Chapter 8
Expanding Horizons: Role of Biotechnology in MAP Research, Production and Utilization

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Abstract Plant tissue culture (PTC) plays a vital role in selection, multiplication, and conservation of the critical genotypes of medicinal plants. These techniques hold immense potential for enhancing the production of high-quality secondary metabolites which form the basis of plant-based medicines. Rapid Biotechnology Based Breeding Methods (BBBMs) have led a revolution in Medicinal Aromatic Plant (MAP) research. Immense contribution has been made through the use of PTC based BBBM's as *Agrobacterium* mediated gene transformation and induction of polyploidy. Metabolic pathway engineering has received a boost using hairy root cultures. The identification of the genes and enzymes mediating the biosynthesis of secondary metabolites, through specific transcriptome detailing, through RNA-sequence analysis, has facilitated better yield of secondary metabolites. Using Next-Generation Sequencing (NGS) techniques like sequence specific nucleases, namely Zinc-Finger Nucleases (ZFNs), Transcription Activator-Like Effector Nucleases (TALENs) and Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR-Cas9) are used for genome editing which can mediate the production of designer MAPs. The application of such genome editing tools have a high potential in MAP research.

Keywords Medicinal aromatic plants · Biotechnology · Next-generation sequencing · Plant cell tissue and organ culture · Secondary metabolites · Micropropagation · Bioreactor

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Abbreviations

2,4-D	2,4-dichlorophenoxyacetic acid
BA	6-benzyladenine
BAP	6-Benzylaminopurine
BBBMs	Biotechnology based breeding methods
Cas	Caspases
COSTREL	Combinatorial super transformation of transplastomic recipient lines
CRISPR	Clustered regularly interspaced short palindromic repeats
DArTTM	Diversity array technology
EST	Expressed sequence tags
GA3	Gibberellic acid
GDA	Gene-driven array
IAA	Indole-3-acetic acid
IBA	Indole-3-butyric acid
IMPPAT	Indian Medicinal Plants, Phytochemistry and Therapeutics Kyoto Encyclopedia of Genes and Genomes
Kn	Kinetin
LS	Linsmaier and Skoog
MAPs	Medicinal Aromatic Plants
MS	Murashige and Skoog
NAA	1-naphthaleneacetic acid
NGS	Next-generation sequencing
PCTOC	Plant cell tissue and organ culture
PGRs	Plant growth regulators
PTC	Plant tissue culture
QTL	Quantitative trait loci
RAPD	Random amplified polymorphic DNA
SDA	Subtracted diversity array
SH	Schenk and Hildebrandt
SNP	Single nucleotide polymorphism
TALE	Transcriptional activator-like effector
TDZ	Thiadiazuron
WPM	Woody plant medium
ZFNs	Zinc-finger nucleases

8.1 Introduction

Traditional systems of medicine practiced globally rely on plants and their products for maintaining health through the repertoire of biologically active constituents present in them. India is amongst the most ancient civilizations and one of the richest repositories for developing knowledge on medical sciences. Shushruta, the

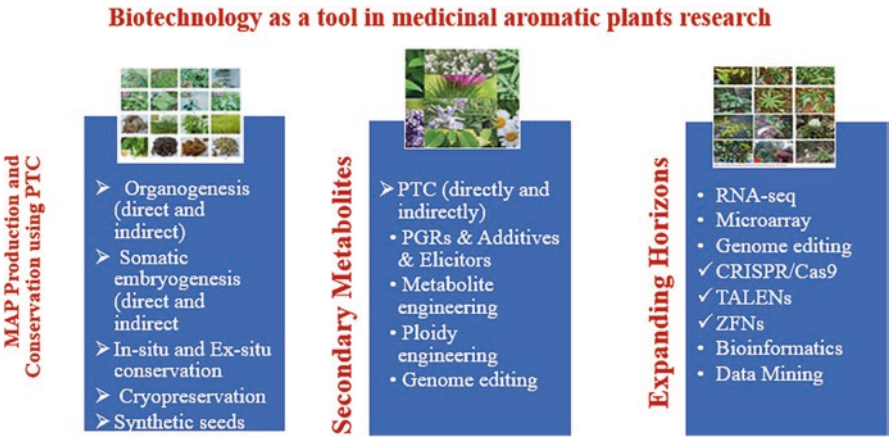

Fig. 8.1 Biotechnology as a tool in Medicinal Aromatic Plants research

Indian physician, referred across the globe as 'Father of surgery' and many alike have established a strong foundation to use medicinal plants for preserving and rectifying good health.

Over the years MAPs have attracted not only researchers but farmers, traders, economists as well as health professionals, as they are natural biological resources for multiple secondary metabolites with potential uses ranging from cosmetics, fragrances, insecticides dye etc.

Though traditionally these plants were grown in wild, the over-exploitation of many species has today lead to close to 4000 MAPs being listed as endangered species (El Meskaoui 2013). Thus, the need of the hour is MAP selection and cultivation using cutting edge biotechnological tools to cater to the increased demand (Canter et al. 2005) (Fig.8.1).

8.2 Plant Cell Tissue and Organ Culture

Use of plant cell tissue and organ culture (PCTOC) technology has reported success as an efficient tool in selection of MAPs as typically MAPs, offer less yield and they are highly sensitive to biotic stress (Isah et al. 2018). This is also an efficient tool for preservation of rare MAP species (Kayser and Wim 2007) and also for production of phytochemicals and secondary metabolites (Nagata and Ebizuka 2002).

The pragmatic approach towards successful PCTOC techniques is dependent on choice of explant, supplementation of medium with phytohormones along with the physical environment. Biotechnological approaches have facilitated *in-vitro* propagation and growth of MAP's through micropropagation, callogenesis, organogenesis, embryo or anther culture, somatic and asexual embryogenesis. The choice of the technique is dependent on plant species and determines the rate of success (Bhojwani and Razdan 1996).

8.2.1 Explants Used

Nodal, internodal, apical segments, leaves and its segments, rhizomes, seeds and shoot tips are the types of explants generally used. For *Acorus calamus,* using rhizome as explant, 73% shoot organogenesis was observed along with IAA-BAP treatment (Bhagat 2011). Tejovathi et al. (2011), successfully used media supplemented with BAP, IBA and GA3 with shoot tips as explant for *Commiphora wightii*. The very useful *Rauvolfia serpentina* responded well to *in-vitro* regeneration with juvenile leaf explants (Singh et al. 2009). Similarly maximum proliferation was achieved using nodal explants in *Thymus hyemalis* (Nordine et al. 2013); apical meristem as explants for *Psoralea corylifolia* Linn (Pandey et al. 2013) and stem explants in *Scrophularia striata* (Lalabadi et al. 2014). Trimmed shoot segments bearing two nodes as explants of *Thymus bleicherianus* Pomel (Aicha and Abdelmalek 2014) and internode explants of *Thymus persicus* cultured on MS medium also gave maximum callus induction (Bakhtiar et al. 2016).

8.2.2 Media Requirements

The most popular media used is Murashige and Skoog (MS) supplemented with vital nutrients. The medium should provide a source for carbon, both macro- as well as micro-nutrients, along with a constant source for growth regulators and vitamins. Phytohormones facilitate regulation of plant physiological as well as morphological processes and are commonly termed Plant Growth Regulators (PGRs). The quantity of PGRs to be supplemented in the media is determined by the capability of explant to itself provide the same. Some species growing successfully in PCTOC without external medium supplements have also been reported. (Murthy et al. 2014).

A high-frequency clonal propagation procedure was developed for *Curcuma angustifolia* Roxb, resulting in a yield of 14.1 ± 0.55 shoots per explants by Jena et al. (2018) using 13.3 μM 6-benzyladenine (BA) fortified MS medium along with 5.7 μM IAA and 135.7 μM adenine sulphate (Ads) within 60 days of inoculation. Thus, a variation in culture media facilitates effective mass propagation thereby enriching commercial application. The effect of thidiazuron (TDZ) on multiple shoot induction from nodal segments of *Allamanda cathartica*, was noted by Khanam and Anis (2018). For shoot proliferation accompanied by shoot elongation, the TDZ exposed cultures were further cultured on MS medium containing different concentrations of 6-benzyladenine (BA) and Kinetin (Kn) but without TDZ. Thus, changing the constituents of culture medium can facilitate rapid clonal propagation of MAPs.

PCTOC is also potent tool for preservation of endangered species. Somatic embryogenesis of an endangered native of Iran, MAP - *Kelussia odorotissima* Mozaff, was facilitated by Ebrahimi et al. (2018). Embryogenic callus induction of

overcotyledonary leaves was observed in a MS media supplemented with 1 mg/l 2,4-dichlorophenoxyaceticacid (2,4-D) and 0.25 mg/l Kinetin. 100% improvement in the conversion rate of the cotyledonary-stage embryos was observed while maintaining genetic stability during *in vitro* multiplication was also assessed with no polymorphic band observed by amplification fragment length polymorphism.

Palmer and Keller (2011) studied the regeneration of plants from the petal explants of *Hypericum perforatum* L. The formation of callus and shoot was induced at 1:10::Cytokinin: auxin concentration using indole-3-acetic acid (IAA), 1-naphthaleneacetic acid (NAA) and indole-3-butyric acid (IBA), though with 2,4-dichlorophenoxyacetic acid (2,4-D) only callus induction resulted in 100% shoot regeneration frequency with 57.4 and 53.4 shoots per explant were obtained with IAA and IBA, respectively, at 1.0 mg/l auxin and 0.1 mg/l kinetin concentration. Kinetin levels of 0.1 and 0.25 mg/l, in absence of auxins lead to low frequency of callus and shoot formation. Rooting and successively flowering with initial use of media without exogenously added auxin was successfully achieved in the greenhouse, suggestive of auxin type being an important governing factor using petal explants.

8.2.3 Environmental Factors

Some desired characteristics, achieved through plant tissue culture (PTC) would be the overexpression of genes leading to synthesis of important secondary metabolites, alterations in the regulatory genes mediating better production of these metabolites along with modifying genes providing better adaptability to stress conditions.

Drought stress tolerance in *Salvia miltiorrhiza* transgenic plants was demonstrated by Liu et al. (2017) through expression of AtEDT1 transcription factor. Another study on the same plant by Zhou et al. (2018), showed an increase in 3,4-dihydroxyphenyllactic acid resulting in better quality of rosmarinic acid produced via rosmarinic acid synthase gene suppression by CRISPR/Cas9-mediated mutagenesis. Kumar et al. (2018a, b) studied over-expression of geraniol synthase genes and geranyl diphosphate synthase genes in *Catharanthus roseus* and obtained significant increase in vinblastine and vincristine yields which hold immense utility for cancer therapy. In *Raphanus sativus* L., higher biosynthetic capacity for quercetin and flavonoid was found in *Agrobacterium rhizogenes*-induced hairy roots (Balasubramanian et al. 2018). Another example of similar use of hairy root cultures in *Sphagneticola calendulacea*, reported better yield of flavonoid, phenolic acid and wedelolactone (Kundu et al. 2018). *Agrobacterium rhizogenes*-derived hairy roots have facilitated industrial production of secondary metabolites mediated through induction of polyploidy artificially (Dehghan et al. 2012), bioreactors (Patra and Srivastava 2017), elicitors (Kastell et al. 2018), and CRISPR/Cas9- a method to edit genome (Li et al. 2017).

8.3 *In Vitro* Micropropagation of Medicinal Plants

Micropropagation is the most developed of all PCTOC techniques. It can be accomplished by somatic embryogenesis and organogenesis, through mainly two paths, direct and indirect. The former is a preferred mode due to induction of bipolar structures while the latter requires various PGR's for induction of shoot and root through a two-step method (Hesami and Daneshvar 2018). Multiple factors, viz. explant-its type, size and age, culture media composition, gelling agent, pH and concentration of PGR's along with external conditions as temperature, photoperiod and light intensity, govern the efficiency of the process (Nalawade and Tsay 2004). A chapter in Vol. 1. of the series Medicinal and Aromatic Plants of the World by Máthé et al. (2015) has dealt with this subject in detail.

8.3.1 Explants Used

Khamushi et al. (2019) developed an efficient method to micro propagate and further achieved effective plantlet regeneration of the cypress of Abarkuh, known to be 4000 years old plant. The secondary shoots proliferated on the woody plant medium (WPM) supplemented with sucrose, agar, benzyladenine (BA) and indolebutyric acid (IBA). After elongation in *in-vitro* conditions, roots were inducted by pulse treatment and the plantlets successfully adapted to develop into mature plants in outdoor conditions. The effect of age of explant was investigated by Niazian et al. (2017) on hypocotyl segments of Ajowan (*Carum copticum* L.) which were 5, 10 and 15 day old. The media used was MS supplemented with different concentrations of Kin PGRs and 2,4-D and the best results for somatic embryogenesis were obtained with 15-day-old hypocotyls. Generation of embryogenic calli with combination of Kin PGRs and 2,4-D also showed success in *Sapindus trifoliatus* though use of media free from PGR helped develop induction of somatic embryos into mature plantlets in some MAP's (Asthana et al. 2017).

8.3.2 Organogenesis

Fadel et al. (2010) in *Mentha spicata* L investigated the effect of changing concentration of inorganic salts used in MS media of quarter, half and full strength via *in vitro* organogenesis using nodal segments as explants. The induction of highest number of shoots and the maximum average shoot length was detected in media of half strength, while in full strength highest leaf number and root length was observed.

Using leaf explants in *Coleus forskohlii*, briq direct organogenesis was attempted by Krishna et al. (2010). An MS medium with BAP 5 mg/l and cytokinins, showed

multiple shoot regeneration, followed by use of MS media fortified with cytokinin and combination of BAP 0.1 mg/ l and IAA.

Multiple shoot induction in *Angelica glauca* through direct organogenesis, using rhizomes as explant was conducted by Janhvi et al. (2018). MS medium with 6-Benzylaminopurine and IAA depicted maximum shoots. Roots (average 4.2 roots per shoot) appeared within 14 days in IAA and NAA supplemented medium. These rooted plantlets, in a greenhouse hardened successfully and recorded survival rate of 72% after 45 days when established in field.

Recent research suggests that additives as casein hydrolysate, nanoparticles, glutamine and picloram, in the culture media augment the efficiency of micropropagation. In cultured *Gloriosa superba* L. rhizome explants, silver nanoparticles of *Ulva lactuca* extracts (ULAgNPs) were used along with 0.5 mg/L silver nitrate ($AgNO_3$), 0.5 mg/L ABA, 2 mg/L BAP and 20% *Ulva lactuca* extracts in MS medium, leading to high percent of embryo maturation (Mahendran et al. 2018).

8.3.3 Somatic Embryogenesis

Somatic embryogenesis is an efficient way for regeneration of plants and scores higher than organogenesis, as the shoots and buds formed from a somatic embryo will obligatory form from a single cell which ensures superior genetic stability. Its applications are diverse especially for large-scale multiplication of MAP's and mass production of artificial seeds.

Propagation via somatic embryogenesis using *O. basilicum* leaves as explants was worked upon by Gopi and Ponmurugan (2006). Initial induction of globular embryos was achieved with BA (1 mg/L) and 2, 4-D (0.5 mg/L) while embryo maturation was in NAA (1 mg/L), KN (0.5 mg/L), BA (1 mg/L).

In *Portulaca oleracea L.*, using stem and leaves as explants induction of callus, somatic embryogenesis, as well as regeneration of plant at varying concentrations of 6-Benzylaminopurine (BAP) and kinetin (Kin) along with auxins as IAA, NAA 2,4-D were investigated. Good transformation was achieved with leaf explants which were pre-cultured for 7 days, then co-cultivated at 25 ± 2 °C for 4 days (Sedaghati et al. 2019).

Somatic embryogenesis of an endangered medicinal plant *Kelussia odoratissima* Mozaff was achieved by Ebrahimi et al. (2018). On the cotyledonary leaves, embryogenic callus was induced in MS medium supplemented with 2,4-D and Kinetin. The development and proliferation of somatic embryos showed significant differences under variable sources of carbon supplemented in media along with different light treatments. In absence of polymorphic bands between mother plant and in-vitro plantlets, using the amplification fragment length polymorphism, the results indicate genetic stability was maintained.

8.4 PTC for Conservation of Medicinal Plants

Plant Tissue Culture, in addition to the aforementioned uses in the plant propagation, has acquired an important role in the conservation of medicinal plants, too. *Ex-situ* conservation, Cryopreservation and Somatic embryogenesis are important domains that will be briefly outlined and illustrated with medicinal and aromatic plant examples.

8.4.1 Ex-Situ *Conservation*

MAPs used to grow wild, but were indiscriminately harvested, once their utility was realized. Further, the effect of manmade factors as pollution, afforestation, industrialization and other anthropogenic factors has now put the MAPs, under risk. To safeguard these versatile plants *ex-situ* conservation is required. It forms a substitute for preserving plant germplasm which is likely to be lost through *in vitro* slow growth cultures which facilitates storage of cloned plants through regular subculturing over a period of 1–15 years (Rao 2004). The sub-culturing procedure itself, if not conducted carefully, is the cause of the germplasm getting contaminated and hence the method has a short life. The alternative to long term preservation is cryopreservation.

Capparis spinosa L, *Lavandula dentata* L. and *Rhazya stricta* Decne, plants were grown and the medium time storage was studied. Using axillary buds as explants, *in vitro* propagation of *C. spinosa* L and *L. dentata* L. plants in MS medium with varying concentration as well as combination of auxins and cytokinins was successful. Further, the shoot tips and nodal buds thus developed, were used in the next stage of *in vitro* conservation. 91.1% and 93.33% survival rates, post a year of conservation were observed by *R. stricta* Decne and *C. spinosa* L, respectively, though it was 90% for *L. dentata* at higher supplementation of sucrose. High genetic stability was observed using Random amplified polymorphic DNA (RAPD), suggestive that the technique is successful for the plants under study in MS media with carbon source being sucrose and sorbitol as osmotic agent (Attia et al. 2017).

8.4.2 Cryopreservation

Long term storage by preserving material like seeds, buds, cuttings, roots, rhizomes, at very low temperatures like −196 °C, is termed as cryopreservation. The techniques aim mainly at preservation along with capacity to retain biosynthetic potential, conservation of germplasm and maintaining genetic stability of the clones. For MAPs, the technique should also lead to good survival rate and biochemical

stability for secondary metabolite production. Long term storage using this technique has been successfully implemented for *Eruca sativa* Mill (Xue et al. 2008), *Hypericum perforatum* (Urbanová et al. 2006) and *Dendrobium candidum* (Yin and Hong 2009).

The conventionally used technique for cryopreservation is vitrification. The plant material for short periods is exposed to glycerol-based cryoprotectants, known as plant vitrification solution (PVS). The procedure involves steps as pretreatment, preconditioning, preculture, osmoprotection, dehydration, cooling, warming, dilution, and finally regrowth. Another tool is encapsulation-dehydration wherein the plant is partially dehydrated in calcium alginate beads. In this technique, simplification of the rewarming step is facilitated as the alginate beads once dehydrated does not lead to formation of ice crystals.

In *Dioscorea floribunda*, cryopreservation of shoot tips provided genetic stability, with high survival rate (87%) though the success rate of plant regeneration was 30%. using vitrification technique (Ahuja et al. 2002). Another study using vitrification along with encapsulation–dehydration technique, in the same plant proved that the content of diosgenin remained unchanged in cryopreserved shoots as compared to control plants, thereby suggestive of maintaining biochemical stability (Dixit-Sharma et al. 2005).

The commonly used techniques for cryopreservation, viz. desiccation, vitrification, and encapsulation–dehydration were investigated by Ghaffarzadeh-Namazi et al. (2017) in the young leaves in the callus of *Satureja spicigera*. The regrowth of callus was studied using agents as PVS2, PVS3, DMSO used in vitrification and highest regrowth of 98.7% was observed when vitrification was done with PVS3.

8.4.3 Synthetic Seeds

With the advantages of genetic stability, handling ease and effectiveness of space, time, cost and labour, another new technique is alginate encapsulated seeds or synthetic seeds. Synthetic seeds besides being a method for conservation, is an efficient method to screen as well as maintain a selected genotype with the potential of producing high yield of secondary metabolites, one of the many reasons MAPs are economically important (Lata et al. 2009). Gantait et al. (2015) evaluated the factors which need optimization for success of this technique and suggested that the important ones are selection of explants and the choice of matrix used for encapsulation. The synthetic seed of *Capparis decidua* were employed for *in vitro* generated shoot tips and nodal segments by encapsulation and complexation was done in 3% alginate solution and 100 mM calcium chloride. Generally, micro-cuttings, unipolar or bipolar propagules of vegetative parts, differentiating aggregates, somatic embryos, ranging from 3–5 mm, lead to successful attempts at producing artificial seeds in various medicinal plants (Atanasov et al. 2019).

Al-Qurainy et al. (2014a, b) produced synseeds from *in vitro* cultured shoots and the encapsulated buds were stored at 4 °C for 60 days. Both dry and non-dry synseeds over the period showed growth though 100% conversion was observed upto 30 days of storage. Thereafter, month old plantlets were analyzed for genetic fidelity with no anomaly in comparison to the mother plant was observed either in morphology or molecular profiles of plantlets.

The conversion response of encapsulated and nonencapsulated nodal segments of *Althaea officinalis,* a medicinal plant, post 6 weeks storage at 6 °C, was significantly higher in encapsulated than non-encapsulated nodal segments (Naz et al. 2018).

8.5 Metabolic Pathway Engineering and Hairy Roots

Currently, gene transformation is being mediated by either direct or *Agrobacterium*-mediated indirect approaches, with the latter being most efficient. *Agrobacterium*-mediated gene transformation methods are largely independent of tissue culture and termed *in planta* gene transformations and bear advantage of being free from soma clonal variation, require less time and are simpler than tissue culture based transformation methods. Transformation of *Bacopa monnieri* with *Catharanthus roseus*, strictosidine synthase and tryptophan decarboxylase genes (engaged in pathway associated with terpenoid indole alkaloid), using LBA1119 strain *of Agrobacterium tumefaciens,* resulted in an increase of 25-fold in the tryptophan content in transgenic tissues in comparison to non-transformants. However, intricate developmental regulation involving different organelles, cells and tissues, in the synthesis of industrially used products, using only tissue culture, possess limitations (Sharma et al. 2018a, b).

Ray of light is in use of metabolite engineering for the medicinal plant wherein the technology helps in manipulating overexpression of genes for biosynthesis of secondary metabolites, or inhibition of desired pathways, utilization of regulators of transcription and preventing catabolic activities leading to denaturation of product (Matveeva and Sokornova 2018). The technique utilizes species of *Agrobacterium* as *A. tumefaciens* and *A. rhizogenes,* generally in conjugation with biolistic transformation methods.

This gene transformation technique faces some technical limitations as recalcitrant response, dependency on genotype and the normal problems associated with tissue cultures. Efficiency of *Agrobacterium*-mediated transformation is affected by concentration of *Agrobacterium* measured through optical density, immersion time of inoculation, *Agrobacterium* elimination of antibiotic, concentration of chemical stimulants as acetosyringone as additives and PGR's used (Niazian 2019).

Various explant types have been used for regeneration of MAP's using *Agrobacterium*. Active shoot regeneration in absence of PGR being supplemented

in media using leaf explants of *Nicotiana tabacum* and *Nicotiana benthamiana* were transformed with *A. tumefaciens* GV3101 (Han et al. 2013). *A. tumefaciens* transformation was used to incorporate Cry3A gene of Bt in embryogenic tissue of Norway spruce (*Picea abies*) (Briza et al. 2013).

The LBA1119 *Agrobacterium tumefaciens* strain was used to transfer to *Catharanthus roseus,* the genes for tryptophan decarboxylase and strictosidine synthase, and an increase in terpenoid indole alkaloid metabolite was confirmed using HPLC. The same was attributed to transitory overexpression of *CrTDC* and *CrST R* genes (Sharma et al. 2018a).

Use of *A. rhizogenes* has facilitated the enhancement of secondary metabolite production via induction of hairy roots in MAPs, a major advantage, which can further be used via metabolite engineering. The hairy root cultures possess immense genetic and metabolic stability *in-vitro*, in comparison to culturing of cells or callus (Grzegorczyk-Karolak et al. 2018).

8.6 Techniques to Enhance Secondary Metabolite Production

Plant-based medicines have been employed by *Homo sapiens* since time immemorial, with Weyrich et al. (2017), reporting that Neanderthal's used anti-bacterial natural products for therapy. Medicine is one of the most lucrative prospects of biotechnology. According to Iannicellia et al. (2020) MAPs are treasured natural assets as they can produce different types of secondary metabolites (Fig. 8.2).

With increased commercialization, there has been an increased focus on secondary metabolites (VijayaSree et al. 2010). The advent of advanced biotechnological techniques has paved the way for genetic enhancement of medicinal plants (Tripathi and Tripathi 2003). Kutchan et al. (2015) reported the presence of more than 200,000 known secondary metabolites. Terpenes have been found to be the most abundant closely followed by alkaloids. The synthesis of majority of these secondary metabolites involves defined metabolic pathways and mechanisms (Kutchan et al. 2015). The genes that code for the synthesis of secondary metabolites have been elucidated to be derived from the ones that code for primary cellular metabolites (Ober 2010). The synthesis of many of the secondary metabolites, like flavonoids, involves a synergistic approach between the different metabolic pathways, shikimic and malonic acid, in plant metabolism (Fig. 8.3). Biotechnological advances contribute in the synthesis of MAP production by: (i) development of plant seeds and plantlets on a large scale with desired traits (ii) opportunities available for modifying important phytochemicals to more valued molecules in an *in vitro* environment which is difficult to attain by synthetic processes (iii) serves as a means of in-vitro conservation (Chatterjee 2002).

Fig. 8.2 Techniques to enhance secondary metabolite production

8.6.1 In Vitro *Regeneration*

In vitro propagation of plants from excised tissue under controlled conditions is referred to as micropropagation. This technique utilizes the inherent genetic reproductive ability of the donor plant (Tasheva and Kosturkova 2013). *In-vitro* propagation and/or regeneration of MAPs hold remarkable possibilities for the production of high-quality plant-based medicines (Murch et al. 2000). By controlling nutritional and hormonal parameters, *in vitro* techniques, are reported to have improved considerably the yield of secondary metabolites.

Ramachandra Rao and Ravishankar (2002) have stated two primary pathways that can be effectively considered for micropropagation: shoot organogenesis and somatic embryogenesis. Successful organogenesis can be attained by maintaining the right physical environment, ideal growth medium composition and choice of appropriate explant (Brown and Thorpe 1995). Somatic and zygotic embryos develop and differentiate identically. Both types of embryos have similar

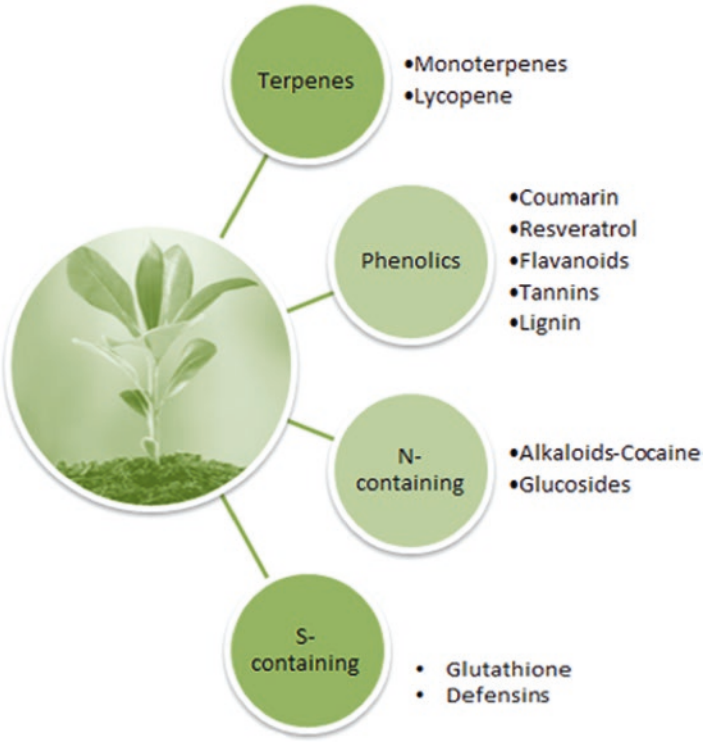

Fig. 8.3 Types of secondary metabolites

developmental stages-proembryo, globular, torpedo (or scutellar stage in monocots), and cotyledonary stages (Bajaj 1995).

8.6.2 Hairy Root Culture

Plant tissue culture facilitates cultivation of medicinal plants and for low-yielding plants and those susceptible to biotic stresses, it is a boon. Grzegorczyk-Karolak et al. (2018) have appropriately stated that PTC leads to successful implementation of both in situ and *ex situ* conservation as well as *in vitro* propagation, polyploidy induction, genetic engineering, and bioreactor applications. PTC has also been employed for successful culture of hairy roots, thus leading to the higher production of secondary metabolites that exhibit enhanced stability in the genetic as well as metabolic set-up when compared with traditional *in vitro* cultures (Roychowdhury et al. 2016). Hairy root culture is involved in large scale production of various secondary metabolites (Kundu et al. 2018). *Agrobacterium rhizogenes* undergoes nucleic acid modifications that induce differentiation of hairy roots. Hairy root

culture has several advantages over the traditional cultivation techniques such as genotypic stability over a long duration, high growth rate and higher yield of secondary metabolite (Srivastava and Srivastava 2007).

The LBA1119 *Agrobacterium tumefaciens* strain was used to transfer to *Catharanthus roseus,* the genes for tryptophan decarboxylase and strictosidine synthase, and an increase in terpenoid indole alkaloid metabolite was confirmed using HPLC. The same was attributed to transitory overexpression of *CrTDC* and *CrST R* genes (Sharma et al. 2018a).

8.6.3 Process Optimization for Producing Secondary Metabolites

Culture systems, which include cell and organ, have wide possibilities for the commercial production of important secondary metabolites. According to Murthy et al. (2014), strain improvement methodologies for maintaining optimum medium and culture conditions, introduction of elicitors for the enhanced synthesis of secondary metabolites, are strategies that have been developed over the years, for the efficient synthesis of secondary metabolites. It is imperative to select a precursor plant that has a high content of secondary metabolites of interest as this would then lead to desirable cell and organ cultures. The genetic makeup of the plant determines the synthesis of secondary metabolites. Recent advanced techniques such as High Pressure Liquid Chromatography and radio-immunioassay can be effectively employed for selection and screening of cell lines that provide a high yield (Matsumoto et al. 1980).

Nutrient medium optimization remains one of the most critical approaches for increased secondary metabolite production by cell/organ culture. The medium composition in turn is affected by several other factors that range from nutrient concentration such as carbon, nitrogen concentration to maintaining the optimum physical as well as fermentation conditions. Since the advent of the science of tissue/cell/organ culture, various media formulations have been introduced, starting from the classic MS (Murshige and Skoog 1962) media to Gamborg's (B5) (Gamborg et al. 1968), Schenk and Hildebrandt (SH) (Schenk and Hildebrandt 1972), Linsmaier and Skoog (LS) (Linsmaier and Skoog 1965), with different media being found to be more suitable for a particular cell/tissue/organ culture. According to the study of Nagella and Murthy (2011) a high concentration MS medium was found to be appropriate for the accumulation of gymnemic acid in culture of *Gymnema sylvestre*, however a 0.75 strength MS medium was found to be better for ginseng adventitious root cultures (Sivakumar et al. 2005a, b). The right composition of medium constituents is vital for the culture of isolated cells/organs/tissues (Murthy et al. 2014). A source of carbon, nitrogen and phosphate is also needed for the proper growth. Among the different carbon sources, sucrose was found to be the most productive in *Gymnema sylvestre* cell cultures.

Other important nutrient, nitrogen, is known to influence metabolite synthesis and accumulation in *in vitro* culture system. Nitrogen is largely available to the plant in the form of either ammonium ion or as nitrate salts. The ratio of the two has been found to markedly affect both biomass as well as secondary metabolite production. Zhang et al. (1996) studied the overall effect of the ratio of these two important nitrogen metabolites on plant cell development as well as production of secondary metabolite, the ginseng saponin, in cell cultures of *Panax notoginseng*. It was reported that saponin biosynthesis was more susceptible to the $NO3^{-}/NH4^{+}$ ratio than that of polysaccharides. Also, ammonium ions were observed to be ameliorative for saponin production.

Phosphate concentration in the media has been found to elicit a positive effect on the biosynthesis of secondary metabolites in cell culture systems. Hagimori et al. (1982) have reported that an elevated phosphate concentration promotes the synthesis of digitoxin in *Digitalis purpurea*. According to the research of Liu and Zhong (1998), a high saponin production can be directly correlated to a high phosphate concentration in *Panax ginseng* and in *Panax quinquefolium*.

Weathers et al. (2005) have dealt in detail on the positive effects of phytohormones as growth regulators for cell/organ culture. The external application of these growth regulators have shown to impact growth and metabolite accumulation in certain hairy root cultures. 2, 4-Dichlorophenoxyacetic acid (2, 4-D), indole acetic acid (IAA) and naphthalene acetic acid (NAA) have exhibited stimulatory effects on the production of anthocyanins and carotenoids in suspension cultures (Seitz and Hinderer 1988; Sahai and Shuler 1984). Another factor that affects secondary metabolite production by cell culture is the size/density of the inoculumn as below a minimum size of the inoculum, the growth is impaired. For every culture type, there will be an optimum inoculum size that would promote cell growth and metabolite accumulation.

Along with the biochemical requirements, physical environment-temperature, hydrogen ion concentration, light intensity, aeration, agitation, also significantly influences the production of secondary metabolites. Usually, a temperature ranges 17–25 °C is considered optimum. However, different plant cultures exhibit different temperature requirements, for example, as per Morris (1986). *Catharanthus roseus* cell line C87 was reported to show enhanced rate of growth at 35 °C. Light intensity has been known to affect plant growth as well as that of plant cell cultures (Zhong 1986). The effect of light intensity, quality and duration has been studied by different scientists on different plant cell cultures which includes studies on the effect of light on anthocyanin pigment production in cultures of *Perilla frutescens*, (Zhong et al. 1991) and *Melastoma malabathricum* (Chan et al. 2010).

For any biochemical process, be it growth or synthesis, pH of the medium ensues to be an important consideration. Generally, extremes of pH are avoided and are usually need to be maintained between pH 5 and 6. Sivakumar et al. (2005a, b) reported a pH of 6–6.5 as ideal for the growth of *Panax ginseng* root cultures. In experiments with low pH, roots failed to thrive.

8.6.4 Elicitors in Tissue Cultures

Various biotic and abiotic stress conditions are known to affect the rate of production of secondary metabolites. These are referred to as elicitors and have been extensively employed in plant cell/tissue/organ culture for the enhanced production of secondary metabolites. (Ramakrishna and Ravishankar 2011). Scientists have been using structural modification studies for developing new elicitors that are crop specific (Qian et al. 2004, 2005). The addition of elicitors promotes the efflux of intracellular products along with easy isolation and purification of the metabolite. Elicitors also promote enhanced production of secondary metabolites per unit mass of biomass. (Halder et al. 2019).

Enhancement in production of ginsenoside in *Panax quinquefolius* was studied with abiotic elicitors as nickel sulphate, cobalt nitrate, hydrogen peroxide, nickel sulphate and sodium nitroprusside, and with biotic ones as filtrates of cultures of *Bacillus circularans*, *Pseudomonas monteili*, *Trichoderma harzianum*, and *Trichoderma atroviridae*. The ginsenosides content doubled with cobalt nitrate in 5 days along with Rc synthesis being induced in plantlets, as against controls wherein same was lacking. Amongst the elicitors studied, *P. monteili* lead to 2.4 times enhancement in yield of saponin, while with *T. atroviridae* or hydrogen peroxide, Rg3 and Rh2 synthesis was induced and highest ginsenosides efficiency of 3.2 times that of control was noticed at a *T. atroviridae* dose of 1.25%v/v dose for 5 days. Thus, it was observed that an increase in panaxadiols was observed with abiotic elicitors while upregulation in the panaxatriol synthesis was observed with biotic elicitors.

Because of these reasons, various plant growth regulators are used to alleviate the biotic and abiotic stress. Different plant growth regulators like auxins, cytokinins, salicylic acid, jasmonic acid, methyl jasmonate, indole acetic acid, gibberellic acid, NAA, brassinosteroids, ethylene etc. were used. Many of the abiotic elicitors can be used for the production of secondary metabolites of the *Withania somnifera* like Plant growth hormones or regulators; Methyl jasmonate, Salicylic acid, Jasmonic acid, Calcium, Polyamines, Nitric oxide, Serotonin, Abscisic acid, Melatonin, Brassinosteroids, Metal ions, Nitrogen source, Carbon source; sucrose, glucose, maltose, fructose etc., Climatic changes; light, temperature, cold stress, drought stress, salinity stress, nutrient stress, chemical stress etc. (Akula and Ravishankar 2011).

8.6.5 Ploidy Engineering

To envisage an effective breeding program for successful production of secondary metabolites, it is important to be aware about the genetic variations prevalent in plants (Niazian et al. 2017). Stability of genome is vital for *in vitro* plant conservation (da Silva et al. 2016). Development of cell lines that give a high yield with

genes of interest have paved the way for increased synthesis of secondary metabolites (Pathak and Abido 2014). Presence of more than one complete genome, polyploidy, is believed to have an evolutionary significance for plants (Madlung 2013). Polyploidy provides an advantage in genetic selection (Levin and Soltis 2018) and is an important tool employed for plant cultivation with the aim to develop genetically improved varieties. One of the most visible effects of polyploidization is enlargement in plant cell size, with a concurrent increase in DNA content as well (Rauf et al. 2006). Lavania (2013) has established a strong correlation between polyploidy induced increase in plant cell size and secondary metabolite production. Plants are susceptible to both biotic as well as abiotic stresses. A polyploid plant species is said to be better suited to adapt to these environmental factors, as it has been found to possess a higher content of antioxidant enzymes (Zhang et al. 2010).

Several researchers have suggested that the quantity of secondary metabolites produced in polyploidy plant species is higher as compared to their diploid counterparts (Pradhan et al. 2018). Thus, artificial induction of polyploidy can be effectively utilized as it not only improves the general health of the plant but also the chemical composition of the medicinal plant (Salma et al. 2017).

Corrêa et al. (2016), induced polyploidy in *Pfafa glomerata* which led to an increase of approximately 31% in the amount of 20-hydroxyecdysone as compared to that in a diploid specie. The most employed inhibitors of mitosis include trifuralin, colchicine and oryzalin, of which colchine has been observed to be the most effective on medicinal plants. Several variable factors such as the type of antimitotic agent, concentration applied, and duration of exposure, ascertain the induction of polyploidy (Salma et al. 2017). Pan-pan et al. (2018) explored that in *Bletilla striata* 0.2% colchicine application for 36 h was found to be most effective for tetraploid development. Biotechnological engineering mechanisms are also being used for inducing haploidy. Different techniques have been explored for haploidy induction in plant tissue culture systems which include androgenesis (Kasha 2005), gynogenesis (Piosik et al. 2016), and wide hybridization-chromosome elimination (Forster et al. 2007). One of the most suitable pathway for introduction of haploidy in medicinal plants has been the androgenesis pathway. (Sharma et al. 2018b). Iannicellia et al. (2016) developed a method for *in vitro* polyploidy development in *Lippia integrifolia* wherein the polyploids exhibited enlarged organ sizes and enhanced production of essential oils. In *Centella asiatica* (L.) Urban, Kaensaksiri et al. (2011) studied morphological changes in the polyploidy plant which were evident by larger stomata and a higher stomatal index in contrast with the normal diploid genotype. Also, there was a visible positive increase in triterpenoid synthesis in the tetraploid species. Zahedi et al. (2014) worked with *Dracocephalum kotschyi* Boiss., an endangered medicinal plant species known to be localized in Iran. Flavanoid content was found to increase approximately 1.2% in the tetraploid species of this plant.

8.6.6 Bioreactors

For enhanced production of secondary metabolites via plant/cell culture, the traditional techniques of cultivation can be further improvised as the cultures have varied physical and chemical requirements with a low rate of germination (Canter et al. 2005). Bioreactor is an eco-sustainable alternative to produce valuable secondary metabolites of medicinal plants in large scale (Werner et al. 2018). A bioreactor is a preferred option for the mass scale production of secondary metabolites, which is also environment friendly. They function as cell culture systems that are continuous and act like biological industries that provide isolation of secondary metabolites in high quality and quantity (Máthé et al. 2015).

Selection of suitable bioreactors plays a crucial role in secondary metabolite production by plant cell cultures. Certain important points need to be considered when designing a bioreactor which include, emphasis on low shear mixing for efficient nutrient transport without sedimentation or clumping of cells, conditions for required aeration, maintenance of sterilization process and optimum light conditions. Choice of bioreactor remains an important consideration. In both suspension cell and hairy root culture, different variants of bioreactors like fluidized bed reactors, stirred reactor, rotating drum reactor, airlift reactor, etc. have been used (Rizvi 2012). For plant cell suspensions, mechanically driven bioreactors are the systems of choice (Eibl et al. 2018). In these bioreactors, an antifoam agent is usually not added as foam is constantly incorporated into the culture broth.

Different strategies have been employed to enhance the production of secondary metabolites in different bioreactor systems. One such strategy is elicitation and co-culture. Wu et al. (2007) studied co-cultivation of adventitious root cultures of *Panax ginseng* and *Echinacea purpurea* in an airlift bioreactor which resulted in increased production of their secondary metabolites like, ginsenosides, chichoric acid, chlorogenic acid and caffeic acid derivatives (Wu et al. 2008). Shin et al. (2002) employed different variants of airlift bioreactors for cultivation of hairy root cultures of *Beta vulgaris* L and a particular type of bioreactor, the cone type, yielded the highest rate of formation of betacyanin.

8.7 Genome Editing

Genetic manipulations have helped in the cheaper production of novel secondary metabolites on a large scale with minimum wastage. The field of genetics with biotechnology, a specialized arm has helped in precise revelation of the biosynthetic pathway followed for secondary metabolite synthesis, identification of the genes involved in the same, as well as the enzymes catalysing the different metabolic steps (Gandhi et al. 2015). Traditional genetic breeding methods have fallen out of practice because of the ambiguity associated with DNA integration, and the appearance of undesirable phenotypic effects (Fig. 8.4). Naqvi et al. (2010) have elucidated that these techniques are not very effective as the changes incorporated are in a few metabolic steps and not in the complete pathway.

Fig. 8.4 New Generation Sequencing Techniques

The technique of knock-out gene has been utilized to reveal the role of genes in the biosynthesis of secondary metabolites. Several plant-based cancer chemotherapeutic medicines such as the *Vinca* alkaloids have been characterized through this technique (Rischer et al. 2006). Also, research undertaken to assess the type of traits that are transmitted from one generation to the other generation, suggest that these traits could be either qualitative or quantitative. Traditional biometrical methods have been employed to elucidate the inheritance of these traits, as many of the traits that determine the chemical composition of the secondary metabolites have been found to be quantitative in nature (Kumar and Gupta 2008). Scientists have extensively employed the quantitative trait loci (QTL) analysis for elucidation of genes that control these quantitative traits by using DNA-based molecular markers (Mary et al. 2013; Collard et al. 2005).

New arenas of molecular biology, like metabolomics, proteomics, transcriptomics and genomics have made it possible to identify genes involved in the synthesis of secondary metabolites. RNA sequencing analysis is frequently employed to identify genes coding for selected secondary metabolites in different MAPs (Tripathi et al. 2016). On elucidation of the complete biosynthetic pathway, genetic biology approaches are adopted and applied for the increased production of the secondary metabolite of interest. Such methodology has been successfully implemented on *Saccharomyces cerevisae* (DiCarlo et al. 2013) and *E. coli* (Mami et al. 2018). In yeast, the earliest successful attempts on employing synthetic biology approach, was for the production of artemisinic acid, a precursor of anti-malarial artemisinin (Paddon et al. 2013).

8.7.1 Bioreactors

To have enhanced production of secondary metabolites via plant/cell culture, the traditional techniques of cultivation are not the right alternative. The cultures have varied physical and chemical requirements and have a low rate of germination (Canter et al. 2005). Bioreactor is an eco-sustainable alternative to produce valuable secondary metabolites of medicinal plants in large scale (Werner et al. 2018). According to Werner et al. (2018), bioreactor is a preferred option for the mass scale production of secondary metabolites, which is also environment friendly. They function as cell culture systems that are continuous and act like biological industries that provide isolation of secondary metabolites in high quality and quantity (Máthé et al. 2015).

8.8 The Future Belongs to the World of 'Omics'

The study of 'Omics' is aimed at analyzing the structure, function, and dynamics of organism/s, through the characterization and quantification of biomolecules, viz. genes, proteins and metabolites. The current perspective and the near future in MAP research is focused on integration of genomics, proteomics, transcriptomics, and metabolomics, broadly categorized as functional genomics (Oksman-Caldentey et al. 2004). The focus thus is to reveal the association of different cellular elements, viz. proteins, genes and metabolites and their function (Rai et al. 2017).

With the cost of sequencing dropping along with the easy availability of groundbreaking procedures, as linked read sequencing, long reads sequencing, optical genome mapping and Hi-C genome linked sequencing, along with the availability of smart sequence assembling, informatics have hastened *de novo* whole genome analysis at the molecular level (Fig. 8.5).

8.8.1 DNA Profiling

A potent tool for detection of genomic DNA and mRNA to further study protein expression is use of DNA microarray. This technique holds significance for MAP's as it can be used for comparative evaluation of the expression of secondary metabolites under different growing conditions. This can be used to hybridize genome fragments and can be a measure for expressed genes. Modifications of this technique are the subtracted diversity array (SDA) and diversity array technology (DArTTM) and are applicable in plants whose sequence information is lacking.

The technique uses an array of DNA probes depicting DNA–DNA and DNA–RNA selective binding for studying profiling of gene expression as well as for comparative genomic (Allen et al. 2010). Various reference sequences are paired to

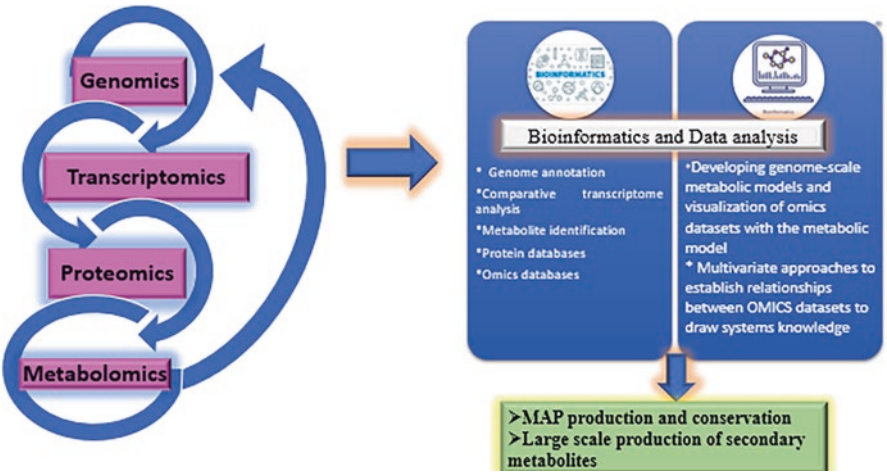

Fig. 8.5 Integrative studies using 'Omics' and Bioinformatics

oligonucleotide probes and the non-overlapping ones are carefully chosen involving 200–300 nucleotide bases of the gene or the cDNA. Another tool for comparative genomics includes expressed sequence tags (EST) (Haq et al. 2014). Such sequence analysis has been accomplished for *C. roseus*, for monoterpene indole alkaloid (MIA) pathway with 3655 unique ESTs reported (Murata et al. 2008) while for *Salvia miltiorrhiza* (Yan et al. 2010), 10,288 ESTs were assembled.

8.8.2 MAP Transcriptomes

For MAP's, the interest is to identify genes coding for proteins associated with the biosynthesis of secondary metabolites (Han et al. 2016). This leads to interest in studying the structural and functional aspects of the genome including the sequencing of mRNAs and *de novo* transcriptome assemblies and analysis (Tripathi et al. 2016).

In *Zanthoxylum planispinum,* an East Asian herb, transcriptome centered studies were conducted by Kim et al. (2019). From the early and maturing fruit stage as also from leaf tissues sequencing of the entire mRNA, was completed and an isoform of the transcriptome was also identified. In the isoforms taken cumulatively, 51,402 unique genes leading to proteins associate with various metabolic pathways and especially ones for secondary metabolite synthesis were short listed.

Using *de novo* transcriptome sequencing, a comparative study of *Ocimum sanctum* and *O. basilicum* was conducted at CIMAP, Lucknow. A Kyoto Encyclopedia of Genes and Genomes (KEGG) analysis of total mRNA- transcripts included 105,470 and 59,648 genes from *O. basilicum* and *O. sanctum,* respectively. The study revealed that 952 transcripts in *O. basilicum* and 501 transcripts in *O. sanctum*

were associated with terpenoids and phenylpropanoids synthesis in the two respective species and the genes associated with these pathways were also found to be linked (Rastogi et al. 2014).

Twenty-seven genes for polyoxypregnane glycosides were distinguished in the transcriptomic studies in *Gymnema sylvestre* using protein databases. Two hundred and thirty five CDSs (coding DNA sequences) were identified through KEGG analysis, of which 19 CDS corresponding to 10 significant enzymes of polyoxypregnane glycoside biosynthesis were analysed (Kalariya et al. 2018).

For *Azadiracta indica*, a comparative study of genome and transcriptomes (four) disclosed that the key steps leading to the secondary metabolite production are similar (Krishnan et al. 2012). Rajakani et al. (2014) established the role of individual cytochrome P450's, involved in isoprenoid synthesis. An analysis of ESTs generated through suppression subtractive hybridization attempts resulted in first full length gene, viz. hydroxyl methyl glutaryl CoA enzyme A reductase (HMGR), the key regulatory for numerous isoprenoids biosynthesis like limonoids (Narnoliya et al. 2014).

Secondary metabolites as essential oil is often stored in trichomes, which play a role in protecting the plant against predators as in *Artemisia annua* L. (Yadav et al. 2014). *A. annua* synthesizes monoterpene oil along with a number of non-volatile sesquiterpenes like artemisinin (anti-malarial) and the biosynthesis of terpenoids and flavonoids in the glandular trichomes, holds significance in plant protection (McKerrow 2015).

Transcriptomic approaches also find utility in comparative profiling of gene expression for different tissue of a plant or between species. In *W. somnifera*, the metabolites in the leaf and root are and the same was analyzed using transcriptomic data (Gupta et al. 2013). A study of different *Panax* species, revealed 19,226 distinctive sequences of P. *notoginseng*, held similarity with *P. quinquefolius* but 11,626 transcripts were distinctive only for *P. notogingseng* while with *P.ginseng*, 19,479 sequences were similar to in *P. notoginseng* and 11,373 sequences were unique to *P. gingseng*.

8.8.3 Molecular Markers and Transcription Factors

Using the study of transcriptomes is a cost-effectual method for molecular markers identification as compared to the conventional methods of plant breeding. With transcriptome data, molecular markers as Single nucleotide polymorphism (SNP) and Single sequence repeat (SSR) can be identified and then used for quantitative trait loci, genetic diversity analysis, gene flow, marker assisted selection, parental analysis and also for evolutionary studies (Zheng et al. 2013). This approach has been utilized in transcriptome analysis of *Withania somnifera* (Gupta et al. 2013) and *Centella asiatica* (Sangwan et al. 2013). In *W. somnifera*, it was found that the dinucleotide SSRs are maximum (2489), with trinucleotide (1681) being next followed by tetra- (92) and finally penta- (20)-nucleotides.

The transcriptional factors (TFs) are DNA binding proteins and are cis regulatory elements. DNA binding domain permits binding with the trans-regulatory region on DNA and thus facilitates regulation of the gene expression resulting in switching the gene on or off under condition thereby enabling the gene expression in a defined and controlled manner. Thus, in MAP's the TF(s) play a vital role and can control the secondary metabolic pathways as often one TF may regulate the entire metabolic pathway. Thus, the identification and isolation of TFs through transcriptome analysis is desirable. TFs as "Zinc finger family", WRKY, F-Box Homeobox, MYB, WD40 repeat family besides others have been worked upon a lot. Such a transcriptome analysis in *C. asiatica*, revealed identification of TFs from 71 different families with "Zinc finger family" being most prominent (Sangwan et al. 2013). Other classes found were F-box Homeobox, AP2, bHLH, GATA, GRAS, MYB, WD40, etc. and for jasmonic acid-treated trichomes, a quantitative expression database was obtained (Spyropoulou et al. 2014).

8.8.4 Combinatorial Supertransformation of Transplastomic Recipient Lines (COSTREL)

Metabolic engineering has influenced the overproduction of useful metabolic products from MAPs. Nuclear transformation has facilitated the same, though recently it has been observed that chloroplast transformation has better advantages. Plasmid or chloroplast transformation provides the potential for multigene transformation and enriched expression of transformed genes. Further, as chloroplast depicts maternal inheritance, the containment of transformed gene is higher. Fuentes et al. (2016, 2018), developed new technique involving both nuclear as well as chloroplast transformation, viz. Combinatorial supertransformation of transplastomic recipient lines (COSTREL).

Industrial scale production of secondary metabolites can be achieved by the transfer from the MAP the entire biosynthetic pathway to another plant which has high biomass. Most researched secondary plant that has been used as the recipient is the tobacco plant. The technique involves conduct of nucleus transformation of an earlier plastid-transformed plant/ cell line. COSTREL was first used to enhance the yield of artemisinin, in *Artemisia annua*. The methodology involved the transfer of genes of artemisinin biosynthetic pathway to the chloroplast of tobacco via "gene-gun" transformation, followed by transformation in transplastomic lines for accessory genes that had the potential to enhance artemisinin production. Such a supertransformation increased the artemisinin production to >120 mg/kg fresh weight in tobacco.

8.8.5 RNA Interference

RNA interference involves manipulating genes for silencing the expression of genes thereby regulating enzymes especially of biosynthetic pathways. Small ribonucleic acid molecules with 20–22 nucleotide, present endogenously termed micro-RNA (mi RNA) along with small interfering RNA (siRNA) are involved in regulation post-transcription, thereby affecting gene expression and the secondary metabolite production can be regulated in MAP's.

In-vitro yield of vincristine and vinblastine from *C. roseus*., which is antineoplastic is low. Pani and Mahapatra (2013), in an *in silico* study projected the role of 2 prospective miRNAs and 12 mRNA targets encoding metabolic enzymes regulating terpenoid indole alkaloids (TIA) pathways and signaling, cell growth and development, and depict perfect complementarity to each other.

In *Podophyllum hexandrum* (Himalayan Mayapple), which produces podophyllotoxin with multiple medicinal properties, Biswas et al. (2016) studied miRNA mediated management of biosynthesis of this secondary metabolite. In the study 60 mature miRNAs and 6 pre-miRNAs were found through pyrosequencing. Validation by quantifiable real-time PCR suggested that the expression of podophyllotoxin was enriched.

Turmeric is a multiversatile herb. Eighteen families of miRNA were identified by Singh and Sharma (2017) of which 16 families revealed their role in regulation of 238 transcripts. These were associated with regulation of rhizome development, biosynthesis of terpenoid backbone, isoquinoline and curcumin along with growth and developmental process of turmeric.

8.8.6 Engineered Sequence-Specific Nucleases

Currently, the engineered sequence-specific nucleases being researched are TALENs (Transcription activator-like effector nucleases), Zinc-finger nucleases (ZFNs), and CRIPR/Cas. These have the potential to generate in the DNA sequence, site-specific double-strand, thereby leading to modification in characteristics of plants. The above mentioned nucleases are fusion proteins and contain two domains namely a sequence-specific DNA-binding domain which is programmable and a nonspecific DNA-cleavage domain, and thus facilitate genetic and metabolic engineering. Such an approach in MAP's can lead to new varieties with the potential to biosynthesize desired secondary metabolites as well as new bio-products of commercial importance (Pouvreau et al. 2018). These techniques mark notable site-specific DNA modification, with an additional advantage that the modification is restricted to gene disruption only and thus the modified plants under regulatory standards do not fall under transgenic plants.

8.8.7 Zinc Finger Nucleases (ZFNs)

The ZFN's are dimers composed of monomers which are a fusion protein of a FokI nuclease domain and DNA binding domain zinc fingers. Recurring cysteine and histidine residues comprise the zinc finger and they generally identify 3 nucleotides. 3/4 zinc fingers comprise a ZFN monomer recognizing 9–12 nucleotides. The possibility to recognize a long section of DNA by incorporating multiple zinc fingers together has been successful as they are modular, though they have elevated toxicity and poor activity.

Construction of desired ZFPs can be achieved from earlier characterized zinc fingers through modular assembly (Kim et al. 2010) and such methods considering framework dependence between nearby zinc fingers can yield functional ZFNs (Bhakta et al. 2013). Modification of wild-type Fok1 domain to yield obligatory heterodimeric Fok1 domain has proved to improve specificity by reducing other undesirable effects (Miller et al. 2007).

The advantage of ZFNs as a genome editing techniques is mainly its high specificity and efficiency. The limitations include the complications associated with technical challenges to design the ZFNs, especially for substitution of larger fragments for knockout development. Moreover, the procedure is expensive and has limited target availability.

8.8.8 Transcription Activator-Like Effector Nucleases (TALENs)

Like ZFNs, TALENS belong to the class of chimeric nucleases obtained through the coupling of FokI endonuclease possessing a cleavage domain, with 13–28 transcriptional activator-like effector (TALE) repeats which are virulence factors developed from *Xanthomonas* plant pathogenic bacteria. Unlike ZFNs, in TALENs, every TALE repeat, targets only one nucleotide providing for a flexible target strategy, thereby increasing potential target sites. Type III secretion system facilitates incorporation of TALEs into the host and theses then trigger transcription of target genes thereby manipulating the normal cellular functions. The TALENS via set of tandem repeats identify and activate DNA sequences upstream to the site of transcription initiation and thus lead to an efficient but selective manipulation of the target DNA (Bogdanove and Voytas 2011).

The limitations associated are that the cDNA encoding TALEN has a size of about 3 kb which makes delivery and expression of TALENs in a cell, difficult. Another bottleneck is the composition of TALE repeat and the effectiveness with which TALENs targets a specific gene, is variable. Additionally, being highly repetitive in nature their compatibility with certain viral vectors, also possess a limitation.

8.8.9 CRISPR/Cas

CRISPR Cas-9 (CRISPR is short for Clustered Regularly Interspaced Short Palindromic Repeats; Cas-9 is a protein in the body) is one of the fastest and most efficient ways to edit genes (Elias 2016). CRISPR-Cas9 is a more robust and simpler tool for targeted genetic editing in agro-based research. It involves a reverse-genetics approach. The endonuclease mechanism, CRISPR, is a useful RNA-guided genome editing tool (Bortesi and Fischer 2015). The Cas9 protein is the endonuclease machinery of the system and comprises of around 1400 amino acids (Song et al. 2019). This mechanism has been extensively studied in various plant species aimed at directed genomic manipulation. The CRISPR-Cas system was for the first time elucidated to function as a defense mechanism in *S. thermophiles* (Barrangou 2007).

Yagiz et al. (2016) have successfully used this approach in *Papaver somniferum* L. in which the biosynthesis of morphine, thebaine etc., was reported to be considerably reduced. Nielsen et al. (2017) utilized this technology to genetically engineer *T. atroroseus* for studying secondary metabolism in this fungus and identified a novel gene that encodes for the production of ZG-1494α, an innovative platelet-activating factor. CRISPR-Cas9 has also been utilised for the manipulation of *P. chrysogenum* genome. CRISPR based techniques of control of gene expression will not only aid in metabolic engineering and secondary metabolite biosynthesis but also enable the development of manipulated genomes (Tong et al. 2019). Based on proteins and accessory RNA, CRISPR/Cas9 genome editing technique is basically categorized into three types (type I, II and III) (Makarova and Koonin 2015). In nature, CRISPR/Cas9 provides protection against viruses in bacteria and archaea. This immunity is attained by integrating short fragments of foreign DNA (called spacer) between two adjoining repeats of the CRISPR locus (Iqbal et al. 2020).

The advantage associated with CRISPR/Cas is that it utilizes a single targeting molecule (gRNA) for genetic sequence manipulation. The recognition sites in ZFNs and TALENs are composed of proteins whereas that in CRISPR are composed of nucleic acid. This allows easy development of CRISPR plasmids. The disadvantage associated with the same is the high incidence of non-specific DNA breakdown.

In *Candida. albicans,* a CRISPR-Cas9-based gene-driven array (GDA) platform by developed by Shapiro et al. (2018) for inserting two different gRNA in the middle of homologous arms to perform genome editing on the adenine biosynthesis gene ADE2. Nødvig et al. (2015) utilized the orthogonal three-function CRISPR system, which combines transcription activation, transcription interference and gene deletion. A three-fold increase in the production of β-carotene was observed with the CRISPR technology (Pan et al. 2016).

Cho et al. (2017), experimented with the CRISPR tool in *Corynebacterium glutamicum*, an important industrial microorganism involved in the production of amino acids, specifically that of γ-aminobutyric acid which is a chemical of high significance. Feng et al. (2018) developed a CRISPR/Cas9 assisted multiplex

genome editing (CMGE) technique in *Escherichia coli* to dissociate transformation from editing, thus leading to an overall increase in the efficiency of the editing process. In this technique, the desired genomic sequences are assembled into replicative plasmids, and Cas9 gene expression is controlled by stringent inducible expression system. The CRISPR/Cas genome editing tool has opened a new era in plant breeding and secondary metabolite production (Malzahn and Lowder 2017). The CRISPR genome editing system has superseded the other genome editing techniques and has countless unexplored applications.

In recent years, there has been increased application of the CRISPR/Cas9 approach in medicinal plants. This system has been successfully employed on the hairy roots of *Salvia miltiorrhiza* to edit a vital gene that is involved in the biosynthesis of tanshinone (Liu et al. 2017). Various research studies have highlighted the application of CRISPR system for altering the chemical composition of certain important metabolites of medicinal plants (Noman et al. 2016).

8.8.10 Bioinformatics and Data Mining

The benefits of metabolomics for metabolic modeling using functional genomics, is a boon due to the advancement via bioinformatics, which has made available many resources and databases. Insight into the metabolome has been facilitated due to the availability of automated tools for analysis of immense high-resolution datasets. Resource databases available for MAP's include Medicinal Plant Metabolomics Resources (MPMRAtMetExpress) (Saito and Matsuda 2010), Metabolome Express (Carroll et al. 2010), Plant Metabolite Network (PMN) (Dreher 2014) and KNApSACK database (Afendi et al. 2012), the Kyoto Encyclopedia of Genes and Genomes (KEGG) and KEGG-plant, METLIN (Smith et al. 2005) and MassBank (Horai et al. 2010).

Immense information on chemical constituents especially essential oils, their GC-MS profile, agro-morphological effect on yield variations, type of fragrance, and bioactivity details of MAPs is found on AromaDb (http://bioinfo.cimap.res.in/aromadb/). The database houses data on 1321 aroma chemical structures and the bioactivities of essential oil/s or the aroma compounds and 357 fragrance types from 166 commercially used plants and 148 high yielding varieties/chemotype. The data base also provides information on the cheminformatics properties as identification, properties as physico-chemical and toxicological and pharmacokinetics (Kumar et al. 2018a, b).

Indian Medicinal Plants, Phytochemistry And Therapeutics (IMPPAT) is a comprehensive online manually curated database of 1742 Indian MAPs mentioning 9596 phytochemicals and 1124 therapeutic utility. The database bridges 27,074 and 11,514 plant-phytochemical and plant-therapeutic associations respectively. The data base comprises of an *in-silico* library of 9596 phytochemicals stating information on chemical structure and identification. The pharmacokinetic, toxicity and similarities in pharmacological properties of the phytochemicals in IMPPAT were

computed using cheminformatic approach (Mohanraj et al. 2018). The IMPPAT database is publicly accessible at: https://cb.imsc.res.in/imppat.

A catalogue of transcriptomic information on MAPs is the EGENES database - a platform for effective plant Expressed Sequence Tags (ESTs) analysis through linking of genomic information with information on functionality (Masoudi-Nejad et al. 2007). The cataloging is done via a process involving sequence cleaning, masking of repeats and vectors, sequence assembling followed by KEGG annotation. Though EGENES lists only few plants, it has attempted to capture on basis of EST information, the reactions and pathways in the plants. Medicinal Plants Genomics Resource (MPGR): http://medicinalplantgenomics.msu.edu/ is another such database, currently for 11 species, with the objective to make accessible information on transcriptome and metabolome of the plant species.

8.9 Conclusions

With increasing concern about mental, as well as physical health, along with the ideology to prevent adverse effects due to long-term use of synthetic drugs, MAPs are being researched as a feasible and viable option. Wild MAPs have been used indiscriminately and thus many of them have become endangered species. Further, to making the cultivation of MAPs profitable, the conventional methods of MAP-breeding need to be upgraded to biotechnology-based breeding methods (Sinha et al. 2019).

PCTOC facilitates not only the faster propagation of these valued plants but also the conservation of the same. Via direct and indirect methods, it can also promote MAP chemical profiles. Important contributions towards the improvement of yields through tissue culture have been achieved through the induction of polyploidy and the use of advanced bioreactors. *Agrobacterium mediated transformation*, also needs a special mention, as it has been reported to facilitate the overexpression of the key-genes of secondary metabolite biosynthesis and to assist in the down-regulation of genes adversely affecting the pathway.

Recently developed techniques like the induction of hairy root cultures and the use of genome editing constructs as ZFNs, TALLENs, and CRISPR/Cas9 have been used to improvise the production and utilization of many MAPs. CRISPR/Cas9 has the potential of being developed as a robust method for the alteration of MAP biochemical profile mediated via induction of targeted mutation in the genome. COSTREL has also facilitated enrichment of secondary metabolism production for commercial activities.

The world of ‘omics’ is the way ahead for MAP’s research. A simplified strategy is being shared which can lead to expanding the horizons in MAP research, production and utilization through tools of biotechnology (Fig. 8.6).

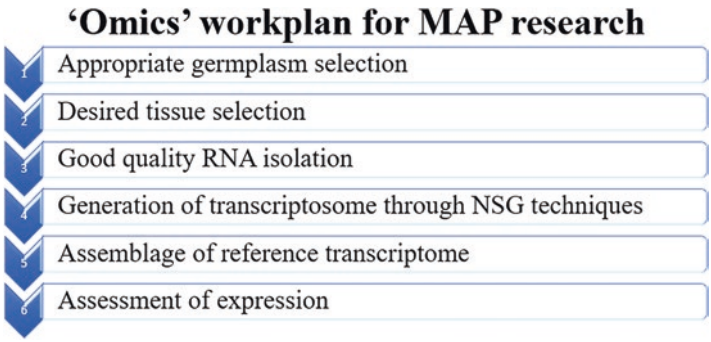

Fig. 8.6 'Omics' workplan for MAP research

References

Afendi FM, Okada T, Yamazaki M, Hirai-Morita A, Nakamura Y, Nakamura K, Ikeda S, Takahashi H, Md. Altaf-Ul-Amin, Darusman LK, Saito K, Kanaya S (2012) KNApSAcK family databases: integrated metabolite–plant species databases for multifaceted plant research. Plant Cell Physiol 53(2):e1. https://doi.org/10.1093/pcp/pcr165

Ahuja S, Mandal BB, Dixit S, Srivastava PS (2002) Molecular, phenotypic and biosynthetic stability in *Dioscorea floribunda* plants derived from cryopreserved shoot tips. Plant Sci 163:971–977

Aicha N, Abdelmalek EM (2014) Rapid *in vitro* regeneration and clonal multiplication of *Thymus bleicherianus* pomel, a rare and threatened medicinal and aromatic plant in Morocco. Med Aromat Plants. 3(1):145–149

Akula R, Ravishankar GA (2011) Influence of abiotic stress signals on secondary metabolites in plants. Plant signaling & behavior 6(11):1720–1731

Allen MJ, Tiwari B, Futschik ME, Lindell D (2010) Chapter 5: Construction of microarrays and their Figapplication to virus analysis. In: Wilhelm SW, Weinbauer MG, Suttle CA (eds) Manual of aquatic viral ecology. ASLO, pp 34–56

Al-Qurainy F, Nadeem M, Khan S, Saleh Alansi, Tarroum M, Gaafar AR (2014a) Storage, regeneration and genetic stability of synthetic seeds of *Ochradenus arabicus*: a high value medicinal plant by PSP. Fresenius Environ Bull 23(5)

Al-Qurainy F, Nadeem M, Khan S, Alansi S, Tarroum M, Al-Ameri A (2014b) Synseed production for storage and conservation of *Ochradenus baccatus delile*. Pak J Bot 46:897–902

Asthana P, Rai MK, Jaiswal U (2017) Somatic embryogenesis from sepal explants in *Sapindus trifoliatus*, a plant valuable in herbal soap industry. Ind Crop Prod 100:228–235. https://doi.org/10.1016/j.indcrop.2017.02.034

Atanasov AG, Waltenberger B, Pferschy J, Ahlawat R, Choudhary AR, Sehrawat (2019) Germplasm conservation via encapsulating in vitro generated shoot tips and nodal segment of Capparis decidua (FORSK.) EDGEW and its regeneration. Rom Biotechnol Lett 24(5):783–788

Attia AO, El Dessoky SD, Al-Sodany Y, Ismail IA (2017) Preservation for some endemic and rare medicinal plants in taif, KSA. Biotechnol Biotechnol Equip 31(5). https://doi.org/10.1080/13102818.2017.1356690

Bajaj YPS (1995) Somatic Embryogenesis and Its Applications for Crop Improvement. Somat Embryol Synth Seed I:105–125. https://doi.org/10.1007/978-3-662-03091-2_8

Bakhtiar Z, Mirjalili MH, Sonboli A (2016) *In vitro* callus induction and micropropagation of *Thymus persicus* (Lamiaceae), an endangered medicinal plant. Crop Breed Appl Biotechnol 16:48–54

Balasubramanian M, Anbumegala M, Surendran R, Arun M, Shanmugam G (2018) Elite hairy roots of *Raphanus sativus* (L.) as a source of antioxidants and flavonoids. 3 Biotech 8:128. https://doi.org/10.1007/s13205-018-1153-y

Barrangou R (2007) CRISPR provides acquired resistance against viruses in prokaryotes. Science (New York) Biotechnol Lett 315:1709–1712

Bhagat N (2011) Conservation of endangered medicinal plant (*Acorus calamus*) through plant tissue culture. J Pharmacog 2(1):21–24

Bhakta MS, Henry IM, Ousterout DG, Das KT, Lockwood SH, Meckler JF, Wallen MC, Zykovich A, Yu Y, Leo H, Xu L, Gersbach CA, Segal DJ (2013) Highly active zinc-finger nucleases by extended modular assembly. Genome Res 23:530–538

Bhojwani SS, Razdan MK (1996) Plant tissue culture: theory and practice. Elsevier Publications

Biswas S, Hazra S, Chattopadhyay S (2016) Identification of conserved miRNAs and their putative target genes in *Podophyllum hexandrum* (Himalayan Mayapple). Plant Gene 6:82–89

Bogdanove AJ, Voytas DF (2011) TAL effectors: customizable proteins for DNA targeting. Science 333(6051):1843–1846

Bortesi L, Fischer R (2015) The CRISPR system for plant genome editing and beyond. Biotechnol Adv 33:41–52. https://doi.org/10.1016/j.biotechadv.2014.12.006

Briza J, Pavingerova D, Vlasak J et al (2013) Norway spruce (Picea abies) genetic transformation with modified Cry3A gene of *Bacillus thuringiensis*. Acta Biochim Pol 60(3):395–400

Brown DC, Thorpe TA (1995) Crop improvement through tissue culture. World J Microbiol Biotechnol 11(4):409–415. https://doi.org/10.1007/BF00364616

Canter PH, Thomas H, Ernst E (2005) Bringing medicinal plants into cultivation: opportunities and challenges for biotechnology. Trends Biotechno 23:180–185. https://doi.org/10.1016/j.tibtech.2005.02.002

Carroll AJ, Badger MR, Harvey MA (2010) The MetabolomeExpress Project: enabling web-based processing, analysis and transparent dissemination of GC/MS metabolomics datasets. BMC Bioinformatics 11:376. https://doi.org/10.1186/1471-2105-11-376

Chan LK, Koay SS, Boey PL, Bhatt A (2010) Effect of abiotic stress on biomass and anthocyanin production in cell cultures of *Melastoma malabarthicum*. Biol Res 43:127–135

Chatterjee SK (2002) Cultivation of medicinal and aromatic plants in India – a commercial approach. Acta Hortic 576:191–202

Cho JS, Choi KR, Prabowo CPS, Shin JH, Yang D, Jang J, Lee SY (2017) CRISPR/Cas9-coupled recombine ring for metabolic engineering of *Corynebacterium glutamicum* Metab Eng 42:157–167. https://doi.org/10.1016/j.ymben.2017.06.010. Epub 2017 Jun 23

Collard BCY, Jahufer MZZ, Brouwer JB, Pang ECK (2005) An introduction to markers, quantitative trait loci (QTL) mapping and marker-assisted selection for crop improvement: the basic concepts. Euphytica 142:169–196

Corrêa JPO, Vital CE, Pinheiro MVM, Batista DS, Saldanha CW, da Cruz ACF, Notini MM, Freitas DMS, DaMatta FM, Otoni WC (2016) Induced polyploidization increases 20-hydroxyecdysone content, in vitro photoautotrophic growth, and *ex vitro* biomass accumulation in *Pfafa glomerata* (Spreng.) Pedersen. Vitro Cell Dev Biol Plant 52:45–55. https://doi.org/10.1007/s11627-016-9746-9

da Silva RL, Ferreira CF, da Silva Ledo CA, de Souza EH, da Silva Paulo H et al (2016) Viability and genetic stability of pineapple germplasm after 10 years of in vitro conservation. Plant Cell Tiss Organ Cult Dordrecht 127(1):123–133. https://doi.org/10.1007/s11240-016-1035-0

Dehghan E, Häkkinen ST, Oksman-Caldentey KM, Ahmadi FS (2012) Production of tropane alkaloids in diploid and tetraploid plants and in vitro hairy root cultures of Egyptian henbane (*Hyoscyamus muticus* L.). Plant Cell Tissue Organ Cult 110:35–44

DiCarlo JE, Norville JE, Mali P, Rios X, Aach J, Church GM (2013) Genome engineering in Saccharomyces cerevisiae using CRISPR-Cas systems. Nucl Acid Res 41(7):4336–4343. https://doi.org/10.1093/nar/gkt135

Dixit-Sharma S, Ahuja-Ghosh S, Mandal BB, Srivastava PS (2005) Metabolic stability of plants regenerated from cryopreserved shoot tips of *Dioscorea deltoidea*—an endangered medicinal plant. Sci Hort 105:513–517. https://doi.org/10.1016/j.scienta.2005.02.011

Dreher K (2014) Putting the Plant Metabolic Network Pathway Databases to Work: Going Offline to Gain New Capabilities. In: Sriram G (ed) Plant Metabolism. Methods in Molecular Biology (Methods and Protocols), vol 1083. Humana Press, Totowa, NJ. https://doi.org/10.1007/978-162703-661-0_10

Ebrahimi M, Mokhtari A, Amirian R (2018) A highly efficient method for somatic embryogenesis of Kelussia odorotissima Mozaff., an endangered medicinal plant. Plant Cell Tiss Organ Cult 132:99–110. https://doi.org/10.1007/s11240-017-1314-4

Eibl R, Meier P, Stutz I, Schildberger D, Hühn T, Eibl D (2018) Plant cell culture technology in the cosmetics and food industries: current state and future trends. Appl Microbiol Biotechnol 102:8661–8675

El Meskaoui A (2013) Plant cell tissue and organ culture biotechnology and its application in medicinal and aromatic plants. Med Aromat Plants 2:3. https://doi.org/10.4172/2167-0412.1000e147

Elias B (2016) Gene editing and the future of genetic conditions. Except Parent (Online) Boston 46(8):17–19

Fadel D, Kintzios S, Economou SA, Moschopoulou G, Constantinidou HA (2010) Effect of different strength of medium on organogenesis, Phenolic accumulation and antioxidant activity of spearmint (*Mentha spicata* L.). Open Hortic J 3:31–35

Feng X, Zhao D, Zhang X, Ding X, Bi C (2018) CRISPR/Cas9 assisted multiplex genome editing technique in *Escherichia coli*. Biotechnol J 13(9):e1700604

Forster BP, Heberle-Bors E, Kasha KJ, Touraev A (2007) The resurgence of haploids in higher plants. Trends Plant Sci 12(8):368–375

Fuentes P, Armarego-Marriott T, Bock R (2018) Plastid transformation and its application in metabolic engineering. Current opinion in biotechnology. 49:10–15

Fuentes P, Zhou F, Erban A, Karcher D, Kopka J, Bock R (2016) A new synthetic biology approach allows transfer of an entire metabolic pathway from a medicinal plant to a biomass crop. elife 5:e13664.

Gamborg OL, Miller RA, Ojima K (1968) Nutrient requirements of suspension cultures of soybean root cells. Exp Cell Res 50:151–158

Gandhi SG, Mahajan V, Bedi YS (2015) Changing trends in biotechnology of secondary metabolism in medicinal and aromatic plants. Planta 241:303–317. https://doi.org/10.1007/s00425-014-2232-x

Gantait S, Kundu S, Ali N, Sahu NC (2015) Synthetic seed production of medicinal plants: a review on influence of explants, encapsulation agent and matrix. Acta Physiol Plant 37:98. https://doi.org/10.1007/s11738-015-1847-2

Ghaffarzadeh-Namazi L, Keller EJ, Senula A, Babaeian N (2017) Investigations on various methods for cryopreservation of callus of the medicinal plant *Satureja spicigera*. J Appl Res Med Aromat Plants 5:10–15. https://doi.org/10.1016/j.jarmap.2016.09.003

Gopi C, Ponmurugan P (2006) Somatic embryogenesis and plant regeneration from leaf callus of *Ocimum basilicum* L. J Biotechnol 126:260–264

Grzegorczyk-Karolak I, Kuźma Ł, Skała E, Kiss AK (2018) Hairy root cultures of *Salvia viridis* L. for production of polyphenolic compounds. Ind Crop Prod 117:235–244. https://doi.org/10.1016/j.indcrop.2018.03.014

Gupta P, Goel R, Pathak S, Srivastava A, Singh SP, Sangwan RS, Trivedi PK (2013) *De novo* assembly functional annotation and comparative analysis of *Withania somnifera* leaf and root transcriptomes to identify putative genes involved in withanolides biosynthesis. PLoS ONE 8:e62714

Hagimori M, Mstomoto T, Obi Y (1982) Studies on production of *Digitalis cardenolides* by plant tissue culture. Plant Physiol 69:653–656

Halder M, Sarkar S, Jha S (2019) Elicitation: A biotechnological tool for enhanced production of secondary metabolites in hairy root cultures. Eng Life Sci 19:880–895

Han ZF, Hunter DM, Sibbald S et al (2013) Biological activity of the tzs gene of nopaline Agrobacterium tumefaciens GV3101 in plant regeneration and genetic transformation. Mol Plant Microbe Interact 26(11):1359–1365

Han R, Rai A, Nakamura M, Suzuki H, Takahashi H, Yamazaki M, Saito K (2016) Chap. 2: De novo deep transcriptome analysis of medicinal plants for gene discovery in biosynthesis of plant natural products. In: O'Connor SE (ed) Methods in enzymology

Haq SU, Jain R, Sharma M, Kachhwaha S, Kothari SL (2014) Identification and characterization of microsatellites in expressed sequence tags and their cross transferability in different plants. Int J Genom

Hesami M, Daneshvar MH (2018) *In vitro* adventitious shoot regeneration through direct and indirect organogenesis from seedling derived hypocotyl segments of *Ficus religiosa* L.: an important medicinal plant. Hort Sci 53:932–936. https://doi.org/10.21273/HORTSCI13058-18

Horai H, Arita M, Kanaya S, Nihei Y, Ikeda T, Suwa K, Ojima Y, Tanaka K, Tanaka S, Aoshima K, Oda Y, Kakazu Y (2010) MassBank: a public repository for sharing mass spectral data for life sciences. J Mass Spectrometry 45(7):703–714

Iannicellia J, Elechosa MA, Juárez MA, Martínez A, Bugallo V, Bandoni AL, Escandóna AS, van Baren CM (2016) Effect of polyploidization in the production of essential oils in *Lippia integrifolia*. Ind Crop Prod 81:20–29

Iannicellia J, Guarinielloa J, Tossib VE, Regaladob JJ, Di Ciaccioa L, van Barene CM, Pitta Álvarez SI, Escandón AS (2020) The polyploid effectin the breeding of aromatic and medicinal species. Sci Hortic 260:108854

Iqbal Z, Iqbal MS, Ahmad A, Memon AG, Ansari MI (2020) New prospects on the horizon: Genome editing to engineer plants for desirable traits. Curr Plant Biol 24:100171. https://doi.org/10.1016/j.cpb.2020.100171

Isah T, Umar S, Mujib A, Sharma MP, Rajasekharan PE, Zafar N, Frukh A (2018) Secondary metabolism of pharmaceuticals in the plant in vitro cultures: Strategies, approaches, and limitations to achieving higher yield. Plant Cell Tiss Organ Cult 2018(132):239–265. https://doi.org/10.1007/s11240-017-1332-2

Janhvi MR, Bhandari A, Mishra S, Rawat B, Dhakad AK, Thakur A, Chandra A (2018) Genetic stability and phytochemical profiling of the *in vitro* regenerated plants of Angelica glauca Edgew.: an endangered medicinal plant of Himalaya Plant Cell, Tissue, and Organ. Culture 135(1):111–118. https://doi.org/10.1007/s11240-018-1448-z

Jena S, Ray A, Sahoo A et al (2018) High-frequency clonal propagation of Curcuma angustifolia ensuring genetic fidelity of micropropagated plants. Plant Cell Tiss Organ Cult 135:473–486. https://doi.org/10.1007/s11240-018-1480-z

Kaensaksiri T, Soontornchainaksaeng P, Soonthornchareonnon N, Prathanturarug S (2011) In vitro induction of polyploidy in *Centella asiatica* (L.) Urban. Plant Cell Tiss Organ Cult 107:187–194

Kalariya KA, Minipara DB, Manivel P (2018) *De novo* transcriptome analysis deciphered polyoxypregnane glycoside biosynthesis pathway in *Gymnemasylvestre*. Biotech 8(9):381

Kasha KJ (2005) Chromosome doubling and recovery of doubled haploid plants. In: Palmer CE, Keller WA, Kasha KJ (eds) Haploids in crop improvement II, vol 56. Springer, Berlin Heidelberg, pp 123–152

Kastell A, Schreiner M, Knorr D et al (2018) Influence of nutrient supply and elicitors on glucosinolate production in E. sativa hairy root cultures. Plant Cell Tiss Organ Cult 132:561–572. https://doi.org/10.1007/s11240-017-1355-8

Kayser O, Wim Q (2007) Medicinal plant biotechnology: from basic research to industrial applications, vol 2

Khamushi M, Dehestani-Ardakani M, Zarei A et al (2019) An efficient protocol for micropropagation of old cypress of Abarkuh (*Cupressus sempervirens* var. horizontalis [Mill.]) under *in vitro* condition. Plant Cell Tiss Organ Cult 138:597–601. https://doi.org/10.1007/s11240-019-01645-z

Khanam MN, Anis M (2018) Organogenesis and efficient in vitro plantlet regeneration from nodal segments of Allamanda cathartica L. using TDZ and ultrasound assisted extraction of quercetin. Plant Cell Tiss Organ Cult 134:241–250. https://doi.org/10.1007/s11240-018-1416-7

Kim J, Lee HJ, Carroll D (2010) Genome editing with modularly assembled zinc-finger nucleases. Nat Methods 7:91–92

Kim JA, Roy NS, Lee I-H, Choi AY, Choi BS, Yu YS, Park N-I, Park KC, Kima S, Yang HS, Choi I-Y (2019) Genome-wide transcriptome profiling of the medicinal plant *Zanthoxylum planispinum* using a single-molecule direct RNA sequencing approach. Genomics 111:973–979

Krishna G, Reddy PS, Nair NA, Ramteke PW, Bhattacharya PS (2010) In vitro direct shoot regeneration from proximal, middle and distal segment of Coleus forskohlii leaf explants. Physiol Mol Biol Plants 16:195–200

Krishnan NM, Pattnaik S, Jain P, Gaur P, Choudhary R, Vaidyanathan S, Deepak S, Hariharan AK, Krishna PGB, Nair J, Varghese L, Valivarthi NK, Dhas K, Ramaswamy K, Panda B (2012) A draft of the genome and four transcriptomes of a medicinal and pesticidal angiosperm *Azadirachta indica*. BMC Genom 13:464–477

Kumar J, Gupta PK (2008) Molecular approaches for improvement of medicinal and aromatic plants. Plant Biotechnol Rep Dordrecht 2(2):93–112. https://doi.org/10.1007/s11816-008-0059-2

Kumar SR, Shilpashree HB, Nagegowda DA (2018a) Terpene moiety enhancement by overexpression of geranyl (geranyl) diphosphate synthase and geraniol synthase elevates monomeric and dimeric monoterpene indole alkaloids in transgenic *Catharanthus roseus*. Front Plant Sci. https://doi.org/10.3389/fpls.2018.00942

Kumar Y, Prakash O, Tripathi H, Tandon S, Gupta MM, Rahman L, Lal RK, Semwal M, Darokar MP, Khan F (2018b) AromaDb: a database of medicinal and aromatic plant's aroma molecules with phytochemistry and therapeutic potentials. Front Plant Sci 13. https://doi.org/10.3389/fpls.2018.01081

Kundu K, Salma U, Ali MA, Hazra AK, Mandal N (2018) Development of transgenic hairy roots and augmentation of secondary metabolites by precursor feeding in *Sphagneticola calendulacea* (L.) Pruski. Indus Crops Prod 121:206–215

Kutchan T, Gershenzon J, Moller BL, Gang D (2015) In: Buchanan BB, Gruissem W, Jones RL (eds) Natural products, in biochemistry and molecular biology of plants. Wiley, Oxford

Lalabadi MA, Jelodar NAB, Bagheri NA (2014) Micro-propagation of Tashnedari Endangered Medicinal Plant *in vitro* culture. Bull Env Pharmacol Life Sci 3:254–257

Lata H, Chandra S, Khan IA, ElSohly MA (2009) Propagation through alginate encapsulation of axillary buds of Cannabis sativa L. an important medicinal plant. Physiol Mol Biol Plant 15:79–86. https://doi.org/10.1007/s12298-009-0008-8

Lavania UC (2013) Polyploidy, body size, and opportunities for genetic enhancement and fixation of heterozygosity in plants. The Nucleus 56(1):1–6

Levin DA, Soltis DE (2018) Factors promoting polyploid persistence and diversification and limiting diploid speciation during the K-Pg interlude. Curr Plant Biol 42:1–7. https://doi.org/10.1016/j.pbi.2017.09.010

Li B, Cui G, Shen G, Zhan Z, Huang L, Chen J, Qi X (2017) Targeted mutagenesis in the medicinal plant *Salvia miltiorrhiza*. Sci Rep. https://doi.org/10.1038/srep43320

Linsmaier EM, Skoog F (1965) Organic growth factor requirements of tobacco tissue cultures. Physiol Plant 18:100–127

Liu S, Zhong JJ (1998) Phosphate effect on production of ginseng saponin and polysaccharide by cell suspension cultures of *Panax ginseng* and *Panax quinquefolium*. Proc Biochem 33:69–74

Liu Y, Sun G, Zhong Z, Ji L, Zhang Y, Zhou J, Zheng X, Deng K (2017) Overexpression of AtEDT1 promotes root elongation and affects medicinal secondary metabolite biosynthesis in roots of transgenic *Salvia miltiorrhiza*. Protoplasma 254:1617–1625. https://doi.org/10.1007/s00709-016-1045-0

Lyle EC (2007) In: Janick J, Whipkey A (eds) Medicinal and aromatic plants – future opportunities. Issues in new crops and new uses. ASHS Press, Alexandria, VA

Madlung A (2013) Heredity; Polyploidy and its effect on evolutionary success: old questions revisited with new tools. Cardiff 110(2):99–104

Mahendran D, Kishor PK, Geetha N, Venkatachalam P (2018) Phycomolecule-coated silver nanoparticles and seaweed extracts induced high-frequency somatic embryogenesis and plant regeneration from *Gloriosa superba* L. J Appl Phycol 30:1425–1436. https://doi.org/10.1007/s10811-017-1293-1

Makarova KS, Koonin EV (2015) Annotation and classification of CRISPR-Cas systems. Springer, CRISPR, pp 47–75

Malzahn A, Lowder L, Qi Y (2017) Plant genome editing with TALEN and CRISPR. Cell Biosci 7:21

Mami Y, Amit R, Naoko Y, Kazuki S (2018) Perspective: functional genomics towards new biotechnology in medicinal plants. Plant Biotechnol Rep Dordrecht 12(2):69–75. https://doi.org/10.1007/s11816-018-0476-9

Mary W, Sandie W, Kay S, Susan MC, Nikki J et al (2013) Identification of quantitative trait loci for cane splitting in red raspberry (*Rubus idaeus*). Mol Breed Dordrecht 31(1):111–122. https://doi.org/10.1007/s11032-012-9775-y

Masoudi-Nejad A, Goto S, Jauregui R, Ito M, Kawashima S, Moriya Y, Endo TR, Kanehisa M (2007) EGENES: transcriptome-based plant database of genes with metabolic pathway information and expressed sequence tag indices in KEGG. Plant Physiol 144(2):857–866

Máthé Á, Hassan F, Kader AA (2015) *In vitro* micropropagation of medicinal and aromatic plants. In: Máthé Á (ed) Medicinal and aromatic plants of the world, vol 1. Springer, Dordrecht, pp 305–336

Matsumoto T, Ikeda T, Kanno N, Kisaki T, Noguchi M (1980) Selection of high ubiquinone 10-producing strain of tobacco cultures by cell cloning technique. Agric Biol Chem 44:967–969

Matveeva TV, Sokornova SV (2018) *Agrobacterium rhizogenes* mediated transformation of plants for improvement of yields of secondary metabolites. In: Pavlov A, Bley T (eds) Bioprocessing of plant in vitro systems. Reference series in phytochemistry. Springer, Cham. https://doi.org/10.1007/82_2018_80

McKerrow JH (2015) Recognition of the role of natural products as drugs to treat neglected tropical diseases by the 2015 Nobel prize in physiology or medicine. Nat Prod Rep 32(12):1610–1611

Miller JC, Holmes MC, Wang J, Guschin DY, Lee Y, Rupniewski I, Beausejour CM, Waite AJ, Wang NS, Kim KA, Gregory PD, Pabo CO, Rebar EJ (2007) An improved zinc-finger nuclease architecture for highly specific genome editing. Nat Biotechnol 25:778–785

Mohanraj K, Karthikeyan BS, Vivek-Ananth RP et al (2018) IMPPAT: a curated database of Indian Medicinal Plants, Phytochemistry And Therapeutics. Sci Rep 8:4329. https://doi.org/10.1038/s41598-018-22631-z

Morris P (1986) Regulation of product synthesis in cell cultures of Catharanthus roseus. Effect of culture temperature. Plant cell reports 5(6):427–429

Murata J, Roepke J, Gordon H, Luca VD (2008) The leaf epidermome of *Catharanthus roseus* reveals its biochemical specialization. Plant Cell 20:524–542

Murch SJ, Krishna Raj S, Saxena PK (2000) Tryptophan is a precursor for melatonin and serotonin biosynthesis in in-vitroregenerated St. John's wort (*Hypericum perforatum* L. cv. Anthos) plants. Plant Cell Rep 19:698–704

Murshige T, Skoog A (1962) A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiol Plant 15(23–28):15

Murthy HN, Dandin VS, Zhong JJ, Paek KY (2014) Strategies for enhanced production of plant secondary metabolites from cell and organ culture. In: Paek K-Y et al (eds) Production of biomass and bioactive compounds using bioreactor technology. https://doi.org/10.1007/978-94-017-9223-3_20

Nagata T, Ebizuka Y (2002) Medicinal and aromatic plants XII. In: Biotechnology in agriculture and forestry. Springer, Heidelberg, USA

Nagella P, Murthy HN (2011) *In vitro* production of gymnemic acid from cell suspension cultures of *Gymnema sylvestre* R. Br. Eng Life Sci 11:537–540
Nalawade SM, Tsay HS (2004) *In vitro* propagation of some important Chinese medicinal plants and their sustainable usage. Vitro Cell Dev Biol Plant 40:143–154. https://doi.org/10.1079/IVP2003504
Naqvi S, Farré G, Georgina S, Capell T, Changfu Z et al (2010) When more is better: multigene engineering in plants. Trend Plant Sci 15(1):48–56
Narnoliya LK, Rajakani R, Sangwan NS, Gupta V, Sangwan RS (2014) Comparative transcripts profiling of fruit mesocarp and endocarp relevant to secondary metabolism by suppression subtractive hybridization in *Azadirachta indica* (neem). Mol Biol Rep 41:3147–3162
Naz R, Anis M, Alatar AA, Ahmad A, Naaz A (2018) Nutrient alginate encapsulation of nodal segments of *Althaea officinalis* L., for short-term conservation and germplasm exchange. Plant Biosyst. https://doi.org/10.1080/11263504.2018.1436610
Niazian M (2019) Application of genetics and biotechnology for improving medicinal plants. Planta 249:953–973
Niazian M, Sadat Noori SA, Tohidfar M, Mortazavian SMM (2017) Essential oil yield and agro-morphological traits in some Iranian ecotypes of ajowan (*Carum copticum* L.). J Essent Oil Bear Plants 20:1151–1156. https://doi.org/10.1080/0972060X.2017.1326849
Nielsen ML, Isbrandt T, Rasmussen KB, Thrane U, Hoof JB et al (2017) Genes linked to production of secondary metabolites in *Talaromyces atroroseus* revealed using CRISPR-Cas9. PLoS One 12(1):e0169712. https://doi.org/10.1371/journal.pone.0169712
Nødvig CS, Nielsen JB, Kogle ME, Mortensen UH (2015) A CRISPR-Cas9 system for genetic engineering of filamentous fungi. PLoS One 10:e0133085. https://doi.org/10.1371/journal.pone.0133085
Noman A, Aqeel M, He S (2016) CRISPR-Cas9: tool for qualitative and quantitative plant genome editing. Front Plant Sci 7:1740. https://doi.org/10.3389/fpls.2016.01740
Nordine A, Bousta D, Khanchoufi AE, Meskaoui AE (2013) An efficient and rapid *in vitro* propagation system of *Thymus hyemalis* lange, a wild medicinal and aromatic plant of Mediterranean region. Int J Pharm Biosci Technol 1(3):118–129
Ober D (2010) Gene duplications and the time thereafter -examples from plant secondary metabolism. Plant Biol (Stuttg) 12:570–577
Oksman-Caldentey KM, Inze D, Oresic M (2004) Connecting genes to metabolites by a systems biology approach. Proc Natl AcadSci USA 101:9949–9950
Paddon CJ, Westfall PJ, Pitera DJ, Benjamin K, Fisher K, McPhee D, Leavell M, Tai A, Main A, Eng D (2013) High-level semisynthetic production of the potent antimalarial artemisinin. Nature 496:528–532
Palmer CD, Keller WA (2011) Plant regeneration from petal explants of *Hypericum perforatum* L Plant Cell. Tissue Organ Cult (PCTOC) 105:129–134
Pan C, Ye L, Qin L, Liu X, He Y et al (2016) CRISPR/Cas9-mediated efficient and heritable targeted mutagenesis in tomato plants in the first and later generations. Sci Rep Nature Publisher Group London 6:24765. https://doi.org/10.1038/srep24765
Pandey P, Mehta R, Upadhyay R (2013) *In-vitro* propagation of an endangered medicinal plant *Psoralea corylifolia* Linn. Asian J Pharm Clin Res 6(3):115–118
Pani A, Mahapatra RK (2013) Computational identification of microRNAs and their targets in *Catharanthus roseus* expressed sequence tags. Genom Data 1:2–6
Pan-pan H, Wei-xu L, Hui-hui L (2018) *In vitro* induction and identification of autotetraploid of *Bletilla striata* (Thunb.) Reichb. f. by colchicine treatment. Plant Cell Tissue Organ Cult 132:425–432. https://doi.org/10.1007/s11240-017-1339-8
Pathak MR, Abido MS (2014) The role of biotechnology in the conservation of biodiversity. J Exp Biol Agri Sci 2(4):352–363
Patra N, Srivastava AK (2017) Mass production of Artemisinin using hairy root cultivation of Artemisia annual in bioreactor. In: Pavlov A, Bley T (eds) Bioprocessing of plant in vitro systems. Planta 249:953–973 971

Piosik Z, Zenkteler E, Zenkteler EM (2016) Development of haploid embryos and plants of Lactuca sativa induced by distant pollination with *Helianthus annuus* and *H. tuberosus* Dordrecht. Euphytica Int J Plant Breed 208(3):439–451

Pouvreau B, Vanhercke T, Singh S (2018) From plant metabolic engineering to plant synthetic biology: the evolution of the design/build/test/learn cycle. Plant Sci. https://doi.org/10.1016/j.plantsci.2018.03.035

Pradhan SK, Gupta RC, Goel RK (2018) Differential content of secondary metabolites in diploid and tetraploid cytotypes of *Siegesbeckia orientalis* L. Nat Prod Res. https://doi.org/10.1080/14786419.2017.1423298

Qian ZG, Zhao ZJ, Xu YF, Qian XH, Zhong JJ (2004) Novel chemically synthesized hydroxyl-containing jasmonates as powerful inducing signals for plant secondary metabolism. Biotechnol Bioeng 86(809–816):116

Qian ZG, Zhao ZJ, Xu YF, Qian XH, Zhong JJ (2005) Highly efficient strategy for enhancing taxoid production by repeated elicitation with a newly synthesized jasmonate in fed-batch cultivation of *Taxus chinensis* cells. Biotechnol Bioeng 90:516–521

Rai A, Saito K, Yamazaki M (2017) Integrated omics analysis of specialized metabolism in medicinal plants. Plant J 90:764–787

Rajakani R, Narnoliya L, Sangwan NS, Sangwan RS, Gupta V (2014) Subtractive transcriptomes of fruit and leaf reveal differential representation of transcripts in *Azadirachta indica*. Tree Genetics & Genomes 10:1331–1351

Ramachandra Rao S, Ravishankar GA (2002) Plant cell cultures: chemical factories of secondary. Metabolites. Biotechnol Adv 20:101–153

Ramakrishna A, Ravishankar GA (2011) Influence of abiotic stress singles on secondary metabolites in plants. Plant Signal Behav 6(1720–1731):106

Rao NK (2004) Plant genetic resources: advancing conservation and use through biotechnology. Afr J Biotechnol 3:136–145. https://doi.org/10.5897/AJB2004.000-2025

Rastogi S, Meena S, Bhattacharya A, Ghosh S, Shukla RK, Sangwan NS, Shasany AK (2014) De novo sequencing and comparative analysis of holy and sweet basil transcriptomes. BMC Genomics 15. https://doi.org/10.1186/1471-2164-15-588

Rauf S, Khan IA, Khan FA (2006) Colchicine-induced tetraploidy and changes in allele frequencies in colchicine-treated populations of diploids assessed with RAPD markers in *Gossypium arboretum* L. Turk. J. Biol. 30:93–100

Rischer H, Oresic M, Seppänen-Laakso T et al (2006) Gene-to- metabolite networks for terpenoid indole alkaloid biosynthesis in *Catharanthus roseus* cells. Proc Natl Acad Sci USA 103:5614–5619. https://doi.org/10.1073/pnas.0

Rizvi Z (2012) Application of artificial neural networks for predicting maximum in vitro shoot biomass production of safed musli (*Chlorophytum borivilianum*). J Med Diagn Methods 1:1–6. https://doi.org/10.4172/scientificreports.464

Roychowdhury D, Halder M, Jha S (2016) *Agrobacterium rhizogenes* mediated transformation in medicinal plants: genetic stability in long-term culture. In: Jha S (ed) Transgenesis and secondary metabolism. Reference series in phytochemistry. Springer, Cham. https://doi.org/10.1007/978-3-319-27490-4_8-1

Sahai OP, Shuler ML (1984) Environmental parameters influencing phenolics production by batch cultures of *Nicotiana tabacum*. Biotechnol Bioeng 26:111–120

Saito K, Matsuda F (2010) Metabolomics for functional genomics, systems biology, and biotechnology. Annu Rev Plant Biol 61:463–489

Salma U, Kundu S, Mandal N (2017) Artifcial polyploidy in medicinal plants: advancement in the last two decades and impending prospects. J Crop Sc Biotechnol 20:9–19. https://doi.org/10.1007/s12892-016-0080-1

Sangwan RS, Tripathi S, Singh J, Narnoliya L, Sangwan NS (2013) *De novo* sequencing and assembly of *Centella asiatica* leaf transcriptome for mapping of structural, functional and regulatory genes with special reference to secondary metabolism. Gene 525:58–76

Schenk RU, Hildebrandt AC (1972) Medium and techniques for induction and growth of monocotyledonous and dicotyledonous plant cell cultures. Can J Bot 50(199–204):17

Sedaghati B, Haddad R, Bandehpour M (2019) Efficient plant regeneration and Agrobacterium-mediated transformation via somatic embryogenesis in purslane (*Portulaca oleracea* L.): an important medicinal plantPlant Cell, Tissue, and Organ. Culture 136(2):231–245. https://doi.org/10.1007/s11240-018-1509-3

Seitz HU, Hinderer W (1988) Anthocyanins. In: Constabel F, Vasil I (eds) Cell culture and somatic cell genetics of plants, vol 5. Academic, San Diego, pp 49–76

Shapiro RS, Chavez A, Porter C, Hamblin M, Kaas CS, DiCarlo JE, Zeng G, Xu X, Revtovich AV, Kirienko NV, Wang Y, Church GM, Collins JJ (2018) A CRISPR-Cas9-based gene drive platform for genetic interaction analysis in *Candida albicans*. Nat Microbiol 3:73–82. https://doi.org/10.1038/s41564-017-0043-0

Sharma A, Verma P, Mathur A, Mathur AK (2018a) Genetic engineering approach using early *Vinca* alkaloid biosynthesis genes led to increased tryptamine and terpenoid indole alkaloids biosynthesis in differentiating cultures of *Catharanthus roseus*. Protoplasma 255:425–435. https://doi.org/10.1007/s00709-017-1151-7

Sharma S, Satardekar KV, Barve SS (2018b) Genetic improvement of medicinal and aromatic plants through haploid and double haploid development. In: Kumar N (ed) Biotechnological approaches for medicinal and aromatic plants. Springer, Singapore. https://doi.org/10.1007/978-981-13-0535-1_24

Shin KS, Murthy HN, Ko JY, Paek KY (2002) Growth and betacyanin production by hairy root cultures of Beta vulgaris L. in air lift bioreactors. Biotechnol Lett 24:2067–2069

Singh N, Sharma A (2017) Turmeric (*Curcuma longa*): miRNAs and their regulating targets are involved in development and secondary metabolite pathways. C R Biol 340(11–12):481–491. https://doi.org/10.1016/j.crvi.2017.09.009

Singh P, Singh A, Shukla AK, Singh L, Pande V, Nailwal TK (2009) Somatic embryogenesis and *In vitro* regeneration of an endangered medicinal plant Sarpgandha (*Rauvolfia serpentina*. *L*). Life Sci J 6(3):74–79

Sinha R, Sharma B, Dangi AK, Shukla P (2019) Recent metabolomics and gene editing approaches for synthesis of microbial secondary metabolites for drug discovery and development. World J Microbiol Biotechnol Oxford 35(11):1–14

Sivakumar G, Yu KW, Hahn EJ, Paek KY (2005a) Optimization of organic nutrients for ginseng hairy roots production in large-scale bioreactors. Curr Sci 89:641–649

Sivakumar G, Yu KW, Paek KY (2005b) Production of biomass and ginsenosides form adventitious roots of *Panax ginseng* in bioreactor cultures. Eng Life Sci 5:333–342

Smith CA, Maille GO, Want EJ, Qin C, Trauger SA, Brandon TR, Custodio DE, Abagyan R, Siuzdak G (2005) METLIN: a metabolite mass spectral database. Ther Drug Monit 27(6):747–751. https://doi.org/10.1097/01.ftd.0000179845.53213.39

Song R, Zhai Q, Sun L, Huang E, Zhang Y, Zhu Y, Guo Q, Tian Y, Zhao B, Lu H (2019) CRISPR/Cas9 genome editing technology in filamentous fungi: progress and perspective. Appl Microbiol Biotechnol 103:6919–6932

Spyropoulou EA, Haring MA, Schuurink RC (2014) RNA sequencing on *Solanum lycopersicum* trichomes identifies transcription factors that activate terpene synthase promoters. BMC Genom 15(1):1

Srivastava S, Srivastava AK (2007) Hairy root culture for mass- production of high-value secondary metabolites. Crit Rev Biotechnol 27(1):29–43

Tasheva K, Kosturkova G (2013). Role of biotechnology for protection of endangered medicinal plant. Additional information is available at the end of the chapter. https://doi.org/10.5772/55024

Tejovathi G, Goswami H, Bhadauria R (2011) *In vitro* propagation of endangered medicinal plant–*Commiphora wightii*. Indian J Sci Technol 4(11):1537–1541

Tong Y, Weber T, Lee SY (2019) CRISPR/Cas-based genome engineering in natural product discovery. Nat Prod Rep 36:1262–1280

Tripathi L, Tripathi JN (2003) Role of biotechnology in medicinal plants. Trop J Pharm Res 2(2):243–253. https://doi.org/10.4314/tjpr.v2i2.14607

Tripathi S, Singh J, Jadaun J, Chandra M, Sangwan NS (2016) Medicinal plant transcriptomes: the new gateways for accelerated understanding of plant secondary metabolism. Plant Genet Res Charac Util 14(4):256–269

Urbanová M, Košuth J, Čellárová E (2006) Genetic and biochemical analysis of *Hypericum perforatum* L. plants regenerated after cryopreservation. Plant Cell Rep 25:140–147. https://doi.org/10.1007/s00299-005-0050-0

VijayaSree N, Udayasri P, Aswanikumar VVY, Ravi Babu B, Phanikumar Y, Vijay Varma M (2010) Advancements in the production of secondary, metabolites. J Nat Prod 3:ISSN 0974–5211

Weathers PJ, Bunk G, McCoy MC (2005) The effect of phytohormones on growth and artemisinin production in Artemisia annua hairy roots. In Vitro Cell Dev Biol Plant 41:47–53

Werner S, Maschke RW, Eibl D, Eibl R (2018) Bioreactor technology for sustainable production of plant cell-derived products. In: Pavlov A, Bley T (eds) Bioprocessing of plant *in vitro* systems: Reference series in phytochemistry. Springer, Cham. https://doi.org/10.1007/978-3-319-32,004-5_6-

Weyrich LS, Duchene S, Soubrier J, Arriola L, Llamas B, Breen J, Morris AG, Alt KW, Caramelli D, Dresely V, Farrell M, Farrer AG, Francken M, Gully N, Haak W, Hardy K, Harvati K, Held P, Holmes EC, Kaidonis J, Lalueza-Fox C, de la Rasilla M, Rosas A, Semal P, Soltysiak A, Townsend G, Usai D, Wahl J, Hudson DH, Dobney K, Cooper A (2017) Neanderthal behaviour, diet, and disease inferred from ancient DNA in dental calculus. Nature 544:357–361

Wu CH, Murthy HN, Hahn EJ et al (2007) Large-scale cultivation of adventitious roots of Echinacea purpurea in airlift bioreactors for the production of chichoric acid, chlorogenic acid and caftaric acid. Biotechnol Lett 29:1179–1182. https://doi.org/10.1007/s10529-007-9399-1

Wu CH, Murthy HN, Hahn EJ, Paek KY (2008) Establishment of adventitious root co-culture of Ginseng and Echinacea for the production of secondary metabolties. Acta Physiol Plant 30:891–896. https://doi.org/10.1007/s11738-008-0181-3

Xue SH, Luo XJ, Wu ZH, Zhang HL, Wang XY (2008) Cold storage and cryopreservation of hairy root cultures of medicinal plant Eruca sativa Mill., Astragalus membranaceus and Gentiana macrophylla Pall. Plant Cell Tissue Organ Cult 92:251–260. https://doi.org/10.1007/s11240-007-9329-x

Yadav RK, Sangwan RS, Sabir F, Srivastava AK, Sangwan NS (2014) Effect of prolonged water stress on specialized secondary metabolites, peltate glandular trichomes, and pathway gene expression in *Artemisia annua* L. Plant Physiol Biochem 74C:70–83

Yagiz A, Gurkok T, Zhang B, Unver T (2016) Manipulating the biosynthesis of bioactive compound alkaloids for next-generation metabolic engineering in opium poppy using CRISPR-Cas 9 genome editing technology. Sci Rep Nature Publisher Group London 6:30910. https://doi.org/10.1038/srep30910

Yan YP, Wang ZZ, Tian W, Dong ZM, Spencer DF (2010) Generation and analysis of expressed sequence tags from the medicinal plant Salvia miltiorrhiza. Sci China Life Sci 53(2):273–285

Yin M, Hong S (2009) Cryopreservation of Dendrobium candidum Wall. ex Lindl. protocorm-like bodies by encapsulation-vitrification. Plant Cell Tissue Organ Cult 98:179–185. https://doi.org/10.1007/s11240-009-9550-x

Zahedi AA, Hosseini B, Fattahi M, Dehghan E, Parastar H, Madani H (2014) Overproduction of valuable methoxylated flavones in induced tetraploid plants of *Dracocephalum kotschyi* Boiss. Bot Stud 55:1–10

Zhang YH, Zhong JJ, Yu JT (1996) Effect of nitrogen source on cell growth and production of ginseng saponin and polysaccharide in suspension cultures of *Panax notoginseng*. Biotechnol Prog 12:567–571

Zhang XY, Hu CG, Yao JL (2010) Tetraploidization of diploid Dioscorea results in activation of the antioxidant defense system and increased heat tolerance. J Plant Physiol 167:88–94. https://doi.org/10.1016/j.jplph.2009.07.006

Zheng X, Pan C, Diao Y, You Y, Yang C, Hu Z (2013) Development of microsatellite markers by transcriptome sequencing in two species of *Amorphophallus* (Araceae). BMC Genom 14:490

Zhong P (1986) Regulation of product synthesis in cell cultures of *Catharanthus roseus*. Effect of culture temperature. Plant Cell Rep 5:427–429

Zhong JJ, Seki T, Kinoshita S, Yoshida T (1991) Effect of light irradiation on anthocyanin production by suspended culture of *Perilla-frutescens*. Biotechnol Bioeng 38:653–658

Zhou Z, Tan H, Li Q, Chen J, Gao S, Wang Y, Chen W, Zhang L (2018) CRISPR/Cas9-mediated efficient targeted mutagenesis of RAS in *Salvia miltiorrhiza*. Phytochem 148:63–70. https://doi.org/10.1016/j.phytochem.2018.01.015