commonly named as "opiate," while another more general term "opioid" is used for drugs having morphine-like properties; however, chemical structure is different from morphine (*Narcotic Drugs* 2014*)*. Some important drugs in the market derived from opium are fentanyl, hydromorphone, methadone, morphine, and pethidine and are used in the treatment of severe pain. Similarly drugs, viz., buprenorphine13 and oxycodone, are used as analgesic for moderate pain. Codeine, dihydrocodeine, and dextropropoxyphene are used as cough suppressants and also fentanyl and fentanyl analogues such as alfentanil and remifentanil as supplement anesthesia. Codeine and diphenoxylate are given for the treatment of gastrointestinal disorders, mainly diarrhea. Buprenorphine and methadone opiates are given to drug addicts as an antidote. Being powerful, opioids such as codeine, morphine, and oxycodone are prescribed when other options of medication do not work or do not give enough relief. Worldwide consumption of opium is approximately 1 million kilograms annually, and the demand is still growing (Roberts 1988). India is the only licit and largest producer of opium, and the majority of its production is exported to meet the national and international demands. The demand for morphine has grown manyfold in the last two decades (Yadav et al. 2006). Since thebaine does not have any narcotic effect, its demand has increased considerably worldwide. In the last three decades with the increasing demand of powerful analgesics, there is a sharp increase (10%) in the production of thebaine-based drugs (Shukla and Singh 2003). Reticuline, a morphine pathway intermediate, is also being used for the manufacture of various compounds which are antimalarial (Camacho et al. 2002; Tshibangu et al. 2003; Angerhofer et al. 1999) and anticancerous (Chen et al. 2002;

Seifert et al. 1996). Morphine and codeine are under international control because of their potential for abuse, while thebaine and oripavine are also under such control because of their convertibility into opioids subject to abuse. Noscapine, papaverine, and narceine are not under international control (*Narcotic Drugs* 2014*)*.

The biosynthetic pathway has been completely elucidated in *Papaver somniferum* for formation of morphine and other alkaloids from its precursor, tyrosine (Fig. 26.2). Battersby and Harper (1958) performed the initial experiments to elucidate the biosynthesis of morphine by using ^{14}C-dl-tyrosine followed by harvesting and analysis of all major alkaloids. The pathway starts with decarboxylation of molecules of L-tyrosine and L-dihydroxyphenylalanine (L-DOPA) to give tyramine and dopamine, respectively (Facchini and De Luca 1994). These steps are considered to be potential rate-limiting steps. Condensation of dopamine with 4-hydroxyphenylacetaldehyde results in the formation of R-reticuline (Schumacher 1983), a tetrahydro benzylisoquinoline which is the starting point of the morphine biosynthetic pathway. In a complex pathway, R-reticuline is converted first to thebaine, from where the pathway bifurcates to form either codeine or morphinone. Both these products can act as substrate for the formation of morphine. The two demethylation steps from thebaine to neopinone and then from codeine to morphine are significant because any change in up- and downregulation of enzyme activity of these demethylation steps results in decrease or increase in substrate or product (Yadav et al. 2006). Apart from that, (S)-reticuline serves as a branch-point

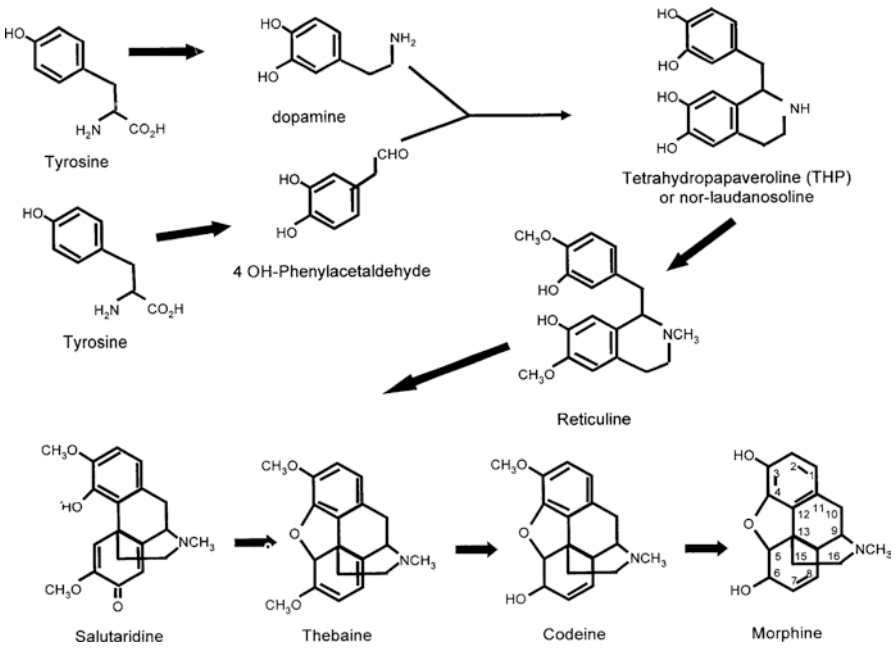


Fig. 26.2 The biosynthetic pathway from L-tyrosine to the common branch-point intermediate (S)-reticuline and then to morphine (Resourced from Meljerink et al. 1999)

intermediate in the biosynthesis of numerous isoquinoline alkaloids. The berberine bridge enzyme (BBE) ([S]-reticuline:oxygen oxidoreductase [methylene bridge forming], EC 1.5.3.9) catalyzes the stereospecific conversion of the N-methyl moiety of (S)-reticuline into the berberine bridge carbon of (S)-scoulerine and represents the first committed step in the pathway leading to the antimicrobial alkaloid sanguinarine (Facchini et al. 1996a, b). Various enzymes catalyzing these pathways have been characterized (Zenk 1985; Gerardy and Zenk 1993; Kutchan and Zenk 1993).

26.4 Genetic Manipulation and Metabolic Engineering of *Papaver somniferum*

Genetic transformation of plant is considered to be a core research tool in plant biotechnology and genetic engineering and a practical tool for improvement of species and cultivars for morphological alteration, disease resistance, and yield traits. In comparison with conventional methods of improvements, genetic engineering of medicinal plants that accumulates biologically active secondary metabolites in their root system has attracted much attention (Sevon and Oksman 2002). Genetic transformation using *Agrobacterium rhizogenes Ri* plasmid is most commonly used for gene transfer into dicotyledonous plants and induction of hairy root in medicinal plants (Tepfer and Casse-Delbart 1987). Hairy roots represent a true engineering platform for horizontal transfer of recombined Ri T-DNAs using *A. rhizogenes* into target host-plant tissue. *Agrobacterium rhizogenes* is a gram-negative bacterium being isolated from natural soil. It causes abnormal behaviors of organ differentiation by formatting rootlike organ, named as hairy root (Chilton et al. 1982; Brillanceau et al. 1989). Growth dynamics and anatomy of hairy roots characterized them as a highly adventitiously highly branched, fast-growing immortal because of their ability to proliferate on culture medium, free from phytohormones. These abnormal but anatomically defined structures known as hairy roots are genetically stable and produce high contents of secondary metabolites as compare to natural roots. Because of stability in culture conditions, these hairy roots are preferred for in vitro production of secondary plant metabolites production over cell suspension culture. Out of the three well-understood strains of *Agrobacterium rhizogenes*, agropine is the most extensively studied and being used for transgenic development worldwide. Other strains are mannopine and cucumopine. Agropine strains contain T-DNA, divided into two regions, i.e., left T-DNA (T_L) and right T-DNA (T_R) (Huffman et al. 1984). White et al. 1985 thoroughly worked out the role of left and right region of root-inducing (Ri) plasmid into plant genome and their subsequent expression. The left region of Ri plasmid contains 18 open-reading frames (ORF). Out of 18 ORFs, 10, 11, 12, and 15 represent the root locus, i.e., *rolA*, *-rolB*, *–rolC*, and *rolD*, respectively (Slightom et al. 1986). These *rol* loci have been found to be important for hairy root formation (White et al. 1985). Out of these, *rol*B plays central and most important

role (Aoki and Syono 1999). The T_R-DNA of the agropine-type Ri plasmid has perfect homology with T_R-DNA of *Agrobacterium tumefaciens* (Huffman et al. 1984). In addition an opine biosynthesis gene known as *ags*-gene is also reported in right T-DNA region of Ri plasmid (Binns and Tomashow 1988). This *ags*-gene is responsible for opine, an energy source for bacterium.

Genetic transformation of natural plant and formation of these abnormal but highly organized structure, i.e., hairy roots, possess the same capability of production and accumulation of biologically active components without affecting the natural root synthesis capacities. Hence, the hairy root system is considered as potential alternative source for the production of secondary plant metabolites in vitro in medicinally important plants (Flores and Medina-Bolivar 1995; Rhodes et al. 1990). In contrast to naturally growing roots, hairy roots are reported to be fast growing and genetically more stable. These can easily be manipulated in large-scale bioreactors and able to produce secondary metabolites at par or higher to intact plants. *Agrobacterium rhizogenes*-mediated transformation of some medicinally important endogenous Chinese plants, its molecular characterization, growth dynamics, and evaluation of biosynthetic productivity of hairy root cultures were studied by Tiwari et al. 2007; 2008.

In addition to its defined role, hairy root cultures may be considered to be an excellent platform for metabolic engineering of secondary plant metabolites by introducing desired genes. A gene 6-b-hydroxylase from *Hyoscyamus muticus* was transferred using *Agrobacterium rhizogenes* to hyoscyamine-rich *Atropa belladonna*. Transformed roots were detected with increased amount of enzyme activity and higher contains of scopolamine (Hashimoto et al. 1993). In another independent study, ornithine decarboxylase gene of yeast overexpressed resulted in accumulation of nicotine in hairy root cultures of *Nicotiana rustica (*Hamil et al. 1990). Tryptophan decarboxylase gene of *Catharanthus roseus* was transferred to *Peganum harmala using* Ri technology. HPLC analysis showed high content of serotonin in hairy root cultures (Berlin et al. 1993). Downs et al. 1994 reported threefold increase in glutamine synthase gene, transferred in *Brassica napus* from soybean. Likewise hairy root cultures of *Rubia peregrina,* containing bacterial isochorismate synthase gene, resulted in double-fold increase in isochorismate synthase activity (Lodhi et al. 1996). These abovementioned examples proved the potential of hairy roots in metabolic engineering of plant-produced secondary metabolites.

26.4.1 Hairy Roots Induction and Metabolic Engineering of Papaver *Species*

Papaver somniferum have been used for long for its biologically active morphinan alkaloids. Benzylisoquinoline alkaloids, which are considered to be largest and highly diverse group of natural plant products, are synthesized in the roots of *P. somniferum* plant species and accumulated in laticifers which are anatomically an

internal secretory system of plant system (Roberts et al. 1983; Nessler et al. 1985; Kutchan et al. 1986). California poppy (*Eschscholzia californica* Cham), a traditional medicinal plant of North America, is an alternative source (Cheney 1964). Roots of *Eschscholzia californica* are the natural source of another benzophenanthridine alkaloid known as sanguinarine. Being a potent antiplaque agent, sanguinarine has been used in oral hygiene products (Dzink and Socransky 1985). Use of biotic fungal elicitors for enhancing production of secondary metabolites especially in cell culture and hairy roots culture has been successfully strategized. In cell suspension of *Eschscholzia californica*, higher content of sanguinarine biosynthesis has been achieved by using fungal elicitors (Schumacher et al. 1987) and methyl jasmonate (Blechert et al. 1995).

An antitumorigenic agent known as noscapine accumulates in roots. However, its concentration was found higher in the shoot latex of opium poppy (Ye et al. 1998). However, due to the requirement for cell type-specific specialization, noscapine and morphine are not reported in cell suspension cultures of opium poppy.

26.4.2 Hairy Root Cultures and Metabolic Engineering in Opium Poppy and California Poppy

Many attempts have been made by various groups to exploit the P. *somniferum* as alternative source for alkaloid production in laboratory under in vitro conditions. However, very low benzylisoquinoline alkaloids were detected in cultures (Kamo et al. 1982; Schuchmann and Wellmann 1983; Yoshikawa and Furuya 1985; Yoshimatsu and Shimomura 1992). Using gene gun bombardment of cell suspension with DNA-coated microcarriers, attempts have been made to understand the mechanism of gene regulation of benzylisoquinoline alkaloids in opium poppy and California poppy. However, using DNA coated with microcarriers has its own disadvantages as several other genes may also be activated due to wound signaling which may lead to misleading results. Hence, investigations on the biosynthesis of alkaloids in *Papaver* species were mainly focused on hairy root cultures as more appropriate model.

Somatic embryos, as potent biological material for production of morphinan alkaloids and papaverine, were also evaluated (Laurain-Mattar et al. 1999). Results opened vista to attend production of these morphinan alkaloids through hairy root cultures. Hairy root cultures were initiated from hypocotyl of P. *somniferum* by using A. *rhizogenes* strain LBA 9402 (Le Flem-Bonhomme et al. 2004). They detected presence of morphine, codeine, and sanguinarine in 1-month-old hairy root cultures. Total content of alkaloid was reported very high in hairy roots than non-transformed roots cultures. Codeine content was detected to have threefold increase in hairy roots cultures. However, sanguinarine was detected only in hairy roots and not in non-transformed roots; perhaps production of these alkaloids is linked with stress-induced response. Cline and Coscia (1988) reported accumulation

of sanguinarine in cell-suspension culture of *Paper bracteatum*. Though sanguinarine accumulates constitutively in *Papaver somniferum* roots, after treatment with fungal elicitors, it was also detected in cell suspension cultures as well (Facchini et al. 1996a).

Park and Facchini (2000a) developed efficient transformation system in both opium poppy and California poppy by using *Agrobacterium rhizogenes*. Opium poppy seedlings showed high percentage of formation of hairy roots, whereas wounded embryogenic callus was found more responsive than seedlings in case of California poppy. Histologically putative hairy roots were nearly identical to non-transformed roots with exception of increase in number and epidermal cell arrangements.

However, unlike other reports, this investigation revealed that concentration of morphine, noscapine, sanguinarine in hairy roots of opium poppy, and sanguinarine in transformed roots of California poppy was reported at par with natural roots.

Larkin et al. (2007) revealed that overexpression of codeinone reductase gene (*CodR*) which is involved in morphinan alkaloid pathway in transgenic *P. somniferum* resulted in marked increase in production of morphinan alkaloids. Above tenfold greater level of CodR transcript in transgenic leaves was detected as compared to non-transgenic plants. The role of various genes involved in metabolic regulations of alkaloids and its possible metabolites engineering applications can be validated by suppression of CodR with RNAi technology in opium poppy which resulted in accumulation of (S)-reticuline in transgenic plants. It happened at the expense of morphine, thebaine, and codeine (Allen et al. 2004). Studies revealed the limitation of metabolic engineering of alkaloid pathways in terms of the availability of cloned genes encoding biosynthetic enzymes and a recalcitrancy of many medicinal plants species in vitro. Hairy roots culture has been exploited for genetic modification of metabolic pathways (Chavadej et al. 1994).

Yun et al. 1992 attempted metabolic engineering on plant alkaloid biosynthesis in *Atropa belladonna* which accumulates hyoscyamine. Interestingly, on transformation with the gene encoding hyoscyamine 6 β-hydroxylase (H6H) from *Hyoscyamus muticus*, *the transgenic roots of Atropa belladonna* were detected with elevated levels of the H6H reaction product scopolamine which was normally not present in non-transformed roots.

Genes encoding benzylisoquinoline alkaloid biosynthetic enzymes were reported by Facchini (2001). Side by side other workers have developed efficient transformation protocols of opium poppy and California poppy (Park and Facchini 2000a, b; Belny et al. 1997). With such advancement of technology, it is now possible to undertake metabolic engineering of benzylisoquinoline alkaloid pathways in plants.

Sang-Un Park et al. (2003) achieved the alternation in benzophenanthridine alkaloid accumulation in transgenic roots of E. *californica* through the modulation of berberine bridge enzyme (BBE) activity. They reported higher levels of berberine bridge enzyme mRNA in transgenic of *Papaver somniferum* roots expressing *BBE* L. Higher contents of proteins, enzyme activity, and benzophenanthridine alkaloids

were noticed. Contrary to this roots transformed with an antisense, berberine bridge enzyme construct from California poppy had lower levels of berberine bridge enzyme mRNA and enzyme activity and low benzophenanthridine alkaloid accumulation as compare to the controls. Further studies showed that cellular pools of certain amino acids were also affected vice versa, thus making California poopy and opium poppy an efficient model for studying metabolic engineering manipulation.

Facchini and Park 2003 worked on aspects of developmental and inducible accumulation of gene transcripts, involved in alkaloid biosynthesis in *Papaver somniferum* L. The basis of study depends on the earlier reports where it was clearly stated that alkaloid biosynthesis in opium poppy is controlled by many developmental and inducible factors (Facchini 2001).

Involvement of specific types of cell alkaloid production is not reported, but it is well understood that opium poppy alkaloids accumulate in laticifers that accompany vascular tissues. Morphine is found in all plant organs, whereas site of accumulation of sanguinarine is root system. Their presence has been detected in developing seedling (Facchini et al. 1996a; Huang and Kutchan 2000). As discussed earlier the presence of sanguinarine is detected in response to fungal elicitors in cell cultures of opium poppy (Facchini et al. 1996b).

Facchini and Park 2003 reached to similar conclusion. A comparative study was conducted on accumulation of gene transcripts encoding eight alkaloid biosynthetic enzymes in opium poppy. A transcript level was recorded with manyfold increase in developing seedlings and high in stems and flower buds as well, whereas it was noticed more variable in roots and leaves of mature plants. Accept for COR, accumulation of other transcript showed a marked induction in response to elicitor treatment or wounding of cultured cells. Specific gene transcript levels are often correlated with the accumulation of morphine or sanguinarine. Result of study reveals that there is some degree of coordination that exists in the developmental and inducible regulation of alkaloid biosynthetic genes in opium poppy. This information helps in understanding of production and accumulation of alkaloids in various organs at different developments stages, and a curtain raises for metabolic engineering of alkaloids in *Papaver* species.

26.4.3 Alkaloid Metabolism in Planta *in* Papaver *Species*

With *Agrobacterium*-mediated genetic transformation, it is possible to modulate the alkaloid metabolism in planta in *Papaver* spp. Thereby, the understanding on alkaloid biosynthesis, enzymatic level for tissue-specific regulations at plant level, may be improved. In an attempt seedling, explants were transformed through *Agrobacterium tumefaciens* containing antisense bbe coding region of P. *somniferum*. Sterilized seed of opium poppy was inoculated with *Agrobacterium tumefaciens* containing antisense bbe coding region followed by selection of transformants, and raising of full plant population through embryogenic callus cultures was achieved (Susanne et al. 2004). Second generation of self-population was taken for alkaloid analysis.

The alkaloid content in latex and roots was determined which revealed marked increase in concentration of several pathway intermediates from all biosynthetic branches, e.g., reticuline, laudanine, laudanosine, *dehydroreticuline*, salutaridine, and (*S)*-scoulerine. A marked alteration of ratio of morphinan and tetrahydro benzylisoquinoline alkaloids in latex was recoded. However, no change was detected in benzophenanthridine alkaloids in roots. Interestingly, altered alkaloid profile was heritable at least to the next generation. No change in benzophenanthridine alkaloids in roots suggested the role of some other enzymes in the control of sanguinarine biosynthesis in opium poppy. Involvement of enzymes downstream of BBE in the regulation of sanguinarine biosynthesis in opium poppy root would be a possible explanation for the unchanged sanguinarine levels in antisense bbe transgenic plants.

Morphine biosynthesis occurs in the aerial parts of the plants as well as in the roots. The localization of the enzymes leading specifically to the benzophenanthridine alkaloids is not yet known. Transcript bbe, involved in the first step of the biosynthesis, has been found in root, stem, and leaf (Huang and Kutchan 2000). However, detection of bbe transcripts in some aerial organs of poppy (Huang and Kutchan 2000; Facchini and Park 2003) was an unusual phenomenon as sanguinarine was reported to present only in roots (Facchini and Luca 1995). The possible root-specific expression of genes encoding enzymes downstream of BBE could explain the lack of sanguinarine in the aerial parts of opium poppy. Furthermore, in developing seeding, sanguinarine starts to increase 5 days after germination, although bbe mRNAs could be observed at earlier stages of development (Facchini et al. 1996a, b; Huang and Kutchan 2000).

26.4.4 Hairy Root Cultures and Metabolic Engineering in Papaver bracteatum *(Iranian Poppy)*

Genetically engineered hairy roots of *Papaver bracteatum*, transformed with codeinone reductase (CodR) gene, have been assessed for metabolic and molecular regulation of morphinan alkaloids (Sharafi et al. 2013a, b). Before that Larkin et al. (2007) studied and reported up to ten folds higher levels of codeinone reductase transcripts in comparison to the normal plants of *P. somniferum*.

Papaver bracteatum (Iranian poppy) is considered to be the alternative source of morphine and codeine. Thebaine, which is a precursor of codeine, may contribute in high-content codeine. Thebaine can be converted to codeine and then morphine in presence of CodR. However, despite having high content of thebaine, production of codeine and morphine was very low because of low activity of CodR enzyme in *Papaver bracteatum*.

Sharafi et al. (2013a, b) found that hairy roots *of Papaver bracteatum* without having CodR contained only thebaine and a very low amount of codeine. However, in transgenic hairy root lines having CodR, a relatively high level of codeine (>160%) and morphine (>60%) was detected. Overexpression of the CodR gene in transgenic hairy roots is the root cause of decrease in the amount of thebaine and an

increase in codeine and morphine contents in the transgenic hairy roots. Interestingly, Larkin et al. (2007) reported increase of thebaine from 58% to 75% in that dried capsule of transgenic *Papaver somniferum* due to CodR overexpression.

In another independent study, the role of salutaridinol 7-*o*-acetyltransferase (*SalAT*) was investigated by generating *Agrobacterium*-mediated hairy roots. *SalAT* is a key gene in morphinan alkaloids biosynthesis pathway. A r*hizogenes* strain containing *Sal*AT cDNA sequence, derived from P. *somniferum* L, was used for transformation of *Papaver bracteatum* seedlings. It was observed that there was an increase of up to 154% in transcript level of *Sal*AT in transgenic roots and 128% in hairy roots without having *Sal*AT. HPLC analysis showed marked increase in thebaine (1.28 % dry weight), codeine (0.02 % dry weight), and morphine (0.03 % dry weight) compared to those of hairy roots without *SalAT* over expression. Study suggests that *P. bracteatum* hairy roots expressing the *SalAT* gene could be potentially used for the production of valuable morphinan alkaloids. Moreover, 40% greater total alkaloids were detected in transgenic SalAT overexpressing line.

Above studies proved that besides *Papaver somniferum*, other paper species like *Papaver bracteatum* may also be exploited to production of morphinan alkaloids by metabolic engineering and molecular regulations of genes.

26.5 Insilco Analysis of Plant Secondary Metabolites

Several plant secondary metabolites possess powerful pharmaceutical and biotechnological properties. These molecules are not only important for research but also very useful in industry too. Secondary plant metabolites are available in nature but in low concentration. To address this issue, we have solutions in the form of metabolic engineering; it could address issues related to low productivity and availability of plant alkaloid issue (Diamond and Penix 2016).

Alkaloids are nitrogen-containing natural compounds found reported in 20% of plants species. *Papaver somniferum* (opium poppy) and *Catharanthus roseus* (Madagascar periwinkle) are the two most important plants where maximum research was carried out on the production and metabolism of alkaloids. In opium poppy metabolic engineering has been used in the production of alkaloids (gene *Cyp80B3*) (Fechinni and Luca 2008).

26.5.1 Development of Analgesic Drugs Based on Morphine

Morphine and codeine are natural alkaloids found in the latex of opium poppy (*Papaver somniferum*) whose pharmacological effects have been known since antiquity. With the aid of modern chemistry, a lot of variants have been produced, namely, heroin which is more hydrophobic in nature as compared to its natural compound and can traverse across the blood-brain barrier readily. Morphine and

codeine have been applied in medicines as analgesics, drugs that relieve severe pain with side effects like addiction, passivity, and euphoria. Drug developers have long sought to make a drug that is analgesic but with no side effects of addiction. Synthetic variants of morphine are obtained by simplifying its structure either by inferring the minimal pharmacophore required for activity or by dissecting the part that relieves pain away from the part that causes addiction. Levorphanol, benzomorphan, cyclazocine, pentazocine, demerol, and many more are such derivatives. Etorphine and buprenorphine are more powerful analgesic that their parent morphine (Lesk 2000).

A summary and comprehensive up-to-date overview of the tools and databases that are currently available for analyzing biosynthetic pathways, combining genomic and metabonomics data as provided by the newly established Secondary Metabolite Bioinformatics Portal (SMBP), are enlisted in Table 26.2 (Weber and Kim 2016).

26.5.2 The Secondary Metabolite Bioinformatics Portal

The Secondary Metabolite Bioinformatics Portal (SMBP at http://www.secondarymetabolites.org) is a web portal introduced to provide links and a one-stop catalogue to databases and tools available to mine, recognize, and characterize natural product biosynthesis pathways. Different strategies are engaged by genomic mining tools commonly used to sense secondary metabolites; analyzing biosynthetic pathways, combining genomic and metabolomic data, and generating genome-scale metabolic models of the secondary metabolite producers are summarized in the following table (Table 26.2).

Table 26.2 Tools for mining of secondary metabolites gene clusters

S. No	Name of the tool	URL
1.	2metDB	http://secmetdb.sourceforge.net/
2.	antiSMASH	http://antismash.secondarymetabolites.org
3.	BAGEL	http://bagel2.molgenrug.nl/
4.	CLUSEAN	https://bitbucket.org/tilmweber/clusean
5.	ClusterFinder	https://github.com/petercim/ClusterFinder
6.	eSNaPD	http://esnapd2.rockefeller.edu/
7.	EvoMining	http://148.247.230.39/newevomining/new/evomining_web/index.html
8.	GNP/genome search	http://magarveylab.ca/gnp/#!/genome
9.	GNP/PRISM	http://magarveylab.ca/prism
10.	MIDDAS-M	http://133.242.13.217/MIDDAS-M/
11.	MIPS-CG	http://www.fung-metb.net/
12.	NaPDoS	http://napdos.ucsd.edu/
13.	SMURF	http://jcvi.org/smurf/index.php

Table 26.3 Tools used for Metabonomic Analysis

S. No	Name of the tool	URL/Link
1.	RiPPquest	http://cyclo.ucsd.edu
2.	Pep2Path	http://pep2path.sourceforge.net
3.	NRPquest	http://cyclo.ucsd.edu
4.	GNP/iSNAP	http://magarveylab.ca/gnp/
5.	GNPS	http://gnps.ucsd.edu/
6.	Cycloquest	http://cyclo.ucsd.edu

The SMBP is likely to enable users to compare and contrast tools for their utilities and create further contributions to the ground of secondary metabolites. Even though there are significant advancements made on computational approaches used to identify and characterize secondary metabolites, still some challenges have to be addressed in the near future. One among them is the incomplete prediction of the core scaffold structure of a compound, because the biochemical knowledge on these systems is not yet implemented in the software or the suitable biochemical knowledge is not available to be the basis for the functioning of new computational algorithms.

An additional unsolved problem is currently incorrect prediction of gene cluster borders. The most commonly used genome-mining software antiSMASH is affected by this concern. Many more problems are reported conserving different metabolites, in particular, which need to be solved in the upcoming future (Table 26.3).

26.6 Economic Importance

Economically important plant parts are its fruit “capsule” and seeds. Opium poppy plant has immense economic importance.

26.6.1 Seeds

Seeds are used as important culinary item, and a small part is exported mainly to other countries in Asia and Africa. Apomorphine hydrochloride can be used as an emetic in small quantities, and its anti-Parkinson efficacy has been recognized and tested (Bernath 1998). Seeds are widely used in the food industry, and seed oil is considered to be an important raw material in the manufacture of paints and varnishes (Balbi 1960). There are many examples in the literature, which detail the industrial utilization of poppy seeds and oil. The seeds are commonly used for decoration of bakery products (Benk 1987)). Seed oil has been found to have properties similar to sunflower and olive oil. The seeds contain mainly two types of fatty acids oleic acid, a mono-unsaturated fatty acid and linoleic acids. Oleic acid helps in cholesterol management in the body. Seeds are also rich in aspartic and glutamic acids, arginine, and methionine (Singh et al. 1999). The seeds are excellent source of B-complex. They are also considered to be having aphrodisiac and constipating

properties. Poppy seeds have antioxidant, disease-preventing, and health-promoting properties because of its chemical properties. Seed husk is a good source of dietary fiber thereby easing constipation problem (Nergiz and Otles 1994), besides being reported as ant carcinogenic (Aruna and Shivaramakrishnan 1992).

26.6.2 Fruit

Fruit of opium poppy is called capsule. Opium – a milky exudate – is extracted from it. In India, latex is collected by incising capsule. Bulk of latex contains 70% morphine. It contains about 70% of the total morphine; morphine yield is maximum in terminal capsule in comparison with lateral ones. The opium poppy plants produce over 40 alkaloids, which may be grouped in 5 categories. The major two groups are:

(a) Phenanthrene alkaloid-comprising thebaine, codeine, and morphine as major constituents
(b) Benzylisoquinoline alkaloids consisting of important alkaloids as papaverine, narcotine, and narceine (cf. Husain and Sharma 1983)

These alkaloids can be extracted from the plants in two ways:

(a) From the latex (opium), which is a rich and convenient source of all alkaloids
(b) From concentrated poppy straw (CPS), which include capsule and three-fourths part of the peduncle

26.7 Opium in Illicit Use

This plant is cultivated in different parts of the world for illicit trade such as "Golden Triangle" (Thailand, Burma, and Laos) and "Golden Crescent" (Afghanistan, Pakistan, and Iran). According to White and Raymer (1985), world opium poppy cultivation covered 270,000–300,000 ha. However, the official statistical reports of the INCB, Vienna, for 1989–1993 stated only 37,000–56,000 ha under opium poppy cultivation for opium production. Regular use can result in the rapid development of tolerance to these effects, thereby having a high potential of addiction and intense physical dependence. Thus development of withdrawal symptoms upon cessation is generally observed. Potentially serious side effects include a severe respiratory distress and low blood pressure. Other common side effects, which may be encountered while using morphine, can be drowsiness, stomach pain and cramps, dry mouth, headache, nervousness, mood changes, difficulty in urinating, purple skin, changes in heartbeat, agitation, hallucinations, vomiting, weakness, seizures, chest pain, irregular menstruation, etc. It still remains one of the most frequently involved drugs along with heroin, oxycodone, methadone, hydrocodone, fentanyl, alprazolam, diazepam, cocaine, and methamphetamine in overdose deaths in the United States (Warner et al. 2016). It has high risk for addiction, due to its extreme effects in the

brain. Heroin overdoses can cause suppression of breathing, leading to hypoxia (Coffin et al. 2010). Long-term usage of heroin leads to tolerance and dependence, resulting in withdrawal symptoms. The abuse is also associated with many serious health conditions, like spontaneous abortion, hepatitis and HIV, infection of the heart lining and valves, gastrointestinal cramping, and pneumonia (www.drugabuse.gov/publications/drugfacts/heroin).

26.8 Conclusion

Opium poppy plant has significant medicinal importance due to its alkaloids. Among the 40 reported alkaloids, morphine is the principal alkaloid in opium with 9–17% of total alkaloids present in dried latex. Morphine is popular as analgesic and narcotic because of its effects on the central nervous system and on smooth muscle. It is widely used to relieve pain. On the other hand, it has been used as a narcotic drug too. Due to its illicit use, the cultivation of plant is quite restricted and is under control of the government of that country. Poppy production is strictly monitored and controlled by the International Narcotic Control Board (INCB) having powers to increase or decrease supply depending on global production (Hagel et al. 2007). India is the only country where extraction of opium is allowed by lancing of capsule by United Nations, while in other countries, it is from dried capsules only. Monitoring of crop is done throughout the growing season, and proper steps are also taken to ensure suitable treatment of harvest residue. Opium cultivation is facing so many challenges firstly by biotic and abiotic factors. Biotic factors include fungal, bacterial, viral diseases, insect nematodes, etc. Abiotic factors include excessive rainfall, temp, and mineral deficiencies. All these factors lead to yield losses, both seed and alkaloid yield. In addition to that cultivation is being done in restricted areas, that is, only those areas having government license of production. Demand of opium poppy is increasing; however, its supply is quite low. Due to so many regulations, farmers are reluctant in growing plants. Therefore, two strategies could be followed, firstly cultivation of poppy plant without morphine and other alkaloids as chief constituents. But that would incur big loss to pharma industry. Second strategy could be large-scale cultivation to meet out industry demand under strict vigilance of the narcotics department of respective countries.

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Chapter 27
Advances in Genetic Engineering of *Ajuga* Species

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Abstract *Ajuga* genus is among one of the more than 250 genera of Labiatae, cosmopolitan in distribution, and comprises of more than 70 species with the remarkable therapeutic importance. Many species of *Ajuga* including *A. bracteosa*, *A. reptans*, *A. Chamaepitys*, etc. have been used in the traditional system of medicine and are also in use for making formulations in modern medicines. *Ajuga* species offer anticancer, antibacterial, antifungal, antidiabetic, anabolic, antileishmanial, anti-inflammatory, hepatoprotective, immunomodulatory, antimalarial, astringent, anthelmintic, and diuretic properties and are used in the treatment of rheumatism, palsy, and gout. They hold a large number of secondary metabolites which are active principles to combat the foresaid diseases including phytoecdysteroids, withanolides, iridoid glycosides, neo-clerodane di- and triterpenoids, sterols, and a large range of flavonoid and phenolic compounds. Some of the species of *Ajuga* are genetically modified for some of these compounds including *A. bracteosa*, *A. reptans*, *A. multiflora*, etc. Latest development made in the exploration of these compounds is yet dealing with the transformation of *rol* genes and some stress and feeding experiments. We precisely discuss here the details of biotechnological progress that has been made in *Ajuga* species so far.

Keywords Ajuga · Ajuga bracteosa · Ajuga reptans · Secondary metabolites · Phytoecdysteroids · Withanolides · Genetic engineering

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27.1 Medicinal Plants and Their Importance

Therapeutically essential plants are found in abundance in the world. The knowledge of medicinal plants is acquired for multiple reasons like treating human disease and domesticated animals' afflictions, edit assurance, water purification, etc. Though, a revolution has been brought by relying on modern synthetic drugs in order to control various ailments, still more than 80% of the world population that belong to the developing and underdeveloped countries depends upon the folk medicine that is a derivative of plants (Kamboj 2000; Vohra and Kaur 2011). Therapeutic plants have been used in popular traditional medication framework, e.g., Greco-Arab, Ayurveda, Sindha, etc. have utilized the therapeutic plants (Parjapati et al. 2003). Ease of access and acceptance in the public arena are the real purposes behind the mass utilization of natural remedies over the globe particularly in Asian zone (Pal and Shukla 2003).

It is roughly estimated that ~70,000 plant species have been utilized at some point, for the therapeutic medications (Kumar et al. 2010). Common types of preparatory strategies utilize powder, poultice, plant parts, juice, decoction, and implantation for cures made of therapeutic plants (Uprety et al. 2012).

27.2 Genus *Ajuga*

The genus *Ajuga* is from the family Lamiaceae that is commonly referred to as "bugleweed." It includes over 300 species in worldwide appropriation (Atay et al. 2016). *Ajuga* species plays a central role in areas relating pharmacology and horticulture. Some *Ajuga* species are developed solely for ornamental purposes due to their alluring leaves and blooms. Pharmacological studies uncover that *Ajuga* @@ is hostile to destructive, slightly mitigating, antimalarial, antimicrobial, antiarthritic, antitussive, hypoglycemic, and insecticidal properties (Atay et al. 2016; Kayani et al. 2016a). Furthermore, a few other *Ajuga* species have discovered use in conventional pharmaceuticals especially in the treatment of diabetes, liver issues, intestinal problems, pneumonia, skin diseases, toothache, tuberculosis, and wound recuperating (Park et al. 2017). *Ajuga* plants are generally gathered for their characteristic therapeutic potential for phytochemical and pharmacological investigations. A few include *Ajuga boninsimae*, *A. bracteosa*, *A. ciliate*, *A. genevensis*, *A. incisa*, *A. makinoi*, *A. multiflora*, *A. pyramidalis*, *A. shikotanensis*, *A. reptans*, and *A. vestita* are currently incorporated into the imperiled plant list (Inomata et al. 2013). Phytochemical studies suggest that *Ajuga* contains phytoecdysteroids, withanolides, neo-clerodane diterpenoids, iridoids, carotenoids, unsaturated fats, flavonoids, iridoids, phenylethanoid glycosides, steroids, sphingolipids, tocopherols, triglycerides, triterpenoids, etc. (Coll and Tandrón 2008; Kayani et al. 2014).

Regarding propagation, Bugleweed regularly propagates utilizing rhizomes and established cuttings or seeds. The commonly used strategy for proliferating *Ajuga*

is regularly thwarted especially by poor seed germination, moderate vegetative duplication rate, and a lack of solid plant material (Park et al. 2017). In vitro plant culture has picked up significance as an optional method for the preservation, mass clonal spread, and change of uncommon plants. Further, this method can likewise be utilized to deliver bioactive compounds that are available in the *Ajuga* plant. Micropropagation is a viable strategy of abiogenetic propagation, attainable in a brief time span and constrained space (Park et al. 2017). Moreover, this procedure has been utilized in different biotechnological applications, for example, in cryopreservation, in hereditary building, and also in the creation of disease-free plants, auxiliary metabolites, and for somaclonal varieties.

27.3 *Ajuga* Species

Ajuga is one of the 266 genera of the Lamiaceae family and comprises of almost 301 species (Israili and Lyoussi 2009). *Ajuga* is known to contain around more than 40 species, and some very important ones are listed below.

27.3.1 A. turkestanica

A. turkestanica is a therapeutic perpetual plant that is native to Uzbekistan and is found to be rich in phytoecdysteroids (Abdukadirov et al. 2004). As a normal practice, the elevated aerial segment of the plant is dried and after that saturated with heated water, and the stock is ingested to ease serious ailments, for example, coronary illness and muscular and stomach problems (Mamatkhanov et al. 1998). Air-dried leaves of *A. turkestanica* accumulate several phytoecdysteroids, in roots as well as in leaves (Lev et al. 1990). The phytoecdysteroid content of *A. turkestanica* comprises 20-hydroxyecdysone (20-HE), ajugasterone B, cyasterone 22-acetate, turkesterone, ecdysone 2,3-monoacetonide, ajugalactone, and ecdysone along with neo-clerodane diterpenes and iridoids (Grace et al. 2008; Ramazanov 2005). *A. turkestanica* have also been examined to encompass 0.02% 20-HE, and roots have been testified to possess 0.052% turkesterone and 0.045% 20-HE (Lev et al. 1990). Advances in methodologies of plant tissue culture for phytoecdysteroid generation in *A. turkestanica* were researched long ago, including the advancement of callus (Lev et al. 1990). *A. turkestanica* hairy root cultures have been developed and have been found appropriate for producing the phytochemicals of interest by making use of the plant's innate production mechanism (Cheng et al. 2008).

27.3.2 A. reptans

Like other *Ajuga* plants, *Ajuga reptans* has also been reported with many ecdysteroids out of which seven are most important and these include ajugalactone, cyasterone, sengosterone, 29-norsengosterone, 29-norcyasterone, 20-HE, and polypodine B (Tomás et al. 1993). Most interestingly the variation of phytoecdysteroid concentration in different tissues of *A. reptans* plant, both from in vitro and in vivo cultures, makes it a rather more special plant for investigation (Tomás et al. 1992).

27.3.3 A. multiflora

A. multiflora Bunge is a perennial herb about 8–13 cm tall, very broadly distributed in Korea and Russia. It is developed as a decorative groundcover in Korea and is used in a customary pharmaceutical for the treatment of high-grade fever. It is accounted for to contain many phytoecdysteroids (De-fu et al. 2002). The plant is ordinarily propagated by the division of rhizomes. In vitro engendering strategy is being utilized generally for extensive scale creation of many plant species. Up till now, few reports are accessible on in vitro engendering of this particular *Ajuga* species (Sivanesan et al. 2011).

27.3.4 A. bracteosa

Among the *Ajuga* species, *Ajuga bracteosa* Wall. ex. Benth is the most widely used plant in folk medicine. It is a perennial herb that is found at an altitude of 1300 m and grows wild across Kashmir to Afghanistan, Bhutan, Nepal, Himalaya, Malaysia, and China (Chandel and Bagai 2011; Singh et al. 2006). The plant is known locally as "Kori Booti" (Hindko, Punjabi) (Chopra and Nayar 1956), "Jaan-e-Adam" (Urdu, Kashmiri) (Hamayun et al. 2006), and "Nilkanthi" (Sanskrit). Morphologically, *A. bracteosa* is branched diffusely, compacted, prostrate, evergreen and often stoloniferous or decumbent (Fig. 27.1). Its height varies from 10 to 30 cm (Hamayun et al. 2006), and it flowers from September to November. In *A. bracteosa*, the upper leaves are sessile, while the lower leaves are petiolate (Upadhyay et al. 2012). The hermaphrodite flowers of *A. bracteosa* possess a variety of colors, including white, pink, or purplish-violet (Pal and Pawar 2011). Being found inside the axillary whorls, the flowers are suffused characteristically at the lower epidermis and appear as spikes (Hamayun et al. 2006). Calyx is pileus and villous, 4 mm in length, ovate to lanceolate and teeth half the length of the tube. Juvenile corolla is 8–10 mm in length, and lilac or pale blue in color; upper lips flat and short; middle lip largest, dilated, and 2-lobed; and lower lips widening and

Fig. 27.1 *Ajuga bracteosa* general plant morphology

3-lobed. Anthers are 2-celled, whereas stamens are didynamous, of which the lower pair is elongated, exerted, ascending, or embodied. Style is 2-fid; ovary is 4-lobed and decreased in size, the lobes being equal (Upadhyay et al. 2012).

27.4 Ethnobotanical and Ethnopharmacological Applications

A wide range of applications have been reported for *Ajuga.* The antileishmanial, antitrypanosomal, antitumor, antimicrobial, anti-inflammatory, immunomodulatory, and hepatoprotective properties of many species of this genus have been reported to date (Ahmed and Chaudhary 2009). Anopheline and culicine mosquitoes have been actively fought by certain species of *Ajuga* (Pavela 2008). It has been reported by Pal and Pawar (2011) that *A. bracteosa* has found extensive use in ethnomedicine rendered to its medicinal properties, comprising of diuretic, hypoglycemic, antifungal, anthelmintic, astringent, antimycobacterial, and anti-inflammatory roles. *A. bracteosa* leaves possess diuretic and stimulant properties. Amenorrhea, palsy, rheumatism, and gout are being treated with the whole plant (Kirtikar and Basu 1918) which lies in conformity with Ayurvedic medicinal recommendations (Kaithwas et al. 2012). A report by Chauhan (1999) revealed that the blood-purifying properties of the plant are attributed to the extract of its leaves, and powdered material is effective against boils and burns. *A. bracteosa* has traditionally been used in the treatment of phlegm and fever (Chauhan 1999). Besides this, Pal and Pawar

(2011) also described the use of *A. bracteosa* in the remedy of gout, and malaria is mentioned, and the plant has been regarded as an alternate of cinchona. In addition to this, other plants of the genus like *A. reptans* and *A. Turkestanica* have also been used because of their known therapeutic potential. Many important metabolites have been identified in roots, leaves, and other plant parts (Cheng et al. 2008; Tomás et al. 1993). The cures for constipation, hypertension, measles, sore throats, stomach hyperacidity, acne, ear infections, jaundice, headaches, and pimples are accredited to the cooling and blood-purifying properties of the leaves of *A. bracteosa* (Barkatullah et al. 2009; Ibrar et al. 2007; Qureshi et al. 2009).

27.5 Medicinal Importance of *Ajuga* Species

Ajuga bracteosa has found tremendous medicinal importance in ethnobotany, and due to this fact, many researchers around the globe focused for its exploration, paving ways for modern active drugs. Chandel and Bagai (2011) reported that the in vivo blood schizontocidal effect (250–750 mg/kg per day) and antiplasmodial efficiency in vitro (IC_{50} 10 μg/mL) of *A. bracteosa* are highly significant. In Swiss albino mice, after studying aerial parts of *A. bracteosa*, Pal and Pawar (2011) described dose-dependent and significant analgesic effects of aqueous and chloroform extracts, proposing its mediation through opioid receptors. It has been reported by Gautam et al. (2011) that considerable anti-inflammatory activity has been shown by 70% ethanolic extract of *A. bracteosa* being mediated by inhibiting COX-1 and COX-2 enzymes. Out of the five active components that have been isolated from this fraction, viz., lupulin A, ajugarin I, 6-deoxyharpagide, withaferin A, and reptoside, the last one plays a significant role in inhibiting COX-2 enzyme. Moreover, after conducting investigation on chronic immunological arthritis in albino rats via 70% ethanolic extract of *A. bracteosa*, Kaithwas et al. (2012) found significant quantity of dose-dependent inhibitory effects alongside therapeutics much better than standard drug aspirin. The anti-inflammatory potential of *A. bracteosa* have been studied by Hsieh et al. (2011), and they reported that chloroform extract (ABCE) depicts its significance in halting the production of TNF-α and NO, repressed NFκB activation, and consequently reduces nuclear p50 and p65 protein levels. Reduction of plasma-aminotransferase activity in mice animal models further allowed the extract to cushion the liver from injury by alleviating CCl_4-based liver fibrosis through reduced activation of macrophage and curtailing the plasma-based aminotransferase activity in mice models. Thus, the studies mentioned above play supportive role in rheumatism as well as in inflammatory diseases.

Significant DPPH-radical scavenging activity (78%) and antimicrobial activity (MIC 0.33–12.2 mg/mL) at 1.0 mg/mL have been exhibited by the essential oils extracted from *A. bracteosa* (Mothana et al. 2012). Acetone extract of *A. bracteosa* exhibited substantial effect against *Escherichia coli* and methanolic extract administered activity against *Staphylococcus aureus* (Vohra and Kaur 2011). Analgesic properties of aerial regions of *A. bracteosa* have been investigated by Pal and Pawar

(2011) in mice with tail immersion test and acetic acid-induced writhing test, and they reported dose-dependent and significant analgesic effects (200 and 400 mg/kg) from water and chloroform extracts.

27.6 Important Secondary Metabolites

Ajuga isolates a large number of compounds, consisting of di- and triterpenes, withanolides, neo-clerodane-diterpenes, anthocyanidin-glucosides, phytoecdysteroids, iridoid glycosides, triglycerides, and flavonoids. These compounds are attributed to be analgesic, anabolic, anti-inflammatory, antiestrogenic, antimicrobial, antileukemic, antihypertensive, antioxidant, antimycobacterial, antimalarial, cytotoxic, antipyretic, hypoglycemic, cardiotonic and vasorelaxing, antifeedant etc. (Israili and Lyoussi 2009).

27.6.1 Phytoecdysteroids

Ecdysteroids are regarded as natural polyhydroxy steroids and steroidal molting hormones of arthropods. Butenandt and Karlson (1954) detected the first ever ecdysteroid (ecdysone) in 1965, immediately followed by Nakanishi et al. (1966) who carried out isolation of ponasterones A, B, and C from *Podocarpus nakaii*, and 20-hydroxyecdysone was isolated from *Podocarpus elatus* (Galbraith and Horn 1966). Isolation of phytoecdysteroids was accomplished from several plant species in the preliminary years, and soon their widespread nature in plants became evident. (Dinan 2001; Dinan et al. 2001, 2009; Lafont et al. 2010). Saatov et al. (1994) reported that so far, almost 14 species of *Ajuga* were found containing ecdysteroids named *A. austral*, *A. chamaepitys*, *A. chia*, *A. decumbens*, *A. iva*, *A. incisa*, *A. nipponensis*, *A. japonica*, *A. multiflora*, *A. remota*, *A. reptans*, *A. turkestanica*, and *A. ciliata*. Except the species of *Ajuga*, a variety of plant species were explored for ecdysteroids production using biotechnological applications, including *Achyranthes bidentata*, *A. aspera* (Amaranthaceae), *Chenopodium album* (Chenopodiaceae), *Cyanotis arachnoidea* (Commelinaceae), *Lychnis floscuculi* (Caryophyllaceae), *Pfaffia glomerata*, *P. tuberosa* (Amaranthaceae), *Polypodium vulgare* (Polypodiaceae), *Pteridium aquilinum* (Pteridaceae), *Rhaponticum carthamoides*, *Serratula tinctoria* (Asteraceae), *Trianthema portulacastrum* (Aizoaceae), *Vitex glabrata*, V. *negundo*, and *V. trifolia* (Lamiaceae) (Thiem et al. 2017). Many phytoecdysteroids have been isolated from various *Ajuga* species (Table 27.1, Fig. 27.2). Among them, the most studied phytoecdysteroids was 20-hydroxyecdysone (Kayani et al. 2014). Many of the derivatives like 2-*O*-acetyl-20-hydroxyecdysone, 3-*O*-acetyl-20-hydroxyecdysone, 3-*O*-acetyl-29-norcyasterone, and 3-*O*-acetyl-cyasterone are reported from various *Ajuga* species (Calcagno et al. 1995b), 22-dehydrocyasterone (Coll et al. 2007), and 3-epicyasterone, Cyasterone-22-*O*Ac, etc.

Table 27.1 Phytoecdysteroids reported from *Ajuga* species

Compound	Source plant	References
20-Hydroxyecdysone	*A. reptans*, *A. macrosperma*	Calcagno et al. (1995a), Castro et al. (2008), and Tomás et al. (1992)
Ajugalactone	*A. reptans*, *A. remota*, *A. chamaecistus*	Calcagno et al. (1995a), Kubo et al. (1983), and Tomás et al. (1992)
Cyasterone	*A. reptans*, *A. iva*, *A. decumbens*, *A. remota*, *A. chamaecistus*	Calcagno et al. (1995a), Imai et al. (1969), Kubo et al. (1983), Sabri et al. (1981), and Tomás et al. (1992)
Makisterone A	*A. iva*, *A. reptans*, *A. macrosperma*	Calcagno et al. (1995a), Castro et al. (2008), and Sabri et al. (1981)
Polypodine B	*A. reptans*	Calcagno et al. (1995a) and Tomás et al. (1992)
Sengosterone	*A. reptans*	Calcagno et al. (1995a) and Tomás et al. (1993)
Ajugasterone A, ajugasterone B	*A. decumbens*	Imai et al. (1969)
Ajugasterone C	*A. remota*, *A. reptans*, *A. chamaecistus*	Kubo et al. (1983)
29-Norsengosterone, 29-Norcyasterone	*A. reptans*	Calcagno et al. (1995a) and Tomás et al. (1992)
Ajugalide-E, 22-Acetylcasterone	*A. taiwanensis*	Chan et al. (2005)
Reptanslactone A, reptanslactone B, sendreisterone, 24-dehydroprecyasterone	*A. reptans*	Vanyolos et al. (2009)
Breviflorasterone	*A. reptans*, *A. macrosperma*	Castro et al. (2008) and Vanyolos et al. (2009)
Ajugacetalsterones C, ajugacetalsterones D, breviflorasterone	*A. macrosperma*	Castro et al. (2008)
22-dehydrocyasterone 2-glucoside, ajugacetalsterone A-B	*A. nipponensis*	Coll et al. (2007)
ajugacetalsterone C-D	*A. macrosperma*	Castro et al. (2008)
Ecdysterone	*A. iva*, *A. decumbens*	Imai et al. (1969) and Sabri et al. (1981)

Phytoecdysteroids are a source of plant chemical defense against nematodes and insects. Based on the ecdysteroid action, certain plant species have shown defense mechanisms against insects (Browning et al. 2007). The binding between the steroid-based hormone 20-hydroxyecdysone (20-HE) with the cognate nuclear-receptor, tends to trigger the focal development transitions, especially metamorphosis and molting within insects (Browning et al. 2007). Ecdysteroids are attributed to be the main cell differentiation regulators, reproduction, and metamorphosis

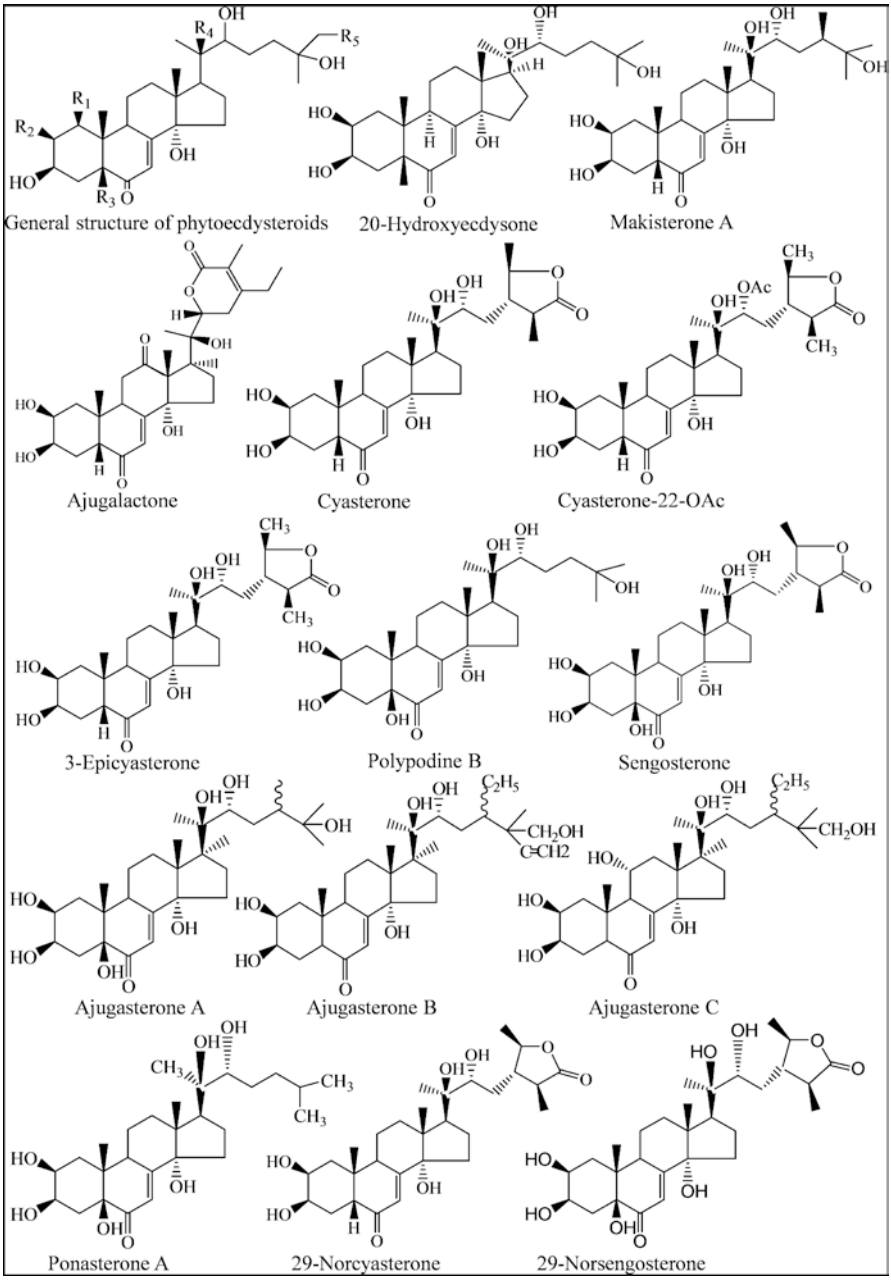

Fig. 27.2 Phytoecdysteroids reported from *Ajuga* species

(Browning et al. 2007). Phytoecdysteroids enhance the polyribosomal activity, thus biosynthesizing proteins, resulting in body mass enhancement (Syrov 1983). Protein-synthesizing processes in rats are increased by the administration of phytoecdysteroids (Otaka et al. 1969) ultimately improving body mass in rats via enhancement of the skeletal muscle and internal organ's mass (Syrov et al. 1996). This enhancement is associated with elevated hemoglobin content, total blood-protein concentration and the erythrocyte number in peripheral blood (Syrov et al. 1996). Kizelsztein et al. (2009) found that plasma insulin levels, body weight gain, glucose tolerance, and body fat mass decrease significantly by the treatment of 20-HE in mice model. Accounting for 115% increase in developing body mass, 20-HE is free of testosterone (Slama et al. 1996). Antidiabetic and anti-obesity effects of 20-HE have been depicted, and it was analyzed that 20-HE starts to illuminate its presumed cellular targets both in vivo and in vitro. Extract from *A. turkestanica* hairy roots comprising of turkesterone, 20-HE plus cyasterone in concentrations of 10 or 20 μg/mL in mouse skeletal cell line enhanced the synthesis of protein by 25.7% and 31.1% (Cheng et al. 2008). The effects of glutamate decarboxylase (Chaudhary et al. 1969; Lupien et al. 1969), alkaline phosphatase (Kholodova 1978), and acetylcholinesterase (Catalán et al. 1984) are elevated by ecdysteroids. In addition to residual nitrogen blood levels and urea levels which are lowered, kidney functioning is improved by phytoecdysteroids. The preparation of ecdysten for difficulties of eye in chronic glomerulonephritis patients is also reported (Saatov et al. 1999). Ecdysteroids are antihyperglycemic (Chen et al. 2006), neuroprotective (Wang et al. 2014), antibacterial, and antifungal (Ahmad et al. 1996). Using ecdysteroids, albuminuria is suppressed (Syrov and Khushbaktova 2000), lipid peroxidation is reduced (Kuzmenko et al. 2001), human lymphocytes are activated (Trenin and Volodin 1999), copulative function is enhanced and sperm quality is improved (Mirzaev et al. 1999), therapeutic effect after lung contusion is exerted (Wu et al. 1997), and myocardial ischemia and arrhythmia are prevented (Wu 2000). The procedure of peroxidation of lipids (POL) is regulated by them in complicated biological systems (Ramazanov 2005). Ecdysterone also plays a role as an anti-inflammatory agent (Kurmukov and Syrov 1988). In order to treat insulin-dependent diabetes, significant effect is produced by Turkesterone (Najmutdinova and Saatov 1999). So far, the biotechnological production of these therapeutically important secondary metabolites has been restricted to in vitro regenerations of *Ajuga* species and hairy roots clones. As we have scarce information about the biosynthetic pathway of phytoecdysteroids, not even a single study has been reported manipulating biosynthetic pathway genes for the scale up production/biosynthesis of these compounds. Some of the pioneer studies performed for the biotechnological productions of ecdysteroids in *Ajuga* species is described in Table 27.2.

Table 27.2 Biotechnological productions of ecdysteroids in *Ajuga* species

Species	Biotechnological approach	Compounds	References
A. Reptans	In vitro root and shoot cultures	Ajugalactone, cyasterone, sengosterone, 29-norsengosterone, 29-norcyasterone, 20-hydroxyecdysone, polypodine B	Tomás et al. (1993)
A. Reptans	In vitro micropropagated plants	29-norsengosterone, 29- norcyasterone, cyasterone, ajugalactone	Tomás et al. (1992)
A. bracteosa	In vitro generated plants, hairy roots, regenerants through *A. tumefaciens* (*rol* genes) and *A. rhizogenes* mediated transformations with strains LBA-9402, A4 and *AR*qua1	20-Hydroxyecdysone, ajugalactone, cyasterone, makisterone A, polypodine B, and sengosterone	Kayani et al. (2016b), Kayani et al. (2017), and Kayani et al. (2014)
A. Reptans	Hairy roots raised through *A. rhizogenes* MAFF 03–01724 strain	20-hydroxyecdysone, cyasterone, isocyasterone, 29-norcyasterone	Fujimoto et al. (2000)
A. Reptans	Hairy roots raised through *A. rhizogenes* MAFF 03–01724 strain	20-hydroxyecdysone	Tanaka and Matsumoto (1993a), Tanaka and Matsumoto (1993b), Uozumi et al. (1993), and Uozumi et al. (1995)
A. Reptans	Hairy roots raised through *A. rhizogenes* MAFF 03–01724 strain	20-hydroxyecdysone, norcyasterone B, cyasterone, isocyasterone	Matsumoto and Tanaka (1991)
A. Reptans	Hairy roots raised through *A. rhizogenes* MAFF 03–01724 strain	20-hydroxyecdysone, cyasterone, 29-norcyasterone	Nagakari et al. (1994a)
A. Reptans	Hairy roots raised through *A. rhizogenes* MAFF 03–01724 strain	20-hydroxyecdysone and some feeding studies	Fujimoto et al. (1997), Fujimoto et al. (2000), Hyodo and Fujimoto (2000), Nagakari et al. (1994a), Nagakari et al. (1994b), Nakagawa et al. (1997), and Okuzumi et al. (2003)
A. multiflora	Hairy roots obtained from *A. rhizogenes* strain A4	20-hydroxyecdysone	Kim et al. (2005)

27.6.2 Withanolides

Withanolides are mentioned as "monopoly of Solanaceous plants" (Khan et al. 1999a), but they are widespread in the plant kingdom. So far, a detailed distribution of withanolides in the plant and their roles is described by Misico et al. (2011). Chemically withanolides are steroidal lactones with an ergostane skeleton. Withanolides exhibited many biological activities such as antitumor, anti-inflammatory, immunomodulating, cytotoxic, antibacterial antifungal, cancer chemopreventive, insecticidal, selective phytotoxicity, antifeedant, etc. (Misico et al. 2011). Except the plants from the Solanaceae family, they have been reported in *Ajuga* species, and almost all the studies regarding their isolation from *Ajuga* are conducted in HEJ research institute in the University of Karachi, Pakistan. The first report of the presence and isolation of a withanolide "ajugin" is reported from *A. parviflora* (Khan et al. 1999a). In the same year, four new withanolides named "ajugin A" and "ajugin B" (Khan et al. 1999b) and "ajugin C" and "ajugin D" were reported from the whole plant extract of the same plant (*A. parviflora*) (Khan et al. 1999c). A few additions in the ajugin list were added when Nawaz et al. (1999) isolated "ajugin E" and "ajugin F" from the whole plant of *A. parviflora.* The same group continued their efforts, and in the subsequent year, they isolated a new withanolide called "coagulins J" which was active against bacteria (Nawaz et al. 2000b) and two withanolides called "withanolide 1" and "withanolide 2" possessing antifungal activities from the same plant (Nawaz et al. 2000a). In the next year, three withanolides "withanolide 1–3" were isolated from *A. bracteosa* possessing acetylcholinesterase activity (Choudhary et al. 2005). Later on, Riaz et al. (2004) isolated three new withanolides from the whole plant of *A. bracteosa* called "bracteosin A," "bracteosin B," and "bracteosin C," and they carry inhibitory activity against cholinesterase enzymes. In the continuation, Riaz et al. (2007) successfully isolated three more withanolides from *A. bracteosa*, named "bractin A," "bractin B," and "bractic acid," and these compounds showed the potential of inhibition against enzyme lipoxygenase. To date, the *Ajuga* species harboring these compounds were not subjected to biological productions. The structures of these compounds are shown in Fig. 27.3.

27.6.3 Iridoid Glycosides/Glucosides

Iridoids are widespread in nature, mainly in dicot plant families, offering a wide range of bioactivities such as neuroprotective, anti-inflammatory, immunomodulator, hepatoprotective, and cardioprotective. They are also found to pertain anticancer, antioxidant, antimicrobial, hypoglycemic, hypolipidemic, and antispasmodic effects (Tundis et al. 2008). They were discovered in a group of ants, and mainly they are related or released as a defense mechanism. Iridoids have been reported in some of the Labiatae family members including *Vitex* and *Ajuga* species. The

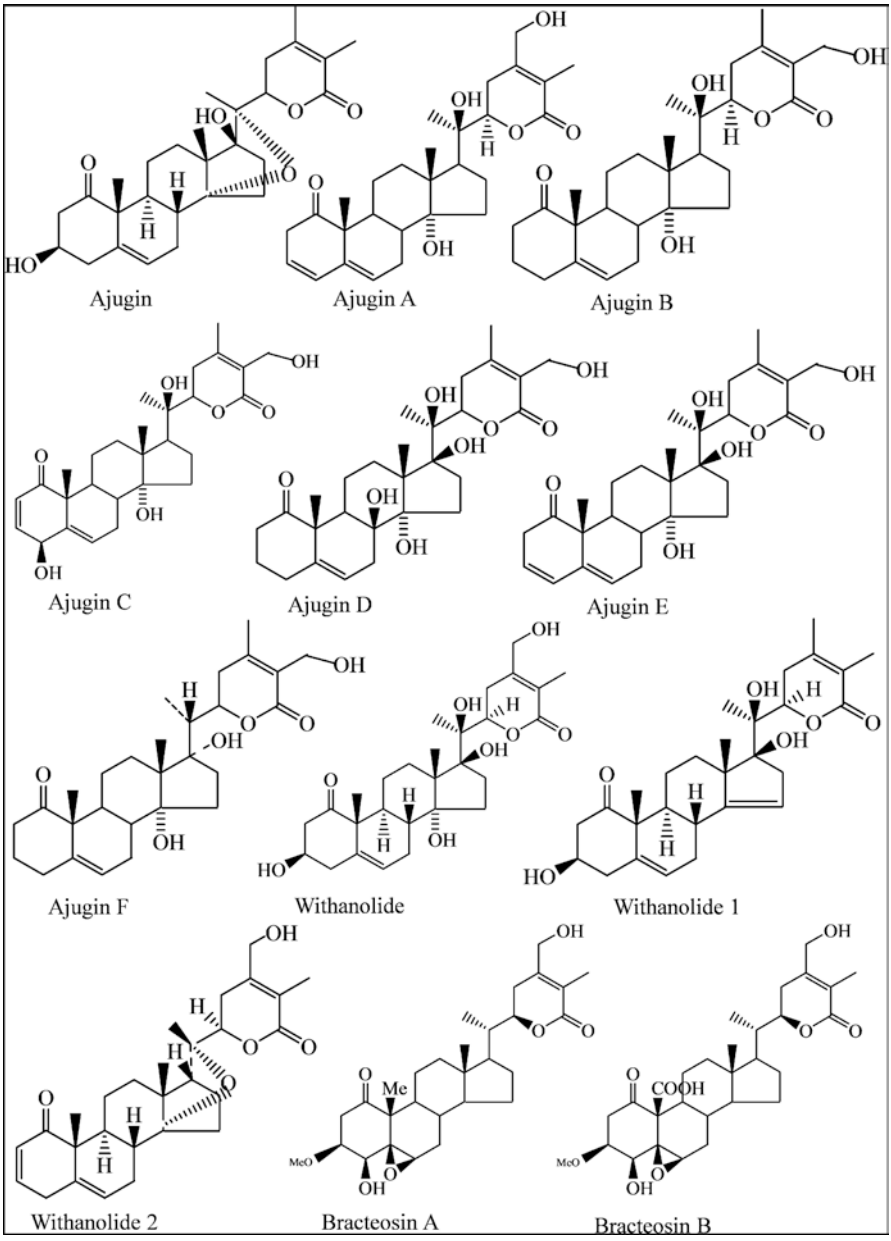

Fig. 27.3 Some of the important withanolides reported from *Ajuga* species

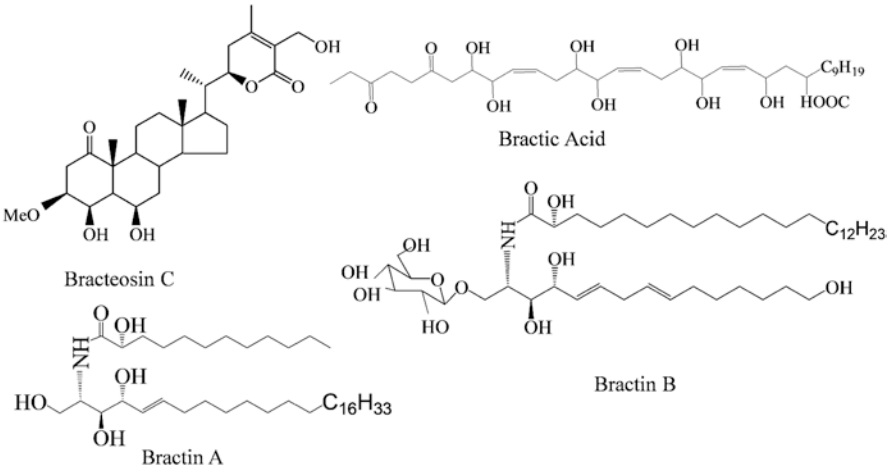

Fig. 27.3 (continued)

presence of iridoids in *Ajuga* species begins when three of the iridoids (reptoside, 8-acetylharpagide, and harpagide) were reported from *A. decumbens* (Fig. 27.4). Interestingly, 8-acetylharpagide displayed the powerful inhibitory effect on Epstein-Barr virus activation (Takasaki et al. 1998). Later on, 8-*O*-acetylmioporoside and ionone glycosides were reported in the *Ajuga* species. They were isolated from the aerial parts of *A. salicifolia* along with corchoionoside C, 8-*O*-acetylmioporoside, harpagide, 8-*O*-acetyl-harpagide, lavandulifolioside, leonosides A and B, and ajugol (Akbay et al. 2003). After 3 years, two iridoid glycosides, 6,8-diacetylharpagide and 6,8-diacetylharpagide-1-*O*-β-(3′,4′-di-*O*-acetylglucoside), were separated from the extracts of aerial parts of *A. remota* along with some known compounds including kaempferol 3-*O*-α-rhamnoside, quercetin 3-*O*-β-glucoside, quercetin 3-*O*-rutinoside, 8-acetylharpagide, and ajugarin I and ajugarin II (Manguro et al. 2006).

Two iridoid glycosides 8-*O*-acetyl harpagide and reptoside have been isolated from *A. bracteosa* by Singh et al. (2006). As significant inhibitory effect is produced by 8-*O*-acetyl harpagide on carcinogenesis test as they suppressed pulmonary tumors in mice, iridoid glycosides have been regarded as novel cancer chemopreventive agents (Konoshima et al. 2000; Takasaki et al. 1999). Manguro et al. (2007) continued their work, and they were successful in isolating five new iridoid glycosides characterized as 6-keto-8-acetylharpagide, 6,7-dehydro-8-acetylharpagide, 7,8-dehydroharpagide, 8-acetylharpagide-6-*O*-β-glucoside, and harpagide-6-*O*-β-glucoside from the aerial parts of *A. remota*. Six years after, four new iridoid glucosides named as ajureptaside A-D were isolated from the whole plant of *A. reptans* along with some known iridoid glucosides, diterpenoid glycoside, aliphatic alcohol glycoside, and ecdysteroids. The diterpenoid glycoside exhibited 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical and H_2O_2 scavenging activities (Ono et al. 2011). Four new iridoid glycosides were identified from the extracts of the root portion of *A. remota* named as 6’-*O*-rhamnosylharpagide, 2′,3′-diacetylharpagide,

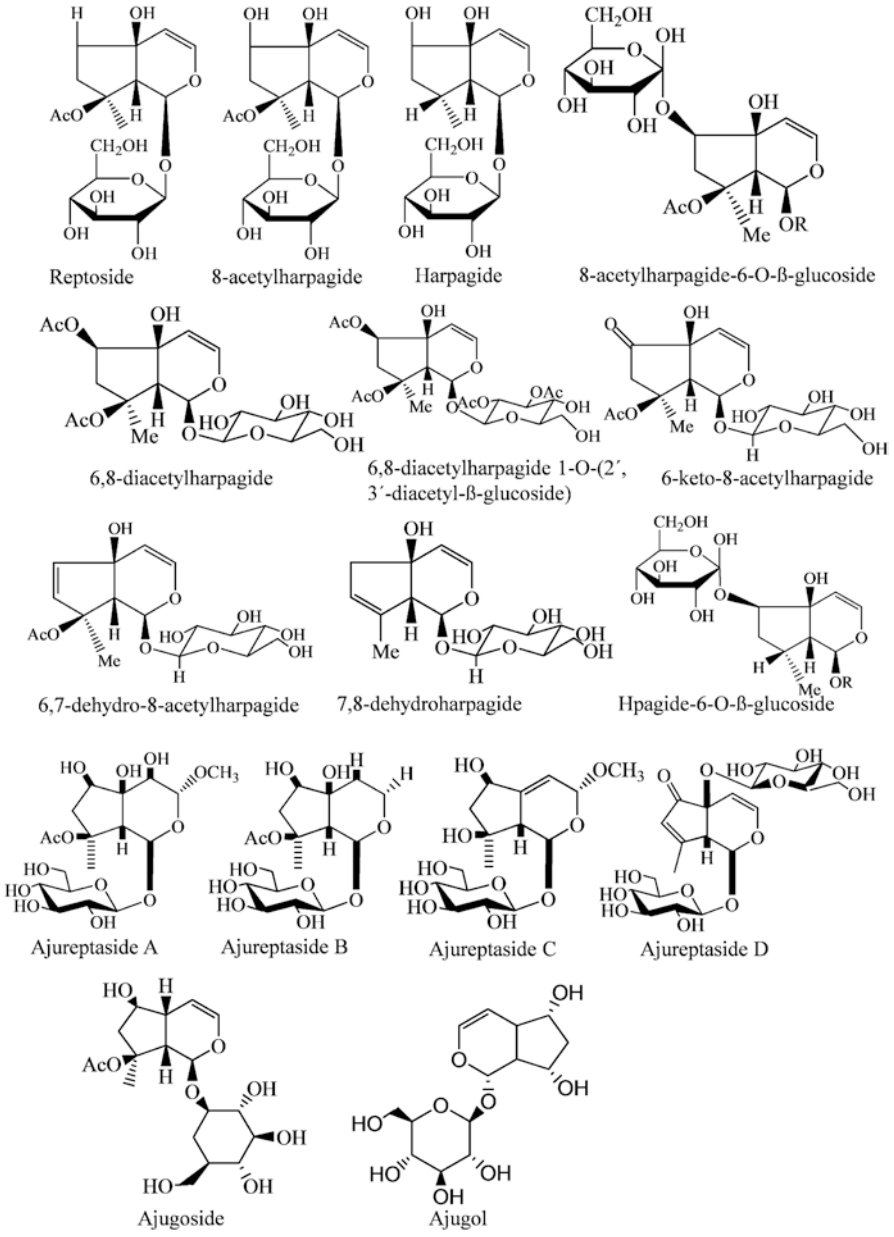

Fig. 27.4 Some of the important iridoid glycosides/glucosides reported from *Ajuga* species

6-*O*-xylosylharpagoside-B, and 6'-*O*-galloyl-7,8-dehydroharpagide together with some known iridoids, phytoecdysteroids, and sterols. Using second instar *Aedes aegypti* larvae in the in vitro larvicidal tests, the EtOAc extract which contained new iridoids was reported to be toxic with LC_{50} of 5.30 ± 1.3 μg/mL (Manguro et al. 2011).

Atay et al. (2016) isolated recently some iridoid glycosides, coumaric acid derivatives, and phenylethanoid glycosides from the aerial parts of Turkish *A. laxmannii*. The extracts exhibited moderate antiparasitic activity, while compound isoorientin which was reported for the first time from genus *Ajuga* displayed the most significant antimalarial potential with an IC_{50} value of 9.7 μg/mL. An endemic Italian medicinal species, *A. tenorei*, was subjected to isolation, and purification studies revealed the isolation of verbascoside, echinacoside, ajugoside, and two already known iridoid glycosides (Frezza et al. 2017). Lots of derivatives of harpagide and other iridoids were discovered whose structures are not given here (Fig. 27.4).

27.6.4 Neo-clerodanes

Neo-clerodanes are a category of compounds isolated from *Ajuga* species and are very potent antifeedants (Coll and Tandrón 2008). Isolation and purification of these compounds in *Ajuga* started when two new neo-clerodane diterpenoids named as ajugapitin and its dihydro derivative (14,15-dihydro-ajugapitin) were isolated (Fig. 27.5) from the whole plant of *A. chamaepitys* (Hernandez et al. 1980). Later on, Camps et al. (1984) isolated two epimeric neo-clerodane diterpenoids named as 2-acetylivain-I and 14,15-dihydro-ajugapitin from the whole plant extract of *A. pseudoiva*. Six years after, two new *neo*-clerodane diterpenoids, ajugachin A and ajugachin B, were isolated from *A. chamaepitys* (Boneva et al. 1990). In the subsequent years, three new clerodane diterpenes, lupulins A–C, were discovered from the whole plant of *A. lupulina*. Together with lupulins A and B, lupulins D also showed promising antibacterial activities against *Staphylococcus aureus*, *Pseudomonas aeruginosa*, and *E. coli* (Chen et al. 1996). A year after, Chen et al. (1997) isolated another structural analogue of lupulin A, and it was potent antibacterial against *P. aeruginosa* and *E. coli*. Bioguided isolation and purification of neo-clerodane continued, and during 1999 three new epimeric neo-clerodane diterpenoids, hativenes A–C, were isolated from *A. pseudoiva* which showed high antibacterial activities toward *P. aeruginosa*, *E. coli*, and *Salmonella typhimurium* (Jannet et al. 1999).

Spanish chemists were the leading scientists working on the bioactivity guided isolation of compounds of *Ajuga* species. They continued digging in for the search of bioactive compounds and found new *neo*-clerodane diterpenes from *A. reptans* and named them ajugatansins (A1, B1 and D1) and some of the previously reported compounds including ajugavensin A (Carbonell and Coll 2001). Another neo-clerodane (bracteonin-A) was added to this list when Verma et al. (2002) were examining the whole plant of *A. bracteosa* with some of known compounds.

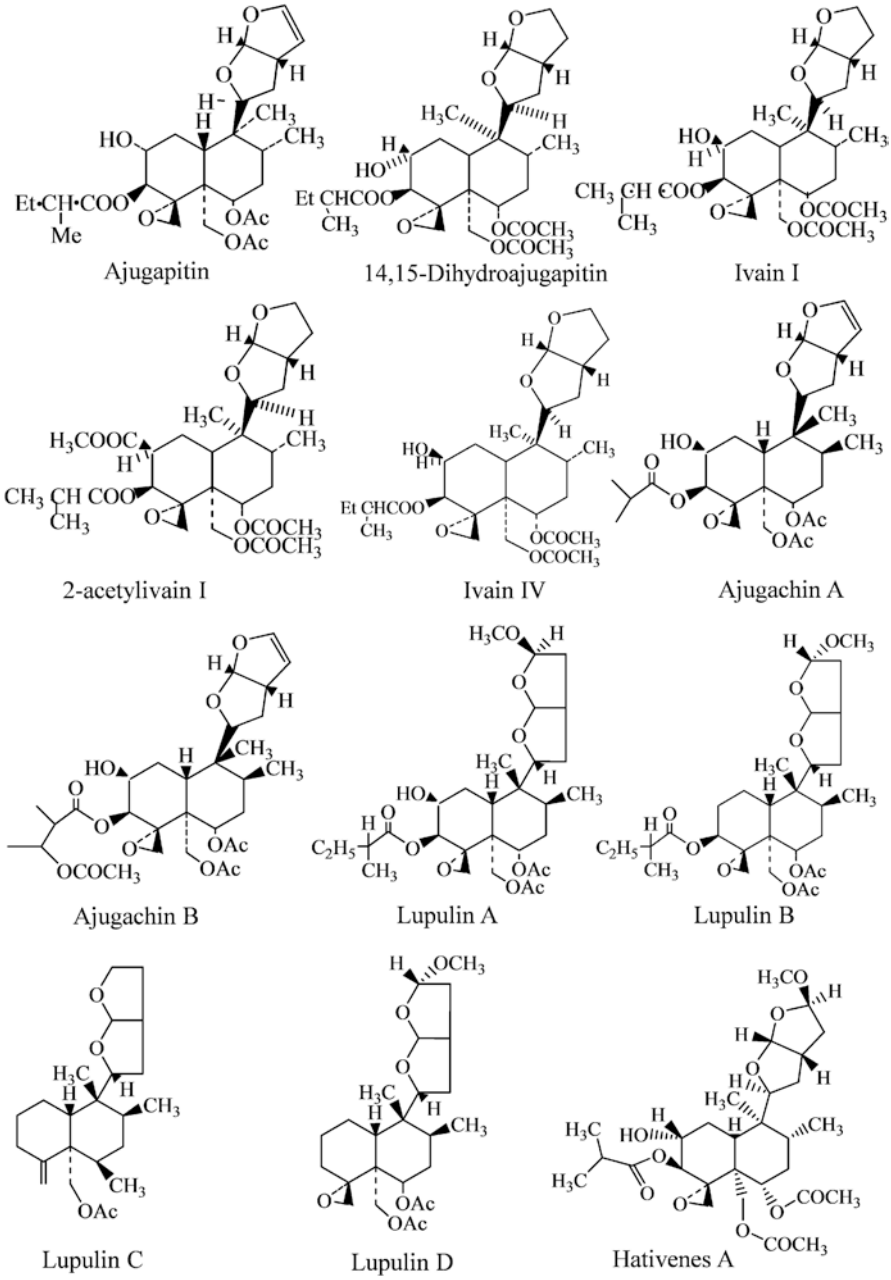

Fig. 27.5 Some of the important neo-clerodane diterpenoids reported from *Ajuga* species

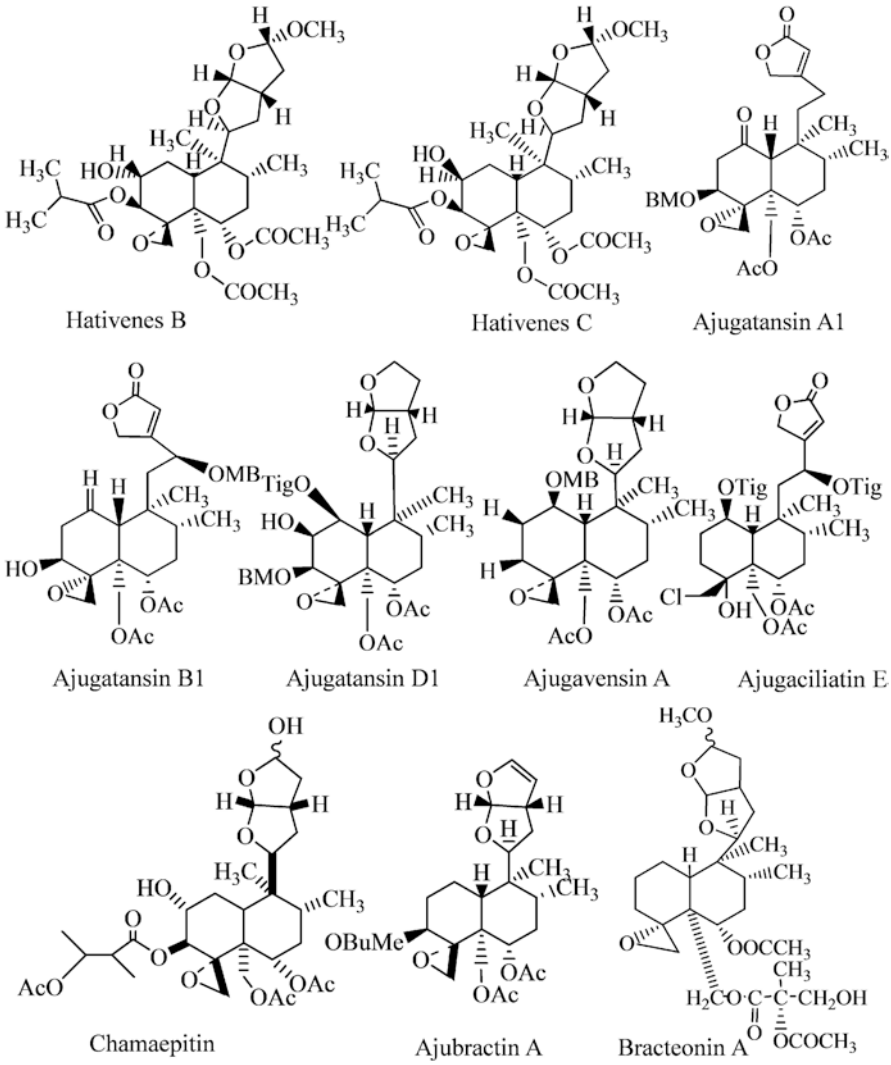

Fig. 27.5 (continued)

Workers at the Institut d'Investigacions Químiques extended their studies and isolated some *neo*-clerodane diterpenes from the aerial parts of *A. remota* called ajugarins I, II, IV, and V and clerodin, ajugapitin, dihydroajugapitin, dihydroclerodin, and deacetylajugarin IV (some of them were already reported from different plants). Compound ajugarins 1, 2, and 4 showed moderate antifeedant activities against *Spodoptera littoralis* (Coll and Tandrón 2005).

Another species, *A. turkestanica*, revealed two novel neo-clerodane diterpenes, 14,15-dihydroajugachin B and 14-hydro-15-methoxyajugachin B, in addition to some known compounds, i.e., chamaepitin, ajugachin B, ajugapitin, and lupulin A

(Grace et al. 2008). In the same way, Huang et al. (2008) isolated four new neo-clerodane diterpenoids from the whole plants of *A. decumbens*, viz., 15-epilupulin A, 6-*O*-deacetylajugamarin, and ajugadecumbenins A and B. After 2 years, chemists from the same institute (Institut d'Investigacions Químiques) isolated different neo-clerodane diterpenoids from dichloromethane extract of *A. bracteosa* including five new compounds called ajubractins A–E along with 12 already reported compounds and their derivatives. All the compounds except the first two displayed moderately high antifeedant activity against *Spodoptera littoralis* larvae on lettuce leaves (*Lactuca sativa*) (Castro et al. 2011). Ten new *neo*-clerodane diterpenes, ajugaciliatins A–J, along with 17 known analogues, were isolated from *A. ciliate* Bunge. They were subjected to evaluation for the neuroprotective effects against neuronal cell death induced by MPP^+- in dopaminergic neuroblastoma SH-SY5Y cells, and some of them demonstrated moderate neuroprotective effects (Guo et al. 2011b). The same group continued their search and found six more *neo*-clerodane diterpenes (three new and three known) from the same plant, and some of them exhibited moderate neuroprotective effects (Guo et al. 2011a). In another study, three new and five known clerodane diterpenes have been isolated from *A. decumbens*. Their effect on the inhibition of LPS-stimulated NO production was examined, and some of them showed significant inhibitory effects (Sun et al. 2012b). The same group continued their work which led them isolation of three new and three known *neo*-clerodane diterpenes from the same plant. Their inhibition of NO production was evaluated in the same way, and majority of them exhibited significant inhibitory effects (Sun et al. 2012a). Coll and Tandrón (2008) documented diverse neo-clerodane diterpenoids from *Ajuga* and concluded that neo-clerodane diterpenoids are responsible for antifeedant activity against pests. They also described in detail the other biological activities of these compounds with structural details.

27.6.5 Other Compounds

Sterols are important metabolites as they are precursor molecules for the steroids metabolism. Stigmasterol and β-sitosterol have been isolated from *A. bracteosa* by Verma et al. (2002). Likewise, isolation of β-pinene, β-phellandrene, limonene, linalool, γ-terpinene, geranyl acetate, Z- β-ocimene, neryl acetate, linalyl acetate, nopyl acetate, borneol, copaen-4-ol, β-sitosterol, and terpinin-4-ol has been carried out by Singh et al. (2006) from the n-hexane fraction of *A. bracteosa*. Moreover, essential oils were extracted from the leaves of *A. bracteosa* by Vohra and Kaur (2011), and the analysis by GC-MS affirmed the presence of α-humulene, limonene, elemol, β-myrcene, β-caryophyllene, camphene, and α-phellandrene. These oils were found active against *Staphylococcus*. The extraction of essential oils from the aerial part of *A. bracteosa* was carried out by Mothana et al. (2012). Following the extraction was analysis by GC/MS and GC and identification of 47 components encompassing high content of aliphatic acids, oxygenated monoterpenes, hexadecanoic acid, and borneol. An ergosterol-5 isolated from the ethyl acetate extract of *A.*

remota (8-endoperoxide) was the active principal with LC_{50} value of 4.40 ± 0.2 against *Aedes aegypti* larvae (Manguro et al. 2011).

A new phthalic acid ester 1,2-benzenedicarboxylic acid bis(2S-methyl heptyl) have been isolated from *A. bracteosa* by Singh et al. (2006). Six flavonol glycosides were isolated from extract of *A. remota* aerial parts (Manguro et al. 2006). From the same plant, three new flavonol glycosides, myricetin 3-*O*-rutinoside-4′-*O*-rutinoside, myricetin 3-*O*-rutinoside-3′-*O*-rutinoside, and isorhamnetin 3-*O*-rutinoside-7-*O*-rutinoside-4′-*O*-β-glucoside, were reported (Manguro et al. 2007). These compounds are attributed for the antioxidant activities of *Ajuga* species.

So far, this was a brief extraction and activity report of the most important categories of compounds from the genus *Ajuga*. The groups which have been involved in the isolation of these compounds also evaluated some of their activities including cancer chemoprevention, insect antifeedant activities, antioxidant activities, etc. Unfortunately, a few of the groups around the world which are working on biotechnological productions of secondary metabolites did focused (to some extent) only on phytoecdysteroids.

27.7 Polyphenols and Antioxidant Properties

Chemically active derivatives of oxygen, reactive oxygen species (ROS) are mediators of intracellular signaling cascades; however their excessive production will lead to oxidative stress which is deleterious to plant cells (Hammerschmidt 2005b; Nordberg and Arner 2001). Damage to vital cellular machinery and enzyme inactivation poses detrimental effects of ROS especially hydroxyl radicals, hydrogen peroxide, and superoxide radicals. Furthermore, highly reactive singlet oxygen is generated as a result of peroxidation of lipids (POL) which consequently generates lipid peroxy radicals and lipid hydroperoxides (Steinberg 1997). Free hydroxyl radicals are strong oxidizing agents devastating significant biomolecules, like DNA (Marnett 2000) and elicit lipid peroxidation (Steinberg 1997). Hence, alleviating the excessive amount of ROS is essential for the plant. An array of radical scavenging antioxidants is a major component of the self-defensive mechanism of plant. Flavonoids, carotenoids, ascorbate, polyamines, phenolics, alkaloids, α-tocopherols, glutathione, etc. comprise the antioxidant defense system (Mullineaux et al. 1997). The defense reactions take place majorly in the intracellular compartments and to certain degree in the apoplast. Enzymes like superoxide dismutases modulate the catalysis of superoxide ($\bullet O_2^-$) to hydrogen peroxide (H_2O_2) and molecular oxygen (Scandalios 1993). Ascorbic acid scavenges ROS (Buettner 1993) and carotenoids help to decrease the singlet oxygen concentration. Moreover, glutathiones have several different functions of recycling ascorbic acid, scavenging singlet oxygen and hydroxyl radicals, and protecting thiol (-SH) groups of enzymes (Foyer et al. 1994). The resistance to the oxidative damages has been enhanced in the plants with higher level of antioxidants. The toxicity to phytophagous organisms or flavor, odor, and

pigment to the plant are imparted by phenolics. The active defense of plants against phytopathogens and activation of genes meant for plant defense are attributed to these diverse groups of secondary metabolites (Hammerschmidt 2005a). Phenolic content is abruptly increased by infected cells in order to get rid of pathogens (Fry 1987). In order to inhibit fungal growth (phytoanticipins), phenolics like phenolic acid, simple phenols, some isoflavones, and flavonols are manufactured by healthy (uninfected) plants. However, a few phenols like phenanthrenes, flavans, furocoumarins, isoflavonoids, and stilbenes are manufactured in response to infection (phytoalexins) (Lattanzio et al. 2001). Sometimes, the stress-induced ROS accumulated by plants are toxic to both plant and pathogen (Baker and Orlandi 1995). ROS is scavenged (antioxidants function), and cell damage is reduced by accumulation of anthocyanins (Kangatharalingam et al. 2002). As compared to ascorbate and tocopherols, more ROS can be scavenged by polyphenols. The antioxidant potentials are determined by the chemical activities of polyphenols like donation of electron or hydrogen (Rice-Evans et al. 1997). Phenolic compounds are released at wounded spot by the plants which are thought to be accountable for the defensive mechanism. Isolation of a new phenolic compound "ajuganane" has been carried out from *A. bracteosa* (Hussain et al. 2012).

Comprising of 15-carbon skeleton, flavonoids form a class of plant secondary metabolites. They consist of flavanols, flavonols, flavanones, flavones, dihydroflavonols, anthocyanidins, dihydrochalcones, and chalcones, which are responsible for a wide variety of biological actions (Parr and Bolwell 2000). Flavonoids are physiologically active secondary metabolites, retaining antifungal (Grager and Harbone 1994) and antimicrobial activities (Yilmaz and Toledo 2004). Microbial enzymes are cross-linked by flavonoids, inhibiting microbial enzymes which breakdown metal ions and cell wall (Skadhauge et al. 1997). It is found that antioxidant potential is also possessed by anthocyanins and flavonoids (Kubo et al. 1999). Significant positive correlation has been found between antioxidant activities and polyphenolic content in plants (Amari et al. 2014). It has been reported that there is a high antioxidant activity of both the roots and the leaves for four *Centella asiatica* accessions. These parts of *C. asiatica* also possessed largest volume of α-tocopherol. Furthermore, a strong correlation was found between total phenolic contents and antioxidant activities (Zainol et al. 2003).

A. bracteosa was recently verified for its antioxidant activities, and maximum was found in reducing power assay in methanol/chloroform extract with 54.0 vitamin C equivalent mg/g. In the same extract, as compared to aqueous extracts of plant, a higher value of total flavonoid content (17.9 mg Quercetin equivalent/g) and total phenolic content (34.1 Gallic acid equivalent/g) was found (Akhtar et al. 2015).

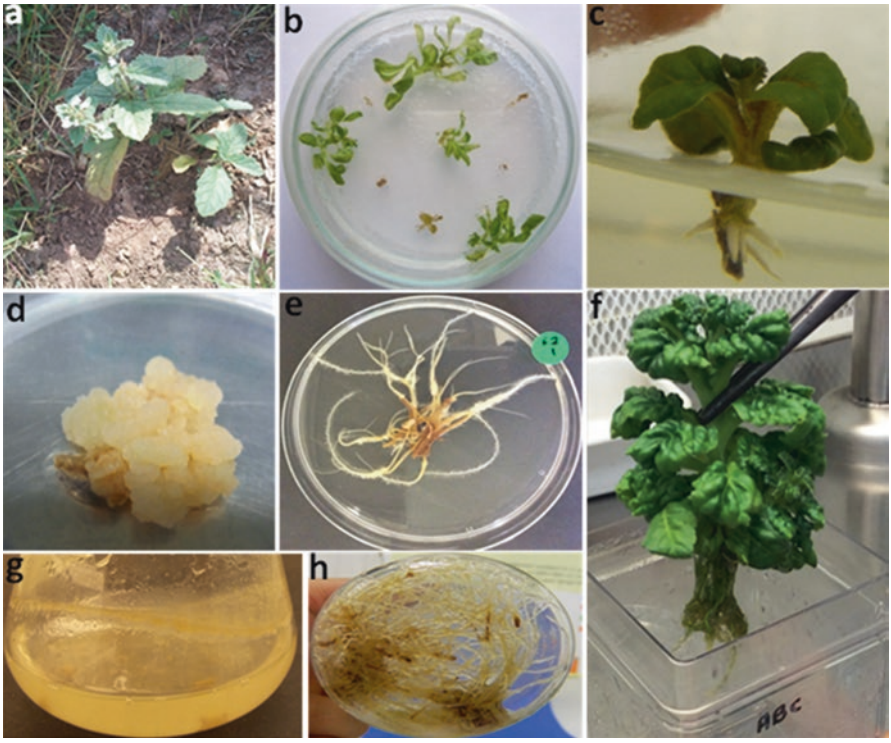

Fig. 27.6 Various biotechnological methods used for the production of valuable secondary metabolites in *A. bracteosa*. (**a**) wild plant, (**b-c**) in vitro tissue culture, (**d**) callus cultures, (**e**) hairy roots cultures in solid medium, (**f**) in vitro grown transgenic plants, (**g**) cell suspension cultures, (**h**) transgenic hairy roots in liquid B5 medium

27.8 Enrichment of Secondary Metabolites

By using biotechnological methods, the secondary metabolites are enhanced, including transformation of the genes which are involved in triggering secondary metabolism. Various biotechnological methods used for the production of valuable secondary metabolites are discussed (Fig. 27.6). Hairy roots are often coupled with elicitors' supplementation, as it is a valuable source for the increased biosynthesis of many secondary metabolites. Although *A. bracteosa* has not been transformed previously, but three other species of *Ajuga* have been investigated and reported for the enhanced production of phytoecdysteroids. *A. reptans* and *A. multiflora* generated transgenic hairy roots, while in order to profile phytoecdysteroids, untransformed cell suspension culture of *A. turkestanica* was investigated. More than 20 hairy root clones of *A. reptans* were established by Matsumoto and Tanaka (1991) after the infection of *A. rhizogenes* strain MAFF 03-01724. As four times greater 20-HE content is produced by an elite hairy root line (Ar-4) than control root, and its weight is enhanced 230 times on culturing for 45 days; close relationship was

found between the production of phytoecdysteroids in these clones and the rate of growth of hairy roots. The hairy roots of *A. reptans* revealed some regenerants which were investigated by Tanaka and Matsumoto (1993a), and they further reported that on comparing with untransformed plants, these regenerants have more number of small-sized leaves, high phytoecdysteroid content, and a high growth rate. Amazingly, the 20-HE content in the mother hairy root lines were higher as compared to the roots of regenerants, further suggesting the high probability of the provision of a sink (leaves) for the accumulation of phytoecdysteroids. When the same hairy roots were cultured on the media supplemented with nutrients deficient in phosphate, a decreased growth rate was observed, but 20-HE content was increased. However, when exogenous indole acetic acid (IAA, 0.1 mg/l) was given, a higher growth rate was observed, but this addition resulted in a decrease in 20-HE content (Uozumi et al. 1995). It was found in an additional batch culture of the same hairy roots that depletion of phosphate ions was carried out after 16 days, and in order to obtain a high cell mass, glucose was the most suitable monosaccharide (Uozumi et al. 1993). When the culture was supplied with naphthaleneacetic acid (0.1 mg/*l*), it was observed that it increased the cell growth rate of the same roots, and when smaller hairy root fragments were supplemented with benzyladenine (10 mg/*l*), the highest plantlet formation frequency in the plantlet formation stage was exhibited. Moreover, β-glucuronidase (GUS) gene was introduced in these hairy roots, depicting the activity of GUS in leaf tissues of the regenerated plants (Uozumi et al. 1996).

In order to establish transformation system for *A. multiflora*, Kim et al. (2005) infected petiole explant with *A. rhizogenes* strain A4 and observed that as compared to the wild type, 20-HE content was increased to tenfold in hairy roots. Mevalonic acid, cholesterol, and acetate are considered as the precursors of phytoecdysteroid biosynthesis. In addition of these precursors in cell suspension cultures of *A. turkestanica*, phytoecdysteroid content was not affected, but 20-HE content was increased to threefold on addition of 125 μM methyl jasmonate (MeJ). On the contrary, an addition of mevalonic acid (150 mg/l), sodium acetate (150 mg/l), and MeJ increased the phytoecdysteroid content to twofold, and a positive response was shown by hairy root cultures (Cheng et al. 2008).

27.9 Conclusions and Future Prospective

The major compounds of *Ajuga* genus are phytoecdysteroids, withanolides, iridoids, neo-clerodane, di- and triterpenoids, and sterols. These compounds possess anabolic, anticancerous, antioxidant, and other wide range of activities. Metabolic pathway of the ecdysteroids is not very well studied in plants as compared to animals (insects). On the other hand, metabolic pathway of withanolides is very well studied in *Withania* species. To date, there is not a single report available mentioning the biosynthetic pathway engineering of any of the species of *Ajuga*. Being the endangered species, a few species of *Ajuga* have been tested for some of the

secondary metabolites especially phytoecdysteroids in the hairy roots and some feeding studies of precursor molecules in culture medium aimed to dig in the biosynthetic pathway. Established phytoecdysteroid biosynthetic pathway genes should be investigated, and metabolic pathways should be engineered using various biotechnological applications like CRISPR-Cas9 for the better yield of the therapeutic metabolites.

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Chapter 28
Laurel (*Laurus nobilis* L.): A Less-Known Medicinal Plant to the World with Diffusion, Genomics, Phenomics, and Metabolomics for Genetic Improvement

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Abstract Medicinal plants have gained the world's attention due to their application in various ways. Laurel (*Laurus nobilis*. L) is a very important medicinal plant of the Mediterranean region. Traditionally this plant has been successfully used in medicine, and its essential oil has great importance. Genomics, breeding, and metabolomics of different crops have remained the main focus of researchers, which made this plant to less known to the world. Most of the researchers only worked about the essential oil and its antibacterial and antioxidant activities. However, still almost no work has been done about the breeding aspects of this important plant. The present review offers an overview about the origin, diffusion, genomics, phenomics, breeding, and metabolomics of laurel. This information would be very helpful for the researchers who are interested in the breeding of this plant.

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28.1 Introduction

Medicinal plants have been used for centuries by the human being for the treatment of various diseases due to presence of therapeutic chemicals (Nostro et al. 2000). In 2008, WHO (World Health Organization) issued a report that clarifies the role of these plants in the life of human being and stated that 80% world population depends on traditional medicines for solving their routine life health problems (Vital and Rivera 2009). Dried leaves and essential oils of several medicinal plants such as *Origanum minutiflorum*, *Foeniculum vulgare*, and *Laurus nobilis* are playing important role as a valuable spice in the culinary and food industry (Dadalioğlu and Evrendilek 2004; Santoyo et al. 2006). Traditional medical system has now become an important part of culture in many developing countries. Till now several thousand plant species having medicinal applications have been investigated in various cultures of the world (Farnsworth and Soejarto 1991; Yaldiz et al. 2018). Lauraceae is one of the largest families of the woody plants containing 2500–3000 spices with a total of 50 genera mainly distributed throughout tropical and subtropical regions of the world. Plants of Lauraceae family are of great economic importance as they are commonly used in construction of house items, as medicinal plant, essential oil, and spices (Oliveira-Filho et al. 2015). *Laurus nobilis* and *L. azorica* (Seub) Franco are the two main species of this family that have been recognized traditionally. *L. nobilis* has great medicinal value and has been used in the treatment of epilepsy, neuralgia, and parkinsonism (Aqili Khorasani 1991).

L. nobilis leaves are mainly used as spice, insecticide, and antiseptic, stomachic, and has been largely used in the rheumatism treatments in European folk medicine. Leaf extracts and essential oil of this plant have been found effective for the treatment of gastric problems like flatulent colic and their anticonvulsive and antiepileptic activities that are now universally proved (El et al. 2014). In addition to medicinal importance, leaves of these plants are in use as flavoring agent and to increase the shelf life of food as they contain higher antioxidant and antimicrobial activity (Cherrat et al. 2014; El et al. 2014).

28.2 Origin and Diffusion

L. nobilis has been used in traditional medicine since Greek empires due to which it is also known as Greek bay. This plant was also related to ancient god of light named Apollo that used to make wreaths for pets, emperors, and generals (Conforti et al. 2006). *L. nobilis* which is commonly called laurel, bay, bay laurel is dioecious, insect-pollinated, and vertebrate-dispersed specie. Evergreen members of Lauraceae family are mainly native to warm regions of the world especially Mediterranean countries (Barla et al. 2007; Arroyo et al. 2010). Laurel is native to Balkans and Asia

Fig. 28.1 Geographical distribution of *L. nobilis* all over the world

Fig. 28.2 Distribution of *L. nobilis* in different areas of Turkey

and then spreads to Mediterranean countries like South France, Spain, Brazil, North Africa, and Corsica Island in Italy, Israel, and Cyprus (Leung and Foster 1999). Figure 28.1 represents the distribution of laurel in the world. Initially laurel plant was present in the form of big forest near the Mediterranean regions when climate was very humid. However, laurel forests disappeared 10,000 years ago and were retreated with the passage of time and were succeeded by present-day drought tolerance sclerophyll plant (Erat et al. 2016).

Turkey is serving as a source of origin, biodiversity, and diffusion of many crops and is playing an important role in the world laurel market (Dadalioğlu and Evrendilek 2004; El et al. 2014). Turkey is leading producer of laurel and holds the 90% of world laurel production. This plant grows naturally in Turkey mainly in the Mediterranean, Marmara, Aegean, and West and Central Black Sea regions (Boza and Hepaksoy 2016) with an altitude of 600–800 meters (Davis 1982). Figure 28.2

represent the distribution of *L. nobilis* in different areas of Turkey. In 1655 this plant was defined as *L. nobilis* by Goodyer, and in Turkey this plant is known as "Daphne" (El et al. 2014). Similarly this plant is famous with the name of "Barg-e Boo" and "Rand" in the Persian people who are using this plant for the treatment of several diseases (Amin et al. 2007).

28.3 The Trade in *L. nobilis*

The flavoring properties of *L. nobilis* have been known since antiquity. The leaves of *L. nobilis* are plucked and dried under shade for use as a flavoring material in a variety of culinary preparations, especially in French cuisine (Kumar et al. 2001). Turkey is the largest producer of the *L. nobilis* worldwide with production of totally 21.788 tons in 2016 (Anonymous 2017) and captures more than 80% world export market. In EU countries, 77% of bay leaves (HS code, 09109950) come from developing countries (Fig. 28.3), nearly all of this (72%) from Turkey. In 2014, EU countries imported bay leaves 20.000 tons from Turkey and 5.000 tons from Morocco. The market for medicinal herbs is interesting because consumers are focused on quality more than on price here. Bay leaves price was € 6.07 per kilogram for importer, but it increased €220–490 per kilogram for retail. There are large differences between prices of importers/wholesalers and retailers because retailers take care of repackaging, branding, marketing, and transportation; besides it was sold in small retail packages instead of bulk (Anonymous 2015). Turkish export of bay leaves is mainly in bulk and raw form.

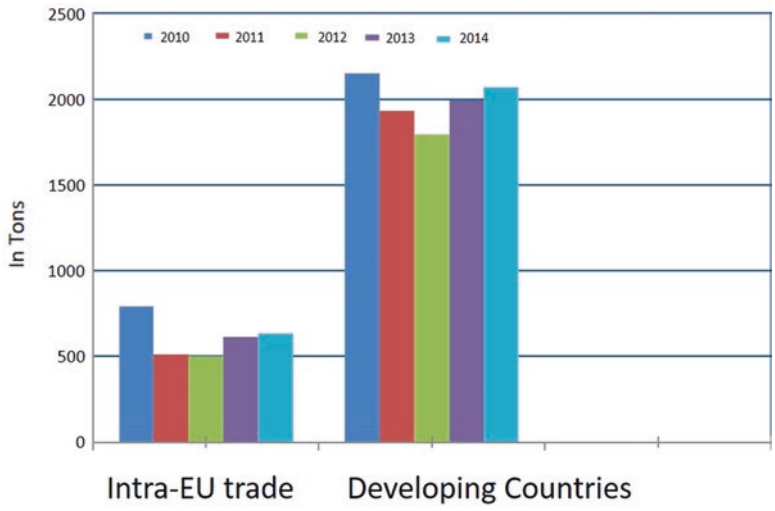

Fig. 28.3 Import of *L. nobilis* in European countries

28.4 Phenomics of *L. nobilis*

Laurel is a small dioecious evergreen tree or shrub that can be distinguished due to its narrowly oblong-lanceolate, alternate, and coriaceous leaves with aromatic smell (Fig. 28.4) (Conforti et al. 2006). Leaves are dark green, leathery, elliptic-lanceolate, and wavy at the margins with 5–8 cm length and 3–4 cm broadness (Afifi et al. 1997). This plant hardly multi-branched and normally attains a height of 20–30 feet (Said and Hussein 2014). This plant makes less branches and contains reddish blue or olive green bark. Leaves of this plant have redolent, balmy, strong, and sweet aromatic fragrance (Shokoohinia et al. 2014). Figure 28.5 shows the leaves of this plant.

Flowering of *L. nobilis* starts in April and fruits ripe in September (Sari et al. 2006). Flowers of this plant are small with four lobed; female contains 2–4 staminodes while 8–12 stamens are present in male plant. Female flowers are pale yellow in color with superior ovary containing single pistil with one ovule and four staminodes which are 4–5 mm in length. Flowering in the male and female plant is shown in Figs. 28.6 and 28.7, respectively. Male flowers are also pale yellow colored having 6–7 mm length, and 8–14 staminodes are present. The opening of a flower occurs at the same time in both male and female plants (8–9 am), and male plants can be distinguished from female on the basis of their flower production. Male plants produce more flowers per single branch as compared to female plant, and the life of a male flower is shorter than the female flower (Pacini et al. 2014). Similarly height of mature flower in male plant is recorded between 5.7 and 6.2 mm that is

Fig. 28.4 *L. nobilis* plant present in the Turkey

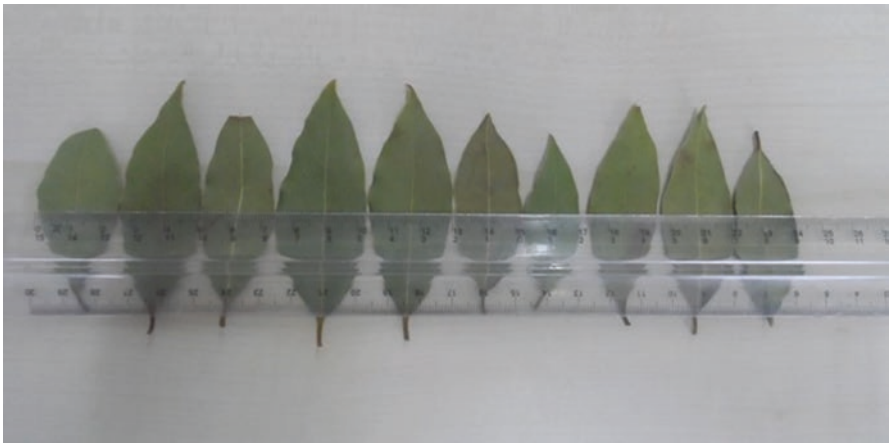

Fig. 28.5 Diversity in the leaves of *L. nobilis* plant

Fig. 28.6 Flowering of male *L. nobilis* plant

approximately double from the flower height in the female plant (Aytürk and Meral 2012). Anthers of *L. nobilis* are opened through valve mechanism which is specific to very few families including Lauraceae family (Hufford 1996), and these valves close the anthers during the rain and high humidity. Angiosperm plants have usually four linear stomia (Hufford 1996); however, anthers of *L. nobilis* contain two U-shaped stomia (Pacini et al. 2014). Pollens of the *L. nobilis* have very thin exine which is covered by very small spines, and intine is very thick as compared to exine. Various monocots of tropical regions (Kress 1986) and some gymnosperms (Pacini et al. 1999) have similar pollen structure such as *Pinus*, *Juniperus*, and *Cupressus*.

Fig. 28.7 Flowering of female *L. nobilis* plant

Fig. 28.8 *L. nobilis* fruit

On the basis of dehydration, Franchi et al. (2011) classified the plants into four groups: recalcitrant pollen and seed, orthodox pollen and seeds, recalcitrant pollen and orthodox seeds, and orthodox pollen and recalcitrant pollens. *L. nobilis* belongs to a group having both recalcitrant pollen and seeds as both pollen and seeds need 30% water contents for their dispersal (Franchi et al. 2011). Viability of *L. nobilis* pollens has been found less than 1 day (Pacini et al. 2014) that is similar to other recalcitrant pollens (Nepi et al. 2001). Ripening of fruit occurs in September–October, and shedding of fruit starts if not collected timely. Fruit has ellipsoid or ovoid shape (Fig. 28.8) with black color and a size of 10–15 mm (Sari et al. 2006; Conforti et al. 2006).

28.5 *L. nobilis* Genetic Resources

Genetic resources are serving as a source of new genes in the breeding programs of plants (Baloch et al. 2014). Efforts have been done to conserve the genetic resources of various crops. However, very few efforts have been done to conserve the genetic resource of dioecious plants on a large scale. *L. nobilis* is mainly distributed in the Mediterranean countries of Spain, Turkey, France, and Italy. Researchers from many countries have performed various studies about the composition and biological activities of essential oils. However, almost no effort has been done in the conservation of genetic resources of this plant. Yalçın et al. 2007 collected the leaves of *L. nobilis* from Northern Cyprus for the determination of essential oil composition. Caputo et al. (2017) used the Italian *L. nobilis*, and they investigated the composition and activities of resulting essential oils. Similarly Damiani et al. (2014), Snuossi et al. (2016), and Derwich et al. (2009) used very limited number of *L. nobilis* plants in their study for the identification of different activities of essential oils. In order to improve the breeding programs of this important plant, there is a need to collect the *L. nobilis* specimens from different climatic zones and should be conserved. Currently Baloch and his team members (co-authors of this study) collected the seeds of around 600 *L. nobilis* genotypes from 300 different geographical points of the Marmara, Aegean, and Mediterranean regions of Turkey at the Aegean Agricultural Research Institute under the umbrella of the General Directorate of Agricultural Research and Politics of Turkish Ministry of Food, Agriculture, and Livestock (TAGEM). A nursery plot was established (Fig. 28.9) at Aegean

Fig. 28.9 Nursery plot of *L. nobilis* established at Aegean Agricultural Research Institute of İzmir, Turkey

Fig. 28.10 *L. nobilis* orchard established at the Aegean Agricultural Research Institute of İzmir, Turkey

Agricultural Research Institute of İzmir (by Kirik and his team), and each genotype would be labelled and used for further genomic and metabolomics analysis.

Laurel orchard was established by germinating the collected seeds. The seedlings were planted and transferred to field conditions at the Aegean Agricultural Research Institute (Fig. 28.10). Each plant was labelled according to collection site and would be used for further genomics and metabolomics studies by the Baloch and his colleagues in near future. The seeds of these samples are conserved at the Aegean Agricultural Research Institute of İzmir with the aim to provide to interested researchers from any part of the world to breed this plant.

28.6 Genomics of *L. nobilis*

L. nobilis is an evergreen shrub with dark green leaves, and different ploidy levels have been identified in *Laurus* (Ehrendorfer et al. 1968), while tetraploidy ($2n = 4x = 48$) has been identified as most frequent karyotype (Arroyo et al. 2010). Genome size of this plant is not investigated till now. Advancement in the field of molecular markers increased the efficiency of breeding activities and helped the scientist to understand and explore the genetic diversity and population structure of any crop (Baloch et al. 2017; Nadeem et al. 2018). Different types of molecular markers have been applied to investigate the population structure and genetic diversity of *L. nobilis*. Arroyo et al. (2010) applied the 20 microsatellite markers for the determination of laurel genetic structure and pattern of gene flow via animal-dispersed seeds and pollen. They used 26 Macaronesian islands (*L. azorica*) and 37 Mediterranean (*L.*

nobilis) genotypes as plant material, and they investigated a total of 222 novel alleles in Macaronesian islands (*L. azorica*) and 196 alleles in *L. nobilis*. Arroyo-Garcia et al. (2001) used AFLP markers for the investigation of genetic similarity between *Laurus* populations and two species collected from various geographical regions. They found that accessions collected from France and Italy expressed lower similarity level with Iberian samples. Accessions from Iberian Peninsula expressed higher genetic similarity with the Canary Islands and Madeira accessions. Rodríguez-Sánchez et al. (2009) used cpDNA for the investigation of historical range shift of 57 laurel populations and three Lauraceae genera. They investigated low sequence variability within laurel and found three lineage groups during their study. A clear divergent group was found in Turkey and near East Asia, while the Aegean region contained a second group and last third one known as Western group containing all populations from Macaronesian and central and western Mediterranean. Their study also expressed a close relationship between the western populations of *L. nobilis* and Macaronesian populations of *L. azorica*. Hajyzadeh et al. (2013) applied a total of 12 SSR markers to identify the genetic diversity and relationship among 40 cherry laurel genotypes with *L. nobilis* collected from various climatic regions of Turkey. Their results showed less genetic variability within cherry laurel, and they found less genetic similarity of cherry laurel with *L. nobilis*. Mohamed et al (2016) used the leaf morphological characters and ISSR markers in order to investigate the genetic differentiations among the eight taxa of Lauraceae collected from various geographical regions of Egypt. They investigated sharp differences in leaf morphology and ISSR markers that showed clear genetic similarity between *L. nobilis* and *L. azorica* by clustering both of these under the same subgroup.

28.6.1 L. nobilis *Breeding*

28.6.1.1 Classical Breeding

Significant efforts have been done to understand the breeding mechanism of angiosperms ranging from self-incompatibility to dioecism and from obligate selfing to obligate outcrossing (Endress 1994). Sexual system is the main component affecting the polyploidy, lineage, evolutionary maintenance, and initial spread of many species (Pannell et al. 2004). Laurel is a small dioecious evergreen perennial tree, and very less work has been done regarding the breeding of this plant. Dioecious nature, difficulties in seed germination, and sexual reproduction are the main factors which hinder the breeding programs of this plant (Souayah et al. 2002). Hybridization method plays a significant role in the breeding of plants especially in dioecious plants. Natural hybridization occurring in *L. nobilis* is possible, but success rate is very low as low rate has been observed in other dioecious plants like date palm. Spontaneous hybridization can also be used if both male and female start flowering at the same time and they are in vicinity so that pollination may occur easily through

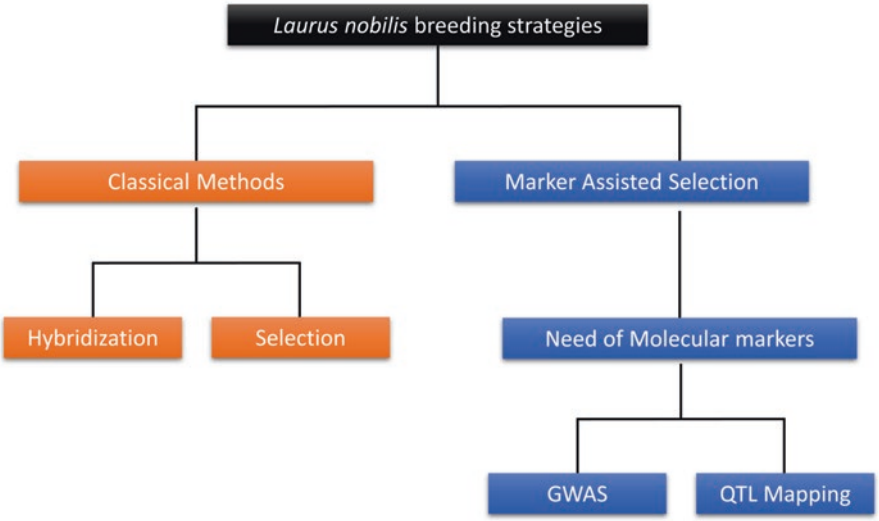

Fig. 28.11 Breeding methodologies used in the breeding programs of *L. nobilis*

wind or insects (Gros-Balthazard 2013). Artificial hybridization gives successful hybrids of plants and has performed in various dioecious plants like *Ocotea catharinensis* Mez (Santa-Catarina et al. 2004), date palm (Gros-Balthazard 2013), and common persimmon (Choi et al. 2003). However, artificial hybridization has also some drawbacks like decrease in the genetic diversity and does not have control over genetic mutations. Hybridization lowers the diversity level by the breakdown of reproductive barriers, extinction of species or populations, and coalition of earlier distinct evolutionary lineages (Buerkle et al. 2003; Vuillaume et al. 201). Low multiplication recovery is a big problem for breeding of this plant, and autumn is the favorable season for its multiplication, through cuttings as the best technique. However, both of these techniques (spontaneous hybridization and artificial hybridization) provide low multiplication recovery (Laurent 2007). Difficulties in pollination and significant loss of seeds by birds result in the small seed yields (Al Gabbiesh et al. 2015). These traditional methods like using seeds, cuttings, and layering are very slow and do not meet the homogeneity levels. Figure 28.11 represents some breeding methodologies that can be used in the improvement of *L. nobilis*.

28.6.1.2 Tissue Culture

Difficulties in the traditional breeding methods have been overcomed by the tissue culture techniques which are promising tools for dioecious plants especially *L. nobilis* demanding conservation (Al Gabbiesh et al. 2015). Somatic embryogenesis derived from calli and direct shoot regeneration are two main tissue culture techniques. However, till now all efforts to obtain shoot induction from calli has been

failed, while somatic embryogenesis has been found successful in other species of *Laurus* (Chen and Chang 2009). Process of zygotic embryo formation results in multiplication of tissues (Al Gabbiesh et al. 2015). Al Gabbiesh et al. (2015) developed a somatic regeneration protocol for the *L. nobilis*, and they had a success rate of 62.5% for the callus regeneration.

Souayah et al. (2002) used various conventional and in vitro techniques for the improvement of this plant. They showed that successful breeding (conventional and in vitro) is clearly dependent on the sampling date, used culture, and type of cuttings. Chourfi et al. (2014) observed that in vitro culture techniques are promising tools to overcome the multiplication problem of this plant. They focused on the germination from seed and micro-cuttings as a tool for multiplication and finally test the acclimatization of these plants.

Organogenesis is another important tissue culture technique and is very effective for dioecious plants like date palm. This technique can be used for *L. nobilis* through the meristem induction, shoot multiplication, shoot elongation, and acclimatization. Induction of organogenic culture is induced from young leaves which are present on the offshoots. Multiplication of shoot requires plant proliferation which is performed in a culture media having specific proportions of auxin and cytokinin. Transformation of these shoot buds in a specific growth medium results in the elongation of offshoots (Al Gabbiesh et al. 2015).

To our best knowledge, even single study is not available about the *L. nobilis* protoplast isolation. Similarly very less work has been done on this technique in other dioecious plants because of rapid protoplast death. There is a need to develop a protocol for the callus formation from the protoplast of *L. nobilis*. Chabane et al. (2007) performed a successful callus formation from the protoplast of date palm. This technique is mainly used to transfer the genes in date palm related to various traits of interest (Chabane et al. 2007), and as *L. nobilis* is very popular for its essential oils, this technique will be very productive in the laurel breeding.

28.6.1.3 Mutation Breeding

For the development of new cultivars, exploitation of genetic diversity is very important. Different methods have been developed to induce the genetic variability. T-DNA insertional mutagenesis, tissue culture-derived variation or somaclonal variation, and physical and chemical mutagens are important methods to induce variations in plants. Gamma radiations are common physical mutagens, and ethyl methane sulfonate (EMS) has been used as chemical mutagen in various crops including *L. nobilis* following a protocol developed by Jain (2012) for date palm. For the generation of mutants, doses of mutagen should be optimized for LD_{50} value. Superior mutants should be selected as they will perform well during regeneration. Some important steps involved in the mutation breeding are:

1. Cell suspension with a 3–4 cell clump size should be prepared in the liquid medium from a fragile callus. Record the cells stationary, growth, and doubling time.
2. Freshly prepared EMS (0.5–1.0%) should be added with very care in the actively growing cell suspension during the growth stage.
3. Very high density (100,000 cells/ml) of cells should be used.
4. Shaking is performed on 100 rpm for 6 h (date palm), and this can be adjusted according to genotype.
5. Centrifuge for 5 min at 5000 or 6000 rpm after transferring the cell solution in centrifuge tubes.
6. Pellet is removed, and fresh culture medium is added and again centrifuged at 5000 or 6000 rpm. Centrifuge should be repeated five to seven times in order to remove mutagens.
7. After the centrifuge, this treated cell solution should be spread on the surface of agar medium for 96 h to recover from mutagen treatment.
8. Thereafter, this mutagen-treated solution should be put under the selection pressure, e.g., fungal toxin for the selection of disease-resistant lines, or some other trait can be tested.
9. For the direct shoot formation or callus production, superior tissues should be subculture repeatedly. These in vitro plantlets should be hardened in greenhouse by managing 70–90% relative humidity.
10. Finally field testing of these plants should be performed in order to evaluate the success.

Mutation breeding gained the concentration by the breeders to produced plants having favorable traits with the help of spontaneous or induced mutation techniques. Both of these techniques have been successfully applied in various dioecious plants (Jain 2012),

Genetic variations are very important to fulfil the needs of breeding in any crop. Somaclonal variations with in vitro selection resulted as promising tool in the breeding of plants especially for biotic and abiotic stress. Somaclonal variations refer to changes occurring during the cell cultures and in the regenerated plants and their progenies (Jain 2012). This technique also has been applied in various plants to develop the new genotypes having more number with good size of fruit and improved texture or taste and for the improvement in the flowering structure (Ahloowalia and Maluszynski 2001; Pedrieri 2001; Witjaksono 2003). Somatic cells can be used as a source of variations that can be used in the breeding programs of *L. nobilis* for the generation of new clones with favorable traits. This method has been successfully used to produce modified clones in various plants like pistachio (Benmahioul et al. 2012), banana (Sahijram et al. 2003), and date palm (Jain 2012). As this technique is applied successfully, it will be very helpful for the generation of new and improved *L. nobilis* plants.

28.6.1.4 Sex Differentiation in the *L. nobilis*

Lauraceae is very interesting family for the study of dicliny as they have separate male and female plants (Pacini et al. 2014). Most of the domesticated plants are monoecious, and only 7% of flowering plants containing 38 families belong to dioecious nature (Renner and Ricklefs 1995). There is a possibility that these dioecious plants have evolved from the hermaphrodites or monoecious through two independent mutations which resulted in the reduced female fertility and male sterility that finally led to the functional dioecy (Charlesworth 1991). Historically, very less breeding program has been done in order to maintain genetic diversity in the dioecious plants because of the absence of accurate way to differentiate the male and female plant (Elmeer and Mattat 2012). Morphological characters like flowering, plant height, flower height, and flower production can be used for the sex differentiation in the *L. nobilis*. Flowering can be used for the sex differentiation of *L. nobilis* as male flower contains 8–14 staminodes while female flower has 2–4 staminodes. Flower production is another criterion that can also be helpful in the sex differentiation. Male plants produce higher numbers of flowers as compared to female plants (Figs. 28.6 and 28.7), and the life of a male flower is shorter than the female flower (Pacini et al. 2014). Flowering height can also be used in the sex differentiation as flower height in mature plant is observed between 5.7 and 6.2 mm which nearly doubles from the height of flowers present in the female plants (Aytürk and Meral 2012). Sex differentiation on the basis of morphological characteristics is very long process because this is a perennial plant and its flowering starts after several years. As compared to the morphological and anatomical characteristics, sex differentiation on the molecular basis can be a more effective and time-saving method with promising, more accurate results. Different molecular markers have been used in different perennial and dioecious plants (Elmeer and Mattat 2012; Kafkas et al. 2001; Deputy et al. 2002). *Litsea cubeba* is an important plant of Lauraceae family, and Wu et al. (2015) applied the SRAP markers for the identification of sex-linked molecular markers. They developed SCAR markers and used them for the sex differentiation of *L. cubeba*. Very recently Khodaeiaminjan et al. (2017) used the SNP marker to identify the sex-linked SNP markers in the pistachio, and they resulted in nine novel sex-linked SNP markers. All of these markers were found homozygous for male and heterozygous for female plants. So now there is a need to utilize these molecular markers in the *L. nobilis* for the precise and early sex differentiation and to improve the breeding program.

28.6.2 Marker-Assisted Breeding

Marker-assisted selection (MAS) is a high-throughput breeding method in which phenotypic selection is made on the basis of genotype of a marker. MAS has changed the fate of plant breeding by overcoming the limitations of classical breeding (Nadeem et al. 2018). In MAS we need markers that can be obtained through the

Fig. 28.12 A systematic approach for the identification of QTLs in the *L. nobilis* for different traits of interest

QTL mapping or genome-wide association mapping (GWAS). QTL mapping is also known as the biparental mapping in which two parents are selected and crossed for the development of mapping populations (Collard et al. 2005; Nadeem et al. 2018). QTL mapping has been done for various traits of interest in many dioecious plants like date palm (Billotte et al. 2010) and *Ginkgo biloba* (Liu et al. 2017). Still no scientific studies have been conducted about QTL mapping in *L. nobilis*, and this can be achieved by selecting two diverse parents having enough compatibility. Crossing of these parents will result in the F1 generation (Nadeem et al. 2018). Normally F2, double haploids, backcross, and inbreed lines are used as mapping populations in annual plants. However, *L. nobilis* is a perennial plant, and development of these mapping populations needs very long period of time. So F1 generation can be used as mapping population in the *L. nobilis*. Methodology of QTL mapping for *L. nobilis* is presented in Fig. 28.12.

QTL mapping has been found very effective in the plant breeding; however, it has some drawbacks like less allelic diversity with lesser recombinations. Development of mapping population is a big drawback of this technique, and it can be a gigantic problem in case of dioecious plants like *L. nobilis.* To overcome these limitations, GWAS emerged as a more handful tool for the breeding of annual and perennial plants.

28.6.2.1 Genome-Wide Association Mapping (GWAS)

Advancement in the field of molecular breeding has resulted in the development of GWAS. Initially GWAS was developed in the human being to identify the different genes controlling various diseases (Brachi et al. 2011). As compared to QTL mapping, here, diverse parents are selected which increase the more recombination, and level of incompatibility is also minimized. Markers identified through the GWAS are very accurate, and they express higher efficiency because phenotypic experiments are conducted in different environmental conditions with several year repetitions (Nadeem et al. 2018). GWAS can be performed in *L. nobilis* through the following steps shown in Fig. 28.13.

Genotyping by sequencing (GBS) has emerged as robust and rapid technique of GWAS, and its low price with high-throughput efficiency has made it a very promising technique (Baloch et al. 2017). Currently Baloch and his team are working on a project for the genome characterization of Turkish *L. nobilis* germplasm using GBS assay that has resulted nearly 50,000 SNP and DArTseq markers separately working under a project (data not shown). Similarly in another project, Baloch and his colleague also used the retrotransposon markers for the investigation of genetic

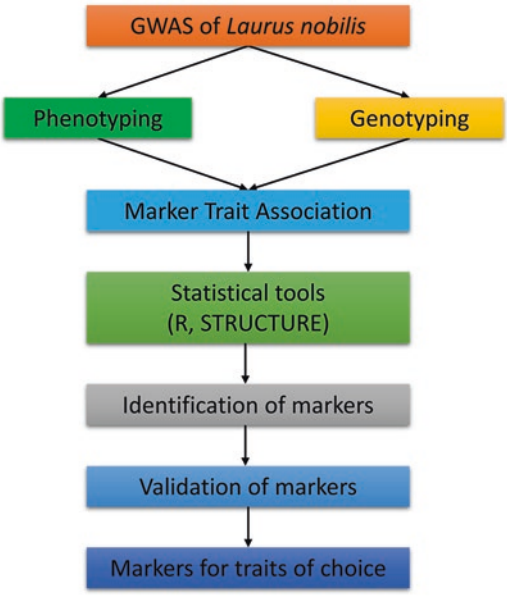

Fig. 28.13 GWAS for the identification of genetic markers in the *L. nobilis*

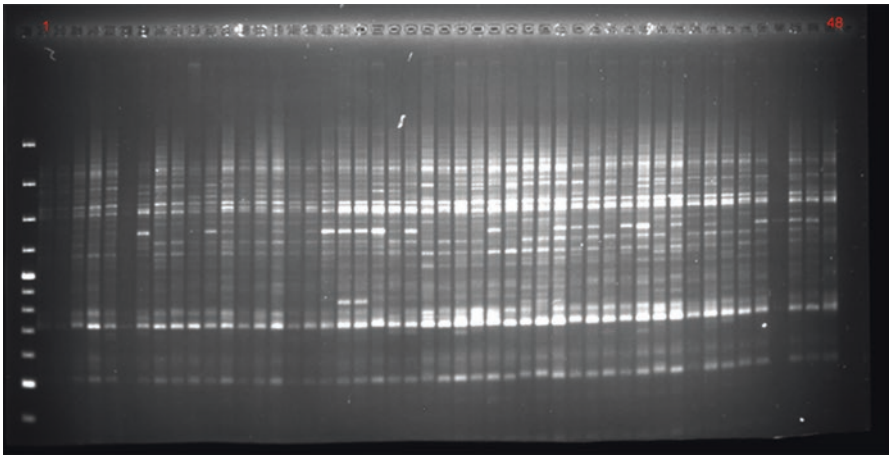

Fig. 28.14 Genetic diversity profil of 48 *L.nobilis* genotypes collected from various geographical regions of Turkey

relationship among and between the Turkish *L. nobilis* germplasm (Fig. 28.14) that will be available very soon (data not shown). These results pertaining to DArTseq and SNP markers will be used in future association mapping of *L. nobilis*. Markers obtained through this study will be used in the breeding programs of *L. nobilis* and will establish the starting point for the breeding of *L. nobilis*.

28.7 Chemical Compositions of Essential Oil

L. nobilis is famous for its essential oil, and normally its leaves have been successfully used as spice and flavoring agent in food industry. Its leaves have been used in the treatment of several diseases (Caputo et al. 2017). Different earlier researchers indicated that bay leaves and their essential oils have beneficial effects against digestive disorders such as flatulent colic, and increasing effect on gastric fluid secretion has been shown in recent studies. Anticonvulsive and antiepileptic activities of the leaf extract have been confirmed. Generally, the yield and composition of the essential oil vary, depending upon the origin, the collection period, and the growth stage of the plant; in Tunisia, essential oil of laurel ranges between 0.65 and 2.2% by weight (Marzouki et al. 2009), but in Turkey, it increased up to 3.73–4.19% (Polat et al. 2009). The essential oil contents of the aerial parts of *L. nobilis* obtained by hydrodistillation were 0.7%, 0.8%, 1.1%, and 0.6% in the vegetative, bud, flowering, and seed-bearing stages, respectively, calculated on a dry-weight basis. The major constituents of this oil were 1,8-cineole (35.7%), trans-sabinene hydrate (9.7%), α-terpinyl acetate (9.3%), methyl eugenol (6.8%), sabinene (6.5%), and eugenol (4.8%) (Verdianrizi and Hadjiakhoondi 2008). The essential oil has been reported to have bactericidal and fungicidal properties; it also depressed the heart rate and lowered blood pressure in animals; and formulations containing laurel leaf and its volatile oils have been claimed to have antidandruff activities. The oil is used mainly as a fragrance ingredient in creams, lotions, perfumes, soaps, and detergents; maximum use level reported is 0.2% in perfumes (Leung and Foster 2003). And also, its leaves and essential oil are mainly used for the treatment of bacterial and fungal infections and for the treatment of gastrointestinal, flatulence, and eructation problems (Chmit et al. 2014; Dias et al. 2014). Monoterpene hydrocarbons are found in higher concentrations in laurel essential oils; 1,8-cineole, −terpinyl acetate, and terpinene-4-ol are main chemical compounds present in laurel essential oil expressing a great antibacterial, antifungal, and antioxidant activity (Yalçın et al. 2007), while some other important chemicals are α-terpineol, α-pinene, β-pinene, sabinene, and terpin-4-01(Özcan and Chalchat 2005). Different concentrations of 1,8-cineole, −terpinyl acetate in laurel essential oil has been reported from different parts of world like 34.62% in Algeria (Jemâa et al. 2012), Turkey 44.97% (Ekren et al. 2013), 52.43% in Morocco (Derwich et al. 2009), Tunisia 56% (Snuossi et al. 2016) and maximum 58.59% in Cyprus (Yalçın et al. 2007). Juergens et al. (2003) checked the anti-inflammatory efficiency of 1,8-cineole in the asthma patients, and they found that essential oil of laurel is mucolytic agent for the lower and upper airways diseases. Alcaraz-Meléndez et al. (2004) reported that usage of 1,8-cineole can boost up the testosterone hydroxylase level in the human body. Recently Caputo et al. (2017) checked the antimicrobial activity of laurel essential oil on five different bacterial strains and have shown significant antimicrobial activity in laurel essential oil. Dadalioğlu and Evrendilek (2004) studied the chemical composition and antibacterial effects of different Turkish medicinal plants and have shown significant antimicrobial activity in Turkish laurel. Damiani et al. (2014) prepared

plant extract containing significant concentrations of 1,8-cineol by steaming the laurel leaf and resulted this extract as a promising tool for the treatment of different bacterial diseases in honeybee. Food deterioration is very big problem, and Cherrat et al. (2014) investigated the antimicrobial activity of laurel essential oils for the preservation of food and found the strongest antimicrobial activity in the laurel essential oils. El et al. (2014) checked the antioxidant and antimicrobial activity in the essential oils of Turkish laurel, and they also found strong antioxidant and antimicrobial activity which can be used in the preservation of food. Muñiz-Márquez et al. (2013) performed the ultrasound-assisted extraction of different phenolic compounds and noted the presence of significant concentrations of these compounds that can be used as strong antioxidants. Fernandez-Andrade et al. (2016) checked the antifungal activity in the Brazilian laurel, and they noted that laurel essential oil expressed moderate-to-strong activity against various fungal strains. Very recently Peixoto et al. (2017) investigated the antifungal potential of laurel essential oils, and they noted strong antifungal activity in laurel essential oil for *Candida* spp. which causes human oral candidiasis. Bay leaves and their essential oil do not appear to have any significant toxicity; however, sporadic reports have indicated that bay leaves may cause allergic contact dermatitis (Brás et al. 2015).

28.8 Conclusions

Laurel has great medicinal importance, and a lot of studies have been done regarding various scientific aspects of the plants including metabolomics. However, very few studies have been done regarding genetic structure and breeding aspects of this plant as compared to other plants. There is a need to investigate the genetic structure of *L. nobilis* collected from different countries of the world. As a lot of work has been performed on the metabolomics of this plant; now there is a need to improve the concentrations of different important compounds through different breeding programs like genome-wide association mapping and genomic selection. There is a need to determine the chromosome number, polyploidy level, mechanism to investigate the sex differentiation, and whole-genome sequence for the better understanding of this plant.

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Chapter 29
Biological Databases for Medicinal Plant Research

Sonu Kumar and Asheesh Shanker

Abstract Bioinformatics resources serve as an important source of data, knowledge, and information in biological studies, including plants having medicinal properties. Most of the plants found in nature have different medicinal properties; therefore, these are used to cure many human diseases from ancient times all over the world. Plant-derived medicines are an important source of lifesaving drugs. The availability of bioinformatics resources brought a major change in medicinal plant research, in terms of time, money, and labor. In this chapter, we have focused on various biological databases which are helpful in medicinal plant research and may result in a rapid and cost-effective lead generation toward finding remedies from plants.

Keywords Bioinformatics · Database · Drug · Plant · Medicine

29.1 Introduction

Plants are important resource providers of diverse materials that are useful for a variety of purpose including timber, food, and medicine. Plants are considered to be the main source of energy and medicine since ancient times. Medicinal plants contain a variety of ingredients, which are widely used in drug development and synthesis. Presently, medicinal plants are used to produce raw materials for many chemical drugs such as antimalarial and anticancer. A large number of clinical drugs are derived from plant extracts and their derivatives. Medicines derived from plants are an important source in plant-based drug development and about one-third of the drugs developed from natural products (Strohl 2000). In 1980, isolation of quinine, an antimalarial drug, from the bark of *Cinchona* species was reported (Buss et al. 1995). Moreover, flavonoid content has been evaluated for their antioxidant and anticancer activities in *Dryopteris erythrosora* (Cao et al. 2013) and *Litchi chinensis* (Wen et al. 2014). Thus, on the basis of ethnomedicinal data, plants have a huge

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potential for new drugs to be discovered (Fabricant and Farnsworth 2001; Clarkson et al. 2004). Studies on medicinal plants are crucial, not only to understand its potential to cure diseases but also for evolution, plant-based drug design, and development. Unfortunately, the lack of knowledge and information is a major limiting factor in medicinal plant studies. Conventional methods for the plant-based drug discovery are much expensive and time consuming (DiMasi et al. 2003). However, the application of computational approaches helps to speed up the process of plant-based drug design (Harishchander 2017).

Recent development in bioinformatics resources brought a major change in current studies of medicinal plants including plant-based drug discovery. The availability of curated databases of medicinal plants and natural products play a vital role in the area of plant-based drug discovery. In order to understand the biological mechanism of drug-like plant-derived compounds, to analyze, and to interpret the data associated with it, bioinformatics approaches were applied (Kann 2009; Harishchander 2017).

29.2 Common Bioinformatics Resources

In the last few decades, bioinformatics has established itself as an independent discipline that deals with the development and application of computational algorithms, tools, databases, and web servers to solve and understand biological problems. The establishment of the National Center for Biotechnology Information (NCBI; http://www.ncbi.nlm.nih.gov/) in 1988 brought a major change in the field of biological research (Smith 2013). Moreover, GenBank (http://www.ncbi.nlm.nih.gov/genbank/), DNA Data Bank of Japan (DDBJ; http://www.ddbj.nig.ac.jp/), and European Molecular Biology Laboratory's European Bioinformatics Institute (EMBL-EBI; http://www.ebi.ac.uk/embl/) were developed. Altogether, these three constitute the International Nucleotide Sequence Database Collaboration (INSDC; http://www.insdc.org) (Cochrane et al. 2015). Apart from this, several biological databases were also developed which provide platform for the annotation of medicinal plant data (Table 29.1).

In addition to this, bioinformatics also provides a variety of tools and techniques needed to analyze and interpret large amount of biological data generated from biological experiments. Sequence alignment is one of the most important bioinformatics techniques broadly used to find similarity between biological sequences (Mount 2004; Sharma et al. 2016). Different computational algorithms like dynamic programming have been developed to align sequences including global (Needleman and Wunsch 1970) and local alignment (Smith and Waterman 1981). Basic Local Alignment Search Tool (BLAST; Altschul et al. 1990) and FASTA (Pearson 1990) help to search large-scale databases. Multiple sequence alignment (MSA) methods, used to align more than two sequences, are also available (Mount 2004). A list of bioinformatics tools which can be used in the study of medicinal plants is represented in Table 29.2.

Table 29.1 List of commonly used bioinformatics database

S. No.	Database	Description	Uniform resource locator (URL)	References
1.	PIR (Protein Information Resource)	Contains functionally annotated protein sequences data	http://pir.georgetown.edu/	Barker et al. (1998)
2.	GenBank	Contains nucleotide sequence database	http://www.ncbi.nlm.nih.gov/genbank/	Benson et al. (2000)
3.	PDB (Protein Data Bank)	Provides information about experimentally determined protein structures, nucleic acids, and complex assemblies	http://www.rcsb.org/pdb/home/home.do	Berman et al. (2002)
4.	KEGG (Kyoto Encyclopedia of Genes and Genomes)	Provides information about genomes, biological pathways, diseases, drugs, and chemical substances	https://www.genome.jp/kegg/	Kanehisa and Goto (2000)
5.	MMDB (Molecular Modeling Database)	Contains experimentally determined three-dimensional biomolecular structures	http://www.ncbi.nlm.nih.gov/Structure/MMDB/mmdb.shtml	Chen et al. (2003)
6.	UniProtKB/Swiss-Prot	Contains protein sequences along with their functional information	http://www.uniprot.org/uniprot/	Boutet et al. (2007)
7.	Entrez Protein Database	Provides collection of protein sequences from several sources	https://www.ncbi.nlm.nih.gov/protein	Wheeler et al. (2007)
8.	DDBJ	Contains nucleotide sequence database	http://www.ddbj.nig.ac.jp/	Kaminuma et al. (2010)
9.	EMBL-EBI	Contains nucleotide sequence database	http://www.ebi.ac.uk/embl/	Amid et al. (2011)

The various bioinformatics resources covered in this chapter will be helpful in several ways in medicinal plant research. The databases including GenBank, DDBJ, and EMBL-EBI can be used to retrieve genomic sequences of medicinal plant. Moreover, for protein sequence data PIR, UniProt, and Entrez Protein Database can be used as primary resources. Three-dimensional structure of proteins associated with medicinal plants can be retrieved from Protein Data Bank. Besides, experimentally determined three-dimensional structure of molecule associated with the medicinal plant can also be obtained from databases like MMDB. Furthermore, KEGG Database assists to obtain information about genomes, biological pathways, diseases, drugs, and chemical substances related to medicinal plants.

Alignment tools including BLAST and FASTA are widely applied to find sequence similarity between nucleotide and/or protein sequences of medicinal plants, whereas ClustalW can be used for multiple sequences alignment. Molecular modeling and docking are widely used techniques for protein structure prediction

Table 29.2 List of commonly used bioinformatics tools

S. No.	Resource	Description	URL	References
Alignment tools				
1.	BLAST	An alignment tool used to find out similarity between nucleotide or protein sequence	http://ncbi.nlm.nih.gov/BLAST/	Altschul et al. (1990)
2.	FASTA	A pairwise sequence alignment tool used to align nucleotide or protein sequences	https://www.ebi.ac.uk/Tools/sss/fasta/	Pearson (1990)
3.	ClustalW	A multiple sequence alignment (MSA) program	http://ebi.ac.uk/Tools/msa/clustalw2/	Thompson et al. (1994)
Molecular modeling tools				
1.	SWISS-MODEL	An automated protein structure homology-modeling server	http://swissmodel.expasy.org/	Guex and Peitsch (1997)
2.	MODELLER	It is a homology modeling program used to predict 3D structures of protein	http://www.salilab.org/modeller/	Webb and Sali (2014)
3.	CPHmodels	It is a web server for protein 3D structure prediction	http://www.cbs.dtu.dk/services/CPHmodels/	Nielsen et al. (2010)
4.	I-TASSER (Iterative Threading ASSEmbly Refinement)	A protein structure and function prediction program that uses hierarchical approach to predict the structure and function of proteins	http://zhanglab.ccmb.med.umich.edu/I-TASSER/	Yang et al. (2015)
Molecular docking and simulation tool				
1.	AutoDock	It is a suite of automated docking tools commonly used to predict interactions between small molecules and receptor of known 3D structure of protein	http://autodock.scripps.edu/	Morris et al. (2009)
2.	AutoDock Vina	It is a new generation of docking software. It is faster than AutoDock and accomplishes significant perfections in average prediction of the binding mode	http://vina.scripps.edu/	Trott and Olson (2010)
3.	Glide	A ligand-receptor docking program based on exhaustive search	http://www.schrodinger.com/glide	Friesner et al. (2004)
4.	GROMACS (GROningen MAchine for Chemical Simulations)	A molecular dynamics simulation package	http://www.gromacs.org/	Hess et al. (2008)

and molecular interaction studies, respectively, whereas molecular dynamics simulation tools can be used to study the behavior of a molecule with respect to time.

29.3 Bioinformatics Resources for Medicinal Plant

Several specialized biological databases and web resources have been developed which provide information about plants having reported medicinal properties. Here we describe various resources that will be useful for medicinal plant research.

29.3.1 Indian Medicinal Plants Database

Indian Medicinal Plants Database (http://www.medicinalplants.in/aboutfrlhtdb) contains around 7263 botanical names of medicinal plants associated with more than 150000 vernacular names in 10 different Indian languages. Moreover, the database also contains more than 5000 authentic images of medicinal plants linked to the specific botanical entities. The database is organized on the basis of six different traditional Indian medicine systems (Ayurveda, Siddha, Unani, Homeopathy, Sowa-Rigpa, and Folk). Ayurveda includes 2559 botanical names and 1540 species, Siddha contains 2267 botanical names and 1149 species, Unani includes 1049 botanical names and 493 species, Homeopathy covers 460 botanical names and 372 species, Sowa-Rigpa holds 671 botanical names and 250 species, and Folk comprises of 6403 botanical names and 5376 species in the database.

29.3.2 Medicinal Plants Database: MEDDB

MEDDB (http://www.ladydoakcollege.edu.in/meddb/home.html) provides data from 110 different species, belonging to a total of 50 families, reported to be commonly used by tribal people around Madurai, India. The database provides search facility using the scientific name of the plant, Tamil vernacular name, and the disease name. The disease search option displays all the plants used for a particular disease (Mary et al. 2012).

29.3.3 HerbMed

The HerbMed (http://www.herbmed.org) database provides hyperlinked access of scientific data underlying the use of herbs for health. Hyperlinks and dynamic links are made to PubMed and other electronic resources, providing evidence-based

information for healthcare professionals, pharmacists, researchers, and healthcare consumers. The features of this resource are discussed from the perspective of ethnobotanists, field biologists, chemists, and biochemists interested in drug development for infectious diseases. Searching the database yields information that cannot be obtained from PubMed alone. The resource has a breadth and comprehensiveness that enable creative cross-referencing and unexpected links to provide fresh insights (Wootton 2002).

29.3.4 InDiaMed

The database of Indian Medicinal Plants for Diabetes (InDiaMed; http://www.indiamed.info) contains information of Indian medicinal plants which are used for the treatment of diabetes. InDiaMed was developed to explore the claims of Indian medicinal flora and open up the facets of many Indian plants which are being examined for their beneficial role in diabetes. InDiaMed provides biochemical, chemical, geographical, and pharmacological information of the medicinal plants. Additionally it includes scientifically relevant information and the comprehensible research done on diabetes. InDiaMed also comprises the list of polyherbal formulations which are used for diabetes treatment in India (Tota et al. 2013).

29.3.5 IMPPAT

The Indian Medicinal Plants, Phytochemistry, and Therapeutics (IMPPAT; https://www.imsc.res.in/~asamal/resources/imppat/home) database is manually curated and provides information about phytochemical constituents of medicinal plants commonly found in India. The database includes 1742 medicinal plants, 9596 phytochemicals, and 1124 therapeutic uses, which are spread over 27074 plant-phytochemical and 11514 plant-therapeutic associations. Moreover, non-redundant information about 9596 phytochemicals with standard chemical identifiers and structure, as well as 960 potential druggable phytochemicals filtered with the help of cheminformatics approaches, have been incorporated in the database (Mohanraj et al. 2017).

29.3.6 MPD3

MPD3 (http://bioinform.info) is a comprehensive online and downloadable database. It provides information about phytochemicals, activities, structural, and test target of medicinal plants at a single platform. The database contains more than

5000 phytochemicals from around 1000 medicinal plants with 80 different properties, more than 900 literature references, and 200-plus targets. Moreover, the database provides 632 genus and 1022 plant-based information including phytochemicals (7062), targets (271), and activities (91) records. MPD3 database provides four different views (genus, plant, activity, and phytochemicals) to categorize information stored in the database (Mumtaz et al. 2017).

29.3.7 MMDBD

Medicinal Materials DNA Barcode Database (MMDBD; http://www.cuhk.edu.hk/icm/mmdbd.htm) is an integrated medicinal material DNA database which contains information over 1000 species DNA sequences and key references of medicinal materials recorded in Chinese and American herbal pharmacopoeia with other related references. It also contains information about medicinal material, adulterant, resources, medical parts, photos, primers used to obtain the barcodes, and key references. The database provides storage, retrieval, comparison, and analysis of DNA sequences on a single web-based platform to distinguish medicinal substances from their general choices and derivatives (Lou et al. 2010).

29.3.8 NAPRALERT

Natural Products Alert (NAPRALERT; https://www.napralert.org/about) database provides information about natural products, including ethnomedical, pharmacological, and biochemical extracts of organisms. The database also contains secondary metabolites information from natural sources. To date, the database includes more than 200000 scientific literatures, representing organism's name and their geographic origin from all over the world (Loub et al. 1985).

29.3.9 DiaMedBase

DiaMedBase (http://www.progenebio.in/DMP/DMP.htm) database provides information about 389 medicinal plants used in treatment of diabetes including genus *Trigonella* (30), *Momordica* (22), *Gymnema* (19), *Opuntia* (13), *Panax* (13), *Allium* (11), *Aloe* (10), and *Tinospora* (10). In the database, 36% of data are collected from whole plant and the rest of data obtained from leaves (26%), seeds (12%), roots (10%), and fruits (4%) of medicinal plants. DiaMedBase provides unique accession number for each entry with "Disease Link" and also shows the list of diseases other than diabetes (Babu et al. 2006).

29.3.10 Indonesian Medicinal Plants Database

Indonesian medicinal plants database (http://herbaldb.farmasi.ui.ac.id) provides information about medicinal plants and three-dimensional (3D) structures of chemical compounds found in Indonesia. The database contains 3825 species records with 16244 local names and 6776 records documented in 12980 species-contents interaction along with 3D structures of 1412 chemical compounds from medicinal plants. All the data stored in the database is collected from the scientific literature, and source is noted (Yanuar et al. 2011).

29.3.11 Natural Medicines Comprehensive Database

The Natural Medicines Comprehensive Database (http://www.naturaldatabase.com) provides comprehensive and reliable natural evidence-based medicine sources, useful for healthcare professionals and patients. The database contains consensus of scientific information on natural medicines. This database is an excellent reference resource for researchers in which 15 categories of information are provided under each named product, to address the challenges most often encountered during patient care (Hsu 2002).

29.3.12 Super Natural II

Super Natural II (http://bioinformatics.charite.de/supernatural) database contains about 326000 natural compounds with corresponding two-dimensional structures and their physicochemical properties. Moreover, it also provides predicted toxicity and vendor information about 170000 compounds. The database also facilitates pathways information related to synthesis and degradation of the natural products, in addition to their mechanism in relation to drugs with similar structure and their target (Banerjee et al. 2014).

29.3.13 NPCARE

Natural Products CARE (NPCARE; http://silver.sejong.ac.kr/npcare), a database of natural product and fractional extract of a variety of biological resources including plants, bacteria, fungus, and sea creatures, contains information about 6578 natural compounds and 2566 fractional extracts with anticancer activities, confirmed for 34 different types of cancer using 1107 cell lines, isolated from 1952 different resources.

Moreover, each entry in this database is annotated with genus and species name, type of cancer, cell line used to validate anticancer activity, target gene or protein, and PubChem ID (Choi et al. 2017).

29.4 Conclusion

The bioinformatics resources described in this chapter will be helpful in medicinal plant research. Moreover, these resources will assist in enhancing the knowledge and information to cure a particular disease with the help of medicinal plant.

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