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Leading Article

Role of biotechnology in medicinal plants

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Abstract

Medicinal plants are the most important source of life saving drugs for the majority of the world's population. The biotechnological tools are important to select, multiply and conserve the critical genotypes of medicinal plants. In-vitro regeneration holds tremendous potential for the production of high-quality plant-based medicine.

Cryopreservation is long-term conservation method in liquid nitrogen and provides an opportunity for conservation of endangered medicinal plants. In-vitro production of secondary metabolites in plant cell suspension cultures has been reported from various medicinal plants. Bioreactors are the key step towards commercial production of secondary metabolites by plant biotechnology. Genetic transformation may be a powerful tool for enhancing the productivity of novel secondary metabolites; especially by Agrobacterium rhizogenes *induced hairy roots. This article discusses the applications of biotechnology for regeneration and genetic transformation for enhancement of secondary metabolite production in-vitro from medicinal plants.*

Key words**:** *Bioreactors; genetic transformation; regeneration; secondary* metabolites

Abbreviations*:* BA: 6-Benzylaminopurine; TDZ: 1-Phenyl-3-(1,2,3-thiadiazol-5-yl) urea; NAA: α-Naphthaleneacetic acid; IAA: Indole-3 acetic acid; 2iP: 6-(γ-Dimethylallylamino) purine; 2,4-D: 2,4-Dichlorophenoxyacetic acid; GA3: Gibberellic acid

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Introduction

Plants have been an important source of medicine for thousands of years. Even today, the World Health Organization estimates that up to 80 per cent of people still rely mainly on traditional remedies such as herbs for their medicines. Plants are also the source of many modern medicines. It is estimated that approximately one quarter of prescribed drugs contain plant extracts or active ingredients obtained from or modeled on plant substances. The most popular analgesic, aspirin, was originally derived from species of *Salix* and *Spiraea* and some of the most valuable anti-cancer agents such as paclitaxel and vinblastine are derived solely from plant sources[1-3].

Biotechnological tools are important for multiplication and genetic enhancement of the medicinal plants by adopting techniques such as *in-vitro* regeneration and genetic transformations. It can also be harnessed for production of secondary metabolites using plants as bioreactors. This paper reviews the achievements and advances in the application of tissue culture and genetic engineering for the *in-vitro* regeneration of medicinal plants from various explants and enhanced production of secondary metabolites.

In-vitro Regeneration

In-vitro propagation of plants holds tremendous potential for the production of high-quality plant-based medicines[4]. This can be achieved through different methods including micropropagation. Micropropagation has many advantages over conventional methods of vegetative propagation, which suffer from several limitations[5]. With micropropagation, the multiplication rate is greatly increased. It also permits the production of pathogen-free material. Micropropagation of various plant species, including many medicinal plants, has been reported[6-8]. Propagation from existing meristems yields plants that are genetically identical with the donor plants[9]. Plant regeneration from shoot and stem meristems has yielded encouraging results in medicinal plants like *Catharanthus roseus, Cinchona ledgeriana* and *Digitalis* spp, *Rehmannia glutinosa*, *Rauvolfia serpentina*, *Isoplexis canariensis*[10-12].

Numerous factors are reported to influence the success of *in-vitro* propagation of different medicinal plants[9, 13-15]. The effects of auxins and cytokinins on shoot multiplication of various medicinal plants have been reported. Benjamin *et al*. has shown that 6-Benzylaminopurine (BA), at high concentration (1–5ppm), stimulates the development of the axillary meristems and shoot tips of *Atropa belladona*[16]. Lal *et al*. observed a rapid proliferation rate in *Picrorhiza kurroa* using kinetin at 1.0–5.0 mg/l[17]. Direct plantlet regeneration from male inflorescences of medicinal yam on medium supplemented with 13.94 µM kinetin has also be reported[18]. The highest shoot multiplication of *Nothapodytes foetida* is achieved on medium containing thidiazuron (TDZ) at a concentration of 2.2 µM[19]. Similarly, it has been observed that cytokinin is required, in optimal quantity, for shoot proliferation in many genotypes but inclusion of low concentration of auxins along with cytokinin triggers the rate of shoot proliferation[20-23]. Barna and Wakhlu has indicated that the production of multiple shoots is higher in *Plantago ovata* on a medium having 4–6 M kinetin along with 0.05 µM NAA[24]. According to Faria and Illg, the addition of 10 µM BA along with 5 µM indole-3-acetic acid (IAA) or 5 µM NAA induces a high rate of shoot proliferation of *Zingiber spectabile*[25]. Faria and Illg have also shown that the number of shoots/explant depends on concentrations of the growth regulators and the particular genotypes. The nature and condition of explants has also been shown to have a significant influence on the multiplication rate of *Clerodendrum colebrookianum* by Mao *et al*.[26]. Actively growing materials were more responsive to shoot induction than

dormant buds. Also BA was proved superior to 6-(γ-Dimethylallylamino) purine (2ip) and TDZ for multiple shoot induction.

Callus-mediated organogenesis

The induction of callus growth and subsequent differentiation and organogenesis is accomplished by the differential application of growth regulators and the control of conditions in the culture medium. With the stimulus of endogenous growth substances or by addition of exogenous growth regulators to the nutrient medium, cell division, cell growth and tissue differentiation are induced. There are many reports on the regeneration of various medicinal plants via callus culture. Satheesh and Bhavanandan have reported the regeneration of shoots from callus of *Plumbago rosea* using appropriate concentrations of auxins and cytokinins[27]. Mantell and Hugo have also reported a high frequency of shoot, root, and microtuber production from *Dioscorea alata* depending on the culture medium used, the type of explant from which the calli originated, and the photoperiod[28]. Plant regeneration has been achieved from leaf callus of *Cephaelis ipecacuanha* on Murashige and Skoog medium supplemented with 4.5 mg/L kinetin and 0.1 mg/L α-Naphthaleneacetic acid (NAA)[29]. Ghosh and Sen established plant regeneration via callus cultures from different explants of *Asparagus cooper*[30]. The relative importance of genotype, explant and their interactions for *in-vitro* plant regeneration via organogenesis in *Solanum melongena* has been investigated[31]. Basu and Chand achieved shoot bud differentiation from root-derived callus of *Hyoscyamus muticus* in MS medium supplemented with 0.05 mg/L NAA and 0.5 mg/L BA[32]. Saxena *et al*. reported plant regeneration via organogenesis from callus cultures derived from mature leaves, stems, petioles and roots of young seedlings of *Psoralea corylifolia*[33]. *In-vitro* organogenesis of *Zingiber officinale* via callus culture has been described by Rout and Das[20]. Further, Shasany *et al*. reported the influence of different growth regulators on high frequency plant regeneration from internodal explants of *Mentha arvensis*[22]. Successful plant regeneration was reported from stem and leaf-derived callus of *Centella asiatica* on MS medium supplemented with 4.0 mg/L BA, 2.0 mg/L kinetin, 0.25 mg/L NAA, and 20 mg/L adenine sulfate[34]. Rapid regeneration of *Plumbago zeylanica* in callus culture was achieved on MS medium supplemented with 2.0 mg/L BA and 0.5 mg/L IAA[21]. The efficient systems for *in-vitro* regeneration of *Solanum laciniatum* and *Echinacea pallida* have been established from leaf explants on medium supplemented with BA and NAA[35, 36]. Pande *et al*. have also reported the *in-vitro* regeneration of *Lepidium sativum* from various explants on medium supplemented with BA and NAA[37].

Regeneration through somatic embryogenesis

Somatic embryogenesis is a process where groups of somatic cells/tissues lead to the formation of somatic embryos which resemble the zygotic embryos of intact seeds and can grow into seedlings on suitable medium. Plant regeneration via somatic embryogenesis from single cells, that can be induced to produce an embryo and then a complete plant, has been demonstrated in many medicinal plant species. Arumugam and Bhojwani noted the development of somatic embryos from zygotic embryos of *Podophyllum hexandrum* on MS medium containing 2 μM BA and 0.5 μM IAA[32]. Ghosh and Sen reported regeneration and somatic embryogenesis in *Asparagus cooperi* on MS medium having 1.0 mg/L NAA and 1.0 mg/L kinetin[39]. Embryogenic calluses and germination of somatic embryos in nine varieties of *Medicago sativa* has been achieved[40]. Using a medium containing 2,4-Dichlorophenoxyacetic acid (2,4-D) and TDZ, Zhou *et al*. have achieved the induction of somatic embryogenesis in cells from *Cayratia japonica*[41]. Somatic embryogenesis and

subsequent plant regeneration from callus derived from immature cotyledons of *Acacia catechu* has also been achieved on medium supplemented with 13.9 µM kinetin and 2.7µM NAA[42]. Gastaldo *et al*. induced somatic embryogenesis from bark callus of *Aesculus hippocastanum* on MS medium supplemented with 2.0 mg/L kinetin, 2.0 mg/L 2,4-D and 2.0 mg/L NAA[43]. High-frequency somatic embryogenesis and plant regeneration from suspension cultures of *Acanthopanax koreanum* have been reported on a medium containing 4.5 µM 2,4-D[44]. Das *et al*. reported high frequency somatic embryogenesis in *Typhonium trilobatum* on medium containing 1.0 mg/L kinetin and 0.25 mg/L NAA[45]. The suspension culture of *Catharanthus roseus* from stem and leaf explants on medium containing NAA and kinetin has been established by Zhao *et al*.[46]. Chand and Sahrawat have reported the somatic embryogenesis of *Psoralea corylifolia* L. from root explants on medium supplemented with NAA and BA[47].

Efficient development and germination of somatic embryos are prerequisites for commercial plantlet production. Lowering of growth regulator concentrations in culture media has improved embryo development and germination of many medicinal plants[38, 48, 49]. Germination of the somatic embryos is achievable on MS medium without the growth regulator[41, 44]. However, Arumugam and Bhojwani noted that inclusion of BA (2 µM) and gibberellic acid (GA3, 2.8 µM) in the medium stimulated embryo development of *Podophyllum hexandrum*, although 75% of the embryos germinated on basal MS medium devoid of growth regulator[38]. Similar results were reported on the germination of embryos of *Psoralea corylifolia*[47]. Wakhlu *et al*. have reported that the somatic embryos of *Bunium persicum* matured and germinated on the basal MS medium supplemented with 1.0 mg/L kinetin[49]. Further, Kunitake and Mii reported that 30–40% of somatic embryos of *A. officinalis g*erminated after being treated with distilled water for a week; they were subsequently transferred to half-strength MS medium supplemented with 1.0 mg/L IAA, 1.0 mg/L GA3 and 1% sucrose[50]. However, the somatic embryos of *Typhonium trilobatum* have been germinated on MS medium supplemented with 0.01 mg/L NAA and 2% (w/v) sucrose after 2 weeks of culture[45].

Conservation through cryopreservation

The cryopreservation of *in-vitro* cultures of medicinal plants is a useful technique. Cryopreservation is long-term conservation method in liquid nitrogen (–196 °C) in which cell division and metabolic and biochemical processes are arrested. A large number of cultured materials can be stored in liquid nitrogen[51]. Since whole plants can regenerate from frozen culture, cryopresevation provides an opportunity for conservation of endangered medicinal plants. For example, low temperature storage has been reported to be effective for cell cultures of medicinal and alkaloid-producing plants such as *Rauvollfia serpentine, D. lanalta, A. belladonna, Hyoscyamus* spp[52].

When plants are regenerated and no abnormality is seen either in fertility or in alkaloid content, the materials can be stored using cryopreservation methods. Cryopreservation has been used successfully to store a range of tissue types, including meristems, anthers/pollens, embryos, calli and even protoplasts. However, the system will depend on the availability of liquid nitrogen methods.

Production of secondary metabolites from medicinal plants

Plants are the traditional source of many chemicals used as pharmaceuticals. Most valuable phytochemicals are products of plant secondary metabolism. The production of secondary metabolites *in-vitro* can be possible through plant cell culture[53, 54]. Successful establishment of cell lines

capable of producing high yields of secondary compounds in cell suspension cultures has been reported by Zenk[55]. The accumulation of secondary products in plant cell cultures depends on the composition of the culture medium, and on environmental conditions[56]. Strategies for improving secondary products in suspension cultures, using different media for different species, have been reported by Robins[57].

The production of secondary metabolites in plant cell suspension cultures has been reported from various medicinal plants. The production of solasodine from calli of *Solanum eleagnifolium*, and pyrrolizidine alkaloids from root cultures of *Senecio* sp. are examples[58, 59]. Cephaelin and emetine were isolated from callus cultures of *Cephaelis ipecacuanha*[60]. Scragg *et al*. isolated quinoline alkaloids in significant quantities from globular cell suspension cultures of *Cinchona ledgeriana*[61]. Enhanced indole alkaloid biosynthesis in the suspension culture of *Catharanthus roseus* has also been reported[46].

Ravishankar and Grewal reported that the influence of media constituents and nutrient stress influenced the production of diosgenin from callus cultures of *Dioscorea deltoidea*[62]. Parisi *et al*. obtained high yields of proteolytic enzymes from the callus tissue culture of garlic (*Allium sativum* L.) on MS medium supplemented with NAA and BAP[63]. Pradel *et al*. observed that the biosynthesis of cardenolides was maximal in the hairy root cultures of *Digitalis lanata* compared to leaf[64]. The production of azadirachtin and nimbin has been shown to be higher in cultured shoots and roots of *Azadirachta indica* compared to field grown plant[65]. Pande *et al*. reported that the yield of lepidine from *Lepidium sativum* Linn depends upon the source and type of explants[37].

Bioreactors are the key step towards commercial production of secondary metabolites by plant biotechnology. Bioreactors have several advantages for mass cultivation of plant cells:

i. It gives better control for scale up of cell suspension cultures under defined parameters for the production of bioactive compounds;
ii. Constant regulation of conditions at various stages of bioreactor operation is possible;
iii. Handling of culture such as inoculation or harvest is easy and saves time;
iv. Nutrient uptake is enhanced by submerged culture conditions which stimulate multiplication rate and higher yield of bioactive compounds; and
v. Large number of plantlets are easily produced and can be scaled up.

Since the biosynthetic efficiency of populations varies, a high yielding variety should be selected as a starting material. The fundamental requirement in all this is a good yield of the compound, and reduced cost compared to the natural synthesis by the plants.

The bioreactor system has been applied for embryogenic and organogenic cultures of several plant species[66, 67]. Significant amounts of sanguinarine were produced in cell suspension cultures of *Papaver somniferum* using bioreactors[68]. Ginseng root tissue cultures in a 20 tonne bioreactor produced 500 mg/L/day; of the saponin that is considered as a very good yield[69]. Jeong *et al*. have established the mass production of transformed Panax ginseng hairy roots in bioreactor[70]. Hahn *et al*. has observed the production of ginsenoside from adventitious root cultures of *Panax ginseng* through large-scale bioreactor system (1-10 ton)[71].

Bioreactors offer optimal conditions for large-scale plant production for commercial manufacture[72]. Much progress has been achieved in the recent past on optimization of these systems for the production and extraction of valuable medicinal plant ingredients such as ginsenosides and

shikonin. Roots cultivated in bioreactors have been found to release medicinally active compounds, including the anticancer drug isolated from various Taxus species, into the liquid media of the bioreactor which may then be continuously extracted for pharmaceutical preparations[4]. Conventional practices require the harvest of the bark of trees, all approximately 100 years old, to obtain 1 kg of the active compound taxol[73]. Research over the last two decades has established efficient protocols for isolated cell cultures and a large-scale bioreactor system. The acceptance of this process for the industrial production of this invaluable compound has recently been established and will significantly impact the production of the tumor-inhibiting pharmaceutical[73].

Genetic Transformation

The recent advances and developments in plant genetics and recombinant DNA technology have helped to improve and boost research into secondary metabolite biosynthesis. A major line of research has been to identify enzymes of a metabolic pathway and then manipulate these enzymes to provide better control of that pathway. Transformation is currently used for genetic manipulation of more than 120 species of at least 35 families, including the major economic crops, vegetables, ornamental, medicinal, fruit, tree and pasture plants, using *Agrobacterium*-mediated or direct transformation methods[74]. However, *Agrobacterium*-mediated transformation offers several advantages over direct gene transfer methodologies (particle bombardment, electroporation, etc), such as the possibility to transfer only one or few copies of DNA fragments carrying the genes of interest at higher efficiencies with lower cost and the transfer of very large DNA fragments with minimal rearrangement[75-77]. The gram-negative soil bacteria, *Agrobacterium tumefaciens,* and the related species, *A. rhizogenes,* are causal agents of the plant diseases crown gall tumour and hairy root, respectively. These species, which belong to the *Rhizobiaceae*, are natural engineers that are able to transform or modify, mainly dicotyledonous plants, although there are reports on the infection of monocotyledonous plants[78-80]. Virulent strains of *A. tumefaciens* and *A. rhizogenes* contain a large megaplasmid (more than 200 kb) which play a key role in tumor induction and for this reason it was named Ti plasmid, or Ri in the case of *A. rhizogenes*. During infection the T-DNA, a mobile segment of Ti or Ri plasmid, is transferred to the plant cell nucleus and integrated into the plant chromosome. *Agrobacterium tumefaciens* transfers the T-DNA into the nucleus of infected cells where it is then stably integrated into the host genome and transcribed, causing the crown gall disease[81, 82]. T-DNA contains two types of genes: the oncogenic genes, encoding for enzymes involved in the synthesis of auxins and cytokinins and responsible for tumor formation; and the genes encoding for the synthesis of opines.

Agrobacterium rhizogenes has been used regularly for gene transfer in many dicotyledonous plants[78]. Plant infection with this bacterium induces the formation of proliferative multibranched adventitious roots at the site of infection; the so-called 'hairy roots'[83]. This infection is followed by the transfer of a portion of DNA i.e. T-DNA, known as the root inducing plasmid (Ri-plasmid), to the plant cell chromosomal DNA. The research is going for the application of plant transformation and genetic modification using *A. rhizogenes*, in order to boost production of those secondary metabolites, which are naturally synthesized in the roots of the mother plant. Transformed hairy roots mimic the biochemical machinery present and active in the normal roots, and in many instances transformed hairy roots display higher product yields.

Genetic transformation has been reported for various medicinal plants. Naina *et al*. reported the successful regeneration of

transgenic neem plants (*Azadirachta indica*) using *Agrobacterium tumefaciens* containing a recombinant derivative of the plasmid pTi A6[84]. The genetic transformation of *Atropa belladona* has been reported using *Agrobacterium tumefaciens,* with an improved alkaloid composition[85, 86]. *Agrobacterium* mediated transformation of *Echinacea purpurea* has been demonstrated using leaf explants[87].

Genetic transformation would be a powerful tool for enhancing the productivity of novel secondary metabolites of limited yield. Hairy roots, transformed with *Agrobacterium rhizogenes*, have been found to be suitable for the production of secondary metabolites because of their stable and high productivity in hormone-free culture conditions. A number of plant species including many medicinal plants have been successfully transformed with *Agrobacterium rhizogenes*. The hairy root culture system of the medical plant *Artemisia annua* L. was established by infection with *Agrobacterium rhizogenes* and the optimum concentration of artimisin was 4.8 mg/L[88]. Giri *et al*. induced the development of hairy roots in *Aconitum heterophyllum* using *Agrobacterium rhizogenes*[89]. Pradel *et al*. developed a system for producing transformed plants from root explants of *Digitalis lanata*[64]. They evaluated different wild strains of *Agrobacterium rhizogenes* for the productions of secondary products obtained from hairy roots and transgenic plants. They reported higher amounts of anthraquinones and flavonoids in the transformed hairy roots than in untransformed roots. An efficient protocol for the development of transgenic opium poppy (*Papaver somniferum* L.) and California poppy (*Eschscholzia californica* Cham.) root cultures using *Agrobacterium rhizogenes* is reported[90]. Bonhomme *et al*. has reported the tropane alkaloid production by hairy roots of *Atropa belladonna* obtained after transformation with *Agrobacterium rhizogenes*[91]. Argolo *et al*. reported the regulation of solasodine production by *Agrobacterium rhizogenes*-transformed roots of *Solanum aviculare*[92]. Souret *et al*. have demonstrated that the transformed roots of *A. annua* are superior to whole plants in terms of yield of the sesquiterpene artemisinin[93]. Shi and Kintzios have reported the genetic transformation of *Pueraria phaseoloides* with *Agrobacterium rhizogenes* and puerarin production in hairy roots[94]. The content of puerarin in hairy roots reached a level of 1.2 mg/g dry weight and was 1.067 times the content in the roots of untransformed plants. Thus, these transformed hairy roots have great potential as a commercially viable source of secondary metabolites.

Conclusion

Plants have been an important source of medicine for thousands of years. Medicines in common use, such as aspirin and digitalis, are derived from plants, and new transgenic varieties could be created as efficient green production lines for other pharmaceuticals as well as vaccines and anticancer drugs. Tissue culture is useful for multiplying and conserving the species, which are difficult to regenerate by conventional methods and save them from extinction. The production of secondary metabolites can be enhanced using bioreactors. Bioreactors offer a great hope for the large-scale synthesis of therapeutically active compounds in medicinal plants. Since the biosynthetic efficiency of populations varies, a high yielding variety is recommended as a starting material. Genetic transformation may provide increased and efficient system for *in-vitro* production of secondary metabolites. The improved *in-vitro* plant cell culture systems have potential for commercial exploitation of secondary metabolites. Tissue culture protocols have been developed for several plants but there are many other species, which are over exploited in pharmaceutical industries and need conservation.

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