# Chapter 16
# Medicinal Plant Research at Crossroads: Biotechnological Approaches for Conservation, Production and Stability in Tissue Cultures and Regenerated Plants

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**Abstract** Medicinal plants are treasures of nature with almost never-ending resource of unlimited, diverse, complex and valuable natural compounds with a variety of pharmacological properties. They are used worldwide by all human beings to maintain and restore good health since ancient times. The cumulative effect of heterogeneous distribution and availability throughout the world, anthropogenic interferences like habitat destruction, overexploitation due to increasing demand, unsustainable harvesting practices from the wild, introduction of exotic species, climatic changes, lack of knowledge about medicinal properties and domestication, less agronomical preference in comparison with food crops, weakening of biodiversity protection laws and/or tendency to undermine these laws by modern socio-economic forces are responsible for the loss of medicinal plant biodiversity. Classical plant tissue culture (PTC)-based biotechnological approaches have been efficiently utilized as sustainable platforms for the conservation of endangered, disease-prone and recalcitrant medicinal plant species via in vitro micropropagation and in vitro production of pharmaceutically important bio-active phytochemicals in different transformed and/or non-transformed cells, organs or regenerated plants that effectively decrease harvesting pressure from wild populations of medicinal plants. Advancement in genomics, transcriptomics, proteomics, nanoscience and synthetic biology in recent times have started to revolutionize medicinal plant research in many dimensions where PTC techniques play very important role for the implementation of these

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modern fields of science by providing constant aseptic control culture condition and better scope to utilize totipotency in comparison with wild cultivation. Hairy root culture, ploidy engineering, metabolic pathway engineering, transgenic plant development, large-scale production in bioreactors, application of Zinc-finger nucleases, TALENs and CRIPR/Cas9 are promising new biotechnological approaches. In spite of various applications and advantages of the PTC techniques, the broader commercial utilization of in vitro techniques as an important tool for the germplasm conservation of threatened commercially important medicinal plant species via mass proliferation and industrial production of SMs can be challenged by genetic, phenotypic and phytochemical instability often developed by somaclonal variations in long-term in vitro culture. The present paper reviews the recent (from 2010 upto June 2020) achievements in PTC research for conservation of medicinal plants, production of important SMs, and the stability of in vitro cultures along with case studies of two endangered species, *Podophyllum hexandrum* and *Rauvolfia serpentina*. There are immense opportunities to produce pharmaceutical important SMs with minimal threat to the biodiversity by the application of suitable conventional and upcoming PTC techniques along with screening for stability.

**Keywords** Genetic stability · Micropropagation · Podophyllotoxin · *Rauvolfia serpentina* · Secondary metabolite

## Abbreviations

| | |
|---|---|
| 2,4-D | 2,4-Dichlorophenoxyacetic acid |
| ABA | Abscisic acid |
| AFLP | Amplified fragment length polymorphism |
| AgNP | Ag-$SiO_2$ core–shell nanoparticle |
| ARC | Adventitious root culture |
| BAP | 6-Benzylaminopurine |
| BCR | Bubble column reactor |
| BTAB | Balloon-type airlift bioreactor |
| BTBB | Balloon-type bubble (air-lift) bioreactor |
| CAD | Cinnamyl alcohol dehydrogenase |
| CAOMT | Caffeic acid-O-methyltransferase |
| CCOMT | Caffeoyl CoA-O-methyltransferase |
| cDNA | Complementary DNA |
| CRISPR | Clustered regularly interspaced short palindromic repeats |
| Crtdc | Tryptophan decarboxylase gene isolated from *Catharanthus roseus* |
| CSC | Cell suspension culture |
| CuONP | Copper oxide nanoparticle |
| DMSO | Dimethylsulfoxide |
| DTAB | Drum type airlift bioreactor |
| DW | Dry weight |

| | |
|---|---|
| EC | Enzyme Commission |
| EST-SSRs | Expressed sequence tag-derived simple sequence repeat markers |
| FeNP | Iron oxide nanoparticle |
| g | Gram |
| $GA_3$ | Gibberellic acid |
| GC-MS | Gas chromatography mass spectrometry |
| HPLC | High performance liquid chromatography |
| HRC | Hairy root culture |
| IAA | Indole-3-acetic acid |
| IBA | Indole-3-butyric acid |
| ISSR | Inter simple sequence repeat |
| Kn | Kinetin |
| l | Liter |
| MeJA | Methyl jasmonic acid |
| Mg | Miligram |
| MIA | Monoterpene indole alkaloid |
| MP | Medicinal plant |
| MS medium | Murashige and Skoog medium |
| NAA | 1-Naphthaleneacetic acid |
| NADPH | Nicotinamide adenine dinucleotide phosphate |
| NMR | Nuclear magnetic resonance |
| NSB | Nutrient sprinkle bioreactor |
| PGRs | Plant growth regulators |
| PSC | Protocorm suspension culture |
| PTC | Plant tissue culture |
| PVP | Polyvinylpyrrolidone |
| RAPD | Random amplified polymorphic DNA |
| RNA | Ribonucleic acid |
| RNAi | RNA interference |
| ROS | Reactive oxygen species |
| SA | Salicylic acid |
| SC | Shoot culture |
| SCoT | Start codon targeted |
| SE | Somatic embryo |
| SEC | Somatic embryo culture |
| SM | Secondary metabolite |
| SOD | Superoxide dismutases |
| SPAR | Single primer amplification reaction |
| SSR | Simple sequence repeat |
| STB | Stirred tank bioreactor |
| TA | Tropane alkaloid |
| TALENs | Transcription activator-like effector nucleases |
| TDC | Tryptophan decarboxylase |
| T-DNA | Transfer DNA |
| TIA | Terpenoid indole alkaloid |

| | |
|---|---|
| WPM | Woody plant medium |
| ZFNs | Zinc-finger nucleases |
| ZnONP | Zinc oxide nanoparticle |

## 16.1 Introduction

Plants are treasures of nature that act as perpetual sources of diverse groups of compounds. Plants with health-promoting constituents are commonly termed as medicinal plants (MPs), which have been used worldwide by all human beings for their medicinal power to maintain and restore good health since ancient times. The identification of MPs, their bioactive constituents and the source organs/parts were undoubtedly more difficult due to the limited knowledge about the bioactive principles and lack of sophisticated instruments. The gradual progression in science and technology, integrated approach of appropriate analytical techniques, improvement in in vitro and in vivo bioassay models, the study of the molecular targets and networking of the bioactive molecules have led to the isolation and identification of several new MPs and their active constituents with improved healthcare efficacy for the treatment of different health problems.

The overwhelming phyto-diversity is used as a valuable, almost never-ending resource of unlimited novel, diverse and complex natural compounds with a variety of pharmacological properties that offer compound scaffolds in the pharmaceutical industry. It is estimated that 70–80% of the world population relies mainly on traditional and herbal medicine for their primary healthcare needs. Recently, World Health Organization (WHO) estimated that 80% of people in developing countries such as India depend on plant-derived medicines, while more than 25% of the total prescribed drugs used in developed countries such as the USA are also plant-derived medicines (Chen et al. 2016). According to the recent document published from Kew Royal Botanic Garden, out of 28,187 plant species with the potential for medicinal use, only 4478 species are cited in a medicinal regulatory publication (Willis 2017). This clearly indicates the huge opportunity in MP research as many more currently unanalyzed medicinally valuable plant-derived natural products are awaiting to be discovered.

The pharmacological properties of MPs are governed by their bioactive principles which are a wide variety of low molecular weight secondary metabolites (SMs), synthesized from divergent metabolic pathways as a by-product of primary metabolites, mostly in tissue or organ or at developmental stage-specific manner in response to various forms of biotic and abiotic stress in a limited plant species (Halder and Jha 2020; Halder et al. 2019). Plant SMs are a fascinating class of phytochemicals with diverse and complex structures that include alkaloids, terpenoids, flavonoids, quinones, coumarins, glycosides, polyketides, volatile oils, resins, tannins, glucosinolates, cyanogenic glycosides, etc., which provide survival, adaptive and competitive benefits to the plants against different biotic and abiotic factors encountered by

them (Halder and Jha 2020). They also represent highly economically valuable plant-based natural products used as drugs, cosmetics, dietary supplements, fragrances, flavours, dye, etc. (Chandran et al. 2020).

The demand for herbal medicine is gaining more attention globally year by year for the treatment of a wide variety of human ailments mainly due to human population explosion, considered very safe as there is no or minimal side effect compared to several synthetic drugs, their broad spectrum of activity, age and sex independent effect, frequent inadequate availability of synthetic drugs in developing countries, the excessive cost of treatments and development of resistance to currently used drugs for infectious diseases. Additionally, public interest in the preventive medicine and integrated medicine in recent times significantly increased the demand of MPs and natural drug therapy. Moreover, the herbal medicine industry is one of the quickest emerging industries worldwide and it is anticipated that the world trade in MPs and their metabolites will touch US$ 5 trillion in 2050 from US$ 60 billion in the year 2000 with an average yearly growth rate of 7% (Government of India 2000).

The conventional method of commercial production of bioactive, plant-derived SMs relies on the extraction of the metabolite from naturally grown MPs after harvesting from wild habitat. But commercial production of these bioactive SMs from natural population is limited due to regional or global constraint of plant material availability, slow growth and multiplication rates, tissue- or organ-specific metabolite production, low productivity, geographical and seasonal variation in content, purification difficulties, application of chemicals to combat with biotic stress, comparatively large production cycles, economic cost involved in implementation of appropriate high-throughput screening bioassays and continuous reduction in land availability. Additionally, overexploitation, insufficient attempts for replenishment after procurement and limited cultivation resulted in gradual depletion of the wild population of many MPs.

There is an enormous discrepancy between demands of consumers for plant-derived medicines and phytochemicals, and the availability of MPs from natural habitat due to gradual increase in demand against the available synthetic medicine and different problems associated with conventional natural supply. De novo chemical synthesis of these complex SMs is often both arduous and commercially expensive due to stereo-specificity (Nandagopal et al. 2018). There has been an impetus to develop viable, biotechnological methods of production of SMs (Nandagopal et al. 2018). In this context, plant tissue culture (PTC) techniques serve as eco-friendly, potential alternatives that minimize these limitations and offer a viable platform for the rapid mass multiplication and medicinally important SM production. Culture of plant protoplasts, cells, tissues, organs or complete plantlets in in vitro aseptic condition on or in certain artificial culture media under defined, controlled physical conditions like light, temperature and humidity is broadly called PTC. In vitro PTC provides an aseptic, stable and controlled culture condition that is always preferred to determine the relationship among different experimental conditions and plant materials with SMs productivity. Additionally, it is used as an alternative approach for rapid proliferation of rare, endangered and threatened MP species.

It is believed that the journey of PTC started through the introduction of the totipotency concept by Gottlieb Haberlandt in 1902, followed by organogenesis in tobacco pith, morphogenesis in carrot phloem cells, micropropagation of *Cymbidium* using meristem culture, development of germplasm conservation method, development of regeneration and multiplication protocols of various plants, conservation of phytodiversity and production of high-value bioactive metabolites from MPs. Nowadays, transfer of the gene of interest, ploidy and metabolic engineering and production of transgenic culture or plant have been made possible through the integration of PTC techniques and recombinant DNA technology.

In recent times, the trend of PTC-based research on MPs has been shifted from micropropagation and ex-situ conservation by organogenesis, morphogenesis and regeneration to commercial production of various pharmaceutically demanded phytochemicals by the implementation of different strategies like optimization of culture medium and conditions, hairy root culture (HRC), metabolite engineering, polyploidy induction and large-scale production in bioreactors. Publication data shown in Fig. 16.1 clearly shows the gradual increase in PTC-based research in last few years due to immense application potentiality of modern PTC for plant improvement and conservation.

Whole genome projects in plants have significantly risen due to plummeting of sequencing cost and the data of assembled draft whole genome sequence of 225 plants available online in early 2017 (Willis 2017). Nowadays, modern PTC approaches are not only confined to the conservation of MPs and enhancement of the productivity of their active SMs, but also exploit the genome of MPs and synthetic biology strategies for the production of engineered molecules and/or custom-designed MPs (Espinosa-Leal et al. 2018).

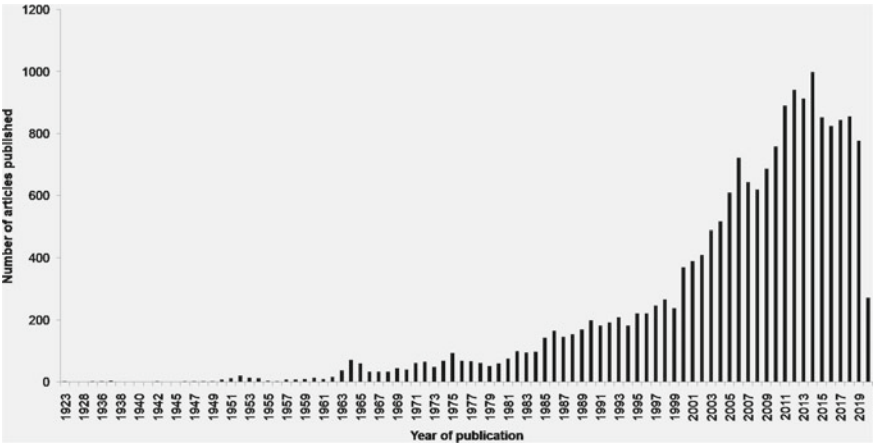


**Fig. 16.1** Graphical representation of year-wise research publication data from 1923–June 2020 based on data search result in PubMed database with keyword "Plant tissue culture"

Although the different domains of PTC-based MP research and their potential applications are very vast, PTC has been mostly exploited as a major platform for in vitro conservation of MPs and to find strategies to enhance the production of SMs within a short span of time, without disturbing their natural populations to fulfil the increasing demand for such compounds. In this context, biochemical and genetic stability of in vitro cultures (transformed or non-transformed cells, tissues, organs, whole plants) are also very important in both conservation and SM production in long-term cultures. Thus, the present review paper intends to assess the recent (2010–June 2020) achievements in PTC research for conservation of MPs and production of medicinally important SMs as well as their class, distribution in plant families and important medicinal uses with special emphasis on their stability. It also includes case studies in two species, *Podophyllum hexandrum* and *Rauvolfia serpentine,* reviewing important achievements over several decades of research.

## 16.2 Problems Associated with Improvement of Medicinal Plants

### *16.2.1 Availability of Resources*

Climatic variation throughout the earth and habitat-specific growth of MPs has led to heterogeneous distribution and availability in all the parts of the world. Many plant species are naturally and exclusively restricted to a specific small geographic area, a province, a nation or a continent and known as endemic plants. These region specific, unique MPs are usually more vulnerable to anthropogenic threats and/or natural changes due to their restricted distribution, one or few populations, small population size, declining population size, excessive collection by humans, short reproduction capacity, specific habitat conditions and necessity of stable and constant environments (Coelho et al. 2020; Fig. 16.2). Hence, endemic species should be carefully monitored and managed, and their conservation is considered a global priority (Coelho et al. 2020).

### *16.2.2 Loss of Biodiversity of Medicinal Plants*

In the recent past, biodiversity of MPs at the level of genes, species and ecosystems encountered enormous pressure throughout this planet due to habitat destruction, rapid increase in human population, rapid urbanization and overexploitation caused by increasing demand coupled with expanding international trade and unsustainable harvesting practices from the wild (Fig. 16.2). Additionally, climate changes, plant

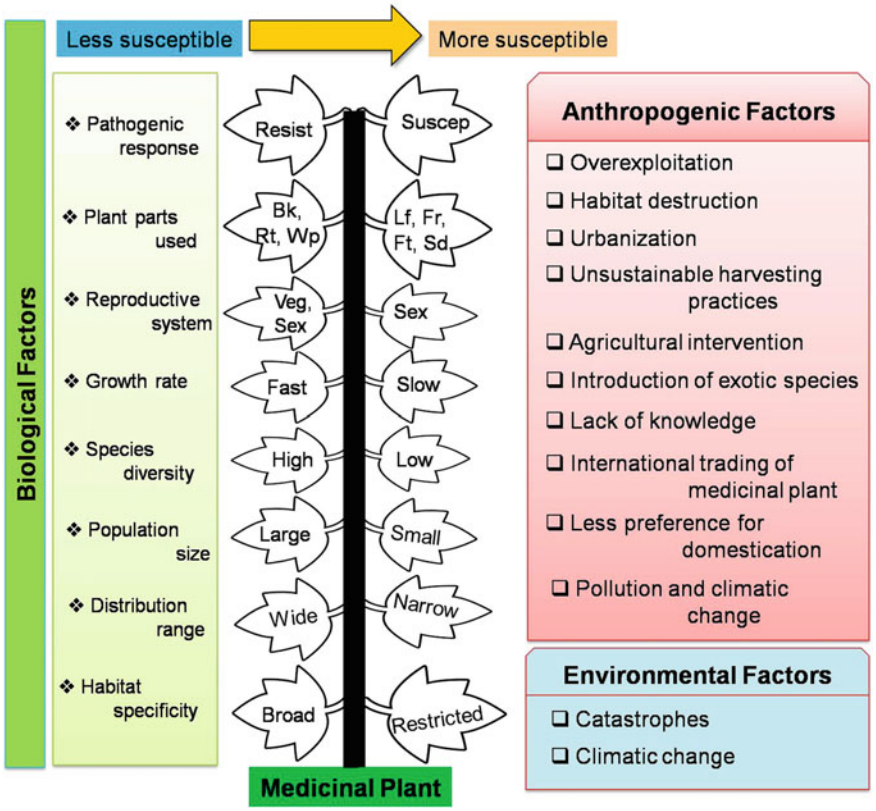


**Fig. 16.2** Major factors that are responsible for loss of biodiversity in medicinal plants (Bk = Bark, Fr = Flower, Ft = Fruit, Lf = Leaf, Resist = Resistance, Rt = Root, Sd = Seed, Sex = Sexual, Suscep = Susceptible Veg = Vegetative, Wp = Whole plant)

diseases, the introduction of exotic species, lack of knowledge of medicinal properties and domestication and agronomical preference of other food crops often create serious threats to many wild populations of economically valuable MPs (Fig. 16.2).

It has been roughly estimated that 20% of wild resources of MPs have already been nearly exhausted with the increase in human population and plant consumption. A highly conservative estimate states that the present loss of plant species is between 100 and 1000 times higher than the expected natural extinction rate that leads to loss of at least one potential major drug every 2 years (Chen et al. 2016). Unfortunately, no reliable estimation of globally threatened MPs is available, only some of the MPs that suffer from genetic erosion and resource destruction have been listed as threatened (Chen et al. 2016). Thus, conservation of biodiversity is an urgent necessity to ensure the sustainability and availability of the variety of MP germplasm that provide raw materials for industries and explore the possibility of future development.

### 16.2.3 Decline in Local Knowledge and Cultural Survival

Since ancient times, MPs are an integral component of the life of tribal communities as these plants support their need for medicine, livelihood security and financial income. They also use these naturally occurring medicinal resources as ethnoveterinary medicine for the maintenance and conservation of the health care of livestock. Apart from the economic importance of conserving biodiversity, several ethnobotanical, cultural, moral and ethical aspects that are associated with the sanctity of all forms of life, also promote conservation. These local communities continually played a pivotal role in the conservation of nature and genetic resources of MPs either through in-situ conservation and/or domestication over several generations. In India, a large number of sacred groves around ancient sacred sites and temples have been preserved by tribal people in several states and these sites act as gene bank of wild plants.

Interestingly, important indigenous knowledge of MPs and their potential applications related to improving health and life privilege associated with these folklore communities have been conserved and transmitted from generation to generation in an undocumented form. These regional cultures and communities are also facing different challenges due to civilization, privatization or nationalization of land, and many of them may have already become extinct or are in the verge of extinction in the near future. Documentation of this knowledge about the MPs, their bioactivity and conservation of those tribal groups are also required.

### 16.2.4 Public Awareness and Need for Further Research Medicinal Plant

A mass extinction of species is occurring currently worldwide, and a significant number of the plants is diminishing at an alarming rate, where anthropogenic reasons being the most dominant weapon that destroying the wild population of MPs. Public awareness and participation are necessary to address the issue, and the conservation and sustainable use of these natural resources is critical to save the diversity of MPs in nature. Involvement of local communities is an important strategy to make conservation sustainable. Effective conservation strategies for MPs should thus take place within four main areas: in-situ conservation, ex-situ conservation, education and research (Sharma and Pandey 2013).

Mother earth has an enormous diversity of plants, and plant cells are the reservoirs of diverse phytochemicals. Only a few diamonds have been collected from the huge pool of bioactive compounds in the plants and a major portion is still not exploited by humans. Recent public awareness towards natural products and advancement in extraction method, purification procedure, detection technique, genomics, transcriptomics, proteomics and metabolomics significantly accelerate research of MPs that can be predicted by the published articles (Fig. 16.1). In the present scenario, the

major concerning areas related to the MP research are the plant identification, their bioactive components, development of sustainable production system through in-situ or ex-situ conservation or through other biotechnological approaches and new product discovery. Availability of the plants, impurity, seasonal variation, tissue or organ-specific production, production restricted to selected taxa, low amount, structural diversity and complexity and lack of universal extraction methods are the major problems in MP research.

## 16.3 Conservation of Medicinal Plants

Nowadays, despite the economical and pharmaceutical demand of MP-derived SMs, indiscriminate use, excessive harvesting, continuous increase of human populations and per capita consumption, deforestation, industrialization, fires, development of land for agriculture, environmental damage and environmental changes create tremendous pressure on the wild population of MPs leading to the extinction and rapid reduction in their indigenous lands, which have put natural populations at risk in future (Fig. 16.2). Besides these anthropogenic factors, the knowledge about multiple biological characters that are correlated with extinction risk of a specific MP is also essential for their sustainable growth in natural habitats. Environmental changes and natural calamities such as floods, fires have also caused loss of biodiversity. The cumulative effect of the above-mentioned factors along with the weakening of customary laws and/or tendency to undermine these laws by modern socio-economic forces is the main causative reasons for the loss of biodiversity throughout the world. According to the IUCN (International Union for Conservation of Nature and Natural Resources) Red List of threatened species, the number of threatened species increases dramatically every year and recent report showed 15,774 threatened plant species out of 38,630 evaluated species, whereas the total number of plant species described is 422,683 (IUCN 2019).

The status of biodiversity of earth greatly relies on the balance between loss of biodiversity and conservation (Fig. 16.3). Both in-situ and ex-situ conservation methods are very effective in biodiversity conservation of plants. These scientific conservation approaches have been implemented or should be implemented globally to prevent the loss of MP biodiversity at the present alarming situation. Protection, preservation, maintenance, conservation and sustainable utilization through scientific conservation could be applied to prevent loss of biodiversity. The in-situ conservation of MPs may be done in naturally occurring forests and non-forest areas such as traditional farming sites and wild nurseries, by clearly demarcating them as “Medicinal Plant Conservation Reserves”, while ex-situ conservation may be done outside natural habitats by cultivating and maintaining plants in botanical gardens, nurseries, other types of gardens (garden of schools, museums and other institutions and personal gardens) or through long-term preservation of seeds in seed banks and long-term preservation of plant propagules in tissue culture repositories. Besides these, identification of new MPs from existing biodiversity or more effective compounds

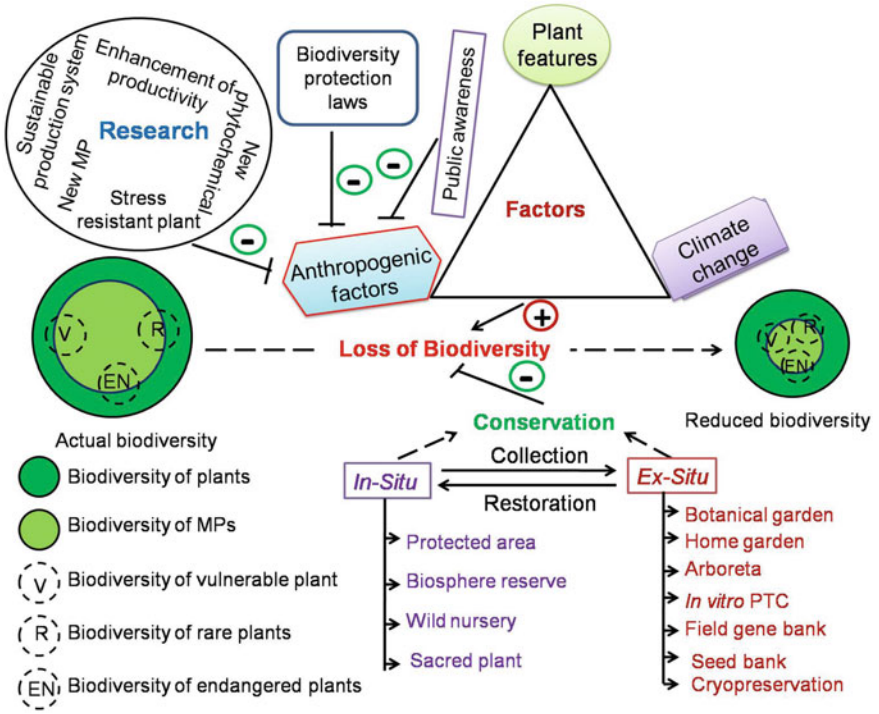


**Fig. 16.3** Schematic representation of the relationship between different positive and negative factors that control medicinal plants biodiversity along with types of conservation

from well-known MPs can stabilize biodiversity by reducing exploitation pressure (Fig. 16.3).

In-situ conservation of MPs is recommended for the sustainable conservation and management of MP as this approach is cost-effective, feasible and do not require special skills or technology as species survive and perpetuate in their own niche or microclimate available in the natural habitat. It may be often challenged by the requirement of robust management systems and gradual increase pressures on land cause disappearance of large wild areas. Additionally, safeguarding via in-situ conservation is not always sufficient to protect valuable MPs in their natural ecological niche. Ex-situ conservation techniques are very important for complementation of in-situ approaches that protect MP populations from the danger of destruction, replacement or deterioration at outside the native habitat, but preferably within the natural areas of distribution by acting as a backup of plant germplasm which might be lost from their wild habitats. In some instances, ex-situ conservation may be the only option for many species which survive and perpetuate optimally only in their own niche or the microclimate.

Although cultivation and maintenance of selected MPs in botanical gardens, arboreta, nurseries, etc., directly strengthen in-situ conservation, generally such sites

have limited numbers of specimens of each species and they are poorly represented in comparison with wild populations. In spite of these, botanic gardens can play major roles in MP conservation through developing propagation and cultivation protocols and undertaking programmes of domestication and variety breeding by the acquisition, implementation and validation of traditional knowledge. Seed banks are quite popular and attractive ex-situ conservation methods of storing the genetic diversity of many plants far distant from their natural habitats under the supervision of the specialist scientists. However, representation of seeds of MPs in seed banks is not always satisfactory.

In this alarming situation, the development and advancement of different biotechnological approaches are promising alternatives for conservation of commercially important, rare, endangered and threatened MP species via rapid mass propagation and germplasm conservation as well as for large-scale production of desirable bioactive phytochemicals and genetic improvement of the MPs. These include different in vitro PTC methods such as micropropagation, somatic embryogenesis, culture of regenerated plants and genetic transformation. During tissue culture and the domestication process of MPs, medicinal efficacy should not be compromised. Conventional, common PTC methods for germplasm conservation and production of bioactive phytochemicals along with route of discovery of new bioactive compound have been shown in Fig. 16.4.

## 16.4 Plant Tissue Culture for Conservation of Medicinal Plants

PTC is a quick and efficient in vitro technique for rapid growth and proliferation of cells, tissues and organs of plants on artificial solid or liquid media under the aseptic condition that has opened extensive areas of research for the conservation of elite germplasms for virtually unlimited time span in limited space by exploring totipotency of plant cell. In vitro PTC approaches for the conservation of plant biodiversity and their utilization are of great interest as they offer several advantages such as season and developmental condition independent collection, rapid multiplication using micropropagation, no geographical and climatic barrier for in vitro propagation and maintenance, production and maintenance of clones of desired elite genotypes, production of disease-free plants, storage of plant germplasm and the scope of genetic manipulation. PTC protocols are very useful for in vitro conservation of various endangered, rare and threatened plant species with slow growth, low abundance, recalcitrant seeds and high susceptibility to replanting diseases. De novo organ development is possible in in vitro culture from mature differentiated cells via direct or indirect organogenesis or somatic embryogenesis by modifying the concentration of exogenous plant growth regulators (PGRs) in the culture medium.

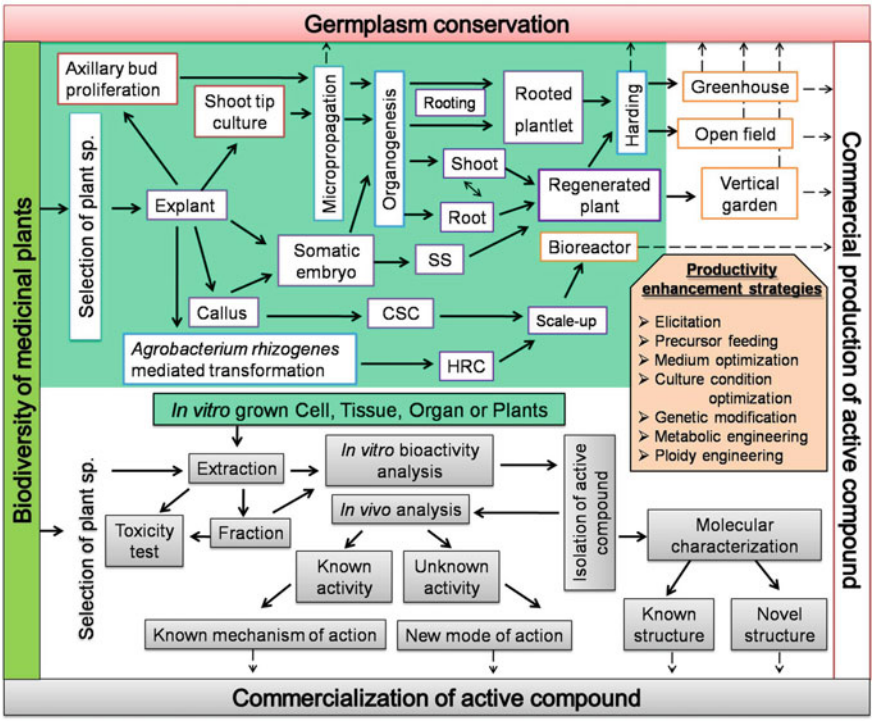


**Fig. 16.4** Schematic representation of possible routes of germplasm conservation, commercial production of SMs and discovery of new phytochemical with bioactivity through the application of different in vitro plant tissue culture-based techniques (CSC = cell suspension culture, HRC= hairy root culture, SS = synthetic seed)

### *16.4.1 Micropropagation of Medicinal Plants and in vitro Conservation*

Micropropagation is a very effective and useful direct application of PTC with a strong potential to conserve endangered, disease-prone and recalcitrant species by rapid multiplication of these plants within a comparatively short time period and limited space. It is concerned with the production of numerous disease-free genetically identical plants for medicinal, conservation, reforestation and commercial purposes. The technique of micropropagation is usually divided into several stages, namely pre-propagation, initiation of explants, subculture of explants for proliferation, shooting, rooting and hardening, which are universally utilized for the mass-scale production of MPs. Recently, the application of computational methods such as artificial neural network and image processing helps researchers to better understand the complex relationship among in vitro micropropagation and different multi-variable factors such as the selection of plant genotype; type, size and age of explants; type and concentration of PGRs; composition and pH of the medium; source

of carbohydrate; gelling agent and culture conditions (temperature, light intensity and photoperiod) in a holistic manner (Niazian 2019; Ray et al. 2020).

In vitro rapid proliferation has been achieved successfully in an array of MPs through micropropagation using nodes, internodes, shoot tips, axillary buds as explants in different tissue culture media and aseptic controlled micro-environmental conditions (Table 16.1). Micropropagated plants are usually with similar genetic characters with respect to plant growth and yield of the target molecule. Genetic homogeneity of micropropagated plants ensures product uniformity which is the primary requirement for commercial production. Additionally, it can be applied for the development of disease-free MPs through meristem culture and can be grown in the fields of the pharmaceutical industries. The report of successful in vitro micropropagation of MPs is attenuated due to the problems associated with the final and critical step of hardening and acclimatization of in vitro raised MPs in natural habitats. Wild grown MPs which have undergone a different growth pathway during in vitro propagation often may show low acclimatization rate (Niazian 2019).

### *16.4.2 Micropropagation via Organogenesis*

Regeneration of whole plants from the explants (cell, tissue or organ) is possible in PTC either directly (emergence of adventitious organs from the explants without callus formation) or indirectly (via formation of callus) through organogenesis that results in the differentiation of monopolar structures, i.e. shoot and/or root, on specific culture medium and in culture conditions. Direct or indirect organogenesis may occur spontaneously on basal culture medium without the application of exogenous PGRs or induced in the culture medium containing exogenous PGRs. Initiation of the development of roots, shoots and complete plants from callus cultures depends on various factors such as type and age of explants, type of callus, type and concentration of PGRs used in the culture medium. Organogenesis and regeneration are most promising tools of PTC-based biotechnology for conservation, improvement and production of desired SMs in MPs.

### *16.4.3 Micropropagation via Somatic Embryogenesis*

Somatic embryogenesis is a cost-effective, high-efficiency, PTC-based useful process for the bulk clonal production of desirable regenerated whole plants from a single or groups of somatic cells/tissues (explants) via a multi-step regeneration process in aseptic condition. The acquisition of embryogenic potentialities of the explants largely depends on dedifferentiation which may be possible either spontaneously or induced by manipulating phytohormone concentration in culture medium that leads to the development of somatic embryo (SE). The differentiation of an embryo occurs either directly with the induction of bipolar SEs (without callus formation)

**Table 16.1** Micropropagation of medicinal plants published during 2010 to June 2020

| Plant (Family) | Micropropagation using preexisting meristem | | Micropropagation via organogenesis | | Micropropagation via somatic embryogenesis | | | References |
|---|---|---|---|---|---|---|---|---|
| | Explant | Survival rate (%) | Explant | Survival rate (%) | Explant | Regeneration frequency (%) | Survival rate (%) | |
| *Acorus calamus* L. (Acoraceae) | Rh | 75 | – | – | – | – | – | Verma and Singh (2012)[1] |
| *Aloe barbadensis* Mill. (Asphodelaceae) | Shoot apical meristem | 100 | Inflorescence axis, L | 80–100 | – | – | – | Sahoo and Rout (2014)[2]; Das et al. (2016)[1] |
| *Aloe peglerae* Schönland (Asphodelaceae) | ST | 100 | – | – | – | – | – | Hlatshwayo et al. (2020)[1] |
| *Alpinia calcarata* Rosc. (Zingiberaceae) | Rh | >90 | – | – | – | – | – | Bhowmik et al. (2016)[2] |
| *Alpinia galanga* L. (Zingiberaceae) | AB | 90 | – | – | – | – | – | Sahoo et al. (2020)[1] |
| *Amomum subulatum* Roxb. (Zingiberaceae) | Rh | 100 | – | – | – | – | – | Purohit et al. (2017)[1] |
| *Ansellia africana* Lindl. (Orchidaceae) | N | 87 | – | – | – | – | – | Bhattacharyya et al. (2017a)[1] |
| *Artemisia annua* L. (Asteraceae) | – | – | S, L | 45 | – | – | – | Zayova et al. (2020)[2] |
| *Artemisia nilagirica* (C.B. Clarke) pamp. (Asteraceae) | – | – | N | ~73 | – | – | – | Shinde et al. (2016)[2] |

(continued)

**Table 16.1** (continued)

| Plant (Family) | Micropropagation using preexisting meristem | | Micropropagation via organogenesis | | Micropropagation via somatic embryogenesis | | | References |
|---|---|---|---|---|---|---|---|---|
| | Explant | Survival rate (%) | Explant | Survival rate (%) | Explant | Regeneration frequency (%) | Survival rate (%) | |
| *Bacopa monnieri* (L.) Wettst. (Scrophulariaceae) | ST, S, N, IN | 100 | ST, S, N, IN, L | 100 | L | 100 | 100 | Khilwani et al. (2016)[3]; Saha et al. (2020)[1,2,3] |
| *Betula platyphylla* (Miquel) Hara (Betulaceae) | – | – | – | – | Mature seed | 88 | 95 | Yang et al. (2020)[3] |
| *Boerhaavia diffusa* L. (Nyctaginaceae) | N | 85 | – | – | – | – | – | Patil and Bhalsing (2015)[1] |
| *Cannabis sativa* L. (Cannabaceae) | N with AB, ST, C, E | 95 | N with AB | 100 | – | – | – | Lata et al. (2016a; b)[2]; Wróbel et al. (2018)[1] |
| *Castilleja tenuiflora* Benth. (Orobanchaceae) | N | 95 | – | – | – | – | – | Martínez–Bonfil et al. (2011)[1] |
| *Catharanthus roseus* (L.) G. Don (Apocynaceae) | ST | 80 | – | – | H | ND | 100 | Yuan et al. (2011)[3]; Kumar et al. (2013)[1] |
| *Celosia argentea* (Var.) Cristata (Amaranthaceae) | – | – | L, S | 90 | – | – | – | Bakar et al. (2014)[2] |
| *Ceropegia noorjahaniae* (Apocynaceae) | N | 85 | – | – | – | – | – | Chavan et al. (2014)[1] |

(continued)

**Table 16.1** (continued)

| Plant (Family) | Micropropagation using preexisting meristem | | Micropropagation via organogenesis | | Micropropagation via somatic embryogenesis | | | References |
|---|---|---|---|---|---|---|---|---|
| | Explant | Survival rate (%) | Explant | Survival rate (%) | Explant | Regeneration frequency (%) | Survival rate (%) | |
| *Chlorophytum borivilianum* Sant. et Fernand. (Liliaceae) | Shoot base | 93–95 | L | 80 | H | 30 | 60–65 | Rizvi et al. (2010)[3]; Samantaray and Maiti (2010)[1]; Basu and Jha (2014)[2] |
| *Cinchona officinalis* L. (Rubiaceae) | N with AB | ND | – | – | – | – | – | Armijos–González and Pérez–Ruiz (2016)[1] |
| *Coelogyne cristata* Lindl. (Orchidaceae) | – | – | Protocorm–like bodies | ND | – | – | – | Naing et al. (2011)[2] |
| *Coleus forskohlii* Briq. (Lamiaceae) | N | 70 | L | 100 | ND | ND | ND | Sahai and Shahzad (2013)[1]; Sreedevi et al. (2013)[2] |
| *Coriandrum sativum* L. (Apiaceae) | – | – | – | – | R | 75 | ~56 | Ali et al. (2017)[3] |
| *Croomia japonica* Miq. (Stemonaceae) | – | – | Rh | 87 | – | – | – | Jiang et al. (2018)[2] |
| *Curcuma amada* Roxb. (Zingiberaceae) | – | – | – | – | Leaf sheath | ~92 | ~83 | Raju et al. (2016)[3] |

(continued)

**Table 16.1** (continued)

| Plant (Family) | Micropropagation using preexisting meristem | | Micropropagation via organogenesis | | Micropropagation via somatic embryogenesis | | | References |
|---|---|---|---|---|---|---|---|---|
| | Explant | Survival rate (%) | Explant | Survival rate (%) | Explant | Regeneration frequency (%) | Survival rate (%) | |
| *Curcuma aromatica* Salisb. (Zingiberaceae) | Rh with AB | ND | – | – | – | – | – | Parida et al. (2020)[1] |
| *Cymbidium giganteum* Wall. Ex Lindl. (Orchidaceae) | – | – | Pseudostem | ND | – | – | – | Roy et al. (2012)[2] |
| *Dendrobium crepidatum* Lindl. & Paxton (Orchidaceae) | – | – | N | 85 | – | – | – | Bhattacharyya et al. (2016b)[2] |
| *D. nobile* Lindl. (Orchidaceae) | – | – | Pseudostem with node | 84.3 | – | – | – | Bhattacharyya et al. (2014)[2] |
| *Digitalis lanata* Ehrh. (Plantaginaceae) | – | – | – | – | L, R | ~95 | 65 | Bhusare et al. (2020)[3] |
| *D. purpurea* L. (Plantaginaceae) | N | 80 | L | ND | – | – | – | Patil et al. (2013)[1]; Pérez–Alonso et al. (2018)[2] |
| *Dorem ammoniacum* D. (Apiaceae) | – | – | R, H, C | 60 | – | – | – | Irvani et al. (2010)[2] |
| *Eclipta alba* (L.) Hassk. (Asteraceae) | N, ST | 80 | – | – | N, L, ST | ~96 | 95 | Bardar et al. (2015)[1]; Salma et al. (2019)[3] |

(continued)

**Table 16.1** (continued)

| Plant (Family) | Micropropagation using preexisting meristem | | Micropropagation via organogenesis | | Micropropagation via somatic embryogenesis | | | References |
|---|---|---|---|---|---|---|---|---|
| | Explant | Survival rate (%) | Explant | Survival rate (%) | Explant | Regeneration frequency (%) | Survival rate (%) | |
| *Elaeocarpus sphaericus* (Gaertn.) K. Schum. (Elaeocarpaceae) | N | 80 | – | – | – | – | – | Saklani et al. (2015)[1] |
| *Embelia ribes* Burm F. (Myrsinaceae) | H | >85 | – | – | – | – | – | Annapurna and Rathore (2010)[1] |
| *Gentiana scabra* Bunge. (Gentianaceae) | ST | 96 | – | – | – | – | – | Huang et al. (2014)[1] |
| *Gloriosa superba* L. (Colchicaceae) | ST | 87 | Tuber | ND | – | – | – | Yadav et al. (2013[2]; 2015[1]) |
| *Gymnema sylvestre* (Retz.) R.Br. ex Sm. (Asclepiadaceae) | N, AB | 85 | – | – | – | – | – | Sharma and Bansal (2013)[1] |
| *Holarrhena antidysenterica* (L.) Wall. (Apocynaceae) | N | 90 | – | – | – | – | – | Kanungo et al. (2012)[1] |
| *Hypoxis hemerocallidea* Fisch., C.A. Mey. & Avé–Lall. (Hypoxidaceae) | – | – | Corm | ND | Corm | 87 | 90 | Moyo et al. (2014)[2]; Kumar et al. (2017)[3] |
| *Justicia gendarussa* Burm. f. (Acanthaceae) | N | 90 | – | – | – | – | – | Thomas and Yoichiro (2010)[1] |

(continued)

**Table 16.1** (continued)

| Plant (Family) | Micropropagation using preexisting meristem | | Micropropagation via organogenesis | | Micropropagation via somatic embryogenesis | | | References |
|---|---|---|---|---|---|---|---|---|
| | Explant | Survival rate (%) | Explant | Survival rate (%) | Explant | Regeneration frequency (%) | Survival rate (%) | |
| *Kelussia odoratissima* Mozaff. (Apiaceae) | – | – | – | – | Germinated seeds without hypocotyls and cotyledonary leaves | 85 | ND | Ebrahimi et al. (2018)[3] |
| *Ledebouria revoluta* (L.f.) Jessop Syn. *Scilla indica* (Wight) Baker (Asparagaceae) | – | – | Scale leaf, leaf lamina, R | 96 | Bulb scale | ~58 | 96 | Haque and Ghosh (2016)[3]; Haque et al. (2018)[2] |
| *Nothapodytes nimmoniana* (Graham) Mabb. (Icacinaceae) | – | – | R, H, CN, AB, terminal bud | 97 | – | – | – | Prakash et al. (2016)[2] |
| *Oplopanax elatus* Nakai. (Araliaceae) | – | – | – | – | L, P, R | 64 | 80 | Moon et al. (2013)[3] |
| *Pelargonium sidoides* DC. (Geraniaceae) | – | – | – | – | Inflorescence shoot, P | ~86 | ~71 | Duchow et al. (2015)[3] |
| *Picrorhiza kurroa* Royel ex Benth. (Scrophulariaceae) | N, ST | 80 | N, L, R | 81 | – | – | – | Rawat et al. (2013a)[1,2] |
| *Piper nigrum* L. (Piperaceae) | – | – | L | ND | – | – | – | Ahmad et al. (2010b)[2] |

(continued)

**Table 16.1** (continued)

| Plant (Family) | Micropropagation using preexisting meristem | | Micropropagation via organogenesis | | Micropropagation via somatic embryogenesis | | | References |
|---|---|---|---|---|---|---|---|---|
| | Explant | Survival rate (%) | Explant | Survival rate (%) | Explant | Regeneration frequency (%) | Survival rate (%) | |
| *Pittosporum eriocarpum* Royle (Pittosporaceae) | N | 73 | – | – | – | – | – | Thakur et al. (2016)[1] |
| *Plumbago rosea* L. (Plumbaginaceae) | – | – | – | – | In, L, P | ND | ND | Borpuzari and Borthakur (2016)[3] |
| *P. zeylanica* L. (Plumbaginaceae) | N | ND | N | 99 | – | – | – | Jain et al. (2018)[1]; Sharma and Agrawal (2018)[2] |
| *Podophyllum hexandrum* Royle (Berberidaceae) | – | – | Rh | ND | ZE | 79–91 | 38 | Rajesh et al. (2014b; c)[3]; Tariq et al. (2015)[2] |
| *Rauvolfia serpentina* (L.) Benth. ex Kurz., (Apocynaceae) | N | 95 | – | – | – | – | – | Ahmad et al. (2015)[1] |
| *R. tetraphylla* L. (Apocynaceae) | – | – | ST, L, R | 86 | – | – | – | Rohela et al. (2019)[2] |
| *Rhazya stricta* Decne. (Apocynaceae) | N with AB | NM | – | – | – | – | – | Mohamed et al. (2014)[1] |

(continued)

**Table 16.1** (continued)

| Plant (Family) | Micropropagation using preexisting meristem | | Micropropagation via organogenesis | | Micropropagation via somatic embryogenesis | | | References |
|---|---|---|---|---|---|---|---|---|
| | Explant | Survival rate (%) | Explant | Survival rate (%) | Explant | Regeneration frequency (%) | Survival rate (%) | |
| *Rumex nepalensis* Spreng. (Polygonaceae) | N | 90 | L | 97 | – | – | – | Ahmad et al. (2010a)[2]; Bhattacharyya et al. (2017b)[1] |
| *Ruta graveolens* L. (Rutaceae) | ST | 80 | – | – | – | – | – | Faisal et al. (2018)[1] |
| *Saussurea involucrata* (Kar. et Kir.) (Asteraceae) | Shoot base | 100 | – | – | – | – | – | Kuo et al. (2015)[1] |
| *Solanum aculeatissimum* Jacq. (Solanaceae) | – | – | L | >90 | – | – | – | Ghimire et al. (2012) |
| *Spilanthes oleracea* L. (Asteraceae) | – | – | N | ~77 | – | – | – | Dandin et al. (2014)[2] |
| *Stevia rebaudiana* Bertoni. (Asteraceae) | N | 70 | – | – | L, IN | 63 | ND | Soliman et al. (2014)[1]; Keshvari et al. (2018)[3] |
| *Swertia chirayita* (Roxb.) H. Karst. [Gentianaceae] | – | – | L | 70–80 | – | – | – | Kanwar et al. (2017)[2] |
| *Tylophora indica* (Burm. f.) Merr. (Asclepiadaceae) | ST, N, AB | 80–100 | L, S, N, P, R | 62–100 | L, S, IN | ND | 88–90 | Da Silva and Jha (2016)[1,2,3] |

(continued)

**Table 16.1** (continued)

| Plant (Family) | Micropropagation using preexisting meristem | | Micropropagation via organogenesis | | Micropropagation via somatic embryogenesis | | | References |
|---|---|---|---|---|---|---|---|---|
| | Explant | Survival rate (%) | Explant | Survival rate (%) | Explant | Regeneration frequency (%) | Survival rate (%) | |
| *Uraria picta* (Jacq.) DC. (Fabaceae) | N | 100 | – | – | – | – | – | Rai et al. (2010)[1] |
| *Veronica anagallis–aquatica* L. (Scrophulariaceae) | N | 80 | – | – | – | – | – | Shahzad et al. (2011)[1] |
| *Vitex trifolia* L. (Verbenaceae) | N with AB | 95 | – | – | N | 89.6 | 90 | Ahmad et al. (2013)[1]; Alatar et al. (2017)[3] |
| *Withania somnifera* (L.) Dunal (Solanaceae) | CN | 84 | ST, R, N | 95 | – | – | – | Chakraborty et al. (2013)[2]; Nayak et al. (2013)[1] |
| *Zingiber zerumbet* Smith (Zingiberaceae) | – | – | Rhizome bud | 80 | – | – | – | Faridah et al. (2011)[2] |
| *Zygophyllum potaninii* Maxim. (Zygophyllaceae) | | | | | C, H | 100 | 55 | Bayarmaa et al. (2018)[3] |

1 = Reference for micropropagation using preexisting meristem, 2 = Reference for micropropagation via organogenesis, 3 = Reference for micropropagation via somatic embryogenesis
(Axillary bud = AB, Apical node = AN, Cotyledon = C, Cotyledonary node = CN, Epicotyls = E, Hypocotyl = H, Leaflet/Leaf = L, Node = N, Internode = IN, Petiole = P, Rhizome = Rh, Root = R, Shoot tip = ST, Stem = S, Zygotic embryo = ZE; ND = not determined)

or indirectly with the induction of embryogenic callus. Restoration of embryogenic competence via dedifferentiation is followed by the proliferation of the embryogenic material either through adventitious budding on the SEs in secondary embryogenesis or embryogenic suspensions/calli that favour large-scale embryogenic cell proliferation before the subsequent embryo differentiation. SEs undergo various changes at morphological, physiological, metabolic, biochemical and gene expression levels during the maturation phase which are followed by desiccation and plant regeneration phase.

SE has the potential for rapid and large-scale multiplication of a wide range of economically important MP species with non-viable or recalcitrant seeds or has limited and rare seed production capability for the ex-situ conservation of germplasms. Moreover, it can be used for the production of mutants and artificial seeds, genetic engineering and germplasm cryopreservation. The innovation of synthetic seed technique in carrot using alginate encapsulated SE in 1982 significantly enhanced the application of SEs by providing the benefits of rapid pathogen-free clonal propagation by avoiding the heterozygosity problem of natural botanical seeds, their short-term storage and transportation for conservation and restoration purposes. Synthetic seeds or artificial seeds can be produced by encapsulating a suitable (~3–5 mm long) plant propagule such as unipolar vegetative propagules, micro-cuttings, or bipolar vegetative propagules such as SEs and protocorm-like bodies (PLBs) within artificial nutrient-filled soft matrix or hydrogel that is able to germinate and produce a healthy plantlet (Gantait et al. 2015; Bayarmaa et al. 2018; Bhusare et al. 2020).

Several researchers explored and assessed the potential of somatic embryogenesis and artificial seed technology as an effective approach to support conservation and rehabilitation programmes for many MP species. Genetic stability, ease in handling and transportation, effectiveness in terms of space, labour, time and cost are the advantages of synthetic seeds containing SE. In addition to conservation, synthetic seeds are ideal to maintain a screened and selected genotype of MPs that contain a high level of desired SMs. Explant selection, treatment with proper concentration and type of PGRs, manipulations of light, temperature and pH, concentrations of encapsulating agent and matrix often act as key factors that should be optimized for successful application of SE and synthetic seeds in MPs (Gantait et al. 2015). Interestingly, Piątczak and Wysokińska (2013) and Rawat et al. (2013b) extended the conception of synthetic seeds by incorporating hairy roots fragments as a novel approach in synthetic seed development of MPs like *Centaurium erythraea* and *Picrorhiza kurroa* with 86% and 73% regeneration, respectively (Gantait et al. 2015).

## 16.5 In vitro Approaches for Secondary Metabolite Production

In addition to conservation, PTC-based biotechnological approaches can be used for the successful production of concerned SMs, reduction of toxic compounds and production of novel chemical compounds (Gandhi et al. 2015). Several PTC-based SM production platforms have been successfully developed for several MP species in the last three decades to cope up with different problems associated with traditional field culture including scarcity of raw material, low productivity, geographical, seasonal and environmental variation of SM content, lack of uniformity in product quality and yield, comparatively large production cycles, biotic and abiotic stress, effect of application of pesticides and herbicides and continuous reduction in land availability for large-scale cultivation in contrast to the gradual increase in demand for plant-derived SMs.

The objective of the study and the availability of the plant are two key factors of consideration for the appropriate selection of plant material and tissue culture method for the production of desired SMs from the diverse groups of MPs and the different methods of PTC available. Non-crop wild plants are usually used for the production of pharmaceutically important compounds, whereas crop plants are used more frequently for the production of bioengineered compounds such as vaccines, antibodies, enzyme replacement and albumin serum (Espinosa-Leal et al. 2018).

The eco-friendly PTC-based bioprocessing methods for important pharmacologically active SMs include cell culture, callus culture, organ culture, micropropagation of whole plants and HRC, which have great potential to serve as attractive alternatives to large-scale SM production from field-grown plant materials (Halder et al. 2019). Apart from the traditional PTC approaches, various biotechnological interventions such as meticulous screening and selection of high-yielding lines, optimization of culture media composition and physical parameters, precursor feeding, elicitation, large-scale cultivation in the bioreactor, plant cell immobilization and biotransformation have been assayed to evaluate their effectiveness towards enhancement of SM production utilizing in vitro cell culture and/or organ culture of different MP species (Halder et al. 2018). In spite of several thousand papers that have been published on in vitro SM production, still there is an enormous area to exploit due to massive biodiversity of plants and their diverse biosynthetic potential. Moreover, enhancement in SM productivity and/or de novo synthesis of novel chemical compounds could be made possible in this omics era using different in vitro techniques through metabolic engineering.

### *16.5.1 Non-transgenic Approaches for Secondary Metabolite Production*

#### 16.5.1.1 Unorganized Callus and Cell Suspension Culture (CSC) of Medicinal Plants for Secondary Metabolite Production

Callus is a dedifferentiated state of tissue that can be initiated from various differentiated and organized plant tissue explants on plant species-specific culture medium which generally comprise of nutrient medium supplemented with different PGRs in in vitro cultures (Halder and Jha 2020). Plant CSC is characterized by free cells or small groups of cells that have generally been established by transferring undifferentiated callus in the liquid nutrient medium supplied with constant aeration agitation on a rotary shaker at a specific rpm. Simple, zero seasonal variation, the ability to maintain cells in a non-differentiating condition, infection-free production system with higher mass production per unit of time and area, cost-effective efficient downstream processing, feasibility of continuous culture in a chemostat, application of elicitation and precursor feeding and scope of large-scale production in the bioreactor are the major advantages associated with CSCs. However, genetic and biochemical instability, low synthesis ability, irregular response to elicitors are the main constraints of undifferentiated and unorganized cultures.

The biosynthetic capabilities of unorganized callus and CSC of different plants have been greatly exploited by several researchers during the last decade, and a wide range of plant-derived pharmaceutically important SMs such as ajmalicine, anthocyanins, artemisinin, camptothecin, colchicine, podophyllotoxin, reserpine, resveratrol, shikonin, taxol, withanolides, vinblastine and vincristine have been successfully produced (Table 16.2). Interestingly, spontaneous production of some SMs at a comparable amount to intact plants have been reported in undifferentiated and disorganized cultures, whereas SMs like morphinan alkaloids, tropane alkaloids (e.g. hyoscyamine and scopolamine), quinoline alkaloids, dimeric monoterpene indole alkaloids (e.g. vinblastine and vincristine), which are localized in morphologically specialized tissues or organs of native plants are either detected in very low amounts or not detected in CSCs, which might be due to loss of tissue-specific function during their dedifferentiation. Implementation of a number of strategies such as the selection of a parent plant with an elite genotype that ensures high-yielding capacity, screening and selection of high-yielding cell clones from the heterogeneous cell populations, elicitation, precursor feeding, optimization of medium and/or culture conditions, immobilization on beads can increase productivity by several folds.

#### 16.5.1.2 Root Organ Culture of Medicinal Plants for Production of Secondary Metabolites

Roots of few MPs are of great interest in the pharmaceutical industry and in the traditional pharmacopeias worldwide due to their ability to accumulate some unique

**Table 16.2** Unorganized undifferentiated callus culture and cell suspension culture of medicinal plants for production of secondary metabolites published from 2010 to June 2020

| Secondary metabolites | Chemical nature | Plant source [Family] | Main use | Culture type; strategy | References |
|---|---|---|---|---|---|
| Ajmalicine | TIA | *Catharanthus roseus* (L.) G. Don [Apocynaceae] | Anti-hypertensive | Leaf-derived CSC; Elicitation | Mandagi et al. (2017) |
| Ajmalicine | TIA | *Rauvolfia serpentina* (L.) Benth. ex Kurz., [Apocynaceae] | Anti-hypertensive | Leaf-derived CSC; Elicitation | Zafar et al. (2020) |
| Andrographolide | Diterpene lactone | *Andrographis paniculata* (Burm.f.) Nees [Acanthaceae] | Anti-cancer, anti-HIV, anti-inflammatory | Shoot and root-derived CSC; Elicitation | Gandi et al. (2012) |
| Artemisinin | Sesquiterpene lactone | *Artemisia annua* L. [Asteraceae] | Anti-malarial, Anti-snake venom | Leaf, stem and germinated seedling-derived CSC; Elicitation | Zebarjadi et al. (2018); Salehi et al. (2019) |
| Asiaticoside, Asiatic acid | Triterpenoid saponin | *Centella asiatica* (L.) Urb. [Apiaceae] | Anti-psoriasis, anti-leprosy, anti-eczema | Leaf-derived CC and CSC; Elicitation | Krishnan et al. (2019) |
| Azadirachtin | Tetranortriterpenoid limonoid | *Azadirachta indica* A. Juss. [Meliaceae] | Anti-plasmodia, anti-parasitic, anti-diabetic | Leaf-derived CSC; Effect of PGRs | Farjaminezhad and Garoosi (2019) |
| Bacosides | Triterpenoid saponin | *Bacopa monnieri* (L.) Wettst. [Plantaginaceae] | Memory enhancer, anti-neoplastic, anti-Alzheimer's | Leaf-derived CSC | Bansal et al. (2017) |
| Camptothecin | MIA | *Nothapodytes nimmoniana* (J. Grah.) Mabb. [Icacinaceae] | Anti-cancer | Leaf, hypocotyl and root-derived CC and CSC; Elicitation | Isah (2017) |

(continued)

**Table 16.2** (continued)

| Secondary metabolites | Chemical nature | Plant source [Family] | Main use | Culture type; strategy | References |
|---|---|---|---|---|---|
| Camptothecin | MIA quinoline alkaloid | *Ophiorrhiza mungos* L. [Rubiaceae] | Anti-cancer | Leaf-derived CSC; Elicitation | Deepthi and Satheeshkumar (2017) |
| Camptothecin | MIA | *Camptotheca acuminata* Decne. [Nyssaceae] | Anti-cancer | Leaf, stem, root-derived CSC; Elicitation | Yang et al. (2017) |
| Capsaicin | Alkaloid | *Capsicum chinense* Murray, *C. frutescens* L. [Solanaceae] | Counterirritant | Hypocotyl-derived CSC; Elicitation | Kehie et al. (2016) |
| Catharanthine, Vindoline | Indole alkaloid | *Catharanthus roseus* (L.) G. Don [Apocynaceae] | Anti-hypertensive | Cambial meristem-derived CSC; Elicitation | Zhou et al. (2015) |
| Chicoric, Rutin, Rosmarinic acid. Isoquercetin | Phenolic compounds | *Ocimum basilicum* L. [Lamiaceae] | Antiseptic, analgesic | Leaf-derived CSC; Elicitation | Açıkgöz (2020) |
| Colchicine | Tropolone alkaloid | *Gloriosa superba* L. [Colchicaceae] | Anti-tumour | Rhizome-derived CSC; Elicitation | Mahendran et al. (2018) |
| Echitamine, Tubotaiwine, Picrinine, Acetylechitamine | Indole alkaloid | *Alstonia scholaris* (L.) R. Br. [Apocynaceae] | Anti-cancer, anti-tuberculosis | Tender leaf-derived CC; Optimization of medium, elicitation and precursor feeding | Jeet et al. (2020) |
| Forskolin | Labdane diterpene | *Coleus forskohlii* (Willd.) Briq. [Lamiaceae] | Anti-asthmatic, anti-glaucoma | CSC; Elicitation | Swaroopa et al. (2013) |
| Ginsenosides | Triterpenes saponin | *Panax ginseng* C. A. Mey. [Araliaceae] | Hepatoprotective, anti-depressant | CSC | Le et al. (2019) |

(continued)

**Table 16.2** (continued)

| Secondary metabolites | Chemical nature | Plant source [Family] | Main use | Culture type; strategy | References |
|---|---|---|---|---|---|
| Gymnemic acid | Triterpenoid saponin | *Gymnema sylvestre* (Retz.) R.Br. ex Sm. [Asclepiadaceae] | Anti-diabetes | Leaf-derived CSC; Elicitation | Chodisetti et al. (2015) |
| Hydroxybenzoic, Hydroxycinnamic acid derivatives | Phenolic acid | *Hypoxis hemerocallidea* Fisch., C.A. Mey. & Avé-Lall. [Hypoxidaceae] | Cardio-protective, anti-impotency, anti-psoriasis | Corm-derived CC | Moyo et al. (2014) |
| Paclitaxel (taxol), Taxanes | Diterpenoid | *Taxus baccata* L. [Taxaceae] | Anti-cancer | Stem-derived CC | Sarmadi et al. (2019) |
| Plumbagin | Naphthoquinone | *Plumbago europaea* L., *P. zeylanica* L. [Plumbaginaceae] | Anti-tumour, anti-microbial | Stem and leaf-derived CSC | Patidar et al. (2015); Beigmohamadi et al. (2019) |
| Podophyllotoxin | Aryl tetralin lignan | *Podophyllum hexandrum* Royle [Berberidacae] | Anti-cancer | Leaf-derived CSC; Precursor feeding | Bhattacharyya et al. (2012); Majumder (2012) |
| Reserpine | TIA | *Rauvolfia serpentina* (L.) Benth. ex Kurz., [Apocynaceae] | Anti-hypertensive | Leaf-derived CSC; Elicitation | Zafar et al. (2017; 2020) |
| Resveratrol | Polyhydroxy-stilbene | *Vitis labrusca L.* [Vitaceae] | Cardio-prodective, anti-cancer | CSC | Nivelle et al. (2017) |
| Rhamnetin | Flavonol | *Vernonia anthelmintica* (L.) Willd. [Asteraceae] | Anthelmintic, diuretic, anti-asthmatic | Leaf-derived CSC; Elicitation | Rajan et al. (2020) |
| Syringin, Rutin | Flavonoid | *Saussurea involucrate* Matsum. & Koidz. [Asteraceae] | Anti-inflammatory, cardiotonic, anti-fatigue | Petiole, leaf and root-derived CC; Elicitation | Kuo et al. (2015) |

(continued)

**Table 16.2** (continued)

| Secondary metabolites | Chemical nature | Plant source [Family] | Main use | Culture type; strategy | References |
|---|---|---|---|---|---|
| Vincristine, Vinblastine | Alkaloid | *Catharanthus roseus* (L.) G. Don [Apocynaceae] | Anti-tumour, Anti-cancer | Leaf and hypocotyl-derived CC and CSC; Elicitation | Fatima et al. (2015); Mekky et al. (2018) |
| Withanolides | Steroidal lactone | *Withania somnifera* (L.) Dunal [Solanaceae] | Adaptogenic, diuretic, anti-inflammatory, immunomodulatory | Root-derived CSC; Elicitation and precursor feeding | Sivanandhan et al. (2014) |

Callus culture (CC), Cell suspension culture (CSC), Monoterpene indole alkaloid (MIA), Terpenoid indole alkaloid (TIA)

root-specific SMs with medicinal applications. Non-transformed adventitious root cultures of various MP species have been developed since early days of PTC as an organ culture system on solid or in liquid medium supplemented with PGRs and used as one of the important alternative source for the production of plant-derived SMs like andrographolide (Singh et al. 2018), chlorogenic acid (Lee et al. 2015), colchicine (Ghosh et al. 2015), eleutheroside B and E (Lee et al. 2015), ginsenoside (Yu et al. 2016; Le et al. 2019; Wang et al. 2019), hypericin (Wu et al. 2014), kaempferide (Han et al. 2019), podophyllotoxin (Rajesh et al. 2014a), steroidal saponins (Basu and Jha 2013), saikosaponins (Kusakari et al. 2012), steviol glycosides (Ahmad et al. 2018), plumbagin (Roy and Bharadvaja 2019), tanshinone (Zaker et al. 2015) and withanolides (Rangaraju et al. 2019). However, the slow growth rate, the requirement of exogenous supply of PGRs in the medium, biochemical and genetic instability in long-term culture and low productivity have limited its broader and commercial applications for SM production.

#### 16.5.1.3 Adventitious Shoot Cultures and Regenerated Medicinal Plants for Production of Secondary Metabolites

Besides the great implementation of shoot culture and shoot culture-derived plants for the conservation of MPs, they also serve as a viable system for PTC-based SM production. Interestingly, few reports confirmed that micropropagated plants retain their ability to produce SMs even after successful field transfer. Non-transformed in vitro shoot cultures or shoot culture-derived micropropagated MPs have been used as an important alternative source for the production of SMs including catharanthine, vindoline and vinblastine from *C. roseus* (Sharma et al. 2019), cardenolides from *D. purpurea* (Pérez-Alonso et al. 2018), bacosides from *B. monnieri* (Saha et al. 2020), tylophorine from *T. indica* (Da Silva and Jha 2016), hypericin from *Hypericum hookerianum* (Sooriamuthu et al. 2013), camptothecin from *C. acuminata* (Sankar-Thomas and Lieberei 2011), reserpine from *R. serpentina* (Panwar and Guru 2015), silymarin from *Silybum marianum* (Sherif et al. 2013), stevioside and rebaudioside A from *Stevia rebaudiana* (Bayraktar et al. 2016), withanolides from *W. somnifera* (Chakraborty et al. 2013), etc.

### *16.5.2 Transgenic Approaches*

#### 16.5.2.1 Transformed Callus and Cell Culture of Medicinal Plants for Secondary Metabolite Production

HRC or Ri- or Ti-transformed plants often showed *rol* gene(s) induced enhancement in SM production due to the over-expression of one or several key regulatory genes involved in biosynthesis pathways. *rol* genes are the potential activators of SMs and cause hypersensitive stress responses. A relatively new biotechnological approach

that relies on *rol* gene specific activation of SM production has been applied successfully in a few plant species to produce transformed callus or cell culture having single or multiple *rol* genes isolated from plasmids of *Agrobacterium* (Sarkar et al. 2018; Halder and Jha 2020). Sarkar et al. (2018) reviewed the effects of *rol* genes of *A. rhizogenes* on morphogenesis and SM accumulation in MPs. Although few reports of transformed CSCs are also available, here only recent reports of the transformed unorganized cultures and their ability to produce SMs have been discussed.

Dubrovina et al. (2010) reported enhancement of 11.9-fold resveratrol accumulation as a result of insertion of *rolC* gene in callus culture of *Vitis amurensis* via *A. tumefaciens*-mediated transformation. Similarly, *rolB*- and *rolC*-transformed callus cultures of *Maackia amurensis* showed production of isoflavonoids at significantly higher levels in comparison with empty vector control callus (Grishchenko et al. 2013; 2016). The *rolC*-transformed calli contained three times higher caffeoylquinic acid in *Cynara cardunculus* var. *altilis*. Stable 3-fold caffeoylquinic acid production was reported in *rolC*-transgenic callus cultures of *C. cardunculus* var. *altilis* compared to the control calli (Vereshchagina et al. 2014). In *rolA*-transformed cultures of *Artemisia dubia* plants, artemisinin and its derived compounds were comparable to that of the non-transformed plant (Amanullah et al. 2016). The potential elicitation capability of *rolA* gene in plant secondary metabolism has been well-observed in *rolA*-transgenic *R. cordifolia* calli, which stimulate anthraquinone yield by the activation of ruberitrinic acid biosynthesis (Veremeichik et al. 2019).

#### 16.5.2.2 Hairy Root Culture of Medicinal Plants for Secondary Metabolite Production

*A. rhizogenes*-derived HRCs have evolved as a most popular, promising, convenient and extensively used PTC-based biotechnological approach for the long-term production of commercially important pharma molecules in comparison with other forms of conventional in vitro cultures due to the major gifted strengths like rapid growth and large biomass accumulation potential, hormone-autotrophy, similar or greater bio-production capacity for SMs as compared to their parent plants, organ-based PTC method, genetic and biosynthetic stability for long-term culture, feasibility of large-scale production in the bioreactor system and often show de novo synthesis of SMs (Roychowdhury et al. 2017).

Productivity of SMs in HRCs of different plant species can be improved by the application of various biotechnological strategies which include the selection of high-yielding rhizoclone(s), optimization of culture medium and culture conditions such as optimum levels of salt, sugar, nitrogen, phosphate and physical factors such as temperature, illumination, light quality, pH of the medium, agitation, aeration and environmental gas (e.g. oxygen and carbon dioxide), replenishment of nutrient and precursor feeding, elicitation, application of phytohormones in medium and scale-up to the bioreactors (Halder et al. 2019).

Besides the production of valuable SMs of MPs, HRC platform also offers application of elicitation (Gabr et al. 2016; Srivastava et al. 2016a; Akhgari et al. 2019),

large-scale production of desired phytochemicals (Bauer et al. 2015; Patra and Srivastava 2016; Kochan et al. 2017; Thakore et al. 2017), induction of artificial polyploidy (Banerjee 2018) and CRISPR/Cas9 mediated genome editing (Li et al. 2017). Several review articles clearly demonstrated the successful establishment of HRCs of several plant species by utilizing different strains of nature's own genetic engineer *A. rhizogenes* and production of target biopharmaceuticals (Halder et al. 2019; Gutierrez-Valdes et al. 2020; Li and Wang 2020). Table 16.3 enlists few selected recent reports of successful SM production using HRC platform of MPs.

#### 16.5.2.3 Transgenic Medicinal Plants for Secondary Metabolite Production

*Agrobacterium rhizogenes* mediated genetic manipulation has undergone several developments with adoption of different binary vector system depending upon target of research (Bahramnejad et al. 2019). Different transgenic approaches like transgenic plants with only T-DNA genes of *A. rhizogenes* or *A. tumifaciens* or with novel genes that modify target metabolic pathways or with genes that improve expression of endogenous pathways or with genes not involved in biosynthetic pathways have been applied successfully in MPs to enhance the production of SMs (Kayani et al. 2018).

Regenerated viable Ri- and Ti-transformed plants have been established in a number of plant species such as *T. indica, B. monnieri, Atropa belladonna, Taraxacum platycarpum, P. rosea, Linum usitatissimum, Aesculus hippocastanum*, *Cichorium intybus* through spontaneous direct, spontaneous indirect, induced direct or induced indirect methods (Roychowdhury et al. 2017; Halder and Jha 2020). These Ri- and Ti-transformed plants also showed similar or higher amount of SMs in comparison with the mother plants. Additionally, few reports showed successful hardening and in vivo growth of these transgenic plants with stable potentiality to produce SMs.

### *16.5.3 Metabolic Pathway Engineering in Medicinal Plants for Production of Secondary Metabolites*

PTC not only offers efficient direct or indirect conservation strategies for MPs and enhancement or improvement methods of the production of valuable bioactive phytochemicals, at the same time, it also provides a platform for metabolic engineering, decipherence of biochemical pathways and production of novel compounds through genetic manipulation. PTC can help produce new forms of plant SMs and/or custom-designed MPs valuable for food, pharmaceutical and other industries (Niazian 2019). Metabolic engineering is a promising method to overcome the limitations of conventional PTC for *in planta* overproduction of some pharmaceutically important SMs by the manipulation of endogenous metabolic pathways in an

**Table 16.3** List of some selected scientific publications on hairy root culture of medicinal plants for production of secondary metabolites published during 2010 to June 2020

| Secondary metabolite | Chemical nature | Plant source [Family] | *Agrobacterium rhizogenes* strain, explant | Content/ fold increased | References |
|---|---|---|---|---|---|
| Ajmalicine | TIA | *Rauvolfia serpentina* (L.) Benth. ex Kurz. [Apocynaceae] | A4; Leaf | 14.8 | Srivastava et al. (2016a) |
| Anisodine[a], Anisodamine[b] | TA | *Przewalskia tangutica* Maxim [Solanaceae] | ATCC10060, MSU440; Hypocotyl | a = ~14*<br>b = ~5* | Lei et al. (2018) |
| Artemisinin | Sesquiterpene | *Artemisia annua* L. [Asteraceae] | LBA301; Apical meristem | 2.44 | Patra and Srivastava (2016) |
| Atropine | TA | *Przewalskia tangutica* Maxim [Solanaceae] | ATCC10060, MSU440; Hypocotyl | ~28* | Lei et al. (2018) |
| Camptothecin | MIA | *Ophiorrhiza rugosa* var. *decumbens* (Gardner ex Thwaites) Deb & Mondal [Rubiaceae] | LBA 9402; Axenic plant | 0.009% DW | Kamble et al. (2011) |
| Chrysin[c], Baicalein[d], Wogonin[e] | Flavone | *Scutellaria bornmuelleri* Hausskn. ex Bornm. [Lamiaceae] | A4, A13, MSU440, ATCC15834; Stem, petiole, leaf | e = ~11<br>c = ~9<br>d = ~13 | Gharari et al. (2020) |
| Emodin[h], Physcion[i] | Anthraquinone | *Polygonum multiflorum* Thunb. [Polygonaceae] | KCTC 2703; Internode, leaf | h = ~4<br>i = 3.5 | Thiruvengadam et al. (2014) |
| Eupalitin | Flavonol glycoside | *Boerhaavia diffusa* L. [Nyctaginaceae] | ATCC 15834, A4, SA79; Leaf | 1.44 | Gupta et al. (2016) |

(continued)

**Table 16.3** (continued)

| Secondary metabolite | Chemical nature | Plant source [Family] | *Agrobacterium rhizogenes* strain, explant | Content/ fold increased | References |
|---|---|---|---|---|---|
| Farnesiferol B | Sesquiterpene | *Ferula pseudalliacea* Rech.f. [Apiaceae] | ATCC 15824, 1724; Leaf, stem, cotyledon, embryo | 0.7–0.75 mg100 $mg^{-1}$ | Khazaei et al. (2019) |
| Glycyrrhizin | Triterpenoid saponin | *Glycyrrhiza glabra* L. [Fabaceae] | A4; Leaf | ~9 | Srivastava et al. (2019) |
| Gymnemic acid | Triterpenoid saponin | *Gymnema sylvestre* (Retz.) R.Br. ex Sm. [Asclepiadaceae] | KCTC 2703; Cotyledon, leaf | ~8 | Praveen et al. (2014) |
| Hyoscyamine[j], Scopolamine[K] | TA | *Hyoscyamus reticulatus* L. [Solanaceae] | A7; Cotyledon | l = 5<br>k = 5 | Moharrami et al. (2017) |
| Mangiferin | Xanthone | *Swertia chirayita* (Roxb.) H. Karst. [Gentianaceae] | LBA9402; Node, internode | 2 | Samaddar et al. (2019) |
| Plumbagin | Naphthoquinone | *Plumbago zeylanica* L [Plumbaginaceae] | LBA9402; Node, internode | ~6.69 $mgg^{-1}$ DW | Basu et al. (2015) |
| Reserpine | TIA | *Rauvolfia serpentina* (L.) Benth. ex Kurz. [Apocynaceae] | LBA9402; Leaf, stem | ~3.11 mg $g^{-1}$ DW | Ray et al. (2014a) |
| Resveratrol | Trihydroxystilbene | *Arachis hypogaea* L [Fabaceae] | LBA9402, A4, R1000; Leaflet, petiole | 19 | Halder and Jha (2016) |
| Rosmarinic acid | Phenolic acid | *Ocimum basilicum* L. [Lamiaceae] | A4, ARqua1-pTSC5, 8196, 11325; Leaf, hypocotyl, cotyledon | 2 | Srivastava et al. (2016b) |

(continued)

**Table 16.3** (continued)

| Secondary metabolite | Chemical nature | Plant source [Family] | *Agrobacterium rhizogenes* strain, explant | Content/ fold increased | References |
|---|---|---|---|---|---|
| Rosmarinic acid | Phenolic acid | *Dracocephalum moldavica* L. [Lamiaceae] | A4; Shoot, leaf | 10 | Weremczuk-Jeżyna et al. (2013) |
| Sanguinarine | Quaternary benzylisoquinoline alkaloid | *Macleaya cordata* (Willd.) R.Br. [Papaveraceae] | 10060; Leaf, stem | 2.3 | Huang et al. (2018) |
| Scopolamine | TA | *Przewalskia tangutica* Maxim [Solanaceae] | ATCC10060, and MSU440; Hypocotyl | ~3* | Lei et al. (2018) |
| Silymarin | Flavonolignan | *Silybum marianum* (L.) Gaertn [Asteraceae] | A4; Cotyledon | 13.59 $\mu g g^{-1}$ DW | Gabr et al. (2016) |
| Solasodine | Steroidal glycoalkaloid | *Solanum khasianum* C.B. Clarke [Solanaceae] | A4; Leaf | 4 | Srivastava et al. (2016a) |
| Solasodine | Steroidal glycoalkaloid | *Solanum mammosum* L. [Solanaceae] | ATCC31798, A4; Leaf | 12 | Ooi et al. (2016) |
| Stevioside | Diterpene glycoside | *Stevia rebaudiana* Bertoni. [Asteraceae] | A4; Leaf | ~1.72 $mg g^{-1}$ DW | Pandey et al. (2016) |
| Stigmasterol[m], Hecogenin[n] | Sapogenin | *Chlorophytum borivilianum* Sant. et Fernand. [Liliaceae] | MTCC2364, MTCC532, PRT Gus; Rhizome | m = 21<br>n = ~2 | Bathoju et al. (2017) |
| Swertiamerin[o], Amarogentin[p] | Secoiridoid glycoside | *Swertia chirayita* (Roxb.) H. Karst. [Gentianaceae] | LBA9402; Node, internode | o = 4<br>p = ~2 | Samaddar et al. (2019) |
| Thebaine[q], Morphine[r], Codeine[s] | Morphinan alkaloid | *Papaver orientale* L. [Papaveraceae] | ATCC15834, R1000, C58C1; Hypocotyl, Shoot, cotyledon | q = ~3<br>r = ~6<br>s = ~3 | Hashemi and Naghavi (2016) |

(continued)

**Table 16.3** (continued)

| Secondary metabolite | Chemical nature | Plant source [Family] | *Agrobacterium rhizogenes* strain, explant | Content/ fold increased | References |
|---|---|---|---|---|---|
| Valerenic acid | Sesquiterpenoid derivative | *Valeriana officinalis* L. [Valerianaceae] | A13; Root, hypocotyl, leaf | 7.9 | Torkamani et al. (2014) |
| Vindoline-type alkaloid[f], Ajmalicine[g] | TIA | *Rhazya stricta* Decne. [Apocynaceae] | LBA9402; Leaf | f = 2<br>g = 1.5 | Akhgari et al. (2019) |
| Withanolides | Steroidal lactone | *Withania somnifera* (L.) Dunal [Solanaceae] | A4, 8196, 1600, 11325; Leaf | Withaferin A = 30*<br>Withanolide A = 2*<br>Withanolide B = 5* | Johny et al. (2018); Sivanandhan et al. 2020 |

*Fold was calculated on the basis of given data in the paper by content in HRC/content in control
Monoterpene indole alkaloid (MIA), Terpenoid indole alkaloid (TIA), Tropane alkaloid (TA)

organism using modern biological tools like genomics, proteomicsand metabolomics that involve over-expression or down-regulation of metabolic pathways by diverting common precursors, enzymes, regulatory proteins, rate-limiting steps, stopping the catabolism pathway of the desired product and obstruction of other pathways.

The *in planta* biosynthesis of valuable plant metabolites is a very complicated process, regulated by numerous complex, interrelated metabolic pathways like shikimate pathway (the main source of phenylpropanoids and aromatic compounds—coumarins, flavonoids, lignans, stilbenoids, catechins, vanillin, gallic acid, etc.), terpenoid pathway or isoprenoid pathway that uses methylerythritol 4-phosphate pathway (the main source of monoterpenoids, diterpenoids, hemiterpenoids, tocopherols, plastoquinones, PGRs, naphthoquinones, cannabinoids, furanocoumarines, and TIAs), mevalonic acid pathway (main source of sesquiterpenoids, phytosterols, triterpenoids and polyprenols) and polyketide pathway (main source of acetogenins and anthraquinones). Moreover, the understanding of these complex biosynthetic pathways has become more difficult due to the involvement of different cells, tissues and organelles.

The over-expression of specific genes and utilization of transcriptional regulators involved in biosynthetic pathways of SMs are the most common strategies for plant-derived metabolite engineering using *Agrobacterium*-mediated and/or biolistic transformation methods (Naizian 2019). The down-regulation of specific pathways using antisense RNA, RNA interference (RNAi), co-suppression techniques or increasing catabolism and carbon into competitive pathways are also used in metabolic engineering to ensure minimum synthesis of unwanted metabolites that in turn increase the desired SM production. Biosynthesis of the target metabolites is often hindered due to the sharing of common precursor molecule by different enzymes of different metabolite pathways. This problem can be overcome using metabolic engineering by redirecting the flux of common precursors towards the desired biosynthesis pathway, by inducing over-expression of genes in the precursor pathway or by down-regulation of competitive pathway. Coordination between the distribution of enzyme and substrate of different cells, tissues and cellular compartments is essential to optimize the function of secondary metabolic pathways. Removal of the product (metabolite) from the site of biosynthesis to a specific cell compartment/cell type is very important to protect cells from self-toxicity as well as often to increase the forward reaction.

Transfer of the whole biosynthetic pathway of useful SMs from original MPs to another plant with high biomass product is a preferred genetic engineering strategy to produce large-scale SMs. *Agrobacterium* (both *A. tumefaciens* and *A. rhizogenes*) and particle bombardment mediated transformation techniques have been effectively used for metabolic engineering of several MPs to achieve over-expression of SMs or the de novo synthesis of plant-made pharmaceuticals. Low plant-specific transformation efficiency, lack of reproducible regeneration protocol, unstable integration of the gene of interest, limitation of our knowledge regarding the complexity of biosynthesis pathway and their regulation, difficulties in obtaining licensing, high costs of securing regulatory approval and public acceptance towards products derived

from genetically modified systems are the major challenges of this biotechnological approach, which need to be addressed in future.

## 16.6 Ploidy Engineering of Medicinal Plants

Polyploidy is the possession of three or more complete sets of chromosomes that may provide certain adaptive advantages and serves as an important factor in the speciation and evolution of eukaryotes (Iannicelli et al. 2020). Induction of artificial polyploidy by the application of antimitotic agents such as colchicine in in vitro plants is often known as ploidy engineering and is used as a plant breeding strategy that enables the development of new and improved cultivars through an amazing impact on phenotypical, biochemical and genetic characteristics. Polyploidization of some medicinal aromatic plant species has been explored to assess its effect on superior vigour and phytochemical composition (qualitative and quantitative) in comparison with their diploid genotypes. Type, duration of exposure and concentration of antimitotic agent are the important parameters in this strategy. There are few reports showing enhancement of SMs in tissue culture regenerated polyploid MPs such as *A. annua, Bletilla striata*, *C. roseus, Dioscorea zingiberensis, Hyoscyamus albus, H. muticus, Pfaffia glomerata, S. miltiorrhiza and S. rebaudiana* in contrast to their diploid relatives (Corrêa et al. 2016; Pan-pan et al. 2018; Xia et al. 2018; Iannicelli et al. 2020).

*Cichorium intybus*, a medicinal herb with anti-hepatotoxic, anti-ulcerogenic and anti-inflammatory effects, as well as appetite enhancing, digestive, liver tonic, cardiotonic, diuretic and tonic properties, showed a significant increase in total phenolic compound (1.9-fold) and chlorogenic acid (10-fold) concentrations in leaves of autotetraploids in comparison with diploid plants (Ravandi et al. 2013). Corrêa et al. (2016) reported that induced polyploidy in *Pfaffia glomerata* resulted 31% increase in 20-hydroxyecdysone production. Enhanced production of antiviral and anti-cancer compound podophyllotoxin by the upregulation of expression level and enzyme activity of genes related to its biosynthesis coupled with morphological changes have been reported in colchicine-induced tetraploid *Linum album* in comparison with the diploid genotype (Javadian et al. 2017). Similarly, colchicine-induced tetraploid of *Bletilla striata* showed 26.7% increase in productivity (Pan-pan et al. 2018). Moreover, colchicine-induced polyploids in *A. annua* showed phenotypic alterations, enhanced photosynthetic capacity, artemisinin level and endogenous contents of indole-3-acetic acid (NAA), abscisic acid (ABA) and jasmonic acid (JA) through differential expression of 8763 genes (Xia et al. 2018).

The polyploidization strategy in combination with/without genetic engineering has been implemented in HRCs of different MPs to study the effect of genome duplication on SM production in HR of *A. annua, Datura stramonium*, *H. muticus* (Dehghan et al. 2012; Banerjee 2018). Interestingly, tetraploid *H. muticus* plants showed 200% higher scopolamine than their diploid counterparts, but this result was not observed in induced stable tetraploid HRCs (Dehghan et al. 2012). According to Banerjee (2018), 70% of the reported research articles on influence of ploidy levels

on the biosynthetic potentials of the HRCs showed advantage of tetraploid over diploid HRCs concerning their metabolite yields, which include research articles with better yield at higher ploidy level, enhancement of initial low yield at higher ploidy level by nutrient optimization, yield improvement of SMs in higher ploidy level and transgenic HR at higher ploidy level with better yield (Banerjee 2018). Only 30% of the reported articles showed low yield in hairy roots of higher ploidy in comparison with diploid hairy roots (Banerjee 2018). Judicious screening and selection of the superior hairy root clones of biomass/biosynthetic potentials based on chromosomal count and higher ploidy, assessment of the individual ploidy-based physiological need of hairy root clonal diversity, harmonizing with the elicitation and polyploidization at inter-and intra-ploidy diversity levels of hairy root clones for maximizing their productivities and optimization of ploidy dependant requirements parameters in bioreactor are the most important steps for maximize the desired effect (Banerjee 2018).

In addition to polyploidy, haploid plants can be produced by the application of conventional PTC-based methods such as androgenesis, gynogenesis and wide hybridization-chromosome elimination (Naizian 2019). Androgenesis pathway is the most used method of haploid induction in different MPs (Sharma et al. 2018).

## 16.7 Large-Scale Cultivation of Medicinal Plants in Bioreactors

In spite of great achievements in PTC-based production of pharmaceutically useful SMs utilizing in vitro-derived plant materials (suspension cells, hairy roots and micropropagated plantlets) in laboratory scale, sustainability and yield are the major concerns with respect to the commercial demand. The scale-up of the plant in vitro culture from laboratory scale to large-scale bioreactors is the final destination to fulfil huge public demand. Bioreactor technology has emerged as an important eco-sustainable and automated bioprocessing method for continuous and consistent large-scale production of biomass or valuable therapeutic SMs or transgenic proteins from in vitro cultivation of living cells or tissues or organs under the optimum environmental conditions in the broth medium.

This is the most potential alternative source after the wild population to fulfil the gradual increase in the demand of plant-derived SMs due to better rate of product multiplication, high specificity and minimum cost. Cultivation of plant cells and hairy roots in the bioreactors is more challenging than microbial cultivation in bioreactors. Parameters of cultivation of each type of explant (microorganism, cell suspension, hairy root and animal cell) in bioreactors are unique and depend on their size, aggregation, sensitivity to hydrodynamic stress and viscosity of the culture broth.

There is increasing interest to evaluate the feasibility of SM production at industrial scale through the use of bioreactors of varying sizes and features (Isah et al. 2018). SM production in bioreactors is quite difficult and complex as it requires

several essential optimization and suitable modifications during the design and operation of bioreactors for plant cell and hairy root cultivation. Homogeneous mixing of medium (for efficient nutrient transport and air-bubble dispersion), maintenance of optimum shearing force, optimization of aeration, minimum cell aggregation and adhesion, light supply for phototrophic and mixotrophic cultures, and optimization of temperature, pH, nutrients are required for organ culture and efficient mass transfer in bioreactors due to their larger cell size and shape, shear sensitivity, tendency to aggregate, slow growth rate, lower oxygen demand, limitation in mass transfer and product formation. However, novel computational tools based on mathematical and statistical modelling, neural networks, artificial intelligence, and in silico prediction have been applied recently to predict suitable design and operational parameters for optimal production of SMs in the bioreactors which is a prerequisite for the development of economically feasible bioreactor-based production unit.

Development of various configurations of bioreactors including stirred tanks, airlift, bubble column, orbital shaker, gas-phase bioreactors and mist or spray reactors has been possible by the recent advancements in bioreactor design and construction. Such bioreactors have been used for PTC-based in vitro growth and large-scale production of desired SMs in few plant species under different culture conditions that open up a new platform with great potentiality (Table 16.4).

## 16.8 Application of Nanoparticles in Tissue Culture of Medical Plants

Nanotechnology is a very recent and advanced technology that deals with the production, characterization and application of substances (nanoparticles or nano materials) with a diameter in the nanoscale ($10^{-9}$ m). In recent times, it is a rapidly expanding field of research due to its wide applications in every field of science including plant science. Plant-based nanoparticle synthesis is more popular as it is cost effective, eco-friendly, safe and single-step method when compared to the more complex chemical and physical methods of nanoparticle synthesis. Nanotechnology has been used in PTC as a new elicitor to promote germination efficiency, boost plant growth and SM production. The effects of some important metal oxide nanoparticles like titanium oxide, zinc oxide, iron oxide and copper oxide have been reported for enhancement of SM production in different plants using different PTC systems (Moharrami et al. 2017; Bhardwaj et al. 2018; Ghazal et al. 2018; Chung et al. 2019; Karimzadeh et al. 2019; Moradpour et al. 2019).

Treatment of suspension cells of *Bacopa monnieri* with zinc oxide nanoparticles (ZnONPs) increased around 2-fold total bacoside A possibly through the modification in gene regulation of isoprenoid pathway than mevalonic acid pathway (Bhardwaj et al. 2018). Karimzadeh et al. (2019) demonstrated different effects of nanoparticle on enzyme activity, total phenol and lignan production in CSCs of *L. usitatissimum*, depending on concentration and type of nanoparticles. Improvement in gymnemic

**Table 16.4** List of some selected scientific publications on large-scale cultivation of medicinal plants in bioreactors published during 2010 to June 2020

| Plant Species | Type of Culture | Objective(s) | Type of bioreactor | Volume (l) | References |
|---|---|---|---|---|---|
| *Artemisia annua* L. | HRC | Artemisinin | BCB, NMB, modified NMB | 3–5 | Patra and Srivastava (2016) |
| | | Biomass production | Mist bioreactor | 20 | Sivakumar et al. (2010) |
| *Atropa belladonna* L. | HRC | Scopolamine | Bioreactor | 1.5 | Habibi et al. (2015) |
| *Azadirachta indica* A. Juss | CSC | Azadirachtin | Bioreactor | 3 | Prakash and Srivastava (2011) |
| | HRC | Azadirachtin | NSB, STB, BCB, Modified NSB | 3–4 | Srivastava and Srivastava (2012) |
| *Brugmansia candida* Pers | HRC | Hyoscyamin, scopolamine | Modified STB | 1.5 | Cardillo et al. (2010) |
| *Buddleja cordata* Kunth | CSC | Biomass and SM | STR | 2–3 | Vazquez-Marquez et al. (2019) |
| *Bupleurum falcatum* L. | ARC | Saikosaponin | STB, BCB, modified airlift reactor | 10–200 | Kusakari et al. (2012) |
| *Catharanthus roseus* (L.) G. Don | CSC | Ajmalicine | Pilot-scale bioreactor | 100 | Fulzele and Namdeo (2018) |
| | HRC | Ajmalicine | BCB | 3 | Thakore et al. (2017) |
| | SEC | SE mediated mass production | Growtek bioreactor | 0.5 | Mujib et al. (2014) |
| *Coleus blumei* Benth | HRC | Rosmarinic acid | Airlift bioreactor | 1 | Bauer et al. (2015) |
| *Dendrobium candidum* Wall. ex Lindl | PSC | Biomass and SM | BTBB | 3 | Cui et al. (2014) |
| *Dracocephalum forrestii* W. W. Smith | SC | Rosmarinic acid | NSB | 10 | Weremczuk-Jezyna et al. (2019) |
| *Eleutherococcus koreanum* Nakai | ARC | Eleutherosid, chlorogenic acid | Airlift bioreactor | 3 | Lee et al. (2015) |

(continued)

**Table 16.4** (continued)

| Plant Species | Type of Culture | Objective(s) | Type of bioreactor | Volume (l) | References |
|---|---|---|---|---|---|
| *Hyoscyamus niger* | HRC | Anisodamine scopolamine, hyoscyamine, cuscohygrine | BCB, HB | 1.5 | Jaremicz et al. (2014) |
| *Hypericum perforatum* L. | ARC | Hypericin | BTAB | 5 | Wu et al. (2014) |
| *Oplopanax elatus* Nakai | ARC | Phenolics, flavonoids | BTAB | 5 | Jiang et al. (2015) |
| *Panax quinquefolius* L. | ARC | Ginsenoside | BTAB | 5 | Yu et al. (2016) |
| | HRC | Ginsenoside | NSB | 1.5 | Kochan et al. (2017) |
| *Polygonum multiflorum* Thunb | ARC | Phenolic compounds | BTBB, pilot-scale tank bioreactor | 3, 5, 20, 500 | Ho et al. (2017) |
| *Rauvolfia serpentina* (L.) Benth. ex Kurz | HRC | Reserpine | Modified airlift bioreactor | 5 | Mehrotra et al. (2015) |
| *Salvia officinalis* L. | HRC, SC | Rosmarinic acid | NSB | 5 | Grzegorczyk and Wysokinska (2010) |
| *Silybum marianum* (L.) Gaertn | HRC | Silymarin | STB | 2.7 | Rahimi et al. (2012) |
| *Vitis amurensis* Rupr | SEC | Resveratrol | BTBB | 3 | Sun et al. (2016) |
| *V. labrusca* L. | CSC | Resveratrol | STB | 14 | Nivelle et al. (2017) |
| *Withania somnifera* (L.) Dunal | CSC | Withanolides | Bioreactor | 7 | Sivanandhan et al. (2014) |

Adventitious root culture (ARC), Balloon-type airlift bioreactor (BTAB), Balloon-type bubble (airlift) bioreactor (BTBB), Bubble column bioreactor (BCB), Bubble column/spray hybrid bioreactor (HB), Cell suspension culture (CSC), Drum type airlift bioreactor (DTAB), Hairy root culture (HRC), Nutrient sprinkle bioreactor (NSB), Protocorm suspension culture (PSC), Shoot culture (SC), Stirred tank bioreactor (STB), Somatic embryo culture (SEC)

acid II and phenolic compounds production in CSCs of *G. sylvestre* was reported by using copper oxide nanoparticles (CuONPs) as a new generation of elicitors (Chung et al. 2019). Copper and gold nanoparticles (1:3) application showed a positive effect in enhancing biomass and SM production in adventitious root cultures of *S. rebaudiana* (Ghazal et al. 2018).

Application of Ag-$SiO_2$ core–shell nanoparticles (AgNPs) with an average size of 101.8 ± 8.9 nm stimulated 3.9-fold artemisinin production over the control in the HRCs of *A. annua* by induction of oxidative stress that result in lipid peroxidation and enhanced catalase activity (Zhang et al. 2013). When HRCs of *Hyoscyamus reticulatus* were elicited with different concentrations of iron oxide nanoparticles (FeNPs)

for different time periods, a 5-fold enhancement of hyoscyamine and scopolamine content was observed in comparison with the control (Moharrami et al. 2017). Application of titanium dioxide ($TiO_2$) nanoparticles on HRC of *S. rebaudiana* showed a maximum 24.28 $mgg^{-1}$ DW stevioside and 21.28 $mgg^{-1}$ DW rebaudioside production (Moradpour et al. 2019).

## 16.9 Application of Genome Editing Strategies and Plant Synthetic Biology in Tissue Culture of Medicinal Plants

The efficacy of conventional genetic engineering to change the secondary metabolic pathway in MPs is often limited by the lack of knowledge of the concerned metabolic pathways, unmanageable transcriptional regulation of endogenous genes, copy number variability and random insertion of recombinant DNA construct during conventional gene transformation. In recent times, synthetic promoters, 'tunable' transcription factors, genome editing tools and site-specific recombinases are available that can help plant biotechnology to improve plants faster and more accurately (Niazian 2019). The co-transformation of plant tissues with DNA constructs encoding engineered sequence-specific nucleases like Zinc-finger nucleases (ZFNs), transcription activator-like effector nucleases (TALENs) and Clustered Regularly Interspaced Short Palindromic Repeats and its associated Cas9 protein (CRISPR/Cas9) which have customizable DNA-binding domain and desired foreigner DNA leads to precise site-specific integration of foreign DNAs (Niazian 2019).

Additionally, CRISPR/Cas9 is one of the newly emerging, suitable, promising, powerful and precise genome editing tools that can be used for gene mutation, deletion, insertion and transcriptional activation/repression of targeted gene of interest in a selected genome. Although the use of CRISPR/Cas9 system in MPs has been limited due to lack of availability of sufficient sequence information in many MPs, the application of it gradually increased in recent years due to its promising potential to regulate plant metabolic networks and improve the quality of MPs (Alok et al. 2018; Shabir 2020). Recently, Shabir (2020) reported the present status of CRISPR/Cas9-mediated genome editing in medicinal and aromatic plants as well as the applications and challenges of this new technique. For example, the expression and metabolomic analysis of edited hairy root lines of *Salvia miltiorrhiza* developed by CRISPR/Cas9-mediated targeted mutagenesis showed suppression of rosmarinic acid synthase gene and decrease in the content of rosmarinic acid and lithospermic acid B (Zhou et al. 2018). Similarly, a rapid and efficient approach for targeted genome modification in *Dioscorea zingiberensis* was also reported using CRISPR/Cas9 system via *A. tumefaciens*-mediated transformation (Feng et al. 2018).

## 16.10 Morphological, Phytochemical and Genetic Stability of in vitro cultures of Medicinal Plants

In spite of various applications and advantages of the PTC techniques, its broader commercial utilization as an important tool for the germplasm conservation of threatened commercially important MP species via mass proliferation and industrial production of SMs can be limited by genotypic and phytochemical variability developed by somaclonal variations in long-term cultures. Somaclonal variations are mostly useless, uncontrollable, unpredictable and may be developed due to inheritable cryptic genetic defects that include chromosomal aberrations, polyploidy, chromosomal rearrangements, DNA hypomethylation, single-gene mutations, genome adaptation to differential microelement environments and the presence of hot spots (Bhattacharyya et al. 2017a; b). Such genetic changes may result in morphological and biochemical variations in uniform in vitro cultures or regenerants.

The potential chance of development of somaclonal variations may be influenced by several factors like length of culture period, genotype and nature of explants, type and concentration of PGRs applied in culture media, culture conditions and pathway of development of the tissue cultured plants. Although the unorganized cultures such as callus and CSC are used as a platform for both micropropagation and SMs production, in many cases, biochemical and genetic instability have been reported when cultured for long term (Halder and Jha 2020). Moreover, phenotypic, biochemical and genetic instability have also been reported in tissue culture regenerants, though the frequency of such report is very low. Generally, proliferation or regeneration via induced indirect organogenesis is more prone to somaclonal variation in comparison with the clonal propagation or regeneration through proliferation of the axillary buds or direct SEs. Thus, the occurrence of somaclonal variations among the regenerates (clones) derived from the same donor mother plant during in vitro micropropagation and unorganized cultures are a major issue of concern as it disrupts the expected homogeneity of the product which is important for the conservation of elite germplasm as well as industrial production of desired phytochemicals.

Commercial utilizations demand long-term maintenance of the desired phenotypic, biochemical and genetic characters and uniformity among all raw materials (cell, tissue, organ or plant population) utilized in the preparation of medical formulations to achieve the maximum advantage of in vitro culture system. Otherwise, commercial products will slow down consumers' confidence. Thus, to avoid the potential drawback of somaclonal variations, the stringent monitoring and evaluation of the genetic stability/clonal fidelity are the prerequisites for the long-term in vitro cultures and regenerated plants for commercial applications.

Clonal fidelity and genetic stability of in vitro grown callus, cell, organ and micropropagated regenerants of MPs can be assessed by evaluating cytological parameters such as chromosome number and morphology, isozyme profile or PCR-based DNA fingerprinting profiles such as random amplified polymorphic DNA (RAPD), inter-simple sequence repeats (ISSR), simple sequence repeats (SSR) and most recently

start codon targeted (SCoT) polymorphism (Thakur et al. 2016; Jiang et al. 2018; Rohela et al. 2019; Jena et al. 2020; Kudikala et al. 2020; Sahoo et al. 2020).

Cytological studies may be used to assess the genetic variability and stability by traditional cytological analysis (determination of chromosome number and morphology) or by flow cytometric analysis (determination of ploidy level and genome size/ DNA content) (Shinde et al. 2016; Ali et al. 2017b). Among molecular markers, RAPD is one of the most common, simple, quick and cost-effective DNA-based molecular markers of analysing the genetic fidelity of tissue culture materials (Rathore et al. 2014; Purohit et al. 2017; Faisal et al. 2018). But in spite of the various advantages, RAPD has a major issue with reproducibility. ISSR markers are polymorphic, reproducible, resolvable, informative, usually developed based on the non-coding regions of the DNA and are widely used in assessing the genetic homogeneity of tissue culture-derived plants (Prakash et al. 2016; Rohela et al. 2019; Jena et al. 2020; Kudikala et al. 2020; Sahoo et al. 2020). SCoT polymorphism is a novel, extremely reliable, cost-effective gene-targeted molecular marker technique derived from flanking ATG translation codon in the plant gene that largely resolved the various limitations of the conventional molecular markers which target a specific region of the genome (Bhattacharyya et al. 2017a). SCoT polymorphism is more sensitive than other conventional molecular markers and can detect even minute degrees of genetic variability. In the recent past, SCoT marker has been widely used in the assertion of clonal fidelity in various MPs and it is considered to be more authentic in assessing genetic homogeneity (Bhattacharyya et al. 2014; Bhattacharyya et al. 2017a; Rohela et al. 2019; Kudikala et al. 2020). It can correlate to functional genes and their corresponding traits (Bhattacharyya et al. 2014, 2017a). Some researchers used molecular markers along with flow cytometry for the assessment of genetic uniformity of in vitro cultures (Jiang et al. 2018; Jena et al. 2020).

Besides the assessment of genetic stability of cell, organ or plant cultures, the biochemical or phytochemical stability assessment is important for commercial production of SMs as genetic stability does not always assure the potential of stable drug yielding traits and stable bioactivities (Pérez-Alonso et al. 2018; Sahoo et al. 2020). Genetic variability originating via somaclonal variations in cultured cells or tissues or plants can disturb the phytoconstituents. Therefore, assessment of the SM yielding potential and bioactivity of long-term in vitro culture is preferred.

### *16.10.1 Morphological, Biochemical and Genetic Stability of in vitro Non-transformed Culture*

From a commercial point of view, higher multiplication rate coupled with enhanced clonal stability and SM production must be ensured by the non-transformed cultures and non-transformed regenerants of important species of MP. Some of the selected studies of genetic fidelity of non-transformed regenerants has been discussed in Table

16.5. Beside these, CSCs or CCs are also evaluated for their biochemical and genetic stability. For unorganized cultures and organ cultures, genetic stability is commonly assessed by cytological and/ or flow cytometric analysis. Use of proper markers and data analysis using a variety of statistical tools provide better insight into g

### *16.10.2 Morphological, Biochemical and Genetic Stability of Transformed Cultures of Medicinal Plants*

Different MPs have been transformed with *A. rhizogenes* or *A. tumifeciens* to develop hairy roots, transformed galls and Ri- or Ti-transformed plants to synthesize important SMs. Similar to the non-transformed cultures, assessment of genetic stability/integrity along with biochemical stability is very important for the long-term transformed cultures. HRCs are usually stable and can be used for long-term culture. Morphological, biochemical and genetic stability of HRCs and Ri-transformed medicinal plants in long-term in vitro cultures have been discussed in the review article of Roychowdhury et al. (2017). Although somaclonal variations and chromosomal abnormalities (including both aneuploidy and polyploidy) are reported in the long-term cultures specially in some unorganized cultures, apart from the variations among different clones of HRC of a single species, the instability in morphology, growth kinetics, biosynthetic potential, chromosome number and loss of integrated T-DNA gene or its expression are very rare in HRCs (Roychowdhury et al. 2017).

*rolA*-transgenic callus cultures of *R. cordifolia* showed stable and elevated growth with increased anthraquinone production over 14 years (Veremeichik et al. 2019). Similarly, *rolC*-transformed callus cultures of *Maackia amurensis* showed genetic and biochemical stability (Grishchenko et al. 2013) after 4 years of culture.

Long-term stability in biomass and production of TIAs by HRC of *R. serpentina* were reported by Pandey et al. (2014). Cytogenetic characterization *A. rhizogenes* transformed root lines of *R. serpentina* showed genetical stability (Ray et al. 2014b).

Morphological and biochemical stability was also reported in *crypt*-transformed *B. monnieri* plants (Paul et al. 2015) and 6-year-old *T. indica* plants grown in in vitro and ex vitro conditions (Roychowdhury et al. 2013). An interesting study, on 5 years in vitro maintained Ti- and Ri-transformed *C. roseus* plants showed that Ri-transformed plants are more genetically stable than Ti-transformed plants whereas biochemically both are stable (Verma et al. 2015).

**Table 16.5** List of some selected scientific publications on stability of in vitro grown or regenerated plants published during 2010 to June 2020

| Plant species | Method used (no. primer used) | Plant materials (no. of sample used) | Result of stability Study | | | References |
|---|---|---|---|---|---|---|
| | | | Genetic | Biochemical/bioactivity | Morphological | |
| *Aloe barbadensis* Mill | RAPD (20), ISSR (15) | In vitro grown leaf-regenerated plantlets (24) | No polymorphism | ND | Normal growth and no sign of morphological variation | Sahoo and Rout (2014) |
| *Alpinia calcarata* Rosc | RAPD (20), ISSR (10) | 6-month-old hardened plants which derived from rhizome via direct shoot organogenesis (7) | 95% and 100 % monomorphism for RAPD and ISSR respectively | ND | ND | Bhowmik et al. (2016) |
| *Alpinia galanga* L. | RAPD (25), ISSR (15); GC–MS | 6-year-old micropropagated plantlets (295- over a period of 6 years in an interval of 6 months by randomly taking at least 20 plants in every time) | No genetic variations | No significant variations in the essential oil yield, total phenolic and flavonoid contents, and bioactivities | ND | Sahoo et al. (2020) |
| *Amomum subulatum* Roxb | RAPD (40) | Tissue culture raised plantlets (10) | 3 primers produced polymorphic bands | ND | Morphological variation observed | Purohit et al. (2017) |
| *Annona reticulata* L. | SCoT (10), ISSR (10) | Node-derived in vitro regenerated plantlet (6) | Regenerated plantlets are monomorphic and true-to-type with mother plant | ND | ND | Kudikala et al. (2020) |

(continued)

**Table 16.5** (continued)

| Plant species | Method used (no. primer used) | Plant materials (no. of sample used) | Result of stability Study | | | References |
|---|---|---|---|---|---|---|
| | | | Genetic | Biochemical/bioactivity | Morphological | |
| *Anoectochilus formosanus* Hayata | ISSR (50) | 5-year-old micropropagated plantlets derived from axillary bud (20) | Low risk of genetic instability, cluster analysis showed 2.76% polymorphism rate | ND | ND | Zhang et al. (2010) |
| *Ansellia africana* Lindl | SCoT (45) | 4-month-old tissue culture raised plants (NM) | Polymorphism detected | ND | No morphological abnormalities | Bhattacharyya et al. (2017a) |
| *Artemisia nilagirica* (C.B. Clarke) pamp | Cytological analysis, ISSR (15) | 5-month-old node-derived regenerated plants (10) | No change in chromosome number and no polymorphism | ND | Morphologically similar | Shinde et al. (2016) |
| *Bacopa monnieri* (L.) Wettst | SPAR, ISSR (103), RAPD (35), Flow cytometry | In vitro propagated plants (NM) | No polymorphism | ND | ND | Khilwani et al. (2016); Saha et al. (2020) |
| *Boerhaavia diffusa* L. | RAPD (14) | Node-derived regenerated field-grown plants (NM) | No polymorphism | ND | ND | Patil and Bhalsing (2015) |

(continued)

**Table 16.5** (continued)

| Plant species | Method used (no. primer used) | Plant materials (no. of sample used) | Result of stability Study | | | References |
|---|---|---|---|---|---|---|
| | | | Genetic | Biochemical/bioactivity | Morphological | |
| *Cannabis sativa* L. | ISSR (18); GC-FID | 8-month-old in vivo grown regenerated plants after up to 30 passages in culture (11) | No genetic polymorphism | No significant difference in cannabinoids content | Morphologically similar | Lata et al. (2016a, b) |
| *Capparis decidua* (Forsk.) Edgew | RAPD (8) | Long-term culture leaf-derived regenerated plantlet (8) | No polymorphism | ND | ND | Tyagi et al. (2010) |
| *Carum copticum* L. | Flow cytometry | Regenerated plants via indirect somatic embryogenesis and indirect shoot regeneration (NM) | DNA content, and genome size stability of regenerated plants | ND | ND | Niazian et al. (2017) |
| *Catharanthus roseus* (L.) G. Don | RAPD (20) | Node-derived regenerated plants (21) | No polymorphism | ND | Morphologically similar | Kumar et al. (2013) |
| *Chlorophytum borivilianum* Sant. et Fernand | RAPD (100), DNA Fingerprinting profiles, Cytology | In vitro and in vivo grown shoot bud-derived micropropagated plants (15) | Monomorphism with the mother plant, somatic chromosome number stable | ND | No gross morphological variation | Samantaray and Maiti (2010); Basu and Jha (2014) |

(continued)

**Table 16.5** (continued)

| Plant species | Method used (no. primer used) | Plant materials (no. of sample used) | Result of stability Study | | | References |
|---|---|---|---|---|---|---|
| | | | Genetic | Biochemical/bioactivity | Morphological | |
| *Cleome gynandra* L. | RAPD (24) | Node-derived micropropagated plants grown in green house (7) | No polymorphism | ND | ND | Rathore et al. (2014) |
| *Coriandrum sativum* L. | Flow cytometry | Root-derived regenerated plants via primary and secondary somatic embryogenesis (NM) | Genetically stable and genome size similar | ND | ND | Ali et al. (2017) |
| *Croomia japonica* Miq. | SSR (10), Flow cytometry | In vitro rhizome-derived regenerants (39) | No polymorphism and stable ploidy level | ND | ND | Jiang et al. (2018) |
| *Curcuma zedoaria* (Christm.) Roscoe | ISSR (27), Flow cytometry; GC–MS, HPLC | Axillary bud-derived in vitro propagated plants (20) and in vivo grown plants (20) | No polymorphism and low level of difference in their nuclear DNA content | Similarity in phytochemical profile was observed with total of 49 components in leaf and 57 compounds in rhizome | No significant morphological changes | Jena et al. (2020) |

(continued)

**Table 16.5** (continued)

| Plant species | Method used (no. primer used) | Plant materials (no. of sample used) | Result of stability Study | | | References |
|---|---|---|---|---|---|---|
| | | | Genetic | Biochemical/bioactivity | Morphological | |
| *Cymbidium giganteum* Wall. Ex Lindl | RAPD (40) | Pseudostem-derived micropropagated plantlets (17) | Overall 5.81% change in the regenerants detected, 17 primers produce polymorphism | ND | TDZ derived plants showed phenotypic variation depending TDZ concentrations used | Roy et al. (2012) |
| *Dendrobium nobile* Lindl | RAPD (80), SCoT (35); colorimetric method | Pseudostem-derived micropropagated plantlets (NM) | 97% genetic fidelity among regenerants | In vitro raised plants exhibited a higher degree of SMs and free radical scavenging activity than the mother plant | ND | Bhattacharyya et al. (2014) |
| *Digitalis purpurea* L. | RAPD (8); HPLC | De novo regenerated plantlets (12) via direct organogenesis from leaf | No genetical variation | No differences on cardenolidecon tent | ND | Pérez-Alonso et al. (2018) |
| *Eclipta alba* (L.) Hassk | RAPD (40) | In vitro regenerated plant (12) | No genetical variation | ND | ND | Bardar et al. (2015) |
| *Gloriosa superba* L. | ISSR (30), RAPD (50) | After 100 days of hardening micropropagated plants (8) | Genetically identical and stable | ND | No morphological variations | Yadav et al. (2013) |

(continued)

**Table 16.5** (continued)

| Plant species | Method used (no. primer used) | Plant materials (no. of sample used) | Result of stability Study | | | References |
|---|---|---|---|---|---|---|
| | | | Genetic | Biochemical/bioactivity | Morphological | |
| *Kelussia odoratissima* Mozaff | AFLP (10) | Regenerated plantlets via somatic embryogenesis (12) | No polymorphism | ND | ND | Ebrahimi et al. (2018) |
| *Ledebouria revoluta* (L.f.) Jessop Syn. *Scilla indica* (Wight) Baker | Cytological analysis | Callus and 1-year-old in vitro and ex vitro plants derived from callus mediated shoot organogenesis (NM) | No variation in ploidy level | All the tissue culture-derived ex vitro field-grown plants have little better anti-microbial activity as compared to naturally propagated in vivo parental plants | Flowering and no morphological variation | Haque et al. (2018) |
| *Nothapodytes nimmoniana* (Graham) Mabb | ISSR (60); HPLC | Field transferred mature regenerated plants (10) | Except two primers, no monomorphic band | No significant variation in camptothecin content | Phenotypically similar | Prakash et al. (2016) |
| *Ocimum basilicum* L. | RAPD (56), ISSR (17) | Node-derived in vitro-raised plantlets (40) | In vitro-raised plantlets produce 100% monomorphic | ND | ND | Saha et al. (2014) |

(continued)

**Table 16.5** (continued)

| Plant species | Method used (no. primer used) | Plant materials (no. of sample used) | Result of stability Study | | | References |
|---|---|---|---|---|---|---|
| | | | Genetic | Biochemical/bioactivity | Morphological | |
| *Pelargonium sidoides* DC | HPLC | Roots of 2-year-old plants that were propagated via somatic embryogenesis (NM) | ND | No variation in coumarin content | ND | Duchow et al. (2015) |
| *Phyllanthus fraternus* Webster | RAPD (15), ISSR (15) | Acclimatized synseed-derived regenerated plants (16) | Polymorphism were detected with 3 RAPD primers | ND | Flowering and morphologically similar | Upadhyay et al. (2014) |
| *Pittosporum eriocarpum* Royle | SCoT (20), ISSR (15), RAPD (15) | Node-derived in vitro-raised plantlets (9) | One RAPD primer showed polymorphic bands in 3 regenerates, UPGMA analysis revealed 97% similarity | ND | ND | Thakur et al, (2016) |
| *Podophyllum hexandrum* Royle (Berberidaceae) | RAPD (10) | Rhizome regenerated plants (NM) | No polymorphism detected | ND | ND | Tariq et al. (2015) |
| *Rauvolfia serpentina* (L.) Benth. ex Kurz | Flow cytometry | In vitro regenerated plants (NM) | No significant variation in nuclear DNA content | ND | ND | Zafar et al. (2019) |

(continued)

**Table 16.5** (continued)

| Plant species | Method used (no. primer used) | Plant materials (no. of sample used) | Result of stability Study | | | References |
|---|---|---|---|---|---|---|
| | | | Genetic | Biochemical/bioactivity | Morphological | |
| *Rauvolfia tetraphylla* L. | SCoT (10), ISSR (10), RAPD (10) | Leaf callus-based (4) and stem callus-based (3) acclimated regenerants | No polymorphism and no significant variation in nuclear DNA content | ND | ND | Rohela et al. (2019) |
| *Rhazya stricta* Decne | ISSR (16), RAPD (20) | In vitro micropropagated plants | Low frequency of polymorphism in RAPD and ISSR profile | ND | Phenotypically similar | Mohamed et al. (2014)[1] |
| *Rumex nepalensis* Spreng | SCoT (36), RAPD (45) | In vitro DSO and IDO mediated regenerated plants (10 each) | DSO-propagated plants showed higher genetic stability that ISO-propagated plants | In both DSO and ISO-regenerated plants the yield of the phytochemicals increased significantly in comparison with the mother plant | Plants developed normal inflorescence and flower morphologically similar | Bhattacharyya et al. (2017b) |
| *Ruta graveolens* L. | ISSR (10), RAPD (10) | Shoot tip-derived in vitro plants (10) | No polymorphism | ND | Morphological variation absent | Faisal et al. (2018) |
| *Solanum aculeatissimum* Jacq | Flow cytometry | Leaf-derived regenerated plantlets (30) | Ploidy stability | ND | Uniform morphology and normal flowering | Ghimire et al. (2012) |

(continued)

**Table 16.5** (continued)

| Plant species | Method used (no. primer used) | Plant materials (no. of sample used) | Result of stability Study | | | References |
|---|---|---|---|---|---|---|
| | | | Genetic | Biochemical/bioactivity | Morphological | |
| *Spilanthes oleracea* L. | RAPD (60); HPLC | Node-derived in vitro regenerated plantlets (6) | No polymorphism | No significant difference in scopoletin content | Flowering with no morphologically variation | Dandin et al., (2014) |
| *Stevia rebaudiana* Bertoni | ISSR(15) | Tissue culture-derived plants (NM) | Low genetic variations | ND | ND | Soliman et al. (2014) |
| *Tylophora indica* (Burm. f.) Merr | RAPD (20), ISSR, chromosomal | In vitro regenerants (14) | Majority reports showed genetic stability, whereas other reported 37.5–62.1% polymorphism | ND | ND | Da Silva and Jha (2016) |
| *Uraria picta* (Jacq.) DC | RAPD (20), HPLC | Node-derived in vitro regenerants (NM) | Genetically identical | No significant difference in isoflavonones content | Flowering and no morphological variation | Rai et al. (2010) |
| *Vitex trifolia* L. | RAPD (40), ISSR (13) | Acclimatized node-derived regenerated plants (10) | Genetically uniform | ND | No morphologically variation | Ahmad et al. (2013); Alatar et al. (2017) |
| *Withania somnifera* (L.) Dunal | RAPD (20), ISSR (10) | In vitro grown regenerated plantlets (14) | No polymorphism | ND | ND | Nayak et al. (2013) |

AFLP = Amplified fragment length polymorphism, GC–MS = Gas chromatography–mass spectrometry, HPLC = High-performance liquid chromatography, ISSR = Inter-simple sequence repeat, RAPD= Random amplified polymorphic DNA, SCoT= Start codon targeted, SPAR= single primer amplification reaction, SSR= Simple sequence repeat. All regenerated plants were selected randomly for stability determination.
DSO = Direct shoot organogenesis, ISO = Indirect shoot organogenesis, ND = not determined, NM = not mentioned

## 16.11 Case Study 1: *Podophyllum hexandrum*

*Podophyllum hexandrum* Royle (syn. *P. emodi* Wall., family Berberidaceae) commonly known the Himalayan Mayapple or the Indian Podophyllum is a medicinally important herbaceous plant. It is the commercially exploitable plant source of podophyllotoxin ($C_{22}H_{22}O_8$) (Fig. 16.5), a pharmaceutically active lignan. Podophyllotoxin is used as a precursor for the synthesis of important anti-tumour drugs like etoposide (VP-16-213) and teniposide (VM-26) which are used in the treatment of lung cancer, testicular cancer, a variety of leukemias and other solid tumours (Imbert 1998; Gordaliza et al. 2004).

*P. hexandrum* is an erect, glabrous, succulent herb, 35–60 cm high with creeping perennial rhizome having numerous roots, found in the Himalayan alpine and subalpine zones from Kashmir to Sikkim at altitudes of 2200–4300 m in India (The Wealth of India 1969). From the Indian Himalayas, it has dispersed to Bhutan, Pakistan, Afghanistan, Nepal and China (Chaurasia et al. 2012). The Indian

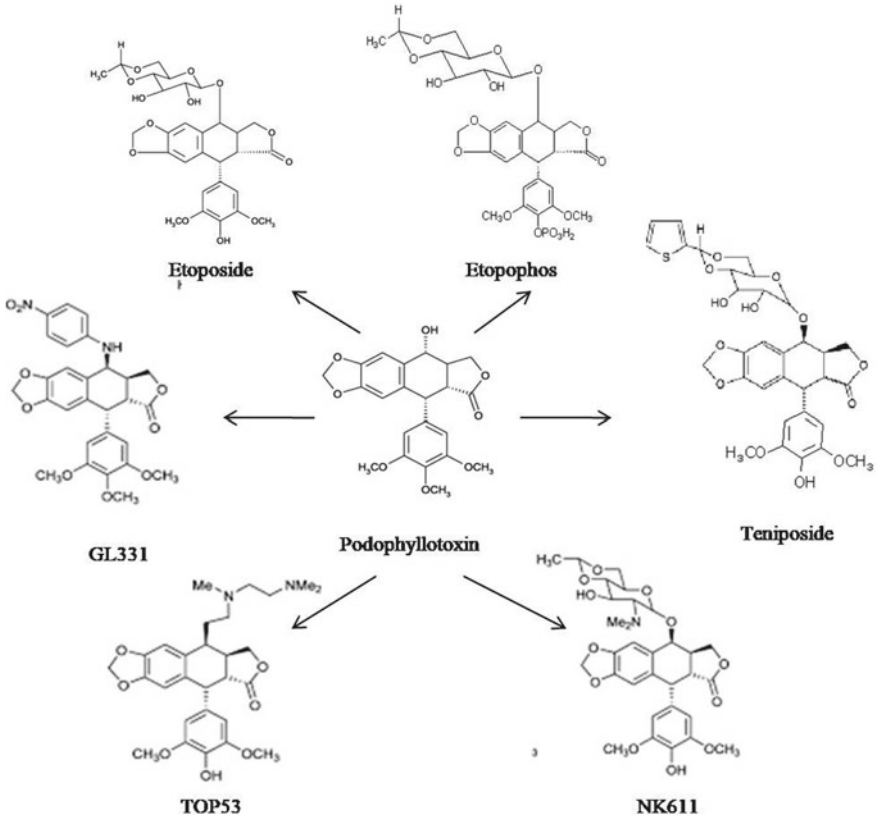


**Fig. 16.5** Chemical structure of podophyllotoxin and some of its derivatives

*Podophyllum* yields 7–15% resin as compared to the American *Podophyllum* or *P. peltatum,* distributed in the Atlantic North America (Chaurasia et al. 2012), which yields only 4–8% resin (Thakur et al. 2010; Qazi et al. 2011). Podophyllotoxin content of rhizomes ranges between 0.36–1.08% DW (Nadeem et al. 2007).

Podophyllotoxin is in high demand in the global market. But *P. hexandrum* is sparse due to its prolonged juvenile stage and poor ability to set fruits. Rhizomes are being indiscriminately collected from the wild to meet the global demand of podophyllotoxin, thus reducing *P. hexandrum* populations. Overexploitation and lack of organized cultivation have made the plant 'critically endangered' (Airi et al. 1997). As podophyllotoxin is immensely important medicinally, new routes for total synthesis of podophyllotoxin have been discovered (Bush and Jones 1995; Berkowitz et al. 2000). But yields are low, and the processes are not viable economically. Thus, the only feasible option is to isolate podophyllotxin from plant sources.

*P. hexandrum* is a slow growing species (Kushwaha et al. 2008). In nature, the seeds remain dormant for about 10 months, an adaptation to get through the harsh climatic conditions of high altitudes (Badhwar and Sharma 1963). The plant also has a low seed number (Kim et al. 2007) and propagates mostly through the rhizomes (Alam et al. 2009). As the species has already acquired a 'critically endangered' tag and the rate of natural propagation is far less than the harvest of the underground parts, safeguarding the existence of this species is of utmost importance. Thus, efforts are given to develop alternative sources and methods of production of podophyllotoxin and biotechnological approaches, particularly plant cell and tissue cultures of *P. hexandrum* appear to be suitable alternatives for producing this pharmaceutically important lignan, together with conserving this immensely important species.

## *16.11.1 In vitro Cultures as Means of Podophyllotoxin Production from* P. hexandrum

### 16.11.1.1 Cell Cultures for Podophyllotoxin Production

Large number of studies have been carried out for enhancing the accumulation of podophyllotoxin in in vitro cultures of *P. hexandrum* by optimizing culture conditions and nutrient levels, addition of elicitors and precursors, immobilization, etc. (Majumder and Jha 2009a; Dhiman et al. 2016; Nandagopal et al. 2018).

*P.hexandrum* is a very recalcitrant species with respect to callus induction in vitro and successful callus cultures of Indian *P. hexandrum* (Fig. 16.6a) were initiated and established from in vitro grown axenic seedling explants and roots and rhizomes isolated from 1-year-old mature plants on B5 and MS media supplemented with growth regulators by Majumder (2008). Cell lines established subsequently differed distinctly in growth rates and podophyllotoxin content and selection of fast-growing cell lines and recloning of such lines led to establishment of cell lines capable of optimum growth, accumulating optimum levels of podophyllotoxin (Majumder and

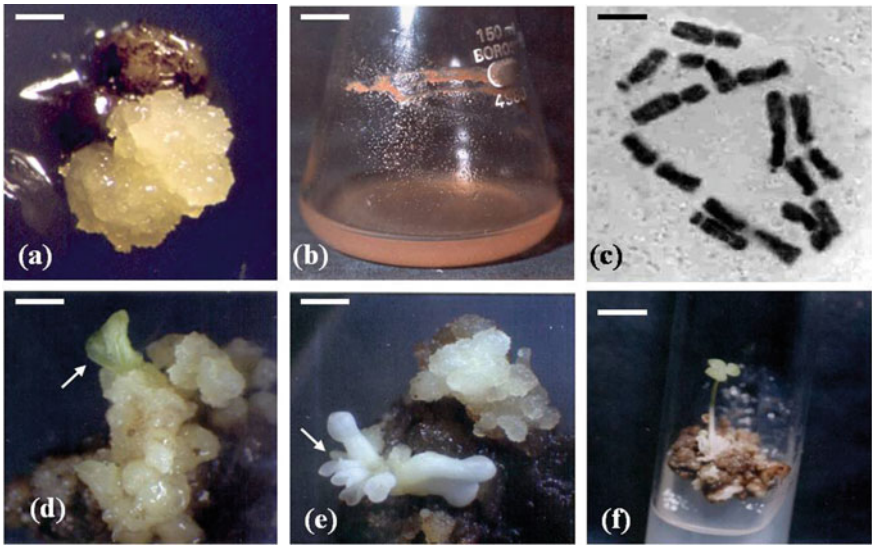


**Fig. 16.6** **a** Mature root-derived callus of *Podophyllum hexandrum* (*Bar: 2.5* mm), **b** Cell suspension culture (*Bar: 10* mm), **c** Diploid plate showing 12 chromosomes from 2-year-old cell line (*Bar: 15* μm), **d** Leaf differentiation (arrow) from shoot bud (*Bar: 3* mm), **e** Development of shoot buds from bud primordia on MS medium supplemented with BA and NAA (*Bar: 3* mm), **f** Elongated microshoot (*Bar: 8* mm)

Jha 2007; Majumder and Jha 2009b). Podophyllotoxin was detected from the leaf induced calli by Chakraborty et al. (2010).

Since podophyllotoxin, a lignan, is a product of the phenylpropanoid pathway, the effects of different concentrations of direct precursors from the pathway (phenylalanine, tyrosine, *trans*-cinnamic acid, *para*-coumaric acid) and one indirect precursor (tryptophan) of podophyllotoxin on CSCs (Fig. 16.6b) of the Indian Podophyllum were studied (Majumder 2008; 2012). A maximum increase in podophyllotoxin accumulation of 4.5-fold over untreated control cultures was noted after the addition of *para*-coumaric acid. Content was also enhanced noticeably (2 to 4-fold over control) after adding tyrosine, tryptophan and phenylalanine to CSCs (Majumder 2008; 2012). Addition of precursors for improving the production podophyllotoxin in CSCs of *P. hexandrum* is also reported by previous workers.

Elicitation is an effective strategy to enhance the production of plant SMs in in vitro cultures. Elicitors are signal molecules which trigger the formation of SMs by activating novel genes encoding enzymes in different biosynthetic pathways. Till date there are very few reports on elicitation in cell cultures of *P. hexandrum*. The effect of two well-known elicitors—salicylic acid (SA) and methyl jasmonate (MeJA)—on CSCs of the *P. hexandrum* was analysed in our laboratory, and a remarkable increase in podophyllotoxin accumulation was noted after the addition of SA (Majumder 2008). In contrast, Bhattacharyya et al. (2012) noted 7–8-fold change

in podophyllotoxin accumulation in a 12-day-old CSC of *P. hexandrum* developed from leaf-derived calli after elicitation with 100 μM MeJA.

#### 16.11.1.2 Organ Cultures, in vitro Regeneration and Propagation

In an earlier study, Sagar and Zafar (2005) reported that mother root explants excised from aseptically germinated seedlings of *P. hexandrum* showed induction of lateral roots and calli on Gamborg's B5 and MS media containing auxins and cytokinins. Roots also regenerated from induced calli on Gamborg's B5 and MS media supplemented mostly with auxins and podophyllotoxin could be detected in root cultures. Later on, in vitro root cultures were initiated from rhizomes isolated from in vitro grown plantlets of *P. hexandrum* on MS medium supplemented with different concentrations of IBA, $GA_3$, hydroquinone and activated charcoal (Li et al. 2009). Podophyllotoxin could be detected in the roots and culture medium. In an attempt to enhance rooting of *P. hexandrum*, in vitro proliferated shoots were cultured on WPM (woody plant medium) supplemented with various auxins (IAA, NAA and IBA) (Guo et al. 2012). The best response was obtained with IAA (1.5 $mgl^{-1}$) and NAA (0.5 $mgl^{-1}$). The plantlets could be transplanted successfully. Rajesh et al. (2014a) explored the potentiality of different explants from in vitro germinated seedlings of *P. hexandrum* in generating adventitious roots on MS medium supplemented with various types of auxins. The authors demonstrated that several nutrient parameters (viz. carbon sources, medium strength, initial medium pH, ammonium and nitrate proportion and phosphate ratio) affected higher biomass and podophyllotoxin accumulation in the adventitious root cultures derived from the root segments.

Strategies for in vitro regeneration and propagation of *P. hexandrum* have been developed. In vitro multiplication via multiple shoot formation from zygotic embryos and subsequent rooting was first reported by Nadeem et al. (2000). Sultan et al. (2006) inoculated roots from in vitro germinated seedlings of *P. hexandrum* on ½ strength Gamborg's medium supplemented with 2,4-D (0.5–1.5 $mgl^{-1}$) and BA (0.2–1.5 $mgl^{-1}$) for induction of callus cultures. When calli were transferred to MS medium with BA (0.5–5.0 $mgl^{-1}$) and IAA (0.5–3.0 $mgl^{-1}$) for shoot regeneration, medium supplemented with 0.5 $mgl^{-1}$ BA and 1.0 $mgl^{-1}$ IAA together with 1% activated charcoal resulted in maximum number of shoots. The regenerated shoots rooted on ½ strength MS medium with activated charcoal, NAA (0.5–2.0 $mgl^{-1}$) and IAA (1.0–2.0 $mgl^{-1}$), which were subsequently hardened and transferred to soil.

Chakraborty et al. (2010) developed an efficient protocol for regenerating plantlets of *P. hexandrum* in vitro through direct organogenesis using rhizome explants. MS medium supplemented with 11.42 μM IAA resulted in maximum rate of multiple shoot formation from rhizome explants followed by a combination of NAA and BAP at higher concentrations. Yellowing of leaves was noted when the shoots were maintained in the culture conditions for more than 4 months. The shoots rooted on IBA supplemented ½ strength liquid MS medium.

Regeneration of shoots was observed again from rhizome segments cultured on MS medium supplemented with various concentrations of auxins (viz. IAA and

NAA) and cytokinins (viz. BAP and TDZ), with high concentrations of BAP and NAA exhibiting maximum shoot regeneration (Tariq et al. 2015). The shoots rooted on ½ strength MS medium with varying concentrations of IBA.

Deb et al. (2018) propagated *P. hexandrum* in vitro using excised embryos from mature seeds. The embryos germinated to give rise to prominent cotyledonary tube with multiple leaves on MS medium supplemented with BA (1.0 μM) and $GA_3$ (0.1 μM). The shoots rooted on IAA (1.0 μM) supplemented MS medium. The in vitro propagated shoots were hardened and transferred to fields.

Plant regeneration via somatic embryogenesis in *P. hexandrum* was first reported by Arumugam and Bhojwani (1990), followed by Nadeem et al. (2000). Recently, Rajesh et al. (2014b) developed another efficient system for in vitro somatic embryogenesis and podophyllotoxin production from seeds collected from three different regions of the Himalayas. SEs were induced from zygotic embryos either directly or through the formation of embryogenic callus. Different types and concentrations of auxins (viz. 2,4-D, NAA, picloram) were used to induce SEs directly from zygotic embryos of which the highest percentage (89.6%) of SE induction was observed in the Milam variety using MS medium supplemented with 1.5 $mgl^{-1}$ 2,4-D. Transfer of the induced SEs to auxin supplemented medium resulted in the formation of embryogenic as well as non-embryogenic callus depending on the type of auxin used. The embryos developed further on 2,4-D free media and germinated on MS basal medium and $GA_3$ supplemented MS medium. The germinated SEs as well as germinated zygotic embryos produced podophyllotoxin. In continuation with their studies, embryogenic callus initiated from the zygotic embryos was also cultured in liquid MS medium supplemented with different concentrations of 2,4-D and NAA along with PVP (Rajesh et al. 2014c). The effect of variable medium strength and carbon source on induction of SEs and podophyllotoxin accumulation was studied. The embryos matured on ABA supplemented 0.75 strength liquid MS medium and synthesized appreciable amounts of podophyllotoxin. Similar to their previous report, the embryos germinated on $GA_3$ supplemented liquid medium and synthesized podophyllotoxin. A survival rate of 44% in growth chamber was noted following proper acclimatization of the regenerated plantlets.

Sporadic shoot organogenesis (Fig. 16.6d–f) was observed in callus cultures maintained on MS basal medium supplemented with BA and NAA for 12 weeks, cultures being derived from mature roots by Majumder (2008). The calli developed nodular structures after 12 weeks which transformed into bud primordia like structures within another 3 weeks of culture. Shoot buds developed upto 1–2 cms with leaf differentiation when transferred onto fresh medium under 16/8 h (light/dark) photoperiod but did not grow further even after trials with other combination and concentration of different cytokinins with or without auxin.

### *16.11.2 Genetic Transformation of* P. hexandrum

Initiation of root cultures in *P. hexandrum* through transformation using *A. rhizogenes* was attempted in our laboratory (Majumder 2008). Three wild-type virulent agropine strains of *A. rhizogenes* (A4, LBA9402, 15834), two supervirulent strains (R1600, R1601) and a mannopine strain 8196 were used for infectivity studies by co-culturing *A. rhizogenes* overnight cultures with mature and immature axenic explants of *P. hexandrum* and 2-year-old callus cultures maintained in the laboratory. However, none of the strains of *A. rhizogenes* used could induce roots in callus cultures or mature and immature axenic explants of *P. hexandrum* under any cultural conditions studied. Wound sites of inoculated mature and immature explants necrosed within 2 weeks of inoculation and after another 2 weeks of culture on antibiotic supplemented medium, whole of the explants turned dark brown. Although the inoculated calli continued to proliferate on medium supplemented with antibiotic, roots were not induced from the callus cultures (Majumder 2008). Giri et al. (2001) reported infection of embryos of *P. hexandrum* with *A. rhizogenes* (strains K599, A4, 15834) and induction of callus producing podophyllotoxin. Successful hairy root cultures of *P. hexandrum* has not been reported to date.

In a more recent attempt, Rajesh et al. (2013) studied different factors affecting the efficiency of transformation (viz. *Agrobacterium* strain, co-cultivation duration and acetosyringone concentration) using embryogenic callus of *P. hexandrum.* Of the three different *A. tumefaciens* strains [LBA 4404, EHA 101 and EHA 105 harbouring the binary vector pCAMBIA 2301 (CAMBIA, Canberra, Australia)] used by the authors, EHA 105 had the highest transformation efficiency (10.79%) followed by EHA 101 (5.50%) and LBA 4404 (2.16%). A notable effect of the strain of *A. tumefaciens* and acetosyringone concentration on transformation efficiency was reported. Another important factor controlling transformation efficiency was the duration of co-cultivation of *Agrobacterium* with the explants, with a period of 3 days to be optimum for a transformation efficiency of 29.64%. Bhattacharyya et al. (2016a) transformed *P. hexandrum* cell cultures and callus by using transformed *A. tumefaciens* (GV3101). The transgenic lines overexpressed the four *PhCAD* isoforms (cinnamyl alcohol dehydrogenase or CAD) with *PhCAD3* favouring the highest accumulation of podophyllotoxin as compared to lignin followed by *PhCAD4* and *PhCAD2*, whereas *PhCAD1* favoured both equally.

Thus *P. hexandrum* is a recalcitrant species with respect to morphogenesis as well as genetic manipulation and more research is required for improving efficiency of genetic transformation in the species.

### 16.11.3 *Genetic and Biochemical Stability in in vitro Cultures of* P. hexandrum

Tariq et al. (2015) could successfully conserve in vitro propagated plants of *P. hexandrum* from rhizome segments, as confirmed by RAPD analysis. The monomorphic banding pattern highlighted the fact that plants propagated in vitro do not necessarily undergo any genetic change.

When cell lines derived following screening and selection of cell suspension cultures of *P. hexandrum* were analysed using 28 random decamer primers (Majumder and Jha 2009b), polymorphic banding patterns were observed, indicating genomic variability among the cell lines. Also, when the cell lines were analysed after 2 years of initiation, considerable variation in podophyllotoxin content was noted between cell lines (0.15–0.45% DW). The content decreased in all the lines till fourth year of initiation (0.03–0.2% DW) after which the lines became stable in producing podophyllotoxin (Majumder and Jha 2009b).

### 16.11.4 *Recent Developments and Application of "-omics" to in vitro Cultures of* P. hexandrum

Podophyllotoxin is a lignan, a compound containing phenylpropanoid dimers ($C_6C_3$), connected by C8 carbon located at the side chain of each unit (Umezawa 2003). In plants, its biosynthesis occurs via the phenylpropanoid pathway (Wankhede et al. 2013). The biosynthetic pathway of podophyllotoxin is more or less elucidated and several cDNAs have been reported (Bhattacharyya et al. 2013). In general, mechanisms of enantioselective dimerization of monolignols are involved in the process of lignan biosynthesis (Bhattacharyya et al. 2013, 2016a).

In an attempt to analyse the cell proteome related to enhanced podophyllotoxin accumulation in elicited CSC of *P. hexandrum*, Bhattacharyya et al. (2012) performed 2-DE proteomic profiling of CSC of *P. hexandrum* elicited with MeJA together with untreated control cultures. Of the various functional groups of proteins identified, 13% were related to SMs. Several enzymes related to monolignol/phenylpropanoid biosynthesis [viz. methyl transferases like CCOMT (caffeoyl CoA-O-methyltransferase) and CAOMT (caffeic acid-O-methyltransferase)] were identified. From their study, the authors suggested that the expression of upstream genes (viz. CCOMT and CAOMT) of monolignol pathway could control the biosynthesis of podophyllotoxin.

Next generation sequencing data of de novo transcriptome analysis can be used for identification of unknown genes in non-model organisms. The transcriptome of *P. hexandrum* cell culture was sequenced by Bhattacharyya et al. (2013). The authors reported 454 pyrosequencing from a CSC of *P. hexandrum* to resolve the genes involved in podophyllotoxin biosynthesis, and nearly all the members of the phenylpropanoid pathway were identified using next generation whole transcriptome

sequencing. In addition, the authors also identified EST-SSRs as molecular markers claiming to be applicable in the conservation of this endangered species.

Of the several factors playing crucial roles in gene regulation are microRNAs or miRNAs which are small (~22 nt) single stranded, non-coding RNA molecules (Bartel 2004). These RNAs play vital roles in several developmental processes in plants. In yet another interesting study, Biswas et al. (2016) used 454 pyrosequencing to identify 60 mature miRNAs and 6 pre-miRNAs in *P. hexandrum* CSCs. The authors utilized different bioinformatics tools to show that the targets of these miRNAs are genes involved in primary and secondary metabolism.

Cinnamyl alcohol dehydrogenase or CAD (EC 1.1.1.95) is an NADPH-dependent enzyme in the phenylpropanoid pathway, catalyzing the synthesis of coniferyl alcohol from coniferaldehyde and sinapyl alcohol from sinapaldehyde. Full length cDNA sequences of the CAD isoforms (*PhCAD1, PhCAD2, PhCAD3* and *PhCAD4*) were deduced by Bhattacharyya et al. (2016a) to isolate CAD isoforms in favour of podophyllotoxin from MeJA elicited CSCs of *P. hexandrum*. Major transcripts of podophyllotoxin biosynthesis were identified using next generation sequencing technology. Each isoform had a higher affinity for coniferaldehyde compared to sinapaldehyde, as indicated by in vitro enzyme assays. Their study revealed transcriptome wide identification and characterization of CAD isoforms specific for podophyllotoxin biosynthesis. The transcriptome data also demonstrated the role of MeJA in controlling different genes and transcription factors in relation to the biosynthesis of podophyllotoxin.

In yet another study, Hazra et al. (2017) demonstrated that MeJA induced the production of reactive oxygen species (ROS) which stimulated the accumulation of podophyllotoxin remarkably in cell cultures of *P. hexandrum.* The mRNA stability of three ROS-responsive biosynthetic genes of podophyllotoxin, viz. *PhCAD3*, *PhCAD4* and *NAC3,* was also increased leading to their upregulation. The authors also noted that other genes involved in the podophyllotoxin biosynthesis pathway, unaffected by MeJA induced ROS, were also upregulated by MeJA. Furthermore, MeJA controlled the genes non-responsive to ROS through the down-regulation of five miRNAs (miR172i, miR035, miR1438, miR2275 and miR8291) specific for biosynthesis of SMs. Through their study, the authors suggested that MeJA controls the biosynthesis of podophyllotoxin by two possible modes (a) by increasing the stability of mRNAs of genes responsive to ROS and (b) up regulation of genes non-responsive to ROS through the down-regulation of some miRNAs non-responsive to ROS.

## 16.12 Case Study 2. *Rauvolfia serpentina*

*Rauvolfia serpentina* (Linn.) Benth. ex Kurz. is an endangered plant having immense medicinal properties, traditionally used for ages. Pathania et al. (2013) created an extensive compilation of 147 plant-derived molecules (PDMs) reported to be extracted from various plant parts of *R. serpentina.* Terpenoid indole alkaloids

(TIAs) of *R. serpentina* currently in clinical use include ajmalicine and serpentine as anti-hypertensive and ajmaline as anti-arrhythmic (Yang and Stöckigt 2010) while reserpine is a major component of various market drugs. Reserpine (3,4,5-trimethyl benzoic acid ester of reserpic acid) is the most common alkaloid which causes depletion in the catecholamine (epinephrine and norepinephrine) and serotonin levels in the brain and peripheral nervous system, thereby leading to sedative, anti-hypertensive and anti-arrhythmic properties (Rolf et al. 2003).

### *16.12.1 Micropropagation as a Means of Production of Secondary Metabolites and Stability in Production*

Micropropagation of the traditional MP *R. serpentina* is being worked upon extensively. Alkaloid extraction from micropropagated plants is a time saving method for commercialization of medicinally important SMs. Bahuguna et al. (2011) reported that total alkaloid content of in vitro multiple shoots (6 weeks old) was similar to the alkaloid content from leaf and stem of field-grown plants (1 year old). Gantait and Kundu (2017) reported the reserpine content of plantlets germinated from synthetic seeds stored at 25 °C and 8 °C to be 203.38 $\mu g g^{-1}$ DW and 249.37 $\mu g g^{-1}$ DW, respectively, indicating that lower temperature is suitable for maintaining alkaloid content during synthetic seed preservation. In vitro cultures thus retained the alkaloid synthesizing capacity which was often enhanced in comparison with that of the parent plant.

### *16.12.2 Genetic Transformation*

*A. rhizogenes* mediated genetic transformation has provided an immense potential for generation of genetically stable, fast-growing hairy root lines having high yield of SMs in hormone-free culture media. This technology has been successfully applied in *R. serpentina* since 1993 by Benjamin et al. and Falkenhagen et al. (1993) and since then extensively exploited till date. Explants from in vitro cultures of *R. serpentina* have responded to different virulent strains of *A. rhizogenes* including 15384 (Benjamin et al. 1993), ATCC SV2, SV4 (Sarma et al. 1997), A4 (Mehrotra et al. 2013a), LBA 9402 (Ray et al. 2014a), MTCC 532 and 2364 (Bhagat et al. 2019). Significant variation in reserpine accumulation was observed in LBA9402 transformed hairy root lines with 3.11 $mg g^{-1}$ DW being the overproduction maxima (Ray et al. 2014a; Fig. 16.7). Researchers have focused on the enhanced SM production in the transgenic root lines. Mehrotra et al. (2013a) reported high reserpine content in roots of spontaneously regenerated plantlets grown for 6 months in green house condition (0.0889% DW) and their parent HRCs (0.0882% DW) which was significantly higher than normal roots (0.0180% DW). Interestingly 90% of the regenerated

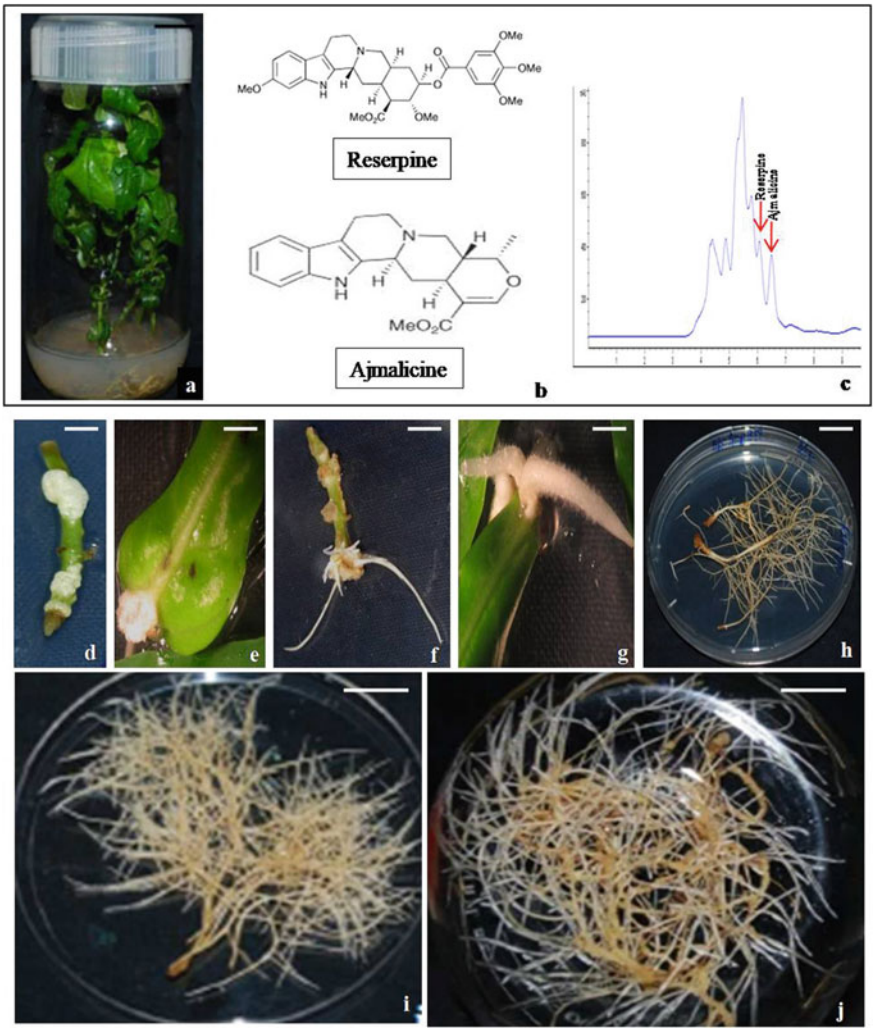


**Fig. 16.7** **a** Multiple shoot cultures of *R. serpentina* maintained on MS medium for 8 weeks under 16/8 h photoperiod (*Bar = 2.0* cm), **b** Chemical structures and **c** HPLC chromatogram of valuable secondary metabolites of *R. serpentina*, **d**, **e** Response of internodal and leaf explants to infection by different strains *A. rhizogenes* (*Bar = 0.8* cm), **f**, **g** Root induction from internodal and leaf explants infected with *A. rhizogenes* strain A4 (*Bar = 0.8* cm), **h** Establishment of transformed root culture on modified MS medium (*Bar = 1.6* cm), **i**, **j** Transformed root line cultured on semi-solid and liquid medium (*Bar = 3.0* cm)

plantlets after acclimatization to field conditions showed morphological features similar to non-transformed plants. Insertion of multiple copies of transgene was attributed as a probable cause of the stunted phenotype of some of the regenerated plantlets.

HRCs have been reported to produce novel alkaloids undetected in plant roots as well as cell cultures including a new subgroup of sarpagine alkaloid, namely 19(S), 20(R)-dihydroperaksine, formed by deacetylation of perakine or raucaffrinoline (Sheludko et al. 2002).

### *16.12.3 Elicitation and Scale-Up*

Biotechnological approaches for elicitation of medicinally active SMs have been extended to both untransformed and transformed cultures involving techniques like optimization of culture conditions, precursor feeding, selection of high-yielding lines and modifying biosynthetic pathways. Problems associated with the limited availability of these compounds can be mitigated to a certain extent by this approach.

Zafar et al. (2017) evaluated effect of $AlCl_3$ on reserpine content and callus growth. Under the influence of 0.15 mM $AlCl_3$ enhancement of reserpine content (0.129 $mgg^{-1}$ DW) was noted in comparison with untreated callus cultures (0.083 $mgg^{-1}$ DW). Verma et al. (2012) carried out co-culture of cell lines leading to increase in biomass and alteration of alkaloid profile the underlying mechanism being that metabolite effluents of one plant is up taken by the other plant for downstream processing to a valuable end product. Observations of *Catharanthus* + *Rauwolfia* co-culture resulted in biomass enhancement by 20–25 times in a 7 l stirred tank bioreactor after a 30-day cycle and presence of two new indole alkaloids in the crude extracts of the co-culture previously unidentified in the individual cell cultures. Variation in reserpine content was noted in whole plant cultures under the effect of precursors and elicitors used either singly or in combination. While enhancement of reserpine content was noted after 6 weeks in the presence of ABA, SA and tryptamine; DMSO treatment had a negative effect on reserpine accumulation. Treatment with a combination of SA and tryptamine unregulated reserpine production (58.04 $mgg^{-1}$ DW) against 18.24 $mgg^{-1}$ DW reserpine obtained from untreated whole plant cultures. However, this treatment resulted in decreased biomass (Panwar and Guru 2015).

Significant enhancement of alkaloids upon application of biotic and abiotic elicitors to HRCs was reported by Srivastava et al. (2016a). They reported 14.8- and 3.1-fold increase in ajmalicine content under 100 mM NaCl and 50 $mgl^{-1}$ mannan, respectively. Similarly, 1.9- and 2.9-fold increase in ajmaline content was recorded upon treatment with 200 mM NaCl and 100 $mgl^{-1}$ mannan respectively. Signal transduction triggered by elicitor–receptor binding at the plant cell membren stimulates SM synthesis as a defence response (Halder et al. 2019). Optimization of bioreactor conditions for effective large-scale production of metabolites is the desired outcome of HRC (Gutierrez-Valdes et al. 2020).

### *16.12.4 Metabolic Engineering*

Metabolic engineering of biosynthetic pathways targets the enhancement of medicinally important SMs as well as generation of new compounds within the biological system. As plants produce such chemicals in low amount, industrial demand necessitates synthesis of chemical analogues. Herein lies the necessity for elucidation of the biosynthetic pathway (primarily the enzyme catalyzed reactions along with the regulatory genes) and its engineering.

The amount and types of alkaloids produced as well as their isolation depend primarily on the nutrient medium, culture conditions as well as the nature of in vitro cultures. *R. serpentina* CSCs and HRCs have been suitably used for the production of more than 35 different alkaloids (Yang and Stöckigt 2010). Generation of hybrid somatic cell line RxR17 (*R. serpentina* x *Rhazya stricta*) resulted not only in production and enhancement of indole alkaloids (obtained from methyl jasmonate supplemented culture media) but also generation of novel alkaloids undetected in either of the parental species (Sheludko et al. 1999). Structural biology techniques involving NMR spectroscopy and X-ray crystallography are used to determine 3D structure of biosynthetic enzymes. These techniques have been extended to *R. serpentina* in analysing the structure of strictosidine synthase (STR1), strictosidine glucosidase (SG), raucaffricine glucosidase (RG), polyneuridine aldehyde esterase (PNAE) and vinorine synthase (VS). Understanding the topology of the substrate-binding sites, paves way for enzyme engineering to produce novel medicinal alkaloids; an approach that has been applied in *C. roseus* (Yang and Stöckigt 2010). Characterization of the TIA biosynthetic pathway gene opens up new possibilities of metabolite pathway engineering. Tryptophan decarboxylase (TDC, EC 4.1.1.28) converts tryptophan into tryptamine, the initial step in the pathway leading to the formation of alkaloids such as such as reserpine, ajmaline, ajmalicine, serpentine, vomiline and yohimbine. In order to enhance the biosynthesis of alkaloids, Mehrotra et al. (2013b) over-expressed tryptophan decarboxylase gene (*Crtdc*) from *C. roseus* in HRCs of *R. serpentina.* Enhancement of reserpine and ajmalicine content was noted among hairy root lines obtained by transformation with *A. rhizogenes* strain A4 harbouring pBI121-Crtdc binary construct (reserpine 0.1202%; ajmalicine 0.0064% DW) over those arising from wild-type A4 transformation (reserpine 0.0596% DW; ajmalicine 0.0011% DW). In a novel approach, Corbin et al. (2017) demonstrated that virus induced gene silencing of phytoene desaturase. Such an approach facilitates characterization of genes involved in the metabolic pathway. Thus, metabolic engineering paves way for production of high-value pharmaceuticals in in vitro systems, which can be scaled up for commercial purposes.

### *16.12.5 Genetic Fidelity of in vitro Cultures of* R. serpentina

One of the aims of in vitro cultures is conservation of genetic material of threatened plant species. Somaclonal variations like chromosomal aberrations, polyploidy and mutations arising in response to stress in culture conditions are a setback in germplasm preservation. Hence, the detection of genetic variation among regenerated plantlets has been targeted by researchers. RAPD profiles of regenerated plants showed homogeneity with donor plants (Senapati et al. 2014). ISSR analysis of indirectly and directly regenerated plantlets and parent explant was performed by Saravan et al. (2011). Plantlets obtained by direct organogenesis showed monomorphism, hence genetic stability, whereas callus regenerated plantlets showed polymorphism for one primer. Somatic clones derived from synthetic seed germination showed genetic stability when assessed by RAPD and ISSR (Faisal et al. 2012). Zafar et al. (2019) tested genetic fidelity among plantlets obtained by direct organogenesis and somatic embryogenesis. Comparative analysis of the estimated 2C DNA content of plantlets derived by direct and indirect organogenesis, somatic embryogenesis and field-grown plant reflected genetic stability as no significant variation was detected by the flow cytometric analysis (Zafar et al. 2019). Minor genetic variation was observed in cell lines after four passages through different media. However, transfer from surface culture to submerged culture resulted in significant genetic variation (Spiridonova et al. 2007) as revealed by RAPD analysis. Decreased polymorphism was observed when transfer to submerged culture was preceded by culture on semi-solid medium possibly due to the nature of surface exposure to the nutrient medium.

Genetic stability was also reported in transgenic cultures. A hairy root line without showing tendency of callusing was maintained for >6 years by Pandey et al. (2014). Interestingly, the authors were able to maintain the long-term cultures in both sucrose and table sugar containing media. Increase in productivity of yohimbine, ajmaline and reserpine was noted in the long-term cultures, in both the carbon source, with up to 296% increase in ajmaline production in sucrose containing media.

## 16.13 Conclusions

MPs with their vast array of pharmacologically active SMs are essential resources of nature that have been used by human beings to maintain and restore good health since ancient times. But, as reviewed, several anthropogenic activities, uneven distribution and availability of MPs all over the earth, inadequately resource management system, lack of public awareness, weakening of biodiversity protection laws, etc., have led to a steady decline in the population of a huge number of these immensely important plants from the wild. Besides, various other factors discussed also affect the availability of phytochemicals from the wild populations of plants. Over the past few decades, a lot of effort has been put by scientists and researchers all over the world to

safeguard the existence of these MPs in their natural habitats either through conventional breeding technologies, ex-situ and in-situ conservation methods or through plant tissue culture approaches. The literature survey demonstrates that various techniques of PTC like micropropagation, somatic embryogenesis, in vitro production of SMs through callus/cell suspension/organ cultures/HRCs or advanced techniques like metabolic pathway/ploidy engineering, nanoparticle application, genome editing have revolutionized the field of MP research. The study further documents the instances of morphological, biochemical and genetic stability/fidelity of non-transformed and transformed in vitro cultures due to their effect on proliferation and SM production. The two case studies presented are examples of the applications of PTC methodologies towards conserving MP biodiversity together with the production of pharmaceutically important SMs. Selection of suitable conventional and upcoming PTC techniques along with screening for stability thus paves way for availability of pharmaceutical SMs from plants with minimal threat to the biodiversity.

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