

Leading Article

Role of biotechnology in medicinal plants

Leena Tripathi* and Jaindra Nath Tripathi

International Institute of Tropical Agriculture (IITA), Nigeria; ^o/o L. W. Lambourn; Carolyn House, 26 Dingwall Rd, Croydon CR9 3EE, UK

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

*For correspondence: Tel: 234-2-241-2626; Fax: 234-2-241-2221; E-mail: l.tripathi@cgiar.org

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 sources¹⁻³.

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 medicines⁴. This can be achieved through different methods including micropropagation. Micropropagation has many advantages over conventional methods of vegetative propagation, which suffer from several limitations⁵. 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 reported⁶⁻⁸. Propagation from existing meristems yields plants that are genetically

identical with the donor plants⁹. 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 belladonna*¹⁶. Lal *et al.* observed a rapid proliferation rate in *Picrorhiza kurroa* using kinetin at 1.0–5.0 mg/l¹⁷. Direct plantlet regeneration from male inflorescences of medicinal yam on medium supplemented with 13.94 μ M kinetin has also been reported¹⁸. The highest shoot multiplication of *Nothapodytes foetida* is achieved on medium containing thidiazuron (TDZ) at a concentration of 2.2 μ M¹⁹. 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 proliferation²⁰⁻²³. 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 μ M NAA²⁴. According to Faria and Illg, the addition of 10 μ M BA along with 5 μ M indole-3-acetic acid (IAA) or 5 μ M 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 cytokinins²⁷. 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 photoperiod²⁸. 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 cooperi*³⁰. The relative importance of genotype, explant and their interactions for *in-vitro* plant regeneration via organogenesis in *Solanum melongena* has been investigated³¹. 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 BA³². 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 Das²⁰. 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 sulfate³⁴. 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 IAA²¹. 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 NAA³⁷.

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 μ M BA and 0.5 μ M IAA³². 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 kinetin³⁹. Embryogenic calluses and germination of somatic embryos in nine varieties of *Medicago sativa* has been achieved⁴⁰. 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 2.7 μM NAA⁴². 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 NAA⁴³. 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-D⁴⁴. Das *et al.* reported high frequency somatic embryogenesis in *Typhonium trilobatum* on medium containing 1.0 mg/L kinetin and 0.25 mg/L NAA⁴⁵. 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 BA⁴⁷.

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 regulator³⁸. 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 kinetin⁴⁹. Further, Kunitake and Mii reported that 30–40% of somatic embryos of *A. officinalis* germinated 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% sucrose⁵⁰. 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 culture⁴⁵.

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 ($-196\text{ }^{\circ}\text{C}$) in which cell division and metabolic and biochemical processes are arrested. A large number of cultured materials can be stored in liquid nitrogen⁵¹. Since whole plants can regenerate from frozen culture, cryopreservation 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 *Rauvolfia serpentina*, *D. lanalta*, *A. belladonna*, *Hyoscyamus* spp⁵².

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 Zenk⁵⁵. The accumulation of secondary products in plant cell cultures depends on the composition of the culture medium, and on environmental conditions⁵⁶. Strategies for improving secondary products in suspension cultures, using different media for different species, have been reported by Robins⁵⁷.

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 reported⁴⁶.

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 BAP⁶³. Pradel *et al.* observed that the biosynthesis of cardenolides was maximal in the hairy root cultures of *Digitalis lanata* compared to leaf⁶⁴. The production of azadirachtin and nimbin has been shown to be higher in cultured shoots and roots of *Azadirachta indica* compared to field grown plant⁶⁵. Pande *et al.* reported that the yield of lepidine from *Lepidium sativum* Linn depends upon the source and type of explants³⁷.

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 bioreactors⁶⁸. Ginseng root tissue cultures in a 20 tonne bioreactor produced 500 mg/L/day; of the saponin that is considered as a very good yield⁶⁹. Jeong *et al.* have established the mass production of transformed Panax ginseng hairy roots in bioreactor⁷⁰. 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 manufacture⁷². 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 preparations⁴. Conventional practices require the harvest of the bark of trees, all approximately 100 years old, to obtain 1 kg of the active compound taxol⁷³. 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 pharmaceutical⁷³.

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 methods⁷⁴. 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 rearrangement⁷⁵⁻⁷⁷. 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 plants⁷⁸⁻⁸⁰. 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,^{81, 82} causing the crown gall disease. 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 plants⁷⁸. 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 A6⁸⁴. The genetic transformation of *Atropa belladonna* has been reported using *Agrobacterium tumefaciens*, with an improved alkaloid composition^{85, 86}. *Agrobacterium* mediated transformation of *Echinacea purpurea* has been demonstrated using leaf explants⁸⁷.

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 artemisinin was 4.8 mg/L⁸⁸. 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 reported⁹⁰. 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 artemisinin⁹³. Shi and Kintzios have reported the genetic transformation of *Pueraria phaseoloides* with *Agrobacterium rhizogenes* and puerarin production in hairy roots⁹⁴. 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.

References

- Katzung BG. *Basic and Clinical Pharmacology*, ed 6, London: Prentice Hall International (UK) Limited, 1995.
- Pezzuto J. Taxol[®] production in plant cell culture comes of age. *Nature Biotechnol.* 1996; 14: 1083.
- Roberts MF. Medicinal products through plant biotechnology. In: Robins RJ, Rhodes MJC. (eds). *Manipulating Secondary Metabolism in Culture*. Cambridge: University Press, 1988 pp 201-16.
- Murch SJ, Krishna Raj S, Saxena PK. Tryptophan is a precursor for melatonin and serotonin biosynthesis in in-vitro regenerated St. John's wort (*Hypericum perforatum* L. cv. Anthos) plants. *Plant Cell Rep.* 2000; 19: 698-704.
- Nehra NS, Kartha KK. Meristem and shoot tip culture: requirements and applications. In: Vasil IK, Thorpe T (eds). *Plant Cell Tissue Culture*. Kluwer Academy, 1994 pp 37-70.
- Murashige T. The impact of plant tissue culture on agriculture. In: Thorpe TA (ed). *Frontiers of Plant Tissue Culture*, University Offset Printing Service, 1978, pp 15-26.
- Skirvin RM, Chu MC, Young HJ. Rose. In: Ammirato PV, Evans DR, Sharp WR, Bajaj YPS (eds). *Handbook of Plant Cell Cultures*, vol. 5, New York: MacMillan, 1990 pp 716-43.
- Withers LA, Anderson PG. *Plant Tissue Culture and its Agricultural Applications*. London: Butterworths, 1986.
- Hu CY, Wang PJ. Meristem, shoot-tip and bud culture. In: Evans DA, Wang WR, Ammirato PV, Yamada Y (eds). *Handbook of Plant Cell Culture*, vol 1. New York: MacMillan, 1983, pp 177-277.
- Paek KY, Yu KJ, Park SI, Sung NS, Park CH.. Micropropagation of *Rehmannia glutinosa* as medicinal plant by shoot tip and root segment culture. *Acta Horticult.* 1995; 390: 113-20.
- Roy SK, Hossain MZ, Islam MS. Mass propagation of *Rauvolfia serpentina* by in-vitro shoot tip culture. *Plant Tissue Cult* 1994; 4: 69-75.
- Perez-Bermudez P, Seitz HU, Gavidia I. A protocol for rapid micropropagation of endangered *Isoplexis*. *In-vitro Cell Dev Biol-Plant* 2002; 38: 178-82.
- Hussey G. In-vitro propagation. In: Ingram DS, Helgeson JP (eds). *Tissue Culture for Plant Pathologists*. Oxford: Blackwell Scientific, 1980, pp 51-61.
- Bhagyalakshmi N, Singh NS. Meristem culture and micropropagation of a variety of ginger (*Zingiber officinale* Rosc.) with a high yield of Oleoresin. *J Horticult Sci.* 1988; 63:321-7.
- Short KC, Roberts AV. *Rosa* spp (Roses): In-vitro culture, micropropagation and production of secondary products. In: Bajaj YPS (ed). *Biotechnology in Agriculture and Forestry, Medicinal and Aromatic Plants. III*, vol 15. Berlin: Springer Verlag, 1991, pp 376-97.
- Benjamin BD, Roja P, Heble MR, Chadha MS. Multiple shoot cultures of *Atropa belladonna*: effect of physicochemical factors on growth and alkaloid formation. *J Plant Nutr.* 1987; 129: 129-35.
- Lal N, Ahuja PS. Plantlet regeneration from callus in *Picrorhiza kurroa* Royle ex Benth. - An endangered medicinal plant. *Plant Tissue Cult.* 1996; 6: 127-34.
- Borthakur M, Singh RS. Direct plantlet regeneration from male inflorescences of medicinal yam (*Dioscorea floribunda* Mart. & Gal.). *In-vitro Cell Dev Biol-Plant.* 2002; 38: 183-5.
- Rai VR. Rapid clonal propagation of *Nothapodytes foetida* (wight) sleumer - a threatened medicinal tree. *In-vitro Cell Dev Biol-Plant.* 2002; 38: 347-51.
- Rout GR, Das P. In-vitro organogenesis in ginger (*Zingiber officinale* Rosc.). *J Herbs, Spices and Med Plants.* 1997; 4: 41-51.
- Rout GR, Saxena C, Samantaray S, Das P. Rapid clonal propagation of *Plumbago zeylanica* Linn. *Plant Growth Reg.* 1999; 28: 1-4.
- Shasany AK, Khanuja SPS, Dhawan S, Yadav U, Sharma S, Kumar S. High regenerative nature of *Mentha arvensis* internodes. *J Biosci.* 1998; 23: 641-6.
- Tsay HS, Gau TG, Chen CC. Rapid clonal propagation of *Pinellia ternate* by tissue culture. *Plant Cell Rep.* 1989; 8: 450-4.
- Barna KS, Wakhlu AK. Axillary shoot induction and plant regeneration in *Plantago ovata* Forssk.. *Plant Cell Tissue Organ Cult.* 1988; 15: 169-73.
- Faria RT, Illg RD. Micropropagation of *Zingiber spectabile* Griff. *Sci Horticult.* 1995; 62: 135-7.
- Mao AH, Wetten A, Fay M, Caligari PDS. In-vitro propagation of *Clerodendrum colebrookianum* Walp: a potential natural anti-hypertension medicinal plant. *Plant Cell Rep.* 1995; 14: 493-6.
- Satheesh Kumar K, Bhavanandan KV. Micropropagation of *Plumbago rosea* Linn. *Plant Cell Tissue Organ Cult.* 1988; 15: 275-8.
- Mantell SH, Hugo SA. Effects of photoperiod, mineral medium strength, inorganic ammonium, sucrose and cytokinin on root, shoot and microtuber development in shoot cultures of *Dioscorea alata* L. and *D. bulbifera* L. yams. *Plant Cell Tissue Organ Cult.* 1989; 16: 23-37.
- Rout GR, Mallick UC, Das P. In-vitro plant regeneration from leaf callus of *Cephaelis ipecacuanha* A. *Adv Plant Sci* 1992; 5: 608-13.
- Ghosh BE, Sen S. Micropropagation of *Asparagus cooperi* as affected by growth regulators. *Biologia Plantarum.* 1994; 36: 527-34.

31. Sharma P, Rajam MV. Genotype, explant and position effects on organogenesis and somatic embryogenesis in eggplant (*Solanum melongena* L.). *J Exp Botany*. 1995; 46: 135-41.
32. Basu P, Chand, S. Regeneration of plantlets from root-derived callus of Egyptian Henbane. *Cell Chromosome Res*. 1996; 19: 31-4.
33. Saxena C, Palai SK, Samantaray S, Rout GR, Das P. Plant regeneration from callus cultures of *Psoralea corylifolia* Linn. *Plant Growth Reg*. 1997; 22: 13-7.
34. Patra A, Rai B. Successful plant regeneration from callus cultures of *Centella asiatica* (Linn.) urban. *Plant Growth Reg*. 1998; 24: 13-6.
35. Okslar V, Strukej B, Kreft S, Bohanec B, Zel J. Micropropagation and hairy root culture of *Solanum laciniatum* Ait. *In-vitro Cell Dev Biol-Plant*. 2002; 38: 352-7.
36. Koroch AR, Kapteyn J, Juliani HR, Simon JE. In-vitro regeneration of *Echinacea pallida* from leaf explants. *In-vitro Cell Dev Biol-Plant*. 2003; 39: 415-8.
37. Pande D, Malik S, Bora M, Srivastava PS. Rapid protocol for in-vitro micropropagation of *Lepidium sativum* Linn and enhancement in the yield of lepidine. *In-vitro Cell Dev Biol-Plant*. 2002; 38: 451-5.
38. Arumugam N, Bhojwani SS. Somatic embryogenesis in tissue cultures of *Podophyllum hexandrum*. *Can J Botany*. 1990; 68: 487-91.
39. Ghosh BE, Sen S. Plant regeneration through somatic embryogenesis from spear callus culture of *Asparagus cooperi* Baker. *Plant Cell Rep*. 1991; 9: 667-70.
40. Fuentes SI, Suarez R, Villegas T, Acero LC, Hernandez G. Embryogenic response of Mexican alfalfa (*Medicago sativa*) varieties. *Plant Cell Tissue Organ Cult*. 1993; 34: 299-302.
41. Zhou J, Ma H, Guo F, Luo X. Effects of thidiazuron on somatic embryogenesis of *Cayratia japonica*. *Plant Cell Tissue Organ Cult*. 1994; 36: 73-9.
42. Rout GR, Samantaray S. Somatic embryogenesis and plant regeneration from callus culture of *Acacia catechu* - a multipurpose leguminous tree. *Plant Cell Tissue Organ Cult*. 1995; 42: 283-5.
43. Gastaldo P, Caviglia AM. Somatic embryogenesis and esculin formation in calli and embryoids from bark explants of *Aesculus hippocastanum* L. *Plant Sci Shannon*. 1996; 119: 157-62.
44. Choi YE, Kim JW, Soh WY. Somatic embryogenesis and plant regeneration from suspension cultures of *Acanthopanax koreanum*. *Plant Cell Rep*. 1997; 17: 84-8.
45. Das P, Palai, SK, Patra A, Samantaray YS, Rout GR. In-vitro Somatic Embryogenesis in *Typhonium trilobatum* Schott. *Plant Growth Reg*. 1999; 27: 95-199.
46. Zhao J, Zhu WH, Hu Q, Guo YQ. Compact callus cluster suspension cultures of *Catharanthus roseus* with enhanced indole alkaloid biosynthesis. *In-vitro Cell Dev Biol-Plant*. 2001; 37: 68-72.
47. Chand S, Sahrawat K. Somatic embryogenesis and plant regeneration from root segments of *Psoralea corylifolia* L., an endangered medicinally important plant. *In-vitro Cell Dev Biol-Plant*. 2002; 38: 33-8.
48. Kumar A. Somatic embryogenesis and high frequency plantlet regeneration in callus cultures of *Thevetia peruviana*. *Plant Cell Tissue Organ Cult*. 1992; 31: 47-50.
49. Waklu AK, Nagari S, Barna KS. Somatic embryogenesis and plant regeneration from callus cultures of *Bunium persicum* Boiss. *Plant Cell Rep*. 1990; 9: 137-8.
50. Kunitake H, Mii M. Somatic embryogenesis and its application for breeding and micropropagation in *Asparagus* (*Asparagus officinalis* L.). *Plant Biotechnol*. 1997; 15: 51-63.
51. Bajaj YPS. *Biotechnology in Agriculture and Forestry 15. Medicinal and aromatic Plants III*. Berlin: Springer-Verlag, 1991.
52. Bajaj YPS. Cryopreservation and the retention of biosynthetic potential in cell cultures of medicinal and alkaloid-producing plants. In: Bajaj YPS (ed). *Biotechnology in Agriculture and Forestry; Medicinal and Aromatic Plants I*, vol 4. Berlin: Springer-Verlag, 1988.
53. Barz W, Ellis B. Potential of plant cell cultures for pharmaceutical production. In: Beal JL, Reinhard E (eds.) *Natural Products as Medicinal Agents*. Stuttgart, Hippokrates, 1981, pp 471-507.
54. Deus B, Zenk MH. Exploitation of plant cells for the production of natural compounds. *Biotechnol Bioeng*. 1982; 24: 1965-74.
55. Zenk MH. The impact of plant cell culture on industry. In: Thorpe TA (ed). *Frontiers of Plant Tissue Culture*, University of Calgary, Int Ass for Plant Tissue Culture, 1978, pp 1-13.
56. Stafford A, Morris P, Fowler MW. *Plant Cell Biotechnology: A perspective*. Enzyme Microbial Tech. 1986; 8: 578-97.
57. Robins RI. Secondary products from cultured cells and organs: Molecular and cellular approaches. In: Dixon RA, Gonzales RA (eds). *Plant Cell Culture*. Oxford: IRL Press, 1994.
58. Nigra HM, Caso OH, Guilietti AM. Production of solasodine by calli form different parts of *Solanum eleagnifolium* Cav. *Plants. Plant Cell Rep*. 1987; 6: 135-7.
59. Toppel G, Witte L, Riebesehl B, Von Borstel K, Hartman T. Alkaloid patterns and biosynthetic capacity of root cultures from some pyrrolizidine alkaloid producing *Senecio* spp. *Plant Cell Rep*. 1987; 6: 466-9.
60. Jha S, Sahu NP, Mahato SB. Production of the alkaloids emetine and cephaeline in callus

- cultures of *Cephaelis ipecacuanha*. *Planta Medica*. 1988; 54: 504-6.
61. Scragg AH. Bioreactors for mass cultivation of plant cells. In: Fowler MW, Warren GS (eds). *Plant Biotechnol.* Oxford: Peragmon Press, 1992.
 62. Ravishankar GA, Grewal S. Development of media for growth of *Dioscorea deltoidea* cells and in-vitrodiosgenin production: Influence of media constituents and nutrient stress. *Biotechnol Lett.* 1991; 13: 125-30.
 63. Parisi M, Moreno S, Fernandez G. Characterization of a novel cysteine peptidase from tissue culture of garlic (*Allium sativum* L.). *In-vitro Cell Dev Biol-Plant.* 2002; 38: 608-12.
 64. Pradel H, Dumkelehmman U, Dietrich B, Luckner M. Hairy root cultures of *Digitalis lanata*. Secondary metabolism and plant regeneration. *J Plant Physiol.* 1997; 151: 209-15.
 65. Srividya N, Devi BPS. Azadirachtin and nimbin content in in-vitrocultured shoots and roots of *Azadirachta indica* A. Juss. *Indian J Plant Physiol.* 1998; 3: 129-34.
 66. Levin R, Gaba V, Tal B, Hirsch S, De Nola D, Vasil K. Automated plant tissue culture for mass propagation. *Biotechnology* 1988; 6:1035-1040.
 67. Preil W, Florek P, Wix U, Beck A. Towards mass propagation by use of bioreactors. *Acta Horticult.* 1988; 226: 99-105.
 68. Park JM, Yoon SY. Production of sanguinarine by suspension culture of *Papaver somniferum* in bioreactors. *J Ferm Bioeng.* 1992; 74: 292-6.
 69. Charlwood BV, Charlwood KA. Terpenoid production in plant cell cultures. In: Harbourne JB, Tomas-Barberan FA (eds). *Ecological Chemistry and Biochemistry of Plant Terpenoids*, Clarendon Press, Oxford, 1991, pp 95-132.
 70. Jeong GT, Park DH, Hwang B, Park K, Kim SW, Woo JC. Studies on mass production of transformed *Panax ginseng* hairy roots in bioreactor. *App Biochem Biotechnol.* 2002; 98: 1115-27.
 71. Hahn EJ, Kim YS, Yu KW, Jeong CS, Paek KY. Adventitious root cultures of *Panax ginseng* c.v. meyer and ginsenoside production through large-scale bioreactor system. *J Plant Biotechnol.* 2003; 5: 1-6.
 72. Kieran PM, MacLoughlin PF, Malone DM. Plant cell suspension cultures: some engineering considerations. *J Biotechnol.* 1997; 59: 39-52.
 73. Mühlbach H. Use of plant cell cultures in biotechnology. *Biotech Ann Rev.* 1998; 4: 113-71.
 74. Birch RG. Plant transformation: Problems and strategies for practical application. *Ann Rev Plant Physiol Plant Mol Biol.* 1997; 48: 297-326.
 75. Gheysen G, Angenon G, Montague MV. Agrobacterium-mediated plant transformation: a scientifically intriguing story with significant application. In: Lindsey K (ed). *Transgenic Plant Research*. Netherlands: Harwood Academic Press, 1998, pp1-33.
 76. Hansen G, Wright MS. Recent advances in transformation of plants. *Trends Plant Sci.* 1999; 4: 226-31.
 77. Shibata D, Liu YG. Agrobacterium-mediated plant transformation with large DNA fragments. *Trends Plant Sci.* 2000; 5: 354-7.
 78. Tepfer D. Genetic transformation using *Agrobacterium rhizogenes*. *Physiol Plant.* 1990; 79: 140-6.
 79. Hiei Y, Ohte S, Komari T, Kumashiro T. Efficient transformation of rice mediated by *Agrobacterium* and sequence analysis of the boundaries of the T-DNA. *Plant J.* 1994; 6: 271-82.
 80. Ishida Y, Saito H, Ohta S, Hiei Y, Komari T, Kumashiro T. High frequency transformation of maize (*Zea mays*) mediated by *Agrobacterium tumefaciens*. *Nature Biotechnol.* 1996; 14: 745-50.
 81. Nester EW, Gordon MP, Amasino RM, Yanofsky MF. Crown gall: a molecular and physiological analysis. *Ann Rev Plant Physiol.* 1984; 35: 387-413.
 82. Binns AN, Thomashow MF. Cell biology of *Agrobacterium* infection and transformation of plants. *Ann Rev Microbiol.* 1988; 42: 575-606.
 83. Chilton MD, Tepfer D, Petit A, David C, Casse-Delbart FT. *Agrobacterium rhizogenes* inserts T-DNA into the genome of the host plant root cells. *Nature.* 1982; 295: 432-4.
 84. Naina NS, Gupta PK, Mascarenhas AF. Genetic transformation and regeneration of transgenic neem (*Azadirachta indica*) plants using *Agrobacterium tumefaciens*. *Curr Sci.* 1989; 58: 184-7.
 85. Yun DJ, Hashimoto T, Yamada Y. Metabolic engineering of medicinal plants: transgenic *Atropa belladonna* with an improved alkaloid composition. *Proc Nat Acad Sci USA.* 1992; 89: 11799-803.
 86. Cucu N, Gabriela, Gavrila L. Genetically modified medicinal plants. II. Transfer and expression of a marker kanamycine resistance gene in *Atropa belladonna* plants. *Rom Biotechnol Lett.* 2002; 7: 869-74.
 87. Koroch AR, Kapteyn J, Juliani HR, Simon JE. In-vitroregeneration and *Agrobacterium* transformation of *Echinecea purpuria* leaf explants. *Trends New Crop New News*, 2002.
 88. Cai G, Li G, Ye H, Li G. Hairy root culture of *Artemisia annua* L. by Ri plasmid transformation and biosynthesis of artemisinin. *Chin J Biotechnol.* 1995; 11: 227-35.
 89. Giri A, Banerjee S, Ahuja PS, Giri CC. Production of hairy roots in *Aconitum heterophyllum*

- Wall. using *Agrobacterium rhizogenes*. *In-vitro Cell Dev Biol- Plant*. 1997; 33: 293-306.
90. Park SU, Facchini PJ. *Agrobacterium rhizogenes*-mediated transformation of opium poppy, *Papaver somniferum* L., and California poppy, *Eschscholzia californica* cham, root cultures. *J Exp Botany*. 2000; 51: 1005-16.
91. Bonhomme V, Laurain-Mattar D, Lacoux J, Fliniaux M, Jacquin-Dubreuil A. Tropane alkaloid production by hairy roots of *Atropa belladonna* obtained after transformation with *Agrobacterium rhizogenes* 15834 and *Agrobacterium tumefaciens* containing rol A, B, C genes only. *J Biotechnol*. 2000; 81:151-8.
92. Argolo AC, Charlwood BV, Pletsch M. The regulation of solasodine production by *Agrobacterium rhizogenes*-transformed roots of *Solanum aviculare*. *Planta Medica*. 2000; 66: 448-51.
93. Souret FF, Weathers PJ, Wobbe KK. The mevalonate-independent pathway is expressed in transformed roots of *Artemisia annua* and regulated by light and culture age. *In-vitro Cell Dev Biol-Plant*. 2002; 38: 581-8.
94. Shi HP, Kintzios S. Genetic transformation of *Pueraria phaseoloides* with *Agrobacterium rhizogenes* and puerarin production in hairy roots. *Plant Cell Rep*. 2003; 21: 1103-7.