REVIEW OF LITERATURE

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This chapter briefly reviews the work done on shoot bud culture, callus culture, peroxidase isoenzymes and some oxidase enzymes.

A. Shoot Bud Culture

There are three common methods for *in vitro* propagation of plants namely shoot bud proliferation, organogenesis and somatic embryogenesis. Among these techniques, axillary bud proliferation is most successful for clonal propagation of angiosperms. Thorpe *et al.* (1991) reported plantlet regeneration from over 70 angiosperms; about 45% via axillary buds, 13% from adventitious buds, 25% indirectly from callus and 17% from somatic embryogenesis. Micropropagation through axillary bud proliferation includes the following stages of development:

I. Explant establishment or initiation of shoots on explants
II. Shoot proliferation and shoot elongation
III. Rooting
IV. Acclimatization of plantlets and field transfer.

During these stages, specially designed chemical and physical environments are required. These requirements also differ from one species to another. The factors that influence *in vitro* plant regeneration at different stages, mostly of hardwood species, have been briefly reviewed here.

I. Explant Establishment:

Establishment of explants is a very important stage for successful propagation of plants *in vitro*. There are several factors that influence the explant establishment Some of the important factors are juvenility and maturity of explant or explant

source, season of explant collection, nutrient medium, growth regulators, phenolic exudation and contamination.

1. Juvenility and maturity of the explant or explant source:

Most of the woody dicot species have been propagated *in vitro* using juvenile explants (Sommer and Wetzstein, 1984). Micropropagation of mature tree species is extremely difficult due to the adult status of explant tissues (D'Souza *et al.*, 1999). Generally, seedling explants establish more easily than mature tree explants. Kanwar *et al.* (1995) reported that explants from 2-year-old plants of *Robinia pseudoacacia* were more easy to establish as compared to the 12- year-old plants. Jones (1978) reported that it was easier to establish explants from one year old apple trees than from 8-year old trees. Organogenesis and plantlet regeneration have been reported from seeds or seedlings of *Achras sepota* (Purohit *et al.*, 1999), *Litchi chinensis* (Das *et al.*, *1999*), *Syzygium cuminii* (Yadav *et al.*, 1990), *Anacardium occidentale* (D'Silva and D'Souza, 1992b), *Diospyros kaki* (Tao *et al.*, 1994), *Thevetia peruviana* (Thind, 1994), *Lagerstroemia parviflora* (Tiwari *et al.*, 1998), *Wrightia tinctoria* (Purohit and Kukda, 1994), *Boswellia serrata* (Purohit *et al.*, 1995), *Garcinia mangostana* (Normah *et al.*, 1995), *Psidium guajava* (Mohamed-Yasseen *et al.*, 1995), *Passiflora edulis* (Kantharajah and Dodd, 1990), *Alnus nepalensis* (Kaur *et al.*, 1993), *Fraxinus excelsior* (Hammatt and Ridout, 1992), *Paulownia fortunei* (Chauhan and Emmanuel, 1998) and *Pterocarpus marsupium* and *P. santalinus* (Anuradha and Pullaiah, 1999). Quraishi *et al.* (1997) studied *in vitro* propagation in *Lagerstroemia parviflora* using nodal segments from seedlings, basal-sprouts and an established tree. They found best axillary shoot development from seedling nodes followed by basal-sprout and established tree. In *Cleistanthus collinus*, best *in vitro* establishment response was exhibited by juvenile nodes (Quraishi *et al.*, 1996), followed by nodal explants from basal sprouts and established trees (Quraishi and Mishra, 1998).

Position of the explant on source tree is also important for establishment of explants. Amin and Jaiswal (1993) observed that the bud explants taken from emerging trunk sprouts in *Artocarpus heterophyllus* invariably produced clumps of multiple shoots, whereas buds from actively growing top branches generally elongated to form a solitary shoot. The frequency of axillary bud formation was higher in explants collected from the top of the chestnut trees than those collected from the lower crown (Sanchez and Vieitez, 1991).

2. Contamination:

Reuveni *et al.* (1990) reported season-dependent contamination in *Carica papaya*. They found lowest frequency of contamination in March and highest in January. In strawberry cvs Douglas and Chandler, use of 1-2 mm long runner tips allowed the establishment of *in vitro* cultures with a 75-100% survival during spring and early summer, while contamination problem occurred with cultures initiated during late summer and autumn (Lopez-Aranda *et al.*, 1994). The nodal segments from field grown seedlings of *Cleistanthus collinus* showed maximum contamination during July to September and minimum contamination during January to June (Quraishi *et al.*, 1996).

Contamination is usually more of a problem with explants from mature trees than with explants from juvenile trees. Generally contamination of sprout explants is less than with mature branches (40% versus 100 % respectively) (Warrag *et al.*, 1990), and the use of enclosed buds avoids many problems (Horgan, 1987).

The contamination in tissue culture can originate from two sources, either through carry over of microorganisms on the surface or in the tissues of explants or through improper procedures in the laboratory (Cassells, 1991). Surface sterilization may be relatively easy, but the systemic infection / endophytic contamination may be extremely difficult to eliminate (Thorpe *et al.*, 1991)

Explanted cells, tissues and organs as well as their environment must be sterile. Working surfaces are best treated with disinfectants to eliminate contamination. The most commonly used surface disinfectants are sodium hypochlorite, calcium

hypochlorite and mercuric chloride (Constabel and Shyluk, 1994). To increase wettability, a small amount of surfactant such as Tween 20, Tween 80, Teepol or Mannoxol, Sodium lauryl sulfate are added to the disinfecting solution. Quick dip of ethyl alcohol was found useful in *Fagus sylvatica* (Meier and Reuther, 1994) and *Alnus nepalensis* (Kaur *et al.*, 1993).

Reddy *et al.* (1998) reported that the micropropagation of 65-year old sandal was unsuccessful due to constant occurrence of both fungal and bacterial contamination in all cultures. They eliminated fungal contamination by using Bavistin, a systemic fungicide, in the sterilization procedure; and isolated gram-negative bacterium, *Pseudomonas,* that showed maximum sensitivity to antibiotic Tetracycline in sensitivity disc test. Kumar *et al.* (1998) successfully decontaminated initial single nodal explants from field grown male and female plants of *Actinidia deliciosa* by a solution of Bavistin and Streptocycline containing 2% Teepol as wetting agent before sterilizing with mercuric chloride. Cardoza *et al.* (1999) effectively controlled contamination in *Anacardium oxidentale* by agitating the explant with 0.1% bavistin on a rotary shaker for 1 hour followed by 0.1% mercuric chloride-sodium lauryl sulfate solution for 15 minutes. They studied contamination in different seasons and observed that in rainy season contamination was high and in summer and winter contamination was very little.

3. Browning of explant and medium:

Generally phenolics are inhibitory substances that should be avoided or eliminated from *in vitro* environments (Krikorian, 1994). The exudation of phenolic substances have been found to depend on the season of explant collection and the age of explant. Das and Mitra (1990) reported that the exudation of phenolics was low if the explants were collected during July to September. In case of *Cleistanthus collinus*, the nodal segments of seedling origin were free from browning problem, but the explants from basal sprouts and

established trees showed excessive exudation of phenolic substances (Quraishi and Mishra, 1998).

Primary explants, especially, of tropical species particularly but by no means exclusively woody ones, are especially likely to darken and it has been more or less been routinely assumed that this darkening is due to polyphenol oxidase activity (Krikorian, 1994). Many agents such as ascorbic acid, sodium hydrosulfite, cystine, diethyldithiocarbamate-DTT, potassium ethylxanthate, thiourea, benzimidazole, sodium bisulfite, polyvinyl pyrrolidone (PVP) soluble, polyclar AT, citric acid, glutathione, bovine serum, albumin, etc. have been used to control browning (Krikorian, 1994).

In case of *Terminalia bellerica,* the problem of leaching was overcome by suspending the explants in PVP and agitating on a rotary shaker (Roy *et al.*, 1987). Suspension of explants in a solution of citric acid and PVP along with addition of these chemicals in nutrient medium effectively checked phenolic exudation in *Lagerstroemia parviflora* (Quraishi *et al.*, 1997) and *Cleistanthus collinus* (Quraishi and Mishra, 1998).

Addition of ascorbic acid in the medium prevented exudate browning of explants in *Eucalyptus citriodora* (Grewal *et al.*, 1980) and *Dalbergia sissoo* (Datta *et al.*, 1982). Ascorbic acid and PVP controlled browning in *Boswellia serrata* (Purohit *et al.*, 1995). Ascorbic acid and citric acid were added in the medium to check the leaching and explant browning in *Anogeissus sericea* (Kaur *et al.*, 1992). In *Feronia limonia*, Purohit and Tak (1992) prevented phenolic exudation by suspending the nodal explants in solution containing ascorbic acid, citric acid and PVP and by addition of these chemicals in the medium. Ziv and Halevy (1983) reported that the control of oxidative browning in *Strelitzia reginae* with 0.04% filter sterilized DTT was pivotal to successful culture.

Frequent subculturing at initiation stage effectively avoided browning of explants in *Melaleuca alternifolia* (List *et al.*, 1996), *Tectona grandis* (Devi *et al.*, 1994), Black berry (Broome and Zimmerman, 1978), Mountain laurel (Lloyd and Mc Cown, 1980) and *Grevillea robusta* (Rajasekaran, 1994).

In case of *Ailanthus malabarica*, phenolic exudation was controlled by incubating the explants on media supplemented with activated charcoal, subculturing explants every two day up to the seventh subculture and incubating the cultures in dark during this period (D'Silva and D'Souza, 1992a).

4. Season of explant collection:

Establishment of explants, particularly those from field grew trees, depends upon the season or time of the year in which explants are obtained from source plant. The best season for explant establishment varies according to type of explant and species. In several micropropagation studies meristem and shoot bud cultures have been initiated in a particular month or season.

The shoot tips of *Carica papaya* explanted between March to June responded in culture better than those explanted during July to September (Litz and Conover, 1981). Quraishi and Mishra (1998) reported that the nodal segments from basal sprouts and terminal twigs of *Cleistanthus collinus* exhibited best bud break response on establishment medium, when explanted in June.

Banko and Stefani (1989) initiated shoot bud cultures from forced axillary shoots of dormant stems of 24-year-old *Oxydendrum arboreum* in March and April and from the nodal explants of growing stems in April. Deora and Shekhawat (1995) reported that the nodal explants harvested during the months of March-April and August-October were best for establishment of cultures of *Capparis decidua*. Das and Mitra (1990) made continuous trials using explants from the elite trees of *Eucalyptus tereticornis* throughout the year. They found that the period between July to September was the best season for rapid and increased multiplication of axillary buds. Devi *et al.* (1995) reported micropropagation of elite 30 years old trees of teak (*Tectona grandis*) plants. They collected shoot buds of different sizes from elite teak plants in different months round the year. They found that the apical shoots (5-9 mm long) collected in December were best to initiate culture on establishment medium. For initiating cultures of *Liquidambar styraciflua*, Sutter and Barker (1985) collected actively growing

shoots from seedlings kept in a greenhouse under lights between October and March and shoot tips from actively growing shoots and dormant buds from both dormant and actively growing shoots from mature trees between February through August. Nobre (1994) collected actively growing 5-6 cm long shoots from field-grown plants of *Myrtus communis* in April, for *in vitro* shoot proliferation. November to January was the best period for establishment of mature tree explants of *Artocarpus heterophyllus* (Amin and Jaiswal, 1993). During this period 100% explants exhibited bud break and only 5% showed death. Lopez-Aranda *et al.* (1994) reported that 75-100% explants of strawberry survived during spring and early summer. Quraishi *et al.* (1996) reported that the nodal explants from *Cleistanthus collinus* seedlings collected during April to June produced more shoots and the shoots were longer than during other times of the year.

5. Medium:

Several media have been formulated for micropropagation of angiosperms but Murashige and Skoog or MS salts (1962) remains the most widely used medium (Harry and Thorpe, 1994). Other media used for micropropagation of angiosperms are Woody Plant Medium or WPM (Lloyd and Mc Cown, 1980), Gresshoff and Doy or GD formulation (1972), Gamborg *et al.* or B5 salts (1968), LS (Linsmaier-Skoog, 1965), IS (Saito and Ide, 1985), DKW (Driver and Kuniyaki, 1984), SH (Schenk and Hildebrandt, 1972), QP (Quoirin and Lepoivre, 1977), and Heller's medium (Vieitez *et al.*, 1989).

Hammatt and Ridout (1992) reported that the cotyledonary nodes of *Fraxinus excelsior* produced best axillary shoots when placed on DKW basal medium, whereas the cultures died on MS medium, and with WPM, the explants developed large amount of callus from their cut ends, and long internodes and small leaves in new axillary shoots. Harda and Murai (1996) studied the effects of two different basal media MS and WPM on shoot proliferation from nodal explants of *Prunus mume*. They found that the shoot proliferation was better on

WPM than MS medium. Ananthakrishnan *et al.* (1999) tested MS, MS half-strength and WP medium for shoot multiplication from embryonic axis with cotyledon segments of *Anacardium occidentale*. They obtained maximum number of shoots from embryonic axis with cotyledon segments cultured on woody plant medium. Anita and Pullaiah (1999) screened MS, B5 and WP media to induce shoot buds in seedling explants of *Sterculia urens*. In this case, the explants oh MS medium exhibited high shoot bud induction frequency without shoot tip necrosis whereas on B5 and WPM they showed low shoot bud induction frequency and shoot tip necrosis. In this case, the MS medium exhibited high frequency of shoot bud induction without shoot tip necrosis whereas B5 and WPM showed low frequency of shoot bud induction with shoot tip necrosis. In cotyledonary nodal explants of *Butea monosperma*, shoot tip necrosis was arrested in 95% of the shoots when fructose was incorporated in half-strength WPM with 30 g/l sucrose and 5 mg/l 6-benzylaminopurine (Kulkarni and D'souza, 2000). Sutter and Barker (1985) reported that the shoot tips excised from seedlings of *Liquidamber styraciflua* grew better on WPM than LS medium. Vieitez *et al.* (1989) found best shoot multiplication from shoot tip and node explants of 50 year old *Camellia japonica* on the micronutrients of WPM and those of the modified Heller's medium. The nodal segments from basal-sprouts and trees of *Cleistanthus collinus* established better on MS medium than on WPM and B5 medium (Quraishi and Mishra, 1998); and the nodal segments from seedlings established better on MS than WPM and White's medium (Quraishi *et al.*, 1996).

Meier and Reuther (1994) found difference in growth of different genotypes of *Fagus sylvatica* explanted on various tissue culture media. They found highest survival rate and growth response of buds from the juvenile genotype on GD and best bud development from mature F-8 explants on MS. In the following subculture steps WPM proved to be more suitable for micropropagation of mature beech material, followed by SH and GD. Nodal explants from Juvenile plants of *Azadirachta indica* showed efficient shoot bud induction and shoot

multiplication on WB medium (Venkateswarlu *et al.*, 1995) and those from 22 year old trees on MS and WB medium (Venkateswarlu *et al.*, 1998).

Among the B5, MS and WPM used, MS medium was found relatively the best suitable medium for shoot regeneration from cotyledonary nodes of *Dalbergia paniculata* (Sreedevi and Pullaiah, 1999). Bergman *et al.* (1985) used MS and LP media for micropropagation of *Salix* sp. For micropropagation of juvenile and adult *Annona squamosa* silver thiosulphate was added at 0.5mg/l in WPM to control leaf abscission (Lemos and Blake, 1996).

6. Growth regulators:

Numerous additional components such as coconut milk, casein hydrolysate, glycine, vitamins, phytohormones and various kinds of agar are added in the medium for meristem and shoot bud culture. The single most important medium additives are the cytokinins (Harry and Thorpe, 1994). Most commonly used cytokinins are benzylaminopurine (BA), 2-isopentyladenine (2-iP), kinetin (KIN) and zeatin (ZEA). The most widely used cytokinin is BA and concentrations vary from 0.1 to 1.0 uM for hardwoods (McCown and McCown, 1987).

Plant growth regulator requirement for meristem and shoot tip culture depends on plant species and culture stage. The growth regulator requirement for meristem and shoot tip culture of diverse dicot and monocot species have been extensively reviewed by Styer and Chin (1983). An extensive review of plant growth regulators used at critical stages in meristem and shoot tip culture is available (Hu and Wang, 1983). The growth regulator requirements for meristem and shoot tip culture of tropical and subtropical fruits and vegetable crops have been reviewed by Litz and Jaiswal (1991); and Seckinger (1991). The culture media and growth hormones used for meristem and shoot tip culture for different crop species have been reviewed recently by Nehra and Kartha (1994).

Most species require exogenous cytokinins to support the growth and development of shoot on establishment medium. Type and concentration of cytokinin in stage I medium are important factors for initiation of culture

Benzylaminopurine is most commonly used cytokinin for meristem, shoot tip and bud-cultures, followed by kinetin and 2-iP (Nair *et al.*, 1979). Benzylaminopurine has been used for initiating the cultures of *Salix* species (Bergman *et al.*, 1985), *Betula pendula* (Besendorfer and Kolevska-Pletikapic, 1990), *Fraxinus excelsior* (Hammat and Ridout, 1992), *Anacardium occidentale* (D'Silva and D'Souza, 1992b), *Robinia pseudoacacia* (Kanwar *et al.*, 1995), *Prunus mume* (Harda and Murai, 1996) and *Cleistanthus collinus* (Quraishi *et al.*, 1996).

Purohit *et al.* (1999) reported that the cotyledonary nodes of *Achras sapota* showed highest bud break frequency and maximum number of shoots on SH medium containing 2.0 mgl-1 BAP followed by MS, WPM, B5 and White's medium. Youn and Ohba (1990) reported that the addition of 1.0 mg / l BAP in IS medium and WPM enhanced shoot development and shoot elongation from axillary buds of 15 year old tree of *Tilia amurensis,* whereas addition of 0.5mg / l was effective with MS medium.

Banko and Stefani (1989) reported that the Zeatin was effective at 4.0 uM, whereas BA and 2-iP were ineffective at the same concentrations, for establishment of shoots forced from dormant stems and nodal explants from current season's growth of a 24 year old tree of *Oxydendrum arboreum.*

With some species, mixed cytokinins have proven to be beneficial for establishment of explants. BA and kinetin were used for establishment of explants from the plants of *Ailanthus malabarica* (D'Silva and D'Souza, 1992a), *Artocarpus heterophyllus* (Amin and Jaiswal, 1993), *Alnus nepalensis* (Kaur *et al.*, 1993), *Tectona grandis* (Devi *et al.*, 1994) and *Pisonia alba* (Jagdishchandra *et al.*, 1999)

Auxins are not essential for stage I culture medium, but beneficial if added at low concentrations (Nehra and Kartha, 1994). BA and IAA have been used in stage I medium for initiation of shoot bud cultures of *Hovenia dulecis* (Echeverrigaray *et al.*, 1998), *Cammellia sinensis* (Banerjee and Agarwal, 1990) and *Anogeissus sericea* (Kaur *et al.*, 1992). Combination of Kinetin, BA and IBA induced bud break in *Dalbergia sisso* (Chauhan *et al.*, 1996). Kinetin, BAP and NAA were used by Purohit and Tak (1992) in *Feronia limonia.*

Cytokinin and auxin have been used together in stage I culture medium for establishment of *Quercus suber* (Manzanera and Pardos, 1990), *Eucalyptus grandis* (Rao and Venkateswara, 1985; Mac Rae and Van Staden, 1990), *Iris ensata* (Yabuya *et al.*, 1991), *Liquidamber styraciflua* (Sutter and Barker, 1985) and *Capparis decidua* (Deora and Shekhawat, 1995).

II. Shoot Proliferation

The main objective of this stage is to produce maximum number of useful propagule units. Axillary shoot proliferation provides genetic stability and it is readily achievable by most plant species. The cytokinin which have been used for proliferation of shoots *in vitro* are BAP, KIN, 2-iP and ZEA. 6-Benzylaminopurine is the most effective synthetic cytokinin used for stimulating axillary shoot proliferation during stage II of micropropagation. The same level of exogenous cytokinin in establishment and proliferation media was used for *in vitro* propagation of *Syzygium cuminii* (Yadav *et al.*, 1990), *Betula pendula* (Besendorfer and Kolevska-Pletikapic, 1990), *Artocarpus heterophyllus* (Amin and Jaiswal, 1993), *Alnus nepalensis* (Kaur *et al.*, 1993) and *Cleistanthus collinus* (Quraishi and Mishra, 1998).

The concentration of BA was increased in stage II medium for multiple shoot induction in *Tectona grandis* (Devi *et al.*, 1994), *Robinia pseudoacacia* (Kanwar *et al.*, 1995) and *Azadiracta indica* (Venkateswarlu *et al.*, 1998).

Cytokinin level has been reduced in stage II medium for shoot proliferation of *Eucalyptus camaldulensis* and *E. torelliana* (Gupta *et al.*, 1983), *E. tereticornis* (Rao, 1998), *Tilia amurensis* (Youn and ohaba, 1990), *Wrightia tinctoria* (Purohit and Kukda, 1994) and *Cleistanthus collinus* (Quraishi *et al.*, 1996).

Sharma and Dhiman (1998) reported estabishment of shoot tips of F1 hybrid of *Paulownia (P. fortunei X P. tomentosa)* on MS basal medium and shoot multiplication on MS medium supplemented with BAP and NAA.

Auxins do not promote shoot proliferation, they are required in stage II culture medium to promote growth of shoots by counteracting the suppressive effect of

high cytokinin concentration on shoot elongation (Lundergan and Janick, 1980). Monette (1986) obtained greast number of shoots of *Actinida deliciosa* on basal medium containing BAP and IBA. Kumar *et al.* (1998) reported that the BA along with IAA elicited best shoot multiplication response in *Actinida deliciosa*.

The addition of adjuvents like biotin along with BAP enhanced the proliferation of shoots in *Artemisia annua* (Usha and Swamy, 1998). Adenine and glutamine with BAP promoted shoot multiplication in *Pterocarpus santalinus* (Patri *et al.,* 1988). Nairn (1987) reported that the 0,5-1.0% activated charcoal in the medium enhanced shoot proliferation.

III. Rooting

When shoots are about 30 mm tall, they can be rooted and acclimatized before transfer to green house or field conditions (Harry and Thorpe, 1994). Rooting is the third and most important stage of micropropagation. Complete plantlet is obtained after this stage. There are three phases of rooting in microshoots: (a) induction, (b) initiation and (c) elongation. Usually induction phase is combined with initiation phase. The stage III culture medium is invariably supplemented with auxin to promote root development in stage II shoots (Nehra and Kartha 1994). The commonly used auxins are indoleacetic acid (IAA), indolebutyric acid (IBA), beta-indolepropionic acid (IPA) and alpha-naphthaleneacetc acid (NAA). The external factors very important to induce rooting in microshoots are as follows:

1. Basal medium.
2. Auxin
3. Method of auxin application
4. Sucrose
5. Agar
6. Other adjuvents
7. Effect of subculturing
8. Ex-vitro rooting

1. Basal medium:

Some times roots are unable to initiate in high salt concentration media regardless of the types of hormone present (Hu and Wang, 1983). When the salt concentration in the medium is lowered to one-half, one third or one fourth of the standard strength, rooting becomes abundant.

Mostly one half strength of MS basal medium is used for rooting. Half-MS has been found suitable for rooting the microshoots of *Artocarpus heterophyllus* (Amin and Jaiswal, 1993), *Cleistanthus collinus* (Quraishi *et al.*, 1996; Quraishi and Mishra, 1998), *Pterocarpus santalinus* (Anuradha and Pullaiah, 1999), *Pisonia alba* (Jagadishchandra *et al.*, 1999), *Azadirachta indica* (Venkateswarlu *et al.*, 1998), *Paulownia fortunei* (Chauhan and Emmanuel, 1998; Sharma and Dhiman, 1998), *Camellia sinensis* (Banerjee and Agarawal, 1990), *Mitragyana parvifolia* (Roy *et al.*,1988) and *Tilia amurensis* (Youn and Ohba, 1990).

MS medium with 1/3 strength of macronutrients has been used for in vitro rooting in *Hovenia dulicis* (Echeverrigaray *et al.*, 1998).

One-fourth strength of MS salts helped in induction of callus free rooting from microshoots of *Boswellia serrata* (Purohit *et al.*, 1995), *Feronia limonia* (Purohit and Tak, 1992), *Wrightia tinctorea* (Purohit and Kukda, 1994), *Alnus nepalensis* (Kaur *et al.*, 1993) and *Canavalia virosa* (Kathiravan and Ignacimuthu, 1999).

San-Jose *et al.* (1990) used half-strength GD medium for *in vitro* rooting of *Quercus petraea*. Das and Mitra (1990) used the Konp's solution to induce rooting in microshoots of *Eucalyptus tereticornis*. White's medium helped in *in vitro* rooting of *Pterocarpus sentalinus* (Reddy and Srivasuki, 1992), *Tectona grandis* (Devi *et al.*,1994) and *Pterocarpus marsupium* (Anuradha and Pullaiah, 1999).

2. Type of Auxin:

The commonly used auxin for rooting are IAA, IBA, IPA and NAA. The kind of auxin has been reported to be most important factor for rooting The auxin

concentration and length of treatment vary depending on explant source. Root induction with IAA has been reported in *Albizzia lebbek* (Gharyal and Maheshwari, 1982), *Eucalyptus* grandis (Rao and Venkateswara, 1985), *Leucaena leucocephala* (Datta and Datta, 1985), Caesalpinia pulcherrima (Rahman *et al.*, 1993), *Melaleuca alternifolia* (List *et al.*, 1996) and *Actinidia deliciosa* (Kumar *et al.*, 1998).

Napthalene acetic acid effectively induced rooting in microshoots of *Episcia cupreata* (Johnson, 1978), *Carries citrange* (Kitto and Young, 1981), *Salix* spp. (Bergman *et al.*, 1985), *Ulmus pumila* (Corchete *et al.*, 1993, Mohan *et al.*, 1995), *Prunus mume* (Harda and Murai, 1996), *Bambusa bambos* (Arya and Sharma, 1998), *Sesamum indicum* (Gangopadhyay *et al.*, 1998) and *Eucalyptus citriodora* (Gupta *et al.*, 1981).

Indole 3-butyric acid is the most effective auxin for rhizogenesis. It has been used for promoting rooting in *Leucosceptrum canum* (Pal, 1983), *Eucalyptus grandis* (MacRae and Van Staden, 1990), *Tilia amurensis* (Youn and Ohba, 1990), *Betula pendula* (Besendorfer and Kolevska-Pletikapic, 1997), *Lagerstroemia parviflora* (Quraishi *et al.*, 1997) and *Artimisia annua* (Usha and Swamy, 1998).

Combination of auxins NAA and IBA induced rooting in *Terminalia bellerica* (Roy *et al.*, 1987), *Syzygium cumini* (Yadav *et al.*, 1990) and *Artocarpus heterophyllus* (Amin and Jaiswal, 1993). Combination of IAA and IBA have been used to induce rooting in *Eucalyptus tereticornis* (Das and Mitra, 1990). Combination of IAA, IBA and NAA have been used for rooting the microshoots of *Mitragyna parvifolia* (Roy *et al.*, 1988), *Eucalyptus tereticornis* (Rao, 1988) and *Dalbergia latifolia* (Rai and Jagdishchandra, 1989). More than three auxins IAA, IBA, NAA and IPA have been used for rooting the microshoots of *Tamarindus indica* (Mascarenhas *et al.*, 1987).

Sharma and Dhiman (1998) have reported that the microshoots of *Paulownia fortunei X P. tomentosa* showed 100% rooting under *in vitro* conditions within ten days without any auxin tratment.

3. Application of auxins:

Besides type and concentration of rooting phytohormone, mode of application and duration of exposure need consideration. Rooting hormones can be applied in three different ways namely continuous, short duration pulse, or a dip in liquid or powder.

In some species, continuous presence of auxin in rooting medium induces callus growth from the base of microshoots. In these species, transfer of microshoots after short time from auxin-containing medium to auxin-free medium proved beneficial. Treatment of microshoots with short duration pulse of auxin has been reported in *Eucalyptus torelliana* (Gupta *et al.*, 1983), *Lagerstroemia indica* (Zhang and Davies, 1986), *Quercus petraea* (San-Jose *et al.*, 1990), *Quercus suber* (Manzanera and Pardos, 1990), *Cornus nuttallii* (Edson *et al.*, 1994) and *Grevillea robusta* (Rajasekaran, 1994).

Use of simultaneous auxin and dark pulses for improvement of rooting has been reported in *Mitragyana parvifolia* (Roy *et al.*,1988), *Terminalia bellerica* (Roy *et al.*, 1987) and *Cleistanthus collinus* (Quraishi *et al.*, 1996).

Rooting of fully developed shoots of *Fagus sylvatica* was achieved by dipping the basal end of the shoot in IBA solution (4.92 μM) for 30 sec and subsequent culture on WPM without plant growth regulators (Meier and Reuther 1994). In this case, 10 year old clones and 38 year old grafted clones showed exhibited 65 and 75 percent rooting, respectively.

4. Effect of sucrose:

Biochemically, root initiation is a high energy process demanding a continuous supply of free sugars from the medium (Greenwood and Berlyn, 1973; Thorpe and Biondai, 1981; Gaspar and Coumans, 1987). The rooting was completely inhibited in microshoots of *Rosa hybrida* on medium without sucrose (Genoud-Gourichon *et al.*, 1996). One percent sucrose helped in callus free rooting in microshoots of *Boswellia serrata* (Purohit *et al.*, 1995). Roy *et al.* (1988) reported

the use of 1.5% sucrose for rooting in micropropagated shoots of *Mitragyna parvifolia*. Youn and Ohba (1990) used 10gm/l sucrose in rooting medium for *in vitro* propagation of *Tilia amurensis*. Gangopadhyay *et al*. (1998) used 20 gm/l sucrose for rooting in *Sesamum indicum*. Purohit and Tak (1992) reported that the higher concentration of sucrose (3%) in the rooting medium also favoured callusing in *Feronia limonia*, whereas lower concentration (1%) supported the growth of developing shoot without callusing.

Manzanera and Pardos (1990) reported that the optimum requirement of sucrose concentration in rooting medium depends on genotype of the clones. They found best rooting of A5 and M13 *Quercus suber* clones on rooting medium containing sucrose 40 and 80 gm / l, respectively.

Harada and Murai (1996) used 3% sorbitol for rooting in *Prunus mume*. Lemos and Blake (1996) reported that the use of galactose instead of sucrose in the rooting medium was beneficial for improvement of rooting in *Annona sequamosa*.

5. Effect of Agar-Agar:

In vitro rooting has usually been done using agar solidified medium as the substrate. For supporting root growth, agar is used as the carrier material to solidify the medium. The agar medium proved superior than sterilized sand for *in vitro* rooting of *Lagerstroemia speciosa* (Lim-Ho and Lee, 1985). Kitto and Young (1981) observed inverse relationship between the rooting ability of *Carrizo citrange* cultures and agar concentration.

6. Effect of adjuvents:

Quraishi *et al*. (1997) reported that addition of antioxidant citric acid and absorbent PVP in the rooting medium was essential to check leaching during rhizogenesis in *Lagerstroemia parviflora*. Ascorbic acid and PVP were incorporated in the medium for rooting in *Boswellia serrata* (Purohit *et al* . 1995) Activated charcoal was added to promote rooting in strawbery (Lopez-Aranda *et*

al., 1994; Damiano, 1978; Mohamed-Yasseen, 1995). In *Annona squamosa,* rooting was obtained when subcultured shoots were preconditioned for 2 weeks in medium with 10 g/l activated charcoal before treatment with 43 μM NAA or 39 μM IBA (Lemos and Blake, 1996).

Activated charcoal reduced apical necrosis and improved quality of roots in *Quercus robur* and *Q. rubra* (Sanchez *et al.*, 1996). Activated charcoal may absorb toxic substances in the medium, thereby improving root regeneration and development (Ziv, 1979; Takayama and Misawa, 1980). Activated charcoal may also absorb residual cytokinin from stage II media (Sanchez *et al.*, 1996).

7. Effect of Subculturing:

The age of the explant source plays a significant role in root regeneration capacity. Repeated subculturing may change the physiological state and gradually rejuvenate the shoot which in turn promotes better rooting (Hu and Wang, 1983; Ecomomou and Read, 1986). The percentage of rooted shoots from adult plant increased with repeated subcultures (Pierik ,1990). Tao *et al.* (1994) reported that the rooting ability of the shoots from adult plants of *Diospyros kaki* increased upto 90% with repeated subcultures.

8. Ex-vitro rooting:

Banko and Stefani (1989) reported 51% rooting success by treatment of the basal ends of the microcuttings with 0.3% IBA in talc (Hormodin No.2). They found successful ex-vitro rooting of *Oxydendrum arboreum* microcuttings in flats containing a medium of 1 peat: 1 fine grade vermiculite (v/v). They maintained high humidity during rooting by enclosure of the flats in clear plastic bags.

IV. Acclimatization of plantlets and field transfer

This phase is critical, because the plantlets undergo a great change in the form of nutrition and in environmental conditions. Before field transfer, special pretreatment, to harden plantlets and improve root growth, are necessary. In case of *Eucalyptus*, plantlets were grown in pots containing a mix of soil : sand : compost (3:3:1) for 15-20 days, transferred to polyethylene bags for further root development and transplanted on soil drenched with fungicide (Mascarenhas *et al.*, 1987).

Basendorfer and Kolevska-Pletikapic (1990) regenerated plants of *Betula pendula* on an artificial horticultural peat/ perlite substrate, subsequently acclimatized them to greenhouse conditions and successfully transferred to a field. Jagadishchandra *et al.* (1999) regenerated plantlets of *Pisonia alba* and acclimatized on sand and soil (1:1) before transferring to the field.

Venkateswarlu *et al.* (1998) reported 85-90% survival of *Azadirachta indica* plantlets transferred to a soil and vermiculite mix. They hardened the plantlets in a mist chamber under 90% humidity. Sharma and Thorpe (1990) reported 100% transplantation success in rooted shoots of *Morus alba* transplanted to sand-vermiculite (1:1) mixture in the greenhouse. Nobre (1994) reported that about 90% of the *Myrtus communis* plants survived when plantlets were subcultured to a horticultural substrate after acclimatization. Normah *et al.* (1995) obtained best (98%) survival of *Garcinia mangostana* plantlets on the mixture of sand, soil and organic material (3 : 2 : 1). Corchete *et al.* (1993) reported 60-70% field survival of *Ulmus pumila* plantlets. Harada and Murai (1996) found that the field survival was relatively low (20-30%) in *Prunus mume*.

Sharma and Dhiman (1998) placed ten days old *in vitro* rooted plantlets of *Paulownia fortunei X P. tomentosa* in high humidity polyethylene chamber for a fortnight for acclimatization. They found that more than 95% plantlets survived during acclimatization.

The rooted plants of *Ailanthus malabarica* (D'silva and D'Souza, 1992a) and *Anacardium occidentale* (D'silva and D'Souza, 1992b) showed highest percentage of survival on a mixtures of sterilized soil, sand and powdered coconut husk (1:1:0.25, v:v:v) in perforated polythene bags.

Successful transfer of regenerated plants of *Tamarindus indica* (Jaiwal and Gulati, 1991) and *Camellia sinensis* (Banerjee and Agarwal, 1990) to soil has been reported. Bunn *et al.* (1989) established the *Leucopogan obtectus* plantlets in pots containing habitat soil. Chauhan and Emmanuel (1998) acclimatized the plantlets of *Paulownia fortunei* in earthen pots and transplanted them in the field. Hammatt and Ridout (1992) successfully established rooted plantlets of *Fraxinus excelsior* in soil. They transferred the plantlets into pots filled with vermiculite and acclimatized them under intermittent mist.

Youn and Ohba (1990) took out *in vitro* regenerated plantlets of *Tilia amurensis* from the culture tubes, washed thoroughly to remove the agar medium and transplanted to 15 X 8 cm vinyl pots containing four non-sterile, different potting mixtures. They placed the potted plantlets in the greenhouse and maintained high humidity by covering the plantlets with transparent plates for 14 days, followed by watering with 0.1% Hyponex every three days. They obtained best results (66.7%) with peat moss:perlite:vermiculite (1:1:1) mixture.

Das and Mitra (1990) transferred 2.5-3.0 cm long rooted shoots of *Eucalyptus teriticornis* to auxin free liquid K-medium. They propped up rooted shoots in the liquid medium with the help of filter paper strips. After 15-20 days, when the plantlets attained a height of 6.0-8.0 cm, they transferred them to pots containing garden soil and sand mixture (1:1) and enclosed the potted plants inside polythene bags. They periodically withdrew the covers to acclimatize the plants and after about one month of transplantation, withdrew the covers permanently. They found 80 % survival of plants transplanted in the field.

Manzanera and Pardos (1990) reported that 11.53 percent plantlets of *Quercus suber* survived after 50 days of transfer to green house. First, they transferred the plantlets to plastic vessels with turf substrate and then placed them in green house where the plantlets were protected from direct sunlight with a net, and periodically fertilized with a liquid solution of Sommer's macroelements. Sharma and Dhiman (1998) reported that the field transferred rooted shoots of *Paulownia fortunei* X *P. tomentosa* attained a height of over 2 m in four months.

B. Callus Culture

Callus is an unorganized mass of proliferating cells. Callus culture may be started from any type of explant material possessing parenchymatous cells capable of renewed cell division. The main factors that influence callus formation are explant type, culture medium and growth regulators.

The importance of the age of the plant is most obvious in initiation of cultures from tree species, where usually callus can be initiated from juvenile tissue, and not explants from mature trees. For *in vitro* plant propagation, the success of callus culture depends on the success of plant regeneration. There are two routes through which plant regeneration may occur, namely organogenesis and somatic embryogenesis.

In spite of its external morphological appearance, callus is not a uniform tissue (Thorpe, 1994). Organic differentiation is controlled by a delicate balance of growth regulators, particularly cytokinins and auxins (Skoog and Miller, 1957). Regeneration capacity is influenced by the pretreatment of the mother shoots, macroelements, hormone concentrations, the gelling agent and the carbohydrate source (Pawlicki and Welander, 1994).

Plantlet regeneration from hypocotyl explants has been achieved in *Aegle marmelos* (Arya *et al.*, 1981), *Albizia amara* and *A. lucida* (Tomar and Gupta, 1988), *A. falcataria* (Sinha and Mallick, 1993), *Sesbania grandiflora* (Khattar and Mohan Ram, 1983) and *Tamarindus indica* (Jaiwal and Gulati, 1991). Plantlet regeneration from stem segments has been obtained in *Acacia nilotica* (Mathur and Chandra, 1983) and *Dalbergia sissoo* (Mukhopadhyay and Mohan Ram, 1981); and from lignotubers in *Eucalyptus citriodora* (Aneja and Atal, 1969).

Somatic embryogenesis can be induced in callus, cell suspension, and protoplast cultures, or directly from cells of organized structures such as a stem segment or zygotic embryo (Thorpe, 1994). The somatic embryogenesis *in vitro* was first reported in carrot by Reinert (1958) and Steward *et al.* (1958). The sequence of steps that leads to plantlets include induction, tissue maintenance, maturation

and germination of somatic embryos, and acclimatization of plantlets before field trials. Factors such as light regimes, concentrations of the basal medium, sucrose, nitrogen level and composition, mineral elements, agar, plant growth regulators and pH affect induction (Tautorus, 1991). In manipulating embryogenesis, it seems clear that two media components, auxin and nitrogen, play crucial roles (Kohlenbach, 1978).

Further studies showed that the process of somatic embryogenesis normally takes place in two stages - first, the induction of cells with embryogenic competence in the presence of high concentrations of auxin: and second, the development of the embryogenic masses into embryos in the absence of, or in the presence of lowered concentration of, auxin. Generally, a high concentration of auxin and a low concentration of cytokinin in the medium promotes abundant cell proliferation with the formation of callus. Often, 2,4-D is used alone to initiate callus. Of all the auxin or auxin like plant growth regulators 2,4-D has proven extremely useful, being used in 57.1% of successful embryogenic cultures (Evans *et al.*, 1981). The removal of phytohormones (used for induction and maintenance) in hardwood somatic embryogenic tissue usually leads to embryo development (Harry and Thorpe, 1994).

Halperin (1966) was the first to recognize the importance of reduced nitrogen in the form of NH4+ for somatic embryogenesis. Activated charcoal, which acts as an adsorbent, has been shown to be useful for stimulate embryogenesis in some species (Ammirato, 1983). Also polyamine involvement in embryogenesis has been reported (Montague *et al.*, 1978; Feirer *et al.*, 1984). Osmotic stress can enhance somatic embryogenesis (Litz, 1986), which could account for the observed effects of high carbohydrate concentrations in the medium (Lu *et al.*, 1983).

Somatic embryogenesis has been reported in several hardwood species such as *Azadirachta indica* (Murthy *et al.*, 1998; Su *et al.*, 1997), *Santalum album* and *S. spicaticum* (Rugkhla *et al.*, 1998), *Quercus acutissima* (Kim *et al.*, 1997), sweetgum trees (Marple *et al.*, 1997), wild cherry *Prunus mum* (Yarin *et al.*, 1997), *Dalbergia sissoo* (Das *et al.*, 1997), *Echinochloa colona* (Somantary *et*

al., 1997), black pepper (Joseph *et al.*, 1996), *Eucalyptus dunnii* (Termigroni *et al.*, 1996), Coffee (Boutel and Berthouly, 1996; Sreenath *et al.*, 1995), rosewood *Dalbergia latifolia* (Rao and Sita, 1996), *Hardwickia binata* Roxb. (Das *et al.*, 1995), *Acacia catechu* (Rout *et al.*, 1995), *Camellia japonica* (Pedroso *et al.*, 1995), *Aesculus hippocastanum* (Gastaldo *et al.*, 1994) and *Qurcus suber* (Dunstan *et al.*, 1993), *Semarouba glauca* (Rout and Das, 1994) and *Thevetia peruviana* (Sharma and Kumar, 1994).

C. Isoenzymes and Enzymes

Electrophoresis is a greek word means 'borne by electricity' (Anbalagan, 1985). In this technique charged molecules are separated by electrical force. Electrophoresis is widely used to separate and characterize proteins by applying electric current (Sadasivam and Manicham, 1997). Bhattacharya and Sengupta (1994) found intervarital differences in electrophoretic profile of total seed proteins of 5 varieties of *Leucaena leucocephala*. Variation in proteins has been observed in rice (Goyal and Sharma, 1998).

Isozymes can be employed as effective markers particularly in studies on differentiation and genetics (Wetter and Dyck, 1983). Isozymes have been used widely in forest genetics research (El-Kassaby and White, 1985). Aravanopoulos *et al.* (1994) studied inheritance of isoenzymes in *Salix eriocephala*. Isozyme markers are important tools in determining the changes taking place during development (Scandalios and Felder, 1971), morphogenesis and regeneration (Rout and Das, 1995; Gupta and Srivastava, 1996; Martinelli *et al.*, 1993).

Gallacher *et al.* (1995) studied alcohol dehydrogenase, peroxidase and phosphoglucomutase isoenzymes in expanded leaf and non-chlorophyllic leaf spindle tissue for rapid discrimination of sugarcane clones. They found that complete discrimination of clones could not be achieved with these markers but identity of mislabeled clones could be checked reliably. Trujillo and Rallo (1995) analyzed pollen samples of 155 *Olea europaea* cultivars from different origins to study isozymatic variability in five enzyme systems. alcohol dehydrogenate

(ADH), esterase (EST), glucose phosphate isomerase (GPI), leucine aminopeptidase (LAP), and malic enzyme (ME) using starch gel electrophoresis. They found polymorphism in all of the isoenzyme systems. Isoenzymes have been successfully used for cultivae identification in several fruit species as apricots (Byrne and Littleton, 1988), mango (Degani and E-Batsri, 1990) apple (Weeden and Lamb, 1985) and grape (Parfitt and Arulsekar, 1989).

Isozyme can be readily detected in angiosperms and gymnosperms, even from minute amounts of material (Cheliak and Pitel, 1984), allowing essentially nondestructive sampling. A variety of tissues can be used; these include leaves, buds, embryo and in conifers megagametophytes. Isozymes have been successfully used on numerous occasions to identify mislabeled clones (Cheliak, 1993).

Guaiacol peroxidase are characterized by their broad specificity with respect to an electron donor, and both guaiacol and pyrogallol have been used as electron donors in assays of their activity (Amako *et al.*, 1994). Guaiacol peroxidase is a typical plant peroxidase, localized in cell walls and vacuoles, but not in chloroplast. This type of peroxidase participates in a great number of physiological processes, such as the biosynthesis of lignin (Halbrock and Grisebach, 1979) and plant development and organogenesis via the degradation of IAA (Schneider and Wightman, 1974; O'Neil and Secot, 1987) or the biosynthesis of ethylene. Many isozymes of guauacol peroxidase have been found in plants, and the expression of specific isozymes has been demonstrated in response to development, senescence (Abeles *et al.*, 1988), wounding and pathogens (Lagrimini and Rothestein, 1987; Albert and Anderson, 1987).

Polyphenol oxidase (PPO), known as catechol oxidases phenolase, or O-diphenol oxygen oxidoreductase, catalyzes the oxidation of o-diphenols to quinones as well as hydroxylation of monophenols (Mayer and Harel, 1979; Naithani *et al.*, 1982). This enzyme primarily has a very high diphenol oxidase to monophenol hydroxylase activity ratio. It is the most well understood form of phenoloxidase found in higher plants. PPO is widely considered as a plastid

enzyme although it was reported to exist in the cytoplasm in degenerating or senescent tissues such as ripening fruit (Flurkey and Jen, 1978).

Polyphenol oxidase (PPO), also known as catechol oxidases or phenolase or O-diphenol oxygen oxidoreductase, is a copper containing enzyme catalyzing the oxidation of o-diphenols to o-diquinones (Rathijen and Robnson, 1992). PPO is encoded in the nucleus and thought to be transported to the chloroplast in an inactive form (Vaughn and Duke, 1984). It is located on thylakoid membranes in healthy green leaves (Vaughn and Duke, 1981). Quninones, the oxidized product of phenols, polymerize to form the familiar brown pigments associated with browning in plants.