
Plant tissue culture: a biotechnological tool for solving the problem of propagation of multipurpose endangered medicinal plants in India

Kuldeep Yadav, Narender Singh* and Sharuti Verma

Plant Tissue Culture Laboratory, Department of Botany, Kurukshetra University, Kurukshetra, Haryana, India-136119

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India has a very rich plant biodiversity, many of which are medicinally useful. The rich resource is disappearing at an alarming rate as a result of over-exploitation. Rapid agricultural development, population growth, urbanization and the indiscriminate collection of medicinal plants from the wild is resulted in an over-exploitation of natural resources. The conventional means of propagation takes a long time for multiplication and also clonal nonuniform. Plant *in vitro* regeneration is a biotechnological tool that offers a tremendous potential solution for the propagation of endangered and superior genotypes of medicinal plants which could be released to their natural habitat or cultivated on a large scale for the pharmaceutical product of interest. After the last four years of intensive research programmes in our laboratory, we are able to micropropagate some of the endangered and valuable medicinal plants species of this region.

Key words: Endangered, *in vitro*, Medicinal plants, Plant tissue culture, Protocols.

Introduction

Biodiversity is the store house of species richness and acts as a cushion against potentially dangerous environmental changes and economic reforms. Plant genetic resources are the major biological basis of the world food security. In all means they support the livelihoods of every life on planet earth. Hence, conservation of such a buffer is considered fundamental and provided priority in all sectors of global development (Tandon *et al.*, 2009). As defined by WHO, health is a state of complete physical, mental, and social well being and not merely the absence of disease or infirmity.

Medicinal plants, since times immemorial, have been used virtually all cultures as a source of medicine. It is estimated that 70-80% people worldwide rely chiefly on traditional, largely herbal, medicines to meet their primary

* Corresponding author: Narender Singh; e-mail: nsheorankuk@yahoo.com

health care needs (Srivastava *et al.*, 1995). Approximately 85% of traditional medicine preparations involve the use of plants or plant extracts (Vieira and Skorupa, 1993).

India has 2.4% of world's area with 8% of global bio-diversity. It is one of the 12 mega-diversity hot-spot regions of the world. Across the country, the forests are estimated to harbour 90% of India's total medicinal plants diversity. Only about 10% of the known medicinal plants of India are restricted to non-forest habitats (Wakdikar, 2004). According to Schippmann *et al.* (1990), one fifth of all the plants found in India are used for medicinal purpose. The world average stands at 12.5% while India has 20% plant species of medicinal value and which are in use. But according to Hamilton (2003), India has about 44% of flora, which is used medicinally. Although it is difficult to estimate the total number of medicinal plants present worldwide, the fact remains true that India with rich biodiversity ranks first in per cent flora, which contain active medicinal ingredient (Mandal, 1999).

Medicinal plants are an integral component of ethno-veterinary medicine also. Farmers and pastoralists in several countries use medicinal plants in the maintenance and conservation of the healthcare of livestock. Intestinal disorders in cows, in Mexico, are treated with herbal extracts of *Polakowskia tacacco*. Dietary supplements such as vitamin A in poultry feeds in Uganda are supplied through enrichments of amaranth (*Amaranthus sp.*). In fact, interest of such use in the veterinary sector has resulted primarily from the increasing cost of livestock maintenance and the introduction of new technology in the production of veterinary medicines and vaccines (Hoareau and DaSilva, 1999).

In the past few decades, there has been an ever-increasing global inclination towards herbal medicine, followed by a belated growth in international awareness about the dwindling supply of the world's medicinal plants (Bodeker, 2002). The plants used in the phyto-pharmaceutical preparations are obtained mainly from the naturally growing areas. The genetic diversity of medicinal plants in the world is getting endangered at alarming rate because of ruinous harvesting practices and over-harvesting for production of medicines, with little or no regard to the future. Also, extensive destruction of the plant-rich habitat as a result of forest degradation, agricultural encroachment, urbanization etc. are other factors, thus challenging their existence (Gupta *et al.*, 1998).

A large sum of money is pumped every year to replenish the lost biodiversity and large numbers of protocols are available at present. Unfortunately, we are not witnessing any improvement in the status of these plant species in nature and the number of threatened plant species is increasing gradually (Tripathi, 2008). Therefore, the management of traditional medicinal

plant resources has become the matter of urgency. To cope up with alarming situation, Biotechnological tools have been increasingly applied for mass propagation, conservation of germplasm, study and production of bioactive compounds and for genetic improvement of the medicinal plants. Tissue culture is useful for multiplying and conserving the species, which are difficult to regenerate by conventional methods and save them from extinction. Micropropagation has superiority over conventional method of propagation because of high multiplication rate. Most of the plant raised through seeds are highly heterozygous and show great variations in growth, habit and yield and may have to be discarded because of poor quality of products for their commercial release. Likewise, majority of plants are not amenable to vegetative propagation through cutting and grafting. Moreover many plants propagated by vegetative means contain systemic bacteria, fungi and viruses (Murch *et al.*, 2000). The *in vitro* propagated medicinal plants are genetically pure elite. Micropropagation techniques are must for conservation of an endangered medicinally important species within short period and limited space. The plants produced from this method are independent of climatic changes or soil conditions.

Efforts have been devoted for *in vitro* mass multiplication of valuable medicinal herbs, *Aegle marmelos*, *Acorus calamus*, *Celastrus paniculatus*, *Commiphora mukul*, *Peganum harmala*, *Prosopis cineraria*, *Simmondsia chinensis*, *Spilanthes acmella*, *Stevia rebaudiana*, *Sapindus mukorossi*. A thorough understanding of economical and ecological importance of the above mentioned important endangered medicinal plants are as follows:

Aegle marmelos (L.) Corr., (Rutaceae) commonly known as “Bael Tree” is a popular vulnerable medicinal plant mostly found in tropical and subtropical regions. Almost all parts of the tree are used in preparing herbal medicine for treating diarrhea, dysentery, dyspepsia, malaria, fever, jaundice, and skin diseases such as ulcers, urticaria, and eczema. The plant is rich in alkaloids, among which aegline, marmesin, marmin, and marmelosin are the major ones (Kala, 2006).

Acorus calamus Linn. (family Araceae) commonly known as “sweet flag” or “Bach” is an important endangered medicinal plant. It is a semiaquatic herb with creeping rhizomes and sword shaped long leaves. The rhizomes possess anti-spasmodic, carminative and anthelmintic properties and also used for treatment of epilepsy, mental ailments, chronic diarrhea, dysentery, bronchial catarrh, intermittent fevers and tumors (Anonymous, 2000).

Celastrus paniculatus Willd. (Celastraceae) commonly known as Malkangni, Jyotishmati, Bitter sweet is a rare and endangered important medicinal plant believed to sharpen the memory and also used to cure a number

of diseases. It is a large, woody, unarmed climbing shrub occurring naturally in hilly parts of India up to an altitude of 1200 m. This plant is widely used to cure depression, paralysis, leprosy, fever, abdominal disorders and cancerous tumors. Chemical constituents of seeds as revealed by phytochemical analysis were sesquiterpene alkaloids like celapagine, celapanigine and celapanine (Sharma *et al.*, 2001).

Commiphora mukul (Hook. ex Stocks) Engl. (Burseraceae), popularly known as “Guggul”, is an important endangered medicinal plant species. It is widely distributed in tropical regions of Africa and Asia. It grows wild in the arid, rocky tracts of north- western regions of India. The plant exudes a medicinal oleo-gum resin (‘Guggul’) from incisions made on the bark in cold season. The latex oozes out through the wound as a yellow fluid which slowly hardens to form the oleo-gum resin. Gum is bitter, acrid, aromatic, pungent, carminative and stomachic stimulating the appetite and improving digestion. It is astringent, expectorant, anthelmintic, antispasmodic, anti-inflammatory, diuretic, depurative, anodyne, vulnerary, thermogenic, antiseptic, nervine tonic, aphrodisiac, stimulant, emmenagogue and diaphoretic (Sosa *et al.*, 1993). It also possesses strong purifying and rejuvenating properties and is said to be a uterine stimulant. The main constituents of guggul include phytosterols, guggulipids and the ketonic steroid compound (guggulsterones) mainly E and Z guggulsterones. These are responsible for the lipid lowering effects of guggul (Singh *et al.*, 1997).

Peganum harmala L. (Syrian Rue), a medicinally important perennial herb of family Nitrariaceae, distributed over semi arid areas of North-West India, North-Africa and central Asia. Medicinally the fruits and seeds of this plant are digestive, diuretic, hallucinogenic, hypnotic, antipyretic, antispasmodic, nauseant, emetic, narcotic and uterine stimulant (Chatterjee, 1997). A red dye obtained from seeds is widely used in Turkey and Iran for colouring carpets. Leaves are useful in asthma, colic, dysmenorrhea, hiccup, hysteria, neuralgia and rheumatism. The plant has also been used as antimicrobial, antitumoral, in curing malaria and has insecticidal potential (Kiritikar, 1995).

Prosopis cineraria (Family: Fabaceae) is a versatile species commonly known as Jhand or Khezri. *Prosopis* species are the dominant species in Indian desert. *P. cineraria* has a very good economic importance in arid regions and is assumed to treat snake bite and scorpion stings. Green pods of this plant are used as food. This species is highly drought tolerant and can withstand in the area having 50mm rainfall annually (Bhandari, 1978).

Sapindus mukorossi (family: Sapindaceae) popularly known as 'Ritha' and 'Soapnut', is a most important deciduous tree of tropical and sub- tropical

regions of Asia. The fruit of this tree contains saponins, the most active secondary metabolites extracted from this plant. It is a good substitute for washing soap and is as such used in preparation of quality shampoos, detergents etc. The fruit is of considerable importance for its medicinal value for treating a number of diseases like common cold, pimples, epilepsy, constipation, nausea etc. It is also used as expectorant and anthelmintic in small doses (Anonymous, 1992).

Simmondsia chinensis (Jojoba) belongs to family Simmondsiaceae, is an evergreen, dioecious desert shrub which grows wildy in Sonora desert of Arizona, northern Mexico, southern and Baja California. Jojoba is now cultivated commercially in Argentina, Australia, Chile, Egypt, India, Israel, Mexico, Peru, South Africa and the USA. Jojoba seed oil is being utilized in industrial lubricants, and in pharmaceutical and cosmetic industries. The liquid wax that makes 40–60 % of seed dry weight has properties similar to sperm whale oil. Due to the ban on the import of sperm whale products into USA and other countries, jojoba is gaining commercial importance at international level (Reddy and Chikara 2010).

Spilanthes acmella Murr. (Family: Asteraceae) commonly known as Akarkara or Toothache plant. This plant is widely distributed in the tropical and sub-tropical regions. The flowers and leaves of this plant have been used as traditional medicine for stammering, toothache, stomatitis and throat complaints. It has potent diuretic activity and the ability to dissolve urinary calculi. The plants have shown anti-inflammatory, antibacterial and antifungal properties. Spilanthol, the most active antiseptic alkaloid extracted from this plant, is found effective at extremely low concentrations against blood parasites, and indeed is a poison to most invertebrates while remaining harmless to warm-blooded creatures (Anonymous, 1989).

Stevia rebaudiana Bertoni, belongs to the Asteraceae family is a natural sweetener perennial herb commonly known as “Sweet Weed”, “Sweet Leaf”, “Sweet Herbs” and “Honey Leaf”. The leaves of this plant are estimated to be 300 times sweeter than sucrose and the sweetness is due to glycosides of which the most abundant is stevioside. The increasing consumption of sugar (sucrose) has resulted in several nutritional and medical problems, such as obesity. Therefore, low caloric sweeteners have been investigated to substitute sugar. The refined extracts of leaves of this plant are officially used as high potency natural-source, low calorie (nonsucrose) sweetener in processed foods, artificial diets and pharmaceuticals. The sweet compounds pass through the digestive process without chemically breaking down, making it safe for diabetic and obese people (Mizutani and Tanaka, 2002).

Table 1. List of the some of the endangered and economically important medicinal plants micropropagated in our laboratory

Plant species	Family	Status/ Use	Explants	Reference
<i>Aegle marmelos</i>	Rutaceae	Vulnerable, Medicinal	Nodal segments and shoot tip	Yadav and Singh (2011 a)
<i>Acorus calamus</i>	Araceae	Endangered, Medicinal	Rhizome tip and Rhizome segments	Yadav <i>et al.</i> , 2011
<i>Celastrus paniculatus</i>	Celastraceae	Rare and Endangered, Medicinal	Seeds, Nodal Segments & shoot tip	Lal and Singh (2010), Lal <i>et al.</i> (2010)
<i>Commiphora mukul</i>	Burseraceae	Vulnerable, Ornamental, Medicinal, Aromatic	Leaf segments, Apical & Nodal segments	Singh <i>et al.</i> (2010b)
<i>Peganum harmala</i>	Nitrariaceae	Medicinal, Dye yielding	Seeds	Goel <i>et al.</i> (2009)
<i>Prosopis cineraria</i>	Fabaceae	Medicinal, Timber, Fodder, Fuel	Seeds	Kumar and Singh (2009)
<i>Simmondsia chinensis</i>	Simmondsiaceae	Medicinal	Nodal segments	Kumar <i>et al.</i> (2010)
<i>Spilanthes acmella</i>	Asteraceae	Endangered, Medicinal, Ornamental	Nodal and intermodal segments	Yadav and Singh (2010), Yadav and Singh (2011b)
<i>Stevia rebaudiana</i>	Asteraceae	Medicinal	Apical and Nodal segments	Kumar and Singh(2009); Singh <i>et al.</i> (2011)
<i>Sapindus mukorossi</i>	Sapindaceae	Medicinal, Ornamental, Fuel	Leaf segments, Apical and Nodal segments	Singh <i>et al.</i> (2010a)

A general overview of beginning of micropropagation of medicinal plants

In vitro culture is one of the key tools of plant biotechnology that exploits the totipotency nature of plant cells (Haberlandt, 1902) and unequivocally demonstrated for the first time in plants by Steward *et al.* (1964). Beyond the discovery of kinetin (Miller *et al.*, 1955), the major work on *in vitro* regeneration has been centered around tobacco (*Nicotiana tabacum* L.) tissue culture, culminating in the first convincing demonstration of the control of differentiation of shoots or roots or both by the kinetin-auxin ratio (Skoog and Miller, 1957) followed by carrot (*Daucus carota* L.) tissue culture and birth of

the concept of totipotency of plant cell with the regeneration of complete flowering plants of carrot from its phloem cells (Steward *et al.*, 1964). Thus, the micropropagation of medicinal plants remained neglected till complete plants of *Rauvolfia serpentine* (L.) Benth., were produced from its somatic callus tissue (Mitra and Chaturvedi, 1970).

Plant tissue culture refers to growing and multiplication of cells, tissues and organs of plants on defined solid or liquid media under aseptic and controlled environment. The commercial technology is primarily based on micropropagation, in which rapid proliferation is achieved from tiny stem cuttings, axillary buds, and to a limited extent from somatic embryos. The process of micropropagation is usually divided into several stages i.e., pre-propagation, initiation of explants, subculture of explants for proliferation, shooting and rooting, and hardening. These stages are universally applicable in large-scale multiplication of plants.

The field performance of these tissue cultured plants depends on the selection of the initial material, media composition, growth regulators, cultivar and environmental factors (Chang *et al.*, 1994). The effects of auxins and cytokinins on shoot multiplication of various medicinal plants have been reported by Skirvin *et al.* (1990). Lal and Ahuja (1996) observed a rapid proliferation rate in *Picrorhiza kurroa* using kinetin at 1.0–5.0 mg/l. Barna and Wakhlu (1998) has indicated that the production of multiple shoots is higher in *Plantago ovata* on a medium having kinetin along with NAA. Faria and Illg (1995) have also shown that the number of shoots per 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. Mao *et al.* (1995) reported that the actively growing materials were more responsive to shoot induction than dormant buds in *Clerodendrum colebrookianum*. Also BAP was proved superior to 6- purine (2ip) and TDZ for multiple shoot induction.

The cultured cells and tissue can take several pathways to produce a complete plant. Among these, the pathways that lead to the production of true-to-type plants in large numbers are the popular and preferred ones for commercial multiplication (Bhojwani and Razdan, 1983; Pierik, 1989).

Regeneration and organogenesis

In this pathway, groups of cells of the apical meristem in the shoot apex, axillary buds, root tips, and floral buds are stimulated to differentiate and grow into shoots and ultimately into complete plants. The explants cultured on relatively high amounts of auxin form an unorganized mass of cells, called callus. 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. Pande *et al.* (2002) have reported the successful *in-vitro* regeneration of *Lepidium sativum* from various explants on MS medium supplemented with 4.0 mg/l BAP and NAA. The role of auxins and cytokinins in callus induction was also advocated by Kumar and Singh (2009) in *Stevia rebaudiana*, Goel and Singh (2009) in *Peganum harmala*, Kumar and Singh (2009) in *Prosopis cineraria*, Lal and Singh (2010) in *Celastrus paniculatus*, Yadav and Singh in *Spilanthes acmella* (2010) and *Aegle marmelos* (2011).

Out of various cytokinins tested, BAP was the most effective for inducing bud break. Effectiveness of BAP was also observed in *Leucaena leucocephala* (Singh and Lal, 2007), *Prosopis cineraria* (Kumar and Singh, 2009), *Spilanthes acmella* (Yadav and Singh, 2010) and *Acorus calamus* (Yadav *et al.*, 2011).

Somatic Embryogenesis

In this pathway, 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. The primary somatic embryos are also capable of producing more embryos through secondary somatic embryogenesis. 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 (Tripathi and Tripathi, 2003). Arumugam and Bhojwani (1990) noted the development of somatic embryos from zygotic embryos of *Podophyllum hexandrum* on MS medium containing BAP and IAA. Efficient development and germination of somatic embryos are prerequisites for commercial plantlet production. Chand and Sahrawat (2002) reported the somatic embryogenesis of *Psoralea corylifolia* L. from root explants on medium supplemented with NAA and BAP. Rooting of shoots was best achieved using different concentrations of auxins. In *A.maemelos*, MS half strength medium supplemented with IAA proved better (Yadav and Singh, 2011). In *P. cineraria*, rooting was achieved on half strength MS medium supplemented with 3.0 mg/l IBA (Kumar and Singh, 2009), while in *L. leucocephala*, NAA resulted in better root formation (Singh and Lal, 2007).

Acclimatization and Transfer of micropropagated plantlets to the soil

Complete regenerated plantlets with sufficient roots were taken were gradually pulled out from the medium and immersed in water to remove the remains of agar-agar particles sticking to the root system by using a fine brush. These plantlets were transferred to pots containing mixture of sterilized soil and sand (3:1). The potted plantlets were covered with a transparent polythene bag to ensure high humidity around the plants. The pots were supplied with MS (half strength) salt solution on alternate days. After about two weeks the polythene bags were removed for 3-4 hours daily to expose the plants to the conditions of natural humidity for acclimatization. These plants were shifted to bigger pots after one month of its transfer and were maintained under green house conditions. Successful acclimatization and field transfer of the *in vitro* regenerated plantlets have also been reported in *Leucaena leucocephala* (Singh and Lal, 2007), *Peganum harmala* (Goel *et al.*, 2009), *Celastrus paniculatus* (Lal and Singh, 2010).

Ex vitro field evaluation of acclimated plants

These recent advances in plant tissue culture have resulted in the development of protocols for micropropagation of many important medicinal plants, but the process of transplantation and acclimatization of micropropagated plants to soil environment continues to be a major bottleneck in the micropropagation of medicinal plants. Acclimatization of a micropropagated plant to a green house or field environment is essential because anatomical and physiological characteristics of *in vitro* plantlets necessitate that they should be gradually acclimatized to the field environment (Hazarika, 2003). Successful acclimatization minimizes the percentage of dead or damaged plants, enhancing the plant growth and establishment (Sha Valli Khan, 2003). Dynamics of the process are related to the acclimatized plant species and both *in vitro* and *ex vitro* culture conditions (Pospisilova *et al.*, 1999). Now days, mycorrhizal technology can be applied to reduce transplantation shock during acclimatization, thus increasing plant survival and establishment rates of micropropagated medicinal plant species (Sharma *et al.*, 2008; Yadav *et al.*, 2011).



Fig. 1. **A.** Shoot formation from nodal explant of *Aegle marmelos* on MS medium + BAP (2.0mg/l); **B.** Root initiation from *in vitro* grown shoot of *Spilanthes acmella* on MS- medium (1/2) + IBA (1.0mg/l); **C.** Callus growth and shoots proliferation from nodal explants of *Prosopis cineraria* on MS medium supplemented with BAP (2.0mg/l); **D.** Rhizome segments of *Acorus calamus* cultured on MS medium + BAP (2.0mg/l).

Conclusion

Medicinal herbs as potential source of therapeutics aids has attained a significant role in health system all over the world for both humans and animals not only in the diseased condition but also as potential material for maintaining proper health. Fresh strategies of afforestation management and restoration of depleting natural resources blending with modern technologies are also required. Biotechnology is a motor of technological advancement in both the developed and developing countries though at different levels in scope and content. In recent years, tissue culture has emerged as a promising technique to obtain genetically pure *elite* populations under *in vitro* conditions rather than

have indifferent populations. Thus *in vitro* cell and tissue culture methodology is envisaged as a mean for germplasm conservation to ensure the survival of endangered plant species, rapid mass propagation for large scale revegetation and for genetic manipulation studies. 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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