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Review Article

Micropropagation: An Important Tool for Conserving Forest Trees

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ABSTRACT

Forest trees are renewable sources of food, fodder, fuel wood, timber and other valuable non-timber products. The ever increasing human and livestock populations have put heavy demands for plant products, resulting in over exploitation of forest trees. Therefore, there is an urgent need for conservation of germplasm and also for propagation of a sustainable utilization of forest trees. Micropropagation of tree species offers a rapid means of producing clonal planting stock for afforestation, woody biomass production and conservation of elite and rare germplasm. This review provides an overview of the success achieved on *in vitro* work done for a number of important forest trees.

Keywords: Micropropagation, multipurpose, *Albizia lebbeck*, *Leucaena leucocephala*, *Prosopis cineraria*

INTRODUCTION

In the past, forests spread over half of the land surface but due to large scale changes in land use, forests covered only 30 per cent of the Earth land area. In particular, the forest and tree cover in India has been reduced by 21 per cent and hence, the forest policy emphasizes on conserving the natural heritage of the country by preserving the remaining natural forests. Forest trees are renewable sources of food, fodder, fuel wood, timber and other valuable non-timber products. Due to the rapid growth in the population and the human desire to progress, there has been a tremendous reduction in the forest and tree cover from the earth's surface, and thus, the increasing demands for biomass fuel wood, timber and pulp for paper industry can no longer be met from the existing natural resources. Consequently, there is an urgent requirement for a large number of improved

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fast growing trees in shortest duration suitable for agro forestry, fuel, timber, and fodder. In order to maintain and sustain forest vegetation, conventional approaches like grafting, layering and cutting have been used for propagation. Nonetheless, these conventional methods of plant propagation and improvement have limited applicability (Yadav & Singh, 2011a; Yadav *et al*., 2012). In general, trees are slow growing, long-lived, sexually self-incompatible and highly heterozygous plants, and limit the use of traditional breeding methods (Williams & Savolainen, 1996). The major constraint with the conventional methods of tree breeding is that these methods are slow, often lead to virus infected material and are less productive, and hence, they cannot be used efficiently for the propagation of trees.

Forest tree biotechnology emerged during the 1980s, and it encompasses a developing collection of tools for modifying tree physiology and genetics to aid breeding, propagation and research (Burdon & Libby, 2006). Advanced biotechnological methods of culturing plant cells and tissues should provide new means for conserving and rapidly propagating valuable, rare and endangered forest tree species. Over the past two decades, various components have been established individually, but all are still considered under the large umbrella of forest tree biotechnology (FAO, 2004). Micropropagation offers a rapid means of aforestation, multiplying woody biomass, conservation of elite and rare germplasm (Bajaj, 1986; Karp, 1994), regeneration of plantlets from both callus cultures and organ cultures (Chalupa, 1987), shortening germination period and developing single cells into callus (Muir *et al*., 1958). The application of micropropagation techniques as an alternative mean of asexual propagation of important trees has increased the interest of workers in various fields. The technique of cell and tissue culture, under controlled and defined conditions, has contributed in raising new plants, manipulation of plant without conventional breeding mechanism and methods, shortening germination and developmental phase of plants. It thus holds a place of unique importance in today's world among plant biologists (Batra *et al*., 2000). Plant tissue culture is also employed in haploid production, production of disease free and resistant plants, elimination of breeding barriers, biosynthesis of secondary metabolites, generation of variability, germplasm conservation and selection of desirable traits (Pijut *et al*., 1990; Karp, 1994; Roja & Rao, 1998). While the main use of propagation technologies has been for forest establishment or clones, there is also a conservation use for those species that are at risk, rare, endangered or of special cultural, economic or ecological value (Benson, 2003). In general, woody trees are difficult to regenerate under *in vitro* conditions. The sticking constraint in the propagation of trees under *in vitro* conditions is the comparatively poor success with mature explants from adult trees. Most of the trees can be propagated by vegetative means during the juvenile phase. As trees grow and attain maturity, the ability of vegetative propagules to root declines. It is well

established that juvenile tissues facilitate propagation of mature trees. Hence, in order to circumvent these impediments, clonal or vegetative propagation has been deployed. Up to 1975, micropropagation involved regeneration of plantlets from callus cultures only, but later organ culture also became quite popular (Chalupa, 1987). The potential benefits of the micropropagation of elite genotypes for production of clonal planting stock for aforestation/reforestation have long been recognized. Plantlets have been regenerated from both juvenile and mature trees (Dunstan, 1988; Thorpe *et al*., 1991). The current extent of the world's plantation forest area is about 187 million hectares (mha) with the annual planting of 4.5 mha. India is one of the largest hardwood plantation resources comprising about 32.5 mha, with *Eucalyptus*, *Acacia* and *Teak* as major species. The annual planting target of India is about 3.0 million hac.

Commercial applications of micropropagation are however generating increasing interest. The potential is huge although, up to now, only several thousand hectares seem to have been established globally using micropropagated material. An overview of the work carried out earlier on the different woody species of forests is given in the following section.

AN OVERVIEW

During the past decade, major advances have been made in this field and now it has become an industrial technology. Great advances in micropropagation have occurred since Harberlandt's exploration of the concept presented in his landmark paper published in 1902. The pioneering experiments were initiated by the father of tissue culture Gottlieb Haberlandt in 1898, in which he chose single cell isolated from the palisade tissue of leaves, epidermis and epidermal hairs of different plants. He grew them on Knop's (1865) salt solution with sucrose and observed the growth in the palisade cells but could not succeed because of handling with highly differentiated cells and lack of proper techniques. Haberlandt had also perceived the concept of growth hormones, which he called "growth enzymes" and felt that these are released from one type of cells and stimulated growth and developments in other cells. From the time Haberlandt presented his paper in 1902 until about 1934, there was hardly any progress was made in the field of plant tissue culture as conceived by Haberlandt. His pioneering experiments inspired other botanists to conduct further work on the morphogenetic potentialities of the living cells and abilities of tissue and organ to develop into complete plant. Kotte (1922), a student of Haberlandt, in Germany and Robbins (1922) were successful in the establishment of excised plant root tips under *in vitro* conditions. Meanwhile, success in the continuously growing cultures of tomato root tips using sucrose, inorganic salts and yeast extract was achieved by White (1943).

Gautheret (1934) observed the proliferation of callus by culturing cambium cells of *Salix* and *Populous* on Knops' solution. Van Overbeek *et al*. (1942) studied

the stimulatory effect of coconut milk on the embryo development and callus formation in *Datura*. These findings set the stage for the large increase in research for tissue culture from this period, advances such as the eradication of viruses through meristem culture (Morel & Martin, 1952), cultivation of single cells and suspension cultures (Muir *et al*. 1954), auxin and cytokinin basis of organogenesis (Skoog & Miller, 1957), somatic embryogenesis (Reinert, 1959), large scale culture of cells (Tulecke & Nickell, 1960), regeneration of plants from single cell (Vasil & Hildebrandt, 1966), uptake of DNA by cells (Ledoux, 1965) and variability of cells in culture (Lutz, 1969) were made. During *in vitro* culture, various intrinsic and extrinsic factors like culture medium (carbohydrates, growth regulators, agar concentration, pH, etc.), culture conditions (photoperiod, temperature), type of explants and their interactions affect the successful growth and development of plant.

This technology of plant tissue culture offers advantages over conventional methods of propagation for a rapid and large scale multiplication of important plants under *in vitro* conditions, irrespective of the season with conservation of space and time (Nehra & Kartha, 1994; Rao *et al*., 1996). The propagation of some commercial plants, which are difficult to reproduce conventionally by seed or vegetative propagules, is realized by *in vitro* tissue culture technique. Thus, advances in biotechnological research have opened new avenues for rapid multiplication of forest trees. Consequently, a large number of horticultural, plantation and forest species, numbers of important fruit trees and medicinal plant are being propagated *in vitro* on commercial scale (Arumugam & Bhojwani, 1990). A large number of woody trees species have been successfully cultured *in vitro* (Table 1).

For micropropagation, beside proper techniques and requirements, various experimental conditions have also been maintained; these are briefly reviewed as follows.

Nutrition

Growth of plants under *in vitro* conditions is largely determined by the composition of the culture medium. The importance of nutrition in plant tissue culture has been reported by Gautheret (1955). The main components of most plants tissue culture media are mineral salts and sugar as carbon source and water. Other components may include organic supplements, growth regulators and gelling agent (Gamborg *et al*., 1968; Gamborg & Phillips, 1995). Although the amounts of the various ingredients in the medium vary for different stages of culture and plant species, the basic MS (Murashige & Skoog, 1962) and LS (Linsmaier & Skoog, 1965) are most widely used media. Media compositions have been formulated for the specific plants and tissues (Nitsch & Nitsch, 1969). Some tissues respond much better on solid media while others on liquid media. As such, no single medium can be suggested as being entirely satisfactory for all types of plant tissues and organs. Different culture media

TABLE 1
Woody trees list

Plant species	Explants used	Medium + PGR's + additives used	Reference
Acacia melanoxylon	Node	MS + BAP (1.0 mg/l) + NAA (0.5 mg/l)	Jones & Smith (1988)
Eucalyptus tereticornis	Node	MS + BAP (1.0 mg/l) + NAA (0.1 mg/l)	Das & Mitra (1990)
Acacia nilotica	Cotyledonary node	B5 + BAP (1.5 mg/l)	Dewan *et al*. (1992)
Euonymus europaeus	Seedling cotyledone	MS + IAA (22.8 µM) + Kn (0.046 µM)	Bonneau *et al*. (1994)
Acacia senegal	Axillary bud	MS + BAP (4.0 mg/l) + NAA (0.5 mg/l) + Ads (25.0 mg/l) + AA (25.0 mg/l)	Gupta *et al*. (1994)
Acacia tortilis	Cotyledonary node	MS + BAP (5.0 mg/l) + NAA (0.1 mg/l)	Macreae (1994)
Fraxinus angustifolia	Shoot tip and node	DKW + BAP (4.4 µM) + IBA (0.98 µM)	Perez-parron *et al*. (1994)
Stryphnodendron polyphythum	Cotyledonary node	MS + BAP (13.3 µM)	Franca *et al*. (1995)
Dalbergia sissoo	Node	MS + BAP (4.4 x 10^{-6} M) + NOA (4.4 × 10^{-7} M)	Gulati & Jaiwal (1996)
Anogeissus latifolia	Cotyledonary node and epicotyl	MS + IAA (0.1 mg/l) + BAP (1.5 mg/l) + Ads (25 mg/l) + L-arginine (25 mg/l) + AA (25 mg/l) + citric acid (25 mg/l) + L-asparagine (1.0 mM) + 200 µM (Fe-EDTA)	Shekhawat *et al*. (2000)
Cardiospermum halicacabum	Cotyledon, hypocotyl, cotyledonary node, leaf, internode and node	MS + BAP (17.9 µM)	Babber *et al*. (2001)
Anogeissus pendula	Cotyledonary node	MS + BAP (4.4 µM) + IAA (5.7 µM) + casein hydrolysate (100 mg/1) + AA (50 mg /1)	Saxena & Dhawan (2001)
Tectona grandis	Apical shoot	MS + BAP (0.28 µM) + Kn (0.46 µM) + Ads (0.27 mM)	Gangopadhyay *et al*. (2003)
Acacia sinuata	Node	MS + BAP (8.9 µM) + TDZ (2.5 µM) + Ads (135.7 µM)	Vengadesan *et al*. (2003)
Prosopis laevigata	Cotyledonary node	MS + 2,4-D (9.05 µM) + BAP (6.62 µM)	Gonzalez *et al*. (2007)

Table 1 (*continued*)

Acacia senegal	Cotyledonary node	MS + BAP (1.0 mg /l)	Khalafalla & Daffalla (2008)
Acacia chundra	Shoot tip and node	MS + BAP (1.5 mg/l) + IAA (0.05 mg/l) + Ads 50 mg/l	Rout *et al*. (2008)
Wrightia tomentosa	Cotyledonary node	MS + BAP (5.0 mg /l)	Joshi *et al*. (2009)
Melia azedarach	Node	MS + BAP (5µM)	Husain & Anis (2009)
Spondias mangifera	Node	MS + BAP (1.0 mg /l)	Tripathi & Kumari (2010)
Michelia champaca	Seedling cotyledone	MS + NAA (2.0 mg /l)	Armiyanti *et al*. (2010)
Acacia auriculiformis	Axillary bud	B5 + coconut milk (10%) + BAP (10^{-6}M)	Girijashankar (2011)
Streblus asper	Node	MS + Kn (4.60 µM) + BAP (4.44 µM)	Gadidasu *et al*. (2011)
Terminalia catappa	Node	MS + BAP (2.0 mg /l)	Phulwaria *et al*. (2012)

proposed by the different scientists from time to time vary from each other in terms of their salt concentrations. Some of the earliest plant tissue culture media were developed by White (1943) and Gautheret (1939). All the subsequent media formulations are based on White's and Gautheret's media. The pH of the medium is also an important factor for tissue culture. The pH of the medium is usually adjusted to between 5 and 5.8 before autoclaving and extremes of pH are avoided. Each plant species has different optimized conditions both for growth of the cells and for production of useful products, so it is necessary to optimize the conditions in each case. Humidity in the culture vessel and osmotic potential of the medium affects the growth and development of plantlets *in vitro* in different ways (Brown *et al*., 1976; Ziv *et al*., 1983).

The MS medium was used either as described originally or with little variation and combination of phytohormones and vitamins, such as *Dalbergia latifolia* (Raghavaswamy *et al*., 1992), *Terminalia arjuna* (Kumari *et al*., 1998), *Sapindus mukorossi* (Philomina and Rao, 1999), *Melia azedarach* (Shahzad and Siddique, 2001), *Azadirachta indica* (Shekhawat *et al*. 2002). Raghavaswamy *et al*. (1992) observed that axillary bud initiation in *Dalbergia latifolia* was better on the MS medium while multiple shoot induction was better on Woody Plant Medium (*WPM*) or MS (reduced major salts) medium. Bhargava *et al*. (2003) reported that globular proembryonic mass of callus was formed on the MS medium after 40-50 days of incubation, and then transferred to B_5 medium for fragile snowy callus in *Phoenix dactylifera*. Sharada *et al*. (2003) used the MS

medium for shoot induction and B_5 or WPM medium for root development in *Celastrus paniculatus*.

There are some complex substances like coconut milk (CM), casein hydrolysate (CH), adenine sulphate (Ads), activated charcoal (AC), which are sometimes required in addition to growth hormones for callus induction and regeneration. For instance, the coconut milk of green nut is very effective in providing an undefined mixture of organic nutrients and growth factors (Gamborg & Phillips, 1995). Raghavaswami *et al*. (1992) reported that the growth adjuvants, like coconut milk, casein hydrolysate and adenine sulphate were also supplemented to the media for direct organogenesis and somatic embryogenesis in *Dalbergia latifolia*. Deb (2001) used 200 mg/l of casein hydrolysate for the induction of embryogenic callus from 3-4 days imbibed seeds of *Melia azedarach*.

Fridberg *et al*. (1978) reported that charcoal had an important role during culture by absorbing toxic compounds released by inoculated explants. Pierik (1987) showed that the addition of AC often has a promoting effect on growth and organogenesis in plant species. Charcoal has been used in regeneration medium for trees like *Dalbergia sissoo* (Gulati & Jaiwal, 1996) and *Areca catechu* (Mathew & Philip, 2000) to prevent browning of culture due to phenolic exudation released by the explants. The beneficial effects of activated charcoal were also found on multiple shoot induction from nodal explants of *Wattakaka volubilis* (Chakradhar & Pullaiah, 2006).

During culture, carbohydrates play an important role and act as an energy source required for growth, maintenance and for synthesis of cell constituents. The most commonly used carbohydrate source is sucrose, but other sugar like glucose, fructose, dextrose, mannitol, sorbitol etc. are also occasionally used. Meanwhile, sucrose also has an important role as it serves as a source of carbon and energy. Sucrose is also required for differentiation of xylem and phloem elements in the cultured cells (Aloni, 1980). Glucose and fructose are also known to support good growth of some tissues and are occasionally used. Sucrose represents the major osmotic component of the medium and is necessary for various metabolic activities. In most plants, 2-3% sucrose is found very effective for optimal growth and morphogenesis. MS medium with 2% sucrose was optimal for culturing of shoot tips in *Tamarindus indica* (Kopp & Nataraja, 1990). In *Eucalyptus sideroxylon*, however, it was observed that 4 to 6% sucrose caused more callus formation during culturing of axillary shoots, while 2-6 per cent sucrose in the MS medium supported roots development (Cheng *et al*., 1992). It was found that 3% sucrose is effective for shoot initiation from cotyledonary node explants in *Stryphnodendron polyphythum* (Franca *et al*., 1995). Twenty per cent sucrose concentration is more effective for the development of globular embryos of *Terminalia arjuna* (Kumari *et al*., 1998). In

Alnus nepalensis, 1.5 % sucrose in WPM medium was optimal for shoot proliferation from terminal axillary buds (Thakur *et al*., 2001). Shahzad and Siddiqui (2001) reported that 3% sucrose was required for callus as well as for shoot proliferation in *Melia azedarach*. Similarly, Shekhawat *et al*. (2002) also advocated the use of 2-3% sugar to obtain multiple shoots in *Azadirachta indica*. Likewise, Chakradhar and Pullaiah (2006) reported that 1.0 per cent of sucrose was necessary for the rooting of regenerated plantlets in *Wattakaka volubili*.

Agar-agar is used as a solidifying agent and assumed to be that of neutral support for callus growth and multiplication. Normally, 0.8 percent agar is used for culture medium. Pasqualatto *et al*. (1986) reported a higher concentration of solidifying agent in the medium reduced vitrification, but in certain cases, an increase in agar amount causes adverse effect, as observed by Lal and Singh (1995).

Plant Growth Regulators

Plant growth regulators directly or indirectly affect the growth and differentiation of plant tissues. Different plant growth regulators have different effects and they vary with the type and quantity to be applied. There are five known major classes of compounds with plant growth regulatory activity. These are auxins, cytokinins, gibberellins, abscisic acid and ethylene. Among various growth regulators, auxins (NAA, IAA, IBA and 2, 4-D), cytokinins (BAP, Kinetin, Zeatin), ABA, gibberellins and ethylene are very important. The nature of organogenic differentiation is determined by the relative concentration of auxins and cytokinins. Higher cytokinins to auxin ratio promote shoot formation, while higher auxins to cytokinins ratio favours root differentiation. Therefore, an auxin/cytokinin ratio plays a critical role in the induction of roots and shoots (Skoog & Miller, 1957). Auxins have an essential role in shoot induction and plant regeneration in most plant species. Auxins also induce somatic embryogenesis from the callus of *Citrus sinensis* (Kochba & Roy, 1973). Cytokinins alone, or in combination with auxins has been generally used in tissue culture. Cytokinins has been used in range of (0.5-30mg/l) and higher concentrations bring about morphological abnormalities and cause hyper hydration. Among cytokinins, BAP is the most commonly used in a variety of explants for shoot regeneration. Goyal and Arya (1979) observed the regeneration in *Prosopis cineraria* on MS medium with different concentrations and combinations of Kinetin, IAA, IBA and BAP. Gamborg's medium was used by Mukhopadhayay and Mohan (1981) for culturing of *Dalbergia sissoo*. Meanwhile, Rumary and Thorpe (1984) reported that in some cases, mixed cytokinins have beneficial role. Multiple shoots were obtained in *Eucalyptus grandis* on the MS medium supplemented with additional thiamine (Lakshmi Sita & Shobha Rani, 1985). It has been reported that the decrease in NAA ensures shoots formation (Rao *et al*., 1984; Sudha Devi & Natreja, 1987). Mittal *et al*. (1989) obtained multiple shoots from the axillary buds of

Acacia auriculiformis on Gamborg's (B_5) basal medium supplemented with coconut milk and BAP. Kopp and Nataraj (1990) regenerated plantlets by supplementing 2.0 mg/l BAP in *Tamarindus indica*. Multiple shoots were obtained from cotyledonary nodes of *Dalbergia latifolia* on MS medium fortified with (2.0 mg/l) BAP (Lakshmi Sita & Raghavaswamy, 1992). BAP also produced longer shoot as compared to kinetin in *Prosopis cineraria* and *Aegle marmelos* (Kumar & Singh, 2009; Yadav & Singh, 2011b), as shown in Fig.1b, 1c, and 1d.

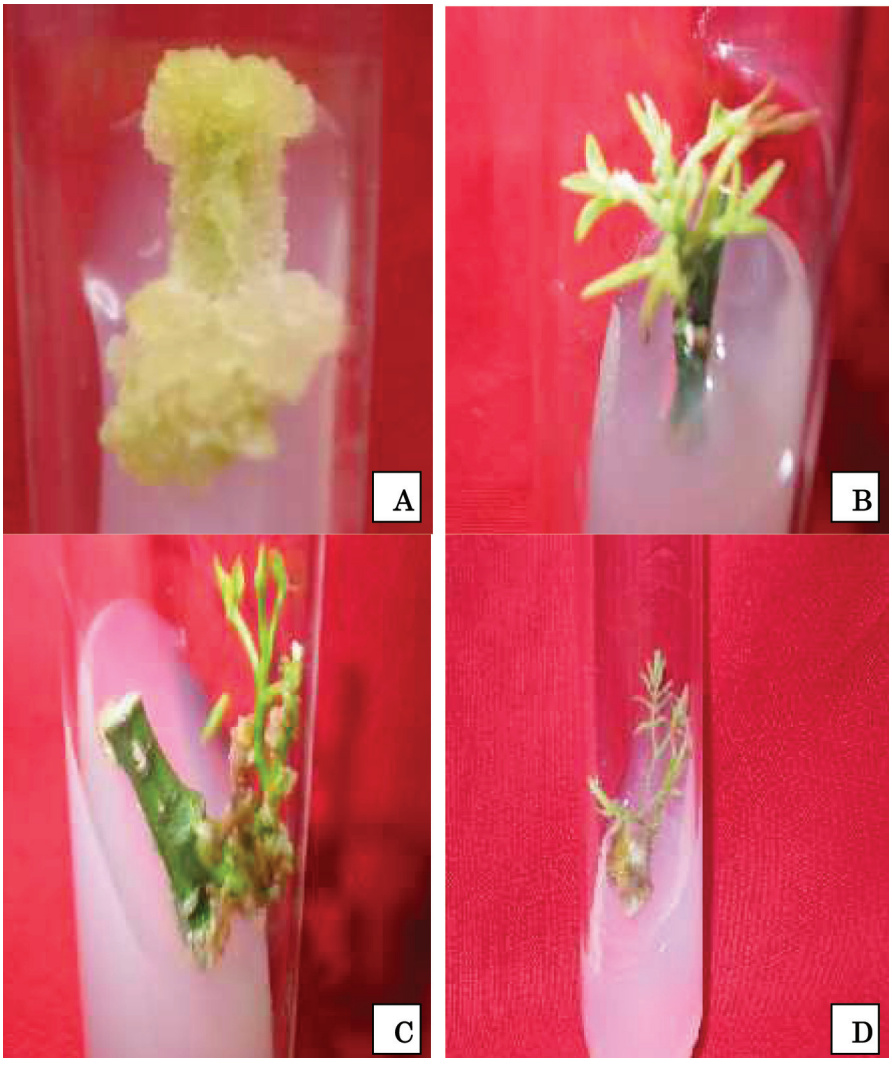

Fig.1: A. Callus induction from internodal segment of *Albizia lebbeck* on MS medium + BAP (2.0 mg/l) + NAA (0.5 mg/l); B. Shoot bud initiation on MS medium + BAP (2.0 mg/l) in nodal explants of *Aegle marmaelos*; C. Shoot formation from nodal explants of *Aegle marmelos* on MS medium with 2.0 mg/l BAP + 1.0 mg/l IAA; D. Callus growth and shoots proliferation from nodal explants of *Prosopis cineraria* on MS medium supplemented with BAP (2.0mg/l).

The MS media supplemented with BAP in combination with NAA supported the highest percentage of callus induction in *Leucaena leucocephala* (Singh & Lal, 2007) and *Albizia lebbeck* (Yadav & Singh, 2011a) (see Fig.1a). In *Eucalyptus camaldulensis*, the highest frequency of somatic embryos were produced from the callus obtained on the MS medium supplemented with 0.5 mg/l of BAP and 0.1 mg/l NAA from mature zygotic embryos (Prakash & Gurumurthi, 2010).

Selection of the Explants

The success in micropropagation from mature plants depends upon the careful selection of explants (Murashige, 1974; Sommer & Caldas, 1981; Williams & Maheswaran, 1986). In *Albizia lebbeck*, stem, root, leaf, rachis, leaflets, hypocotyls and axillary buds were used for regeneration (Arya *et al*., 1978; Gharyal & Maheshwari, 1990). Several factors influence the behaviour of the inoculum in culture (Murashige, 1974). These factors include:

a. The organ that serves as tissue source;
b. The physiological and ontogenic stage of the organ;
c. Season in which explant is obtained;
d. Size of the explants;
e. Overall quality of plant from which explants are to be taken

Huda *et al*. (2007) studied the *in vitro* morphogenic responses of different explants of *Corchorus olitorius*. The different explants viz. leaf segments, internodes and nodal segments showed different morphogenic responses. The nodal segments initiated callus earlier than leaf segments and the internode explants and higher amount of callus were obtained from the leaf segments than internodes and nodal segments. It was also reported by many workers that the quality of explants primarily determines the establishment of *in vitro* culture (Keathley, 1984). The nodal explants from a mature tree of *Heavea brasiliensis* failed to produce plantlets, while the explants taken from 6 to 8 weeks old plant regenerated plantlets (Rehman *et al*., 1981). Similarly, the explants from mature trees of *Eucalyptus citriodora* required pre-treatment for induction of shoot buds but the explants from seedlings did not require any pre-treatment (Gupta *et al*., 1981). Gulati and Jaiwal (1996) reported that the nodal explants taken from coppied shoots of mature *Dalbergia sissoo* exhibited the least phenolic exudation and responded better shoot regeneration, but this was not observed in the explants taken from mature trees. Callus formation and regeneration of plantlets from nodal explants was reported by Nandwani and Ramawat (1991) in *Prosopis juliflora*. Swamy *et al*. (1992) used the nodal explants of *in vitro* grown root suckers from the 60-80 years old tree of *Dalbergia latifolia* for direct organogenesis. In *Fraxinus angustifolia*, shoot tips and nodal segments were used for micropropagation (Perez-parron *et al*., 1994). The maximum number of shoots (9 shoots per explant) in the *Aegle marmelos* from nodal segments were obtained on

the MS medium supplemented with BAP (8.8µM) + IAA (5.7µM) by Pati *et al*. (2008). In *Melia azedarach*, multiple shoots were produced from the nodal segments on the MS medium supplemented with 5 µM of BAP (Husain & Anis, 2009). Tripathi and Kumari (2010) obtained an efficient *in vitro* propagation of *Spondias mangifera* using nodal explants from seedlings.

In addition, season was also found to affect the shoot proliferation and explants contamination. Seasonal conditions at the time of explants collection may influence the *in vitro* growth of explants, phenolics exudation and degree of contamination. The nodal segments of *Eucalyptus tereticornis* collected during July to September were more responsive to micropropagation because of the negligible phenolic exudation from explants as compared to that collected in October-November and May-June due to the high amount of phenolic exudation (Das & Mitra, 1990). Similar effects of season have also been noticed in other plants like *Tactona grandis* (Gupta *et al*., 1980) and *Eucalyptus tereticornis* (Das & Mitra, 1990). Bonneau *et al*. (1994) observed a higher percentage of embryonic callus production from the zygotic embryo explants in *Euonymus europacus* taken during May to September. Similarly, Thakur *et al*. (2001) observed an optimal establishment of axillary and terminal buds of *Alnus nepalensis* cultured during February and March; thereafter, the percentage establishment showed a declining order. Singh and Goyal (2007) reported that the August to October season was the best for explant collection in *Salvadora oleoides* throughout the year. The harvesting time of pods also showed a significant effect on the *in vitro* germination of seeds. Yadav and Singh (2011a) recorded the highest germination (83.3%) for the seeds extracted from dark-yellow pods in *Albizia lebbeck*.

Meanwhile, the size of the explants plays a key role in expressing the morphogenetic potentiality. Among other, Okazava *et al*. (1967) reported that small explants are more likely to form callus while larger explants maintain greater morphogenetic potentiality. This may be due to the available food reserves and growth regulators which have been proven to be useful in the initiation of new growth (Anderson, 1980). The orientation of the explants also plays an important role in giving morphogenic response. The horizontal position of the explants has been reported to promote adventitious shoot formation in many higher plants (Frett & Smagula, 1983; Pierik, 1987).

Cultural Conditions

Light is an important factor for the success of a tissue culture experiment. The intensity, quality and extent of daily exposure of light are the determining factors in the plant tissue culture. Cultures are usually maintained at a constant temperature of 25±2ºC and a photoperiod of 16 hours of light (20 µmol m^{-2} s^{-1} photosynthetic photon flux intensity) and 8 hours of darkness.

Gupta *et al*. (1981) reported multiple shoots production when the terminal buds from twenty years old tree of *Eucalyptus*

citridora were cultured on the MS medium at 15^{0}C in continuous light, followed by culture at 25^{0}C with 16 hours photoperiod. The effects of light and cytokinins interaction on the cultured cotyledon explants of *Radiata pine* were studied by Victor *et al*. (1984). In *Eucalyptus tereticonis*, a high rate of multiplication was achieved on the MS medium at a slightly higher temperature (30-32^{0}C) (Das & Mitra, 1990). Calleberg and Johansson (1993) studied that direct regeneration was mostly stimulated when the anther cultured was incubated at 20^{0}C. The formation of multiple shoots at 25±2^{0}C and 16 hours of light and 8 hours of dark periods under light intensity of 3000-4000 lux has also been reported in *Azadirachta indica* (Shekhawat *et al*., 2002).

Organogenesis

It involves the formation of organized structure like shoot and root from pre-existing structure, i.e. unorganized mass of cells known as callus. The controlled organogenesis under *in vitro* was given by White (1939) who obtained the shoots from the callus of *Nicotiana glauto* and *N. Iongsodorffi* hybrid on a agar-agar solidified medium. Later on a number of reports approved, depicting the formation of shoots and roots either directly from the explants or indirectly, i.e. from the callus.

Organogenesis deals through two pathways, i.e. direct pathway and indirect pathway. Direct pathway occurs through the continuous development of shoot meristems activity from lateral or axillary buds. Indirect pathways deal with the shoot formation *via* callus formation. Indirect regeneration often results in somaclonal variation making the strategy less desirable for large scale clonal multiplication. Therefore, direct regeneration without a callus phase is a reliable method for clone production.

Direct Organogenesis

Direct organogenesis, i.e. without callus formation, has also been reported in many herbeaceous and tree species. Several limitations, such as low shoot proliferation in forest trees, excessive phenolic exudation (Linington, 1991), basal callusing (Marks & Simpson, 1994), vitrification (Monsalud *et al*., 1995), and shoot tip necrosis (Bargchi & Alderson, 1996) are pronounced in tree tissue culture. Further, difficulty in rooting (Harada & Murai, 1996) has also had a negative effect on micropropagation of woody forest tree species.

Micropropagation without an intervening callus phase is advantageous over conventional vegetative propagation in terms of quantity, quality and economics (Altmann & Loberant, 1998). In general, three modes of *in vitro* plant regeneration have been in practice, namely, organogenesis, embryogenesis and axillary proliferation. The difference mainly matters when it relates to the genetic stability of the resulting micropropagated plants, and the obvious option would be the axillary and adventitious shoot proliferation. Meanwhile, *in vitro* micropropagation has been proven in the recent past as a means for supplying planting material for forestry (Ahuja, 1993; Lakshmi Sita & Raghavaswamy,

1998). Multiple shoots could be induced from the nodal segments of *Eucalyptus grandis* (Cresswell & Nitch, 1975). The use of a protocol to promote axillary and apical shoot bud proliferation *in vitro* has been used for the propagation of forest tree species. The multiple shoots of Himalayan oaks were induced from intact embryos and cotyledonary nodes of Himalayan oaks (Purohit *et al*., 2002). A method for adventitious shoot regeneration from leaf explants of micropropagated *Peach* shoots has been developed (Gentile *et al*., 2002). This method involves the utilization of shoot tips, lateral buds and small nodal and internodal cutting as explants and it genetically establishes stable culture without any callus formation. Mittal *et al*. (1989) observed the formation of multiple shoots from the axillary buds from the *in vitro* grown seedlings of *Acacia auriculiformis*. Kopp and Natraraja (1990) regenerated plantlets from shoot tip explants of *in vitro* grown seedlings of *Tamarindus indica.* Das and Mitra (1990) reported 18-22 shoots per explants in *Eucalyptus tereticornis* when nodal explants were inoculated on the MS medium with BAP (1.0 mg/l) and NAA (0.1 mg/l). Singh *et al*. (1993) observed the sprouting of axillary bud in *Acacia nilotica* on MS and WP medium fortified with BAP (1.0 mg/l).

Kumar and Seeni (1998) achieved a rapid clonal multiplication of *Aegle marmelos* by enhancing axillary bud proliferation in single node segment of a twenty five years old tree on the MS medium supplemented with BAP (2.5mg/l) in combination with IAA (1mg/l). Komalavalli and Rao (2000) established the *in vitro* propagation protocol of *Gymnema sylvestre* - a multipurpose plant. The MS medium fortified with growth regulators such as BAP (0.5mg/l) in combination with NAA (0.01mg/l) has been reported to give optimum results in *Utleria salcifolia* (Gangaprasad, 2003). Kumari *et al*. (2005) reported multiple shoot formation from the nodal and shoot tip explants in *Wedelia chilensis* under *in vitro* conditions. Rathore *et al*. (2005) developed protocol for the *in vitro* propagation of *Maerua oblongifolia* using nodal shoots segments on MS medium and achieved a high rate of shoot multiplication. Thidiazuron (TDZ) was used at 1.0 mM and found to be the most effective in inducing bud break and growth, and also in initiating multiple shoot proliferation at the rate of 25 microshoots per nodal explant with axillary buds, after 4 weeks of culture (Ahmad & Anis, 2007). Rathore and Shekhawat (2009) reported 35–40 shoots per culture vessel shoots proliferated on the MS medium supplemented with 4.44 lM BA and 0.57 lM indole acetic acid (IAA) and additives in *Pueraria tuberosa*. The multiple shoot formation was observed to be the highest in the MS fortified with 2 mg/L Benzyl amino purine (BAP) and 0.1 mg/L Naphthalene acetic acid (NAA) in *Acacia auriculiformis* (Girijashankar, 2011).

Indirect Organogenesis

Lakshmi Sita and Vaidyanathan (1979) raised plantlets from the cotyledonary callus of *Eucalyptus citriodora*.

Adventitious shoot regeneration was reported through the callus culture in *Eucalyptus camaldulensis* taken from the shoot culture of mature tree (Murlidharan & Mascarenhas, 1987). Inamdar *et al*. (1990) reported the formation of somatic embryos via callusing from culture of shoot apices of adult *Crataeva nurvala* on MS medium containing 2, 4-D. The callus cultures and plantlet formation *in vitro* were reported in *Prosopis juliflora* by Nandwani and Ramawat (1991). Joshi and Dhar (2003) obtained the maximum shoots from epicotyle explants that were cultured on the MS medium supplemented with (0.25 µm) NAA and (1.0 µm) Kinetin in *Saussurea obvallata*. Thomas and Philip (2005) reported a high frequency shoot organogenesis from the leaf derived callus of *Dalbergia sissoo*. Similarly, Faisal and Anis (2005) developed a protocol for high frequency shoot regeneration and plant establishment of *Tylophora indica* from petiole derived callus. Organogenic Callus was developed from the stem explant of *Ruta graveolens* on the MS medium composed of 2.5 µm BA + 10 µm 2-4-D. Deepa *et al*. (2006) noticed profuse callusing from cotyledon and shoot tip explants in *Pseudarthria viscida* on the MS medium supplemented with 2,4-D (1.5-2 mg/l) and BAP (1-1.5 mg/l). Agrawal and Sardar (2006) described a high frequency shoot regeneration through leaflet and cotyledon derived calli in *Cassia angustifolia*. The MS medium, supplemented with BAP (1.0 mg/l) and IBA (0.5 mg/l), was found to be the most effective combination for shoot bud differentiation in *Jatropha curcus* (Rajore & Batra, 2005). Meanwhile, subsequent shoot regeneration was achieved in the MS medium supplemented with BAP (2mg/l).

The occurrence of genetic variation is a matter of great variation concern where commercial success in micro propagation is dependent mainly on the maintenance of clonal uniformity (Bonga, 1987). However, abnormalities in the tissue culture and the plants produced from them often increase in frequency with the increase in the culture passages.

Rooting of In vitro Regenerated Shoots

For the development of a perfect plantlet, it is essential that the regenerated shoots must develop the roots. The culture medium for rooting varies from tissue to tissue, as well as from species to species. The shoots of certain plant species developed root only on the simple MS medium, i.e. hormone free media, as reported in *Cardiospermum halicacabum* (Babber *et al*., 2001). Among the auxins, IBA with MS medium is the most commonly used to induce rooting (Raghavaswamy *et al*., 1992; Nandwani & Ramawat, 1993; Shahazad & Siddqui, 2001). Ahmed *et al*. (2007) used different concentrations of IBA, NAA and IAA for rooting and the highest rooting percentage (97.66%) was reported on the MS medium with 0.1 mg/l IAA. This shows that IBA is better than NAA and IAA for shoot and root formation. As for the effect of NAA on root formation, data indicated that the percentage of shoots formed roots was 87%

on the MS basal medium without plant growth regulators as compared to the MS basal medium supplemented with NAA at low concentrations of 0.01 and 0.1 mg/l NAA, where the root percentage was 80%, 73% and 53%, respectively. On the other hand, NAA at 1.0 and 1.5 mg/l did not help shoots to form roots. The shoots must be transferred to a rooting medium which is different from the shoot multiplication medium, particularly in its hormonal and salt composition. However, the nutritive medium for rooting varies from tissue to tissue, as well as from species to species. Shoot multiplication was induced on full strength MS medium whereas the salt concentration was reduced to half (Garland & Stoltz, 1981; Zimmerman & Broome, 1981) or a quarter (Skirvin & Chu, 1979) for rooting. Most frequently, IAA, IBA and NAA (0.1-1.0 mg/l) have been used for this purpose; however, IAA and IBA were found to be more effective (George & Sherrington, 1984). In *Moringa pterygosperma,* half strength MS medium supplemented with GA_3 (0.2 mg/l) produced roots within 7 days in 25% cultures. However, a better rooting was developed with 3% sucrose and IBA (0.2 mg/l) only (Mohan *et al*., 1995). Sharma and Padhya (1996) observed root induction in *Crataeva nurvala* within 7 days on the MS medium with a low dose of NAA (0.5 µm). Mustafa and Hariharan (1997) got limited root in the hormone free medium whereas NAA alone was better than the combination of NAA and IBA in *Alpinia galangal.* Sharada *et al*. (2003) achieved 85% rooting on McCown medium (WPM) containing IBA (5 x 10^{-6}M) in *Celastrus paniculatus.* The high percentage of root regeneration in adventitious shoots was obtained on the MS medium supplemented with 0.1 mg/l IAA in *Populus ciliata* (Thakur *et al*., 2005). Sayd *et al*. (2010) reported that all strengths of MS-medium with 1g/l AC produced rooted shootlet but the quarter strength of MS-medium produced the highest rooting, leaf number and shootlet length. *In vitro* developed microshoots were rooted on the MS half strength medium supplemented with 2.46 µM IBA in *Streblus asper* (Gadidasu *et al.,* 2011).

Hardening of Regenerated Plantlets

After rooting, hardening of regenerants prior to transfer in the soil increases the survival rate of transferred plants. So, it is a step which gradually acclimatizes the plant to the harsh natural environment. Spraying, misting and covering with the thin polythene may serve to fulfil the above objective. Various types of substrates have been used during acclimatization such as soil vermiculites mixture sterilized sand and soil (Goyal & Arya, 1981; Gulati & Jaiwal, 1996; Philomina & Rao, 1999; Thakur *et al*., 2001; Sunaina & Goyal, 2000). The *in vitro* developed plantlets of *Dalbergia latifolia* were successfully transferred by Raghavaswamy *et al*. (1992) by keeping the roots in tap water and high humidity. After this, the plantlets were exposed to fresh air for a few hours daily and after 14 days they were transferred into 1:3 courses sand:soil mixture. The plantlets of

4-5 cm long with fresh leaves were slowly transferred to field conditions (Chaudhary *et al*., 2004). Sharma *et al*. (2006) transferred well-developed plantlets with a complete root system into pot containing sterile vermiculite and covered with transferred polythene bags to ensure high humidity. The serving plants were transplanted to field after 2 months. The most crucial step in the micropropagation is the hardening and acclimatization as it is the process which makes the plantlets capable of tolerating the natural environmental conditions. Complete regenerated plantlets with sufficient roots were taken out from the test tubes and washed several times with sterile distilled water to remove traces of the MS medium by putting the roots under continuous slow running water with the help of fine brush. Then, the *in vitro* regenerated plantlets were transplanted in small earthen pots containing sterilized soil and sand mixture (3:1). Each pot was covered with polythene bags with small holes to maintain high humidity and they were kept in the culture room to get acclimatized. The plantlets were initially irrigated with half strength (salts only) MS medium without sucrose on alternate days. The plantlets were exposed to the conditions for natural humidity for 3-4 hours daily to after 10 days of transfer. After about 30 days, the plants were transferred to bigger pots in the greenhouse and were maintained under natural conditions of day length, temperature and humidity. Finally, the plants were transferred to the field conditions. Successful acclimatization and field transfer of *in vitro* regenerated plantlets have also been reported in *Tamarindus indica* (Kopp & Nataraja, 1990), *Eucalyptus tereticornis* (Das & Mitra, 1990), *Prosopis juliflora* (Nandwani & Ramawat, 1991), *Dalbergia latifolia* (Raghavaswamy *et al*., 1992), *Thevetia peruviana* (Kumar, 1992), *Alpinia galangal* (Anand & Hariharan, 1997), *Terminalia arjuna* (Kumari *et al*., 1998), *Sapindus mukorossi* (Philomina & Rao, 2000), *Salvadora persica* (Mathur *et al*., 2002), *Bupleurum disticho-phyllum* (Karuppusamy & Pullaiah, 2007), *Spondias mangifera* (Tripathi & Kumari, 2010), *Acacia auriculiformis* (Girijashankar, 2011), *Streblus asper* (Gadidasu *et al.,* 2011). Hardening and acclimatization of plantlets were done because the plantlets raised under *in vitro* conditions on the synthetic carbohydrate supplemented medium under artificial light failed to abruptly acclimatize to the rigour of the natural environment. So, a careful transfer of the plantlets in the soil after hardening and acclimatization is required.

CONCLUSION

Tissue culture offers unparalleled opportunity for forest tree improvement. Micropropagation techniques have been applied to a wide range of tree species. Successful *in vitro techniques* are dependent upon the strong and intricate interactions between the explant, plant growth regulators, culture conditions, and genotype. The present status of tree tissue culture, however, is adequate to initiate commercialization

programmes. Commercialization has already been proven to be successful in the cases of some trees like Eucalyptus, Rosewood, Poplar and others. In a commercial scale, the improved protocols should be working efficiently with various genotypes, preferably of the mature origin. Meanwhile, the establishment of protocols with reduced steps of developmental pathways may significantly reduce the time and cost. Most of the tree tissue culture research has centred on methods, primarily on the development of culture media and techniques to induce juvenility in trees for micropropagation. The thrust is towards methods, which have commercial use and the potential for patents. Also, unless the cost of plantlet production by tissue culture techniques is brought down considerably to match with or less than the conventional methods of propagation, the efforts are not worthy enough to justify. Nonetheless, clonal fidelity in trees micropropagated by organogenesis has not been adequately tested with many species. Research on micropropagation will stress on the mass propagation of mature trees and reduction of costs. Research on micropropagation by somatic embryogenesis has to be intensified so as to reach the ultimate goal of mass propagation since it also allows automation which will in turn reduce the cost per propagule. Continued research into the use of biotechnology will contribute to improving the vegetative propagation of the species, which will ultimately play an important role in future breeding programmes of forest tree improvement and reforestation programmes.

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