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Review: Biotechnological strategies for conservation of rare and endangered medicinal plants

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ABSTRACT

Rai MK (2010) Review: Biotechnological strategies for conservation of rare and endangered medicinal plants. Biodiversitas 11: 157-166. The use of medicinal plants is as old as human civilization. The biotechnological tools play a crucial role in conservation of rare and endangered medicinal plants. The rapid depletion of plant genetic diversity has made essential to develop new *in situ* and *ex situ* conservation methods. Advances in biotechnology offer new methods for conservation of rare and endangered medicinal plants. The present review is focused on biotechnological tools like *in vitro* culture, micropropagation, mycorrhization, genetic transformation and development of DNA banks. These are imperative and important alternatives for the conservation of rare and endangered medicinal plants.

Key words: biotechnological strategies, DNA banks, medicinal plants, transformation, micropropagation, mycorrhization.

INTRODUCTION

Since the beginning of the civilization medicinal plants have been important resource without which the survival of mankind has not been possible. According to World Health Organization (WHO) up to 80% of people still rely mainly on traditional knowledge-based remedies. As a matter of fact, plants are the main source of modern medicines. It is estimated that 25% medicines are still obtained from the plants (Tripathi and Tripathi 2003). The famous Indian herbal therapy 'Ayurveda' is based mainly on herbal system. India is home to a great variety of medicinal plants, and is ranked sixth among 12 hotspots of mega diversity countries of the world. The Himalayas is designated as one of the global biodiversity hotspots. Unfortunately due to over exploitation, habitat loss and non-judicious use, many species of medicinal plants have become rare, threatened or endangered. In addition to this, the medicinal plants are highly affected by climate change, such as: increase in carbon dioxide concentration which favors C3 plants over C4 plant, increase in diseases and pest, high rain fall and high salt content in soil etc.

In article 8 of The Convention on Biological Diversity (CBD) emphasized on the fundamental requisite of *in situ* conservation of ecosystems and natural habitats. All over the world, the protected areas are the most widely accepted and practically approachable to biodiversity conservation. There are two methods of conservation of medicinal plants: (i) *ex situ* conservation, and (ii) *in situ* conservation, but these techniques are natural and time-consuming. Tripathi and Tripathi (2003) stated that biotechnological tools are important for multiplication and conservation of the critical genotypes of medicinal plants. Therefore, biotechnological techniques can be applied for the conservation of rare and endangered medicinal plants. Biotechnological approaches are imperative for rapid multiplication and genetic improvement of medicinal plants. These include: (i) Micropropagation (ii) Mycorrhization (iii) Genetic transformation, and (iv) Development of the DNA banks.

O'Gara (1996) stated that the in the process of development of sustainable plant production the use of microbial inoculants as substitution for chemical fertilizers and pesticides is getting attention. It is believed that delivery of microbial inoculants via micropropagation is one of the solutions to this problem. In micropropagation practices, the growth substrate lacks microbe and, as a result of this nutrient-rich growth substrate, delicate plants having no interaction with other microorganisms are produced (Dolcet-Sanjuan et al. 1996). Micropropagation is an important technique for the production of elite plants, but due to transient transplantation shock plants require bio- hardening before transplantation. For this reason, mycorrhizal technology can be applied.

Inoculation of arbuscular mycorrhizal fungi (AMF) into the roots of micropropagated plantlets plays a advantageous role (Blal et al. 1990; Schubert et al. 1990; Azcon-Aguilar et al. 1994; Declerck et al. 1994; Varma and Schuepp 1994a,b; Gribaudo et al. 1996; Martins et al. 1996; Vestberg and Uosukaninen 1996; Budi et al. 1998; Naqvi and Mukerji 1998; Gange and Ayres 1999; Vosatka et al. 1999; Sylvia et al. 2003; Voets et al. 2005; Chandra et al. 2010). There are excellent reviews on micropropagation and mycorrhization by Conner and Thomas (1981), Vestberg and Estaun (1994), Varma and Schuepp (1995) and Lovato et al. (1996) and Rai (2001).

This article is focused at biotechnological strategies for conservation of rare and endangered medicinal plants particularly on micropropagation and mycorrhization.

MICROPROPAGATION (*IN VITRO* REGENERATION)

Micropropagation is the technique of *in vitro* multiplication of large number of plants from its part, whether it is leaves, seeds, nodes and tubers etc. *In vitro* propagation is used for the production and multiplication of novel plants, which are genetically similar and virus free. Micropropagation has been proved as an important technique for the multiplication of plants in a large scale. Usually, micropropagation is carried out in two ways: direct and indirect. Callus production from explant followed by shoots and roots is one method, while direct shooting on the auxiliary explant followed by rooting is another method. The concept behind the *in vitro* regeneration is that from the single explant the development of whole plantlet under controlled conditions can be obtained, and thereafter its acclimatization followed by transfer in the field can be achieved. Gottlieb Heberlandt (1854-1945) cultivated plant tissues in culture *in vitro*. He is regarded as father of plant tissue culture (Chawla 2002). Later on, Murashige and Skoog (1962) developed basal medium in which major and minor elements, vitamins, amino and iron sources were considered for the growth of the plant besides the temperature and humidity.

Micropropagation, the application of tissue culture for efficient clonal plant production has been used commercially since the 1960s and is possibly the oldest example of commercial Biotechnology. In the United States, the micropropagation industry has been developed primarily to provide service the temperate and tropical ornamental plant industry. Production of elite stock plants for small fruit and vegetable crops is a secondary area of activity. It should also be applied for the conservation of rare and endangered medicinal plants.

The technique of micropropagation is applied with the objective of enhancing the rate of multiplication. Through tissue culture over a million plants can be grown from small piece of plant tissue within 12 months. Such a prolific rate of multiplication cannot be expected by any of the *in vivo* methods of clonal propagation. Another advantage in propagation through tissue culture is that shoot multiplication cannot be expected by any of the *in vivo* methods of clonal propagation. Moreover, the shoot multiplication usually has a short cycle, results in logarithmic increase in the number of shoots. Tissue culture provides propagules such as minitubers or minicorms for plant multiplication throughout the year irrespective of the season. Using this method stock of germplasm can be maintained for many years. Clonal propagation *in vitro* appears to have permanent advantages in case in which serious problem occur. This is because of the fact that through *in vitro* methods more pathogen free plants can be raised and maintained economically.

Three examples of *in vitro* propagation are *Gloriosa superba* L, *Rauwolfia serpentina* L. Benth. Ex. Kurz. and *Buchanania lanzan* Spreng.

Gloriosa superba L.

Gloriosa superba (Colchicaceae) also known as Malabar glory lily or 'Kembang telang' (Java, Indonesia) is a perennial tuberous climbing herb, widely scattered in the tropical and sub-tropical parts of the India, including the foothills of Himalayas. It is also called as 'Mauve beauty', 'Purple prince', 'Modest', 'Orange gem', 'Salman glow' and 'Orange glow'. It is adapted to different soil texture and climatic conditions. The plant grows in sandy-loam soil in the mixed deciduous forest in sunny weather. It occurs in thickets, forest edges and boundaries of cultivated areas in warm countries upto height of 2530 m. (Neuwinger 1994). *G. superba* is a inhabitant of tropical Africa and now found growing naturally in many countries of tropical Asia including Bangladesh, India, Sri Lanka, Indonesia, Malaysia and Myanmar. In India, it occurs commonly in tropical forests of Bengal and Karnataka (Sivakumar and Krishnamurthy 2002). The plants thrive from the arid Bundelkhand to humid Assam valley.

It is known by different names in India such as 'Kalihari', 'Agnishikha', 'Languliata and 'Nangulika'. Different species of *Gloriosa* includes *G. superba*, *G. abyssinica*, *G. carsonii*, *G. simplex*, *G. grandiflora*, *G. minor*, *G. magnifica*, *G. lutea*, *G. plantii*, G. *latifolia*, *G. longifolia*, *G. rothschildiana*, *G. virescens*, *G. sudanica*, *G. lutea* and *G. baudii*. *Gloriosa superba* is also known as the national flower of Zimbabwe. Except diverse pharmaceutical products and other therapeutic preparations, it is also a popular plant for providing color in greenhouse and conservatories even immature flowers are gorgeous to behold (Kranse 1986; Ghani 2000).

G. superba is a semi-woody herbaceous branched climber, reaching just about five meters in height. One to four stems arise from a single V-shaped fleshy cylindrical tuber. *G. superba* is an essential medicinal plant because all parts are used in the medicine, which contains two important alkaloid, colchicine and colchicoside; leaves are used to treat cancer related diseases, also in ulcer, piles, scrofula (Evans et al. 1981).

Usually *G. superba* is multiplied by corm and seeds but due to low germination capability it restricts for the regeneration. Consequently, in order to safeguard and conserve this important plant, biotechnological approaches would be very useful (Sivakumar and Krishnamurthy 2002). The conventional method of propagation has many drawbacks as 50% of the yield has to be set aside for raising the next crop, transmittance of soil-borne diseases from one crop to the next, and from one location to another and during the 2-3 month storage period between harvest and the raising of next crop (Mrudul et al. 2001). Hassan and Roy (2005) reported 92% of the cultures of apical and axillary buds of young sprout from naturally grown *G. superba* plants regenerate four shoots per culture in MS basal medium fortified with 1.5 mg/L BA + 0.5 mg/L NAA. Custers and Bergervoet (1994) reported tissue culture of *G. superba* by shoot cuttings and explants from node, internodes, leaves, flowers, pedicels and tubers. *G. rothschildiana* and *G. superba* were cultured on MS basal medium with 3% w/v sucrose, 0-10 mg/L Benzyl Adenine (BA) and 0.1 mg Indole Acetic Acid (IAA) and maintained for 24 days under 16 hours photoperiod. Addition of low level of Benzyl Adenine (BA) (1 mg/L) improved plant growth, whereas the high level of BA (10

mg/L) caused proliferation of multiple shoots, from rhizome meristem, by applying alternatively high and low BA level, a method of continued propagation was achieved which resulted in a 4-7 fold multiplication of qualitatively good plantlets every 18 weeks. The resulting shoots were incubated on MS medium, with 3% sucrose and 0-1 mg/L IAA or NAA. Transplantation into soil was only possible after the plants had formed.

In 1993, Samarajeewa and group studied clonal propagation of *G. superba* from apical bud and node segment of shoot tip, cultured on solidified agar (0.8% w/v) Gamborg's B_5 medium containing BA, IAA, Kinetin, NAA, IBA or 2,4-D. The cultures were maintained under fluorescent light at 25-27ºC. Primary cultures were initiated on solid B_5 medium containing 0.5 to 1 mg/L BA and 0.01-0.5 mg/L IAA, IBA, NAA when shoot tip of primary cultures were transferred to shoot multiplication media, shoot proliferation occurred via adventitious bud formation within 4-8 weeks. *In vitro* propagation and corm formation in *G. superba* was reported by Somani et al. (1989). The fresh sprouts were excised from corms of *G. superba* and dissected propagules with shoot and root primordia were placed on MS basal medium containing 3% sucrose and 0.6% agar. Explant germinated on the MS medium producing shoot and root, which formed new corm within one month. For shoot and cormlet regeneration, 1-4 mg/L kinetin was added to the medium. Cultures were maintained at 25ºC in white fluorescent light (2500 lux) with an 8-h/day photoperiod. Sivakumar and Krishnamurthy (2002) reported *in vitro* organogenetic responses of *G. superba.* They used MS medium supplemented with Adenine sulfate) ADS and BA, 98%. The callus induction occurred in non-dormant corm bud explants. The maximum number of multiple shoot (57%) was observed in corm-derived calluses.

Sivakumar and Krishnamurthy (2002, 2004) studied induction of embryoids from leaf tissue of *G. superba*. The nodular calli were observed on Schenk and Hildebrandt (SH) basal medium supplemented with 2, 4-D and 1 isopentyldene. Jha et al. (2005) reported production of forskolin, with anolides, colchicine and tylophorine from plant source by using biotechnological approach.

G. superba is a commercially important medicinal plant which has diverse medicinal applications and eventually due to over-exploitation this plant is facing local extinction in India. It has been affirmed as endangered plant by IUCN and hence there is a pressing need to conserve the plant by *in situ* and *ex situ* multiplication in general and micropropagation and mycorrhization in particular so as to meet the ever-increasing demand from the industries.

Much research has not been carried out on *Gloriosa* due to some problems. Basically the plant is monocot having very low germination capacity (0.001%), and life span is also very short just 2-3 months, conventional propagation is very limited and also slow since one tuber produce only one plant at a time, besides this the plant is the richest source of colchicine, the high priced alkaloid (USD 3600/100 g) along with gloriosin and colchicoside, which has very high demand in pharmacological companies from all over the world. The whole plant is used for the medicinal purposes. Due to overexploitation by the local people his plant is endangered, that is why it is the pressing need to conserve this important medicinal plant.

There are many contributions on micropropagation and secondary metabolites production of *G. superba* (Sivakumar and Krishnamurthy 2004; Jha et al. 2005; Ade and Rai 2009). The research on micropropagation of *G. superba* is in progress in Department. of Biotechnology of SGB Amravati University, Amravati, Maharashtra State, India. We have developed effective protocol and standardized the optimum culture conditions. Moreover, biohardening was done by delivery of mycorrhizal propagues into the roots of the plantlets of *G. superba*. Encouragingly, 90% survival of the plantlets was observed.

***Rauwolfia serpentina* (L.) Benth. ex Kurz.**

Rauwolfia serpentina belongs to Family Apocynaceae and commonly known as 'Sarpagandha' or 'Pulai pandak' (Indonesia). It is a woody perennial shrub and also known as 'Chota chand' and 'Chandrika'. In traditional and ayurvedic medicine, the plant is used for mental disorders, epilepsy and also for sleeplessness.

The plant is grown by means of vegetative propagation using cuttings. The germination of the seeds is poor due to presence of cinnamic acid and its derivatives (Sahu 1979). *R. serpentina* has been overexploited by local people, pharmaceuticals and government agencies of India, and thus it is endangered medicinal plant. Therefore, micropropagation of this plant has been need of the hour. Bhatt et al. (2008) developed protocol for micropropagation of *R. serpentina*. They used shoots and leaves as explant and grew on MS medium supplemented with 2,4-Dicholorophenoxyacetic acid (2,4-D) plus 2-benzyl amino purine (BAP) and Indole-3 butyric acid. They found induction of callus from leaf and stem tissues. The authors reported that combination of IBA (0.125 mg/ml) plus BAP (1.0 mg/L) demonstrated better results.

Boke (2004) biohardened the micropropagated plantlets by using arbuscular mycorrhizal fungi. Two mixtures i.e. sand: soil: cow-dung 1: 1: 1 and sand: soil: vermicompost 1: 1: 1 was taken because it gave best survival rate and then they were combined with *Glomus macrocarpum* and mixture of *Glomus* species. The survival was 98% after 30 days, 93% after 60 days and 85% after 90 days on the mixture of sand: soil: vermicompost in combination with mixture of *Glomus* species containing *G. mosseae, G. fasciculatum* and *G. geosporum* and second combination that resulted best was sand: soil: cow-dung in combination with mixture of *Glomus* species containing *G. mosseae, G. fasciculatum* and *G. geosporum* where 84% survival was observed after 30 days, 60% after 60 days and 55% after 90 days. Consequently, it can be said that sand: soil: vermicompost + mixture of *Glomus* species was the best for biohardening of *in vitro* grown plantlets of *R. serpentina*.

***Buchanania lanzan* Spreng.**

Buchanania lanzan Spreng (Chironji) (Anacardiaceae family) is a commercially useful tree species all over the greater part of India. It is a vulnerable medicinal plant. The seeds are used as expectorant and energizer. The oil extracted from kernels is applied on skin diseases and also

to remove spots and blemishes from the face. The root is used as expectorant, in biliousness and also for curing blood diseases. The juice of the leaves is digestive, expectorant, aphrodisiac and purgative. The gum after mixing with goat milk is used for treating pains. The tribal people collect the fruits of this tree to earn their livelihood, through its sale and are consequently overexploited. During the recent past, due to excessive felling of trees and overgrazing, considerable reduction in the population of the *B. lanzan* in the forest and non-forest areas has been observed (Singh et al. 2002).

There is a problem in the regeneration of *B. lanzan* due to association of fungi with seeds. These fungi include *Alternaria alternata* (Pr.) Keissler, *Aspergillus flavus* Link, *A. ochraceus* Withelm, *A. niger* Van Tiegh, *A. aculeatus* Lizuka, *A. funiculosus* Smith, *Cladosporium* Link ex Fr, *Chaetomium globosum* Kunze and Schum., *Curvularia lunata* (Wakker) Boedijn, *Fusarium moniliforme* var. *subglutinans* Wr. and Rg, *F. semitectum* Berk and Rav, *Macrophomina phaseolina* Ashby, *Mucor varians* Povah, *Penicillium citrinum* Thom, *Trichothecium roseum* Link, *Rhizopus arrhizus* and *Verticillium* species (Sharma et al. 1998). The presence of hard seed coat is another inherent problem which leads to low germinating capability. We have developed technique for the rapid clonal multiplication and establishment of a gene bank *in vitro* (Shende and Rai 2005). The decoated seeds of *B. lanzan* were cultured in MS medium enriched with different auxins and cytokinins alone or in combination. MS medium supplemented with BAP 22.2 µM and NAA 5.37 µM promoted formation of the maximum number of shoots as compared to BAP and IBA. BAP and NAA were found to be superior as compared to BAP and IBA combinations. MS medium with kinetin 23.3 µM induced profuse rooting of the initiated multiple shootlets.

MYCORRHIZATION

Inoculation of mycorrhizal fungi into the roots of plants is referred as mycorrhization. Mycorrhizal fungi are of two kinds: Arbuscular Mycorrhizal Fungi (AMF) and Ectomycorrhizal Fungi. Usually, AMF are used for inoculation in medicinal plants. These are symbiotic fungi and occur in 90% of the plants (Williams et al. 1994). The AMF helps the plant partner by increasing uptake of nutrients in general, and phosphorus in particular (Vestberg and Estaun 1994). The diverse role played by AMF has been extensively studied (Gianinazzi et al. 1990; Ponton et al. 1990; Cargeeg 1992; Arias and Cargeeg 1992; Varma and Schuepp 1995; Lovato et al. 1996; Hindav et al. 1998; Sylvia et al. 2003; Voets et al. 2005; Chandra et al. 2010). AMF reduces the stress and promotes the plant growth for better survival. Associated with nutrition, water/aeration, soil structure, pH, salt, toxic metals, and pathogens (Sylvia and Williams 1992). Vestberg and Estaun (1994) published an admirable review on factors affecting the result of mycorrhizal inoculation. The potential for biocontrol of plant diseases by AM and ectomycorrhizal fungi was reviewed by Linderman (1994) and Duchesne (1994), respectively. The need for mycorrhization and the different role played by AMF was discussed at length by Varma and Schuepp (1995). Lovato et al. (1996) reported different roles played by AMF as bioregulators, bioprotectors, biofertilizers, and stressed on mycorrhizal inoculation of tissue-culture-raised plantlets.

Delivery of mycorrhizal propagules

Colonization of AMF in seedlings grown on agar medium is well experimented (Hayman 1983). AMF establishes symbiosis with root organ culture (Becard and Fortin 1988; Gemma and Koske 1988; Becard and Piche 1989; Chabot et al. 1992a,b; Elmeskaoui et al. 1995; Declerck et al. 1996a, 1998; Pawlowska et al. 1999; Sylvia et al. 2003; Voets et al. 2005; Chandra et al, 2010). In 1994, Vestberg and Estaun emphasized on development of inoculation protocol for each plant species.

In 1986, Trouvelot and his colleagues reported that the inoculation techniques differ depending on the substratum or the nature of the inoculum used. Selection of quantity (Morandi et al. 1979; Daniels et al. 1981; Ravolanirina et al. 1989; Guillemin et al. 1992; Morte et al. 1996) and quality of inoculum is an important point both for *in vitro* and *in vivo* inoculation (Vestberg and Uosukainen 1996). The inoculums should not only be pure, but also be able to exhibit the desired effect on the host-partner. It is noteworthy that hyphae, spores, chlamydospores and mycorrhizal roots have been used as inocula in various *in vitro* and *in vivo* studies.

The sterilization of AMF inoculum is an important part of successful inoculation programs. Healthy spores can be easily separated from the old and deteriorated spores by centrifugation (Furlan et al. 1980). Only the healthy inoculum should be selected for surface sterilization. There are many procedures used for surface sterilization of inocula for establishing *in vitro* symbiosis on agar culture (Tommerup and Kidby 1980; Macdonald 1981; Strullu and Romand 1986; Becard and Piche 1992). Becard and Piche (1992) suggested a method, which is widely used. The mixture of antibacterial agents, such as streptomycin (200 mg) and gentamicin (100 mg), is used for sterilization of spores followed by four rinses (Becard and Piche 1992). The pregerminated spores or pieces of mycorrhizal roots showing hyphal growth are used to inoculate the sterile root system by placing them close to emerging lateral roots. Since, the MS (Murashige and Skoog 1962) rooting medium contains these elements at high concentrations, a change of medium is essential before the tripartite culture stage (Elmeskaoui et al. 1995). Axenically infected mycorrhizal roots can also be used as inoculum to overcome the problem of contamination (Elmeskaoui et al. 1995; Declerck et al. 1996a,b, 1998, 2000; Plenchette et al. 1996). A reliable technique to establish arbuscular mycorrhizal symbiosis in micropropagated plantlets has been developed (Declerck et al. 1998). The tripartite culture system seems to be a potent tool for the commercial production of arbuscular mycorrhizal spores and to get a high percentage of *in vitro* mycorrhized plantlets (Elmeskaoui et al. 1995).

If arbuscular mycorrhizal fungi are inoculated to *in vitro*-grown plantlets, they may augment the competence of transplant shock tolerance and growth during the acclimatization phase. Fortuna et al. (1992) evaluated transplant shock tolerance by inoculation of *Glomus mosseae* and *G. coronatum* into micropropagated *Prunus cerasifera*. After four weeks growth, 100% survival of plants was recorded. Further, they reported that both fungi improved tolerance of plantlets after removal from *in vitro* and *in vivo* systems. Karagiannidis and Hadjisavva-Zinoviadi (1998) found that *G. mosseae* improved plant growth up to 11.6 times and increased grain yield up to 5.4 times as compared to non-inoculated plants.

In 1998, Nowak claimed that the induced resistance response caused by inoculants is due to `biotization'. *In vitro* co-culture of plant tissue explants with beneficial microbes induces developmental and metabolic changes, which enhance their tolerance to abiotic and biotic stresses. Further, he reviewed benefits of *in vitro* biotization of plant tissue cultures with microbial inoculants.

Search for potential mycorrhizal partners

A vast body of literature provides evidence that mycorrhiza researchers used mostly species of *Glomus*, including *G. mosseae, G. fasciculatum. G. etunicatum, G. tenue*, and *Gigaspora margarita* for inoculation to the *in vitro* raised plantlets as these occur frequently. The endophytes show host-specificity and therefore vary in their effectiveness in plant growth promotion. (Guillemin et al. 1992; Vestberg and Estaun 1994; Sylvia 1998; Rajan et al. 1999). Many researchers are of the opinion to screen AM fungal inoculants for their efficacy, and the species with high potential for nutrient uptake should be selected for inoculation programs (Abbott et al. 1992; Azcon-Aguilar et al. 1997; Puthur et al. 1998). Sieverding (1989) remarked that it would be more interesting to find one isolate that is effective with a wide range of plant species, since interactions can occur between different isolates in mixtures. Schubert et al. (1990) screened AMF and found *G. constrictum* to be less effective in enhancement of growth of *Actinidia deliciosa* than *G. caledonium, G. occultum* and *G. versiforme*.

Efficacy of *Glomus deserticola* and *G. mosseae* on the growth and development of tissue-culture-raised plantlets of avocado (*Persea americana* Mill.) was assessed by Azcon-Aguilar et al. (1992). He further reported that the former increased shoot height, leaf number and vigor of the plantlets more than the latter. Arines and Ballester (1992) used *G. aggregatum* and *G. deserticola* for inoculation of micropropagated plantlets of *Prunus avium* with 100% survival of plantlets. The influence of inoculation of *Glomus fasciculatum* (LPA 7), Bouhired et al. (1992) reported positive effect of *G. intraradices* and *Glomus* on the growth of *Phoenix dactylifera* (date palm). Williams et al. (1992) screened more than 80 Finnish inoculants and selected only Finn 98 (*G. intraradices*) and 128 (*Glomus* sp.), for inoculation of micropropagated plantlets of strawberry. *Glomus geosporum* from the Kent collection was chosen as a broad range AMF to include within the trial. *G. intraradices* and *G. mosseae* were selected by Vestberg (1992) for inoculation of strawberry. They reported that the latter was found to be the most efficient fungus, as it increased shoot growth several-fold. Lemoine et al. (1992) screened seven ericoid mycorrhizal fungi against microplants of nine cultivars of *Rhododendron hybrida* and found that use of defined disinfected substrata, combined with specific mycorrhizal fungal strains, is essential for guaranteeing an optimal production of outplanted *Rhododendron* microplants at nursery level.

Guillemin et al. (1992) made a noteworthy contribution by screening many AMF for establishment of symbiosis in micropropagated pineapple plantlets, and reported that Queen and smooth cayenne pineapple plants associated with *Glomus* species (LPA 21) presented better growth than those infected with the other AMF, and the best growth was obtained for the Spanish variety by inoculating plants with *Glomus* sp. (LPA 25). In 1992, Fortuna and colleagues reported the infectivity and effectiveness of *G. mosseae, G. caledonium, G. coronatum* and *Glomus* strain A6, in micropropagated plantlets of plum rootstock (*Prunus cerasifera* Ehrh, clone Mr S 2/5). The authors also evaluated the most and the least infective fungi, *G. mosseae* and *G. coronatum*, respectively, for enhancement of growth of micropropagated *P. cerasifera*. Verma and Jamaluddin (1995) reported a low percentage of infection in seedlings of teak (*Tectona grandis*) inoculated with *G. fasciculatum.* The authors reported that the mixed inoculum of AMF was more efficient for augmentation of growth and biomass of teak. *In vitro* propagation of *Feronia limonia* was carried out by Vyas et al. (2008). The authors inoculated *Piriformospora indica* during *in vitro* rooting and *ex vitro* transfer as a result, the survival percentage increased to 90%. In 2009, Ranjan and his coworkers standardized bio-hardening protocol for *in vitro* regenerated plantlets of Chilli using *G. mosseae, Gigaspora margarita* and mixed arbuscular mycorrhizal fungi (AMF) strains. *In vitro* raised plantlets were treated with AMF, which demonstrated high percentage (97.08%) of plant survival with mixed strain of *G. mosseae* and *G. margarita*.

The efficacy of ectomycorrhizal fungi was not realized by the researchers for inoculation of tissue culture raised plantlets until 1990. Gay et al. (1992) used ectomycorrhizal fungi as a tool to enhance rooting of tissue culture-raised cuttings of *Pinus halepensis* and suggested that an Indole-3-Acetic Acid (IAA) over producer mutant of ectomycorrhizal fungi, such as *Hebeloma hiemale* and *H. cylindrosporum*, could improve the rooting of cuttings of *Cerasus avium* and *Prunus cerasus* up to 95%, which are generally non-ectomycorrhizal. Martins et al. (1996) also reported *Amanita muscaria, Laccaria laccata, Piloderma croceum* and *Pisolithus tinctorius* to be useful on acclimatization of tissue culture-derived plantlets of *Castanea sativa* Mill. They found a positive effect on growth of mycorrhized plants of *C. sativa. P. tinctorius* was most effective in colonizing roots of both micropropagated plants and seedlings, whereas *A. muscaria* and *L. laccata* only colonized a few feeder roots of some plants and *Piloderma croceum* did not form mycorrhizae. The effect of *Hebeloma cylindrosporum* on in vitro rooting of tissue culture raised plantlets of *Prunus avium* and *P.*

cerasus was reported by Grange et al. (1997). The survival percentage was increased from 30 to 100%. Reddy and Satyanarayana (1998) screened *Cenococcum geophilum, L. laccata, Paxillus involutus* and two isolates of *P. tinctorius*, to inoculate micropropagated plantlets of *Populus deltoides* (G 48), and found that *P. involutus* formed mycorrhizae with plantlets of *P. deltoides* while others failed, though they colonized the substrate extensively. The plantlets colonized with *P. involutus* showed appreciably improved growth and dry weights.

Ectomycorrhizal fungi can be utilized efficiently for improvement of growth of the micropropagated plantlets as they are easily available facultative biotrophs. The basic advantage of these fungi is that they can be cultured axenically on artificial medium. Although, the technique of mycorrhization is of greatest importance for the growth and development of the micropropagated plantlets, there are some problems in establishment of mycorrhizal host symbiosis *in vitro*: (i) contamination of inoculum, (ii) behavior of the host *in vitro*, and (iii) obligate nature of the endophyte.

Lovato et al. (1996) opined that a perspective for the near future should be the development of integrated technologies. Not only the mycorrhizal fungi, but also other organisms capable of promoting plant growth or protection, such as, symbiotic or associative bacteria, plant growth promoting rhizobacteria (PGPR), pathogen antagonists, or hypovirulent strains of pathogens would be incorporated into the substrate for micropropagated plant production, they further added. An additional advantage of these fungi is that they are root-colonizers and can be cultivated easily on artificial culture medium. Recently, Ari and Trappe (1998) discussed the ecological role of different DSEs. Sieber et al. (1998), working on DSE in general and DSH (dark septate hyphomycetes) in particular for the past two decades, suggested that fungal endophytes are ubiquitous in trees, and therefore they should be screened for obtaining more and more DSH. Eventually, the most efficient strains of these fungi should be selected for enhancing plant growth. A new hope in 1998, Verma and his group discovered *Piriformospora indica* (named after India), a new fungal endophyte, which belongs to hyphomycetes (Basidiomycota) from sandy desert soil of Rajasthan in north-west India. It is worth mentioning that the fungus can be easily cultured on various media (Verma et al. 1998; Varma et al. 1999). The molecular phylogeny of the fungus revealed that it is closely related to the *Rhizoctonia* group. The characteristic pear-shaped chlamydospores were found to be efficient in successfully colonizing plants like maize, tobacco and tomato in pot cultures. The hyphae generally colonize the surface of the roots and later (about two weeks), the cortex of the plant. The fungus seems to be promising due to its rapid root-colonizing capacity and cultivable nature (Varma et al. 1999). It also promotes growth of medicinal plants (Prasad et al. 2008). These include *Withania somnifera, Spilanthes calva* (Rai et al. 2001) and *Adhatoda vasica* (Rai and Varma 2005) and *Feronia limonia* (Vyas et al. 2008).

GENETIC TRANSFORMATION

Medicinal plants are one the most important source of drugs, as plants contain many secondary metabolites which are mainly responsible for their medicinal properties. Genetic transformation improves yield and quality of medicinal plants, which involve the alteration or introduction of genes which improve the secondary metabolite synthesis in plant. Genetic transformation technology has become a versatile platform not only for trait improvement but also for studying gene function in plants. Genome manipulation is the general aim of the genetic transformation with medicinal plants by developing techniques for desired gene transfer into the plant genome in order to improve the biosynthetic rate of the compounds of interest. An essential strategy in this regard is the choice of the correct marker genes for genetic transformation, as it assists to analyze the transformed cell. Many researchers are mainly focusing on the mechanism of transfer and integration of the marker and reporter genes.

Agrobacterium tumefaciens and *A. rhizogenes* are virulent for plants. They contain a large megaplasmid (more than 200 kb), which plays a key role in tumor induction. 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 and transcribed. Genetic transformation helps to improve secondary metabolite biosynthesis. The main aim is to identify the enzyme in metabolic pathway and then manipulate this enzyme to provide better control of the pathway. Genetic transformation is a powerful tool for enhancing the productivity of novel secondary metabolites of limited yield. Hairy roots, transformed with *A. 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. Genetic transformation facilitates the growth of medicinal plants with multiple durable resistances to pests and diseases. Likewise, transgenes or marker-assisted selection may assist in the development insect, pest, drought, salinity resistant plants, which will be needed to fulfill the worlds need and save land for the conservation of plant biodiversity in natural habitats. There are more than 120 species belonging to 35 families in which transformation has been carried out successfully by using *Agrobacterium* and other transformations techniques (Birch 1997). Yun et al. (1992) and Cucu et al. (2002) reported genetic transformation in *Atropa belladonna* by using *A. rhizogenes*.

Agrobacterium tumefaciens mediated high frequency and simple procedure for genetic transformation of the medicinal plant *Salvia miltiorrhiza* was developed (Yan and Wang 2007). They used Leaf discs and pre-cultured it on MS medium supplemented with 6.6 µmol/L BAP and 0.5 µmol/L NAA for one day and later co-cultured with *A. tumefaciens* strain EHA105 having plasmid pCAMBIA 2301 on the same medium for three days. The regenerated buds on selection medium (60 mg/L kanamycin and 200 mg/L cefotaxime) were transferred to fresh MS medium with 60 mg/L kanamycin for rooting. After 15 days, the rooted plantlets were successfully transplanted to

soil. The transgenicity of the regenerated plants was analyzed by PCR, Southern hybridization and GUS histochemical assay.

Transformation study of the figwort, *Scrophularia buergeriana* (figwort) was done by Park et al (2003). *S. buergeriana* contains bioactive natural products which are used for the treatment of fever, constipation, neuritis, and laryngitis. In transformation study, *S. buergeriana* plants were regenerated from leaf explants and co-cultivated with *A. tumefaciens* strain GV3101. Shoot regeneration was observed on medium supplemented with 2 mg/L 6-BAP and 70 mg/L putrescine. Detection of the NPT gene, and GUS enzyme activity, confirmed the genetic transformation of *S. buergeriana*. Their work demonstrates the potential of using *A. tumefaciens* to transfer foreign genes into important medicinal plant. *Ruta graveolens* L. is important source of active biomolecules such as furocoumarins, furoquinolines and acridone alkaloids. The efficient genetic transformation protocol for *R. graveolens* was developed by using *A. tumefaciens* (Karine et al. 2005). The regeneration and transformation was obtained by co-cultivation of hypocotyls of 2-3 weeks old plants and *A. tumefaciens* strain C58C1Rif^R containing a plasmid NPT and β-glucuronidase genes.

Echinacea purpurea is an important herb which can used to treat cold and act as an imunostimulant and anti-inflammatory remedy. The plant regeneration and method for transformation pCHS (pBI121-based vector having GUS, reporter gene) into *E. purpurea* was firstly reported by Wang and To (2004). Zeef et al. (2000) reported plant transformation system for *Hyoscyamus muticus*, an important medicinal plant of the Solanaceae family. The system used by them consists of plasmid carrying the *npt*II and *gus*A genes. Particle bombardment method was used by them to deliver this gene in to leaf explant.

Mentha spp. belonging to the family Lamiaceae, distributed mostly in the temperate and sub-temperate regions of the world. It is an important crop being the source of essential oils enriched in certain monoterpenes, widely used in food, flavor, cosmetic and pharmaceutical industries. The monogenic basis for conversion of menthone to menthol showed that gene R, either homozygous (RR) or heterozygous (Rr), is responsible for the reduction of menthone to menthol or carvone to carveol. Plant transformation technology has not only played an important role in introducing insecticidal genes into relevant crops but also has become a versatile platform for cultivar improvement as well as for studying gene function in plants.

Most important extensively studied medicinal plant, the *Atropa belladonna*, which is member of the Solanaceae family. This plant is a major source of tropane alkaloids, which is used as antimicrobial compounds in pharmaceutical drugs.

DEVELOPMENT OF THE DNA BANKS

Genetic diversity has significant contribution in conservation of plant genetic resources (PGR). There are approaches which are widely applied with their strength and weaknesses. These include *ex situ* and *in situ* conservation. The maintenance of plant populations in their habitats, where they can naturally occur, grow and reproduce is *in situ* conservation. When they grow outside their natural habitat or production areas is referred to as *ex situ* conservation of germplasm. Depending on the biological nature of the species to be conserved, different types of *ex situ* conservation methods are available (Vicente et al. 2006).

The establishment of DNA banks is one of the *ex situ* conservation method which is planned activity. The extraction of genetic material, and storage should be made readily available for molecular applications. DNA resources can be maintained at ‑20ºC for short- and mid-term storage (i.e. up to 2 years), and at ‑70ºC or in liquid nitrogen for longer periods. These experiments normally aim to obtain knowledge to improve the efficiency of some conservation activities or to scientifically inform decisions related to the conservation of germplasm. Other objectives of the creation of DNA banks may be related to training or distribution to scientists with an interest in different areas of biology. DNA banks assembled as a means to replace traditional methods of conserving genetic resources. This is important to note as conservation of genome fragments or individual genes are quite a different situation from the conservation of entire genotypes, as living organisms, for their future use. DNA may be a cost effective form for conservation of germplasm depending on the objective of the conservation and the type of use to which it would be applied. For many species that are difficult to conserve by conventional means (either as seeds or vegetatively) or that are highly threatened in the wild, DNA storage may provide the ultimate way to conserve the genetic diversity of these species and their populations in the short term, until effective methods can be developed (Dulloo et al. 2006).

Cryopreservation is an important technique for long-term storage of tissues/plants. This requires liquid nitrogen (-196^{o}C). The technique has been proved to be very useful for *A. belladonna, Digitalis lanata, Hyoscyamus* sp., and *R. serpentina*. Sharma and Sharma (2003) studied cryopreservation of shoot tips of *Picrorhiza kurroa* Royle ex Benth (IC 266698), which is also an endangered medicinal plant of India. The authors found that shoot-tips obtained from four weeks old cultures were dehydrated and directly immersed in liquid nitrogen. By vitrification, the shoot tips were cryopreserved and shoot regeneration of cryopreserved shoot tips to 70% and 35%, respectively.

Recently, Mandal et al. (2009) cryopreserved embryogenic cultures of *Dioscorea bulbifera* using an encapsulation-dehydration procedure. They reported 53.3% recovery of growth of embryogenic culture after cryopreservation. On subculturing of these cultures, plantlets were obtained through embryo conversion. The authors reported 80% success of regeneration of cryopreserved embryogenic cultures

DNA banks are kind of “Gene Library” in which DNA samples are stored. These provide vital information to the conservation scientists. DNA samples may be of three kinds: (i) total genomic DNA, (ii) DNA libraries, (iii) individual cloned DNA fragments including RFLP probes,

mini- and microsatellites, etc. Some important DNA banks are as below: (i) The Royal Botanic Garden, Kew, UK, which contains PGR DNA specimens, and presently the world's largest and the most comprehensive PGR DNA bank, consisting of over 20,000 DNA specimens representative of all plant families. (ii) The US Missouri Botanical Garden has collection of more than 20,000 plant tissue samples, and provide raw material for the extraction of DNA for its subsequent use in conservation research. (iii) The Australian Plant DNA Bank of Southern Cross University, which was established in June 2002. It contains representative genetic information from the entire Australian flora. (iv) DNA bank of Leslie Hill Molecular Systematics Laboratory of the National Botanical Institute (NBI) in Kirstenbosch, South Africa, in collaboration with the Royal Botanic Garden, Kew, which preserves genetic material of the South African flora (Rice et al. 2006).

CONCLUSSION

Conservation of rare and endangered medicinal plants needs urgent attention. Although efforts have been made to conserve endangered medicinal plants by *in situ* and *ex situ* methods, the biotechnological strategies would open up new vistas in the field of conservation. Micropropagation of endangered plants like *Aquilaria malaccensis*, *Dioscorea deltoidea*, *Guaicum officinale*, *Hydrastis canadensis*, *Nardostachys grandiflora*, *Panax quinquefolius*, *Picrorhiza kurroa*, *Podophyllum hexandrum*, *Prunus africana*, *Pterocarpus santalinus*, *Rauwolfia serpentina*, *Saussurea costus*, *Gloriosa superba* and *Taxus wallichiana* will be beneficial because these plants will reach critically endangered, or possibly endangered stage.

There is a pressing need to deliver mycorrhizal propagules into the roots of the tissue-culture-raised plantlets of endangered medicinal plants during the acclimatization process because the plantlets are devoid of microbes in sterile medium. Consequently, the plants suffer from 'transient transplantation shock'. In order to avoid this bottleneck and for better survival and sustainable plant production, mycorrhization of the micropropagated plantlets is necessary. *Agrobacterium tumefaciens* and *A. rhizogenes* are the potent biological tools for transformation of endangered medicinal plants for development of varieties resistant to stress conditions and also for over production of secondary metabolites so that exploitation of these plants will be minimized. Cryopreservation is another technique to preserve the endangered medicinal plants. Moreover, DNA banks would be useful for long-term preservation and sustainable plant productions.

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