REVIEW OF LITERATURE

In the last two decades, in vitro plant regeneration studies have been carried out on several forest tree species which has resulted in the accumulation of good amount of literature on the subject. Significant success has been achieved in in vitro plantlet regeneration of many tree species. Mascarenhas and Murlidharan (1989) have reviewed the work carried out on the tissue culture of forest trees of India. It includes 66 tree species belonging to 48 genera and 21 families. Since then investigations have been carried out on several new species. In the present review, a brief account of the work carried out on in vitro regeneration of dicotyledonous forest tree species has been included with emphasis on explant, nutrient media and mode of regeneration. It also includes a brief account of isoenzyme and protein variations.

Vegetative propagation of plants in vitro can
be achieved in three different ways:

A. Regeneration from existing meristems

B. Regeneration from adventitious meristems and

C. Regeneration via somatic embryogenesis.

A. Regeneration from existing meristems:

In this case, the incipient shoot remains in differentiated state, thus, to establish a complete plant, only elongation and root differentiation are required (Hu and Wang, 1983). Plant regeneration from shoot buds is the most common method for clonal propagation of hardwoods (Von Arnold and Wallin, 1988). The reasons for preference of this technique over other techniques are amenability of mature trees to this technique and possibility of production of large number of plantlets from single plant within a short period, that too, with least genotypic variations. Using bud culture technique, successful regeneration of plantlets of several hard-wood species has been reported. A list of some of these species with explant and reference(s) is given in appendix A. Whitehead and Giles (1977) estimated that more than $10^6$ rooted plantlets can be produced per year from the culture of a single bud of *Populus nigra*,


P. yunnanensis and P. hybrida.

The main factors that influence shoot bud culture are the age of tree, position of bud on parent plant, season of explant collection, genotype of parent plant, physiological state of explant, culture medium and plant growth regulators.

Significant effect of age on shoot induction response has been reported by Besendorfer and Kolevska-Pletikapic (1991) in Betula pendula. Axillary bud explants from 2-year-old plant showed adventitious and axillary shoot development, whereas only axillary shoots developed by 4-year-old explant. Decrease in the rate of shoot multiplication with increase in age of tree has been reported by Grewal et al. (1980) in Eucalyptus citriodora.

Explants from lower branches are normally easier to establish than explants from more mature tissues in the upper part of the tree. Purohit and Tak (1992) reported that the nodal explants from basal branches of Feronia limonia are most suitable for multiple shoot proliferation, whereas very young segments from apical portion of fresh branches tend to callus on shoot proliferation medium. Basipetally increasing gradient in growth response of nodal segments has also been reported in Morus alba.
(Sharma and Thorpe, 1990), Eucalyptus tereticornis (Das and Mitra, 1990) and Quercus suber (Manzanera and Pardos, 1990). Amin and Jaiswal (1993) the production of multiple shoots from apical bud explants of emerging trunk sprouts of adult Artocarpus heterophyllus tree, whereas, apical bud from actively growing top branches elongated to form a solitary shoot.

Season of bud collection has been found to influence in vitro plantlet regeneration. Das and Mitra (1990) made month-wise inoculation of nodal explants from elite tree of Eucalyptus tereticornis, throughout the year. They found that July to September was the best period for multiplication of axillary buds. Best season for explant collection differs from species to species. For Artocarpus heterophyllus, November to January (Amin and Jaiswal, 1993); for Betula pendula, February to March (Basendorfer and Kolevska-Pletikapic, 1991); and for Eucalyptus citriodora, February to March and September to October (Grewal et al., 1980; and Gupta et al., 1981) have been reported to be the best season for explant collection.

The genetic make-up of parent plant has been found to greatly influence the prospects of in vitro shoot
multiplication and root induction. Youn and Ohba (1990) studied shoot development and shoot elongation in axillary bud explants of seven plus tree of *Tilia amurensis*. They found significant variation in tissue culturability of different clones. In this case, Kong Won No. 12 showed the best response on WPM with 1 mg l\(^{-1}\) BAP. Sharma and Thorpe (1990) reported variation in the rate of shoot multiplication among different clones of *Morus alba*.

Tree tissue cultures have been maintained on a large variety of different nutrient media (Bonga, 1982). Murashige and Skoog (1962) (MS) medium is the one used most frequently and with great success (Hu and Wang, 1983). The growth and salt concentration requirement in medium vary from species to species especially from one stage of culture development to another.

There are three developmental stages in bud culture. Different nutrition medium and physical conditions are required during these developmental stages. The three developmental stages are:

(i) bud induction and establishment
(ii) multiplication and elongation of shoot
(iii) rooting and hardening
(i) Bud Induction and Establishment:

Age of explant, position of bud on tree and season of explant collection play decisive role during the first stage of micropropagation through shoot bud culture. Besides these, plant growth regulators and polyphenol oxidation are the other major factors influencing induction and establishment of shoot bud.

Requirement of cytokinin in the medium for bud break has been suggested by Bergman et al. (1984). In most of the studies, cytokinin has been included in culture medium for stage 1. The most frequently used cytokinin is 6-benzyl aminopurine (BAP). It has been included in the initial medium for shoot bud culture of Betula pendula (Besendorfer and Kolevska-Pletikapic, 1991), Tilia amurensis (Youn and Ohba 1990), Quercus suber (Manzanera and Pardos 1990), Morus alba (Sharma and Thorpe 1990), Leucaena leucocephala (Datta and Datta 1985), Mitragyna parvifolia (Roy et al. 1988), Lagerstroemia speciosa (Lim-Ho and Lee 1985) and Terminalia bellirica (Roy et al. 1987). Kinetin (Kn), 2-isopentyl adenine (2-ip), zeatin and thidiazuron (TDZ) are other cytokinins used for shoot bud induction and establishment. Kinetin and BAP together have been reported to be
included in stage I medium for micropropagation through bud
culture of *Artocarpus heterophyllus* (Amin and Jaiswal 1993)
and *Eucalyptus torrelliana* (Gupta *et al.* 1983).

Yadav *et al.* (1990) evaluated the performance of Kn, BAP and 2-ip using shoot buds from shoot tip and
nodal segments of *Syzygium cumini* seedlings. They found
best shoot bud induction response on the stage I medium
containing BAP. Zeatin has been reported to be better than
2ip, and BAP for micropropagation of *Oxydendrum arboreum*
(Banko and Stefani 1989).

Sharma and Thorpe (1990) reported bud break
and development of single axillary bud from nodal segments
of *Norus alba* on MS medium alone. However, these shoots
turned yellow and died within 6 weeks.

Exogenous supply of auxin has also found to be
useful for bud induction and establishment of explant *in vitro*. Naphthlene acetic acid (NAA) has been included in
initial medium for micropropagation of *Aegle marmelos*
(Bhati *et al.*, 1992), *Dalbergia latifolia* (Lakshmi Sita and
Raghavaswamy 1992), *Eucalyptus grandis* (Rao and Venkateswara
1985), *E. tereticornis* (Das and Mitra 1990), *Feronia limonia*
(Purohit and Tak 1992) and *Pterocarpus santalinus* (Reddy
and Srivasuki 1992). Indole acetic acid has been included
in stage 1 medium for micropropagation of Prosopis juliflora (Nandwani and Ramawat 1991).

Many woody plants contain phenols, which become oxidized due to tissue injury during dissection. Polyphenol oxidases are responsible for the oxidation of phenols. The oxidized products of phenols, in many cases, have been found to be potent inhibitor of growth. They turn the explant brown and ultimately kill them. The following procedures have been developed to overcome this problem:
(a) addition of antioxidants in culture medium,
(b) presoaking of explants in antioxidant solution,
(c) incubation of primary cultures in reduced light or darkness, and
(d) frequent transfer of explants to fresh medium.

Purohit and Tak (1992) included citric acid (10 mg l⁻¹), ascorbic acid (100 mg l⁻¹) and polyvinyl pyrrolidone (PVP) (50 mg l⁻¹) in culture medium to prevent browning of nodal explants of Feronia limonia. Gupta et al. (1980) succeeded in reducing the blackening of culture medium during bud culture of Tectona grandis by suspending the explants in a solution of different antibrowning agents in 0.058 M sucrose and agitating on a rotary shaker for 45 min.
before incubation onto MS solid medium. The antibrowning agents used were \( \text{H}_2\text{O}_2 \) (5%), ascorbic acid (0.28 mM), soluble PVP (0.7%) and Polyclar AT (0.7%). Although blackening was reduced by all the agents, multiple shoots were formed only from explants treated with polyclar AT (an insoluble PVP), which combines with phenolics by H-bonding.

Das and Mitra (1990) found exudation of phenolic compounds from the cut ends of axillary bud explants of *Eucalyptus tereticornis*, when placed in either photoperiod or in complete darkness. They transferred the explants 2–3 times into fresh medium at two day interval to facilitate quick exudation of phenolics and establishment of shoot bud culture.

During explant establishment, all phenols do not inhibit the growth or damage the explant. Phenol exudates origination from seedling stimulated *in vitro* growth of *Betula pendula* buds (Welander 1988).

ii) Multiplication and Elongation of Shoots:

Presence of cytokinin in nutrition medium is the main requirement during multiplication of shoots. It is one of the most important steps in micropropagation technique because economic viability of the technique depends on
the number of propagules regenerated \textit{in vitro}.

Sometimes, the shoots multiplied \textit{in vitro} remain small and give bushy appearance. Rooting of such shoots is difficult. Therefore, before rooting, shoot elongation is necessary.

The cytokinins which have been used for proliferation of shoots \textit{in vitro} are BAP, Kn, 2ip and Zeatin.

Banko and Stefani (1989) studied \textit{in vitro} propagation of \textit{Oxydendrum arboreum} from nodal explants of 24-year-old tree. They found best proliferation of shoots from nodal segments on WPM supplemented with 4 \textmu M zeatin, whereas BAP and 2ip were ineffective at the same concentrations.

Youn and Ohba (1990) reported best shoot development from axillary buds of \textit{Tilia amurensis} on TS medium and WPM supplemented with 1.0 mg l\textsuperscript{-1} BAP and best shoot multiplication on WPM and IS medium supplemented with 0.2 mg l\textsuperscript{-1} BAP.

The most commonly used cytokinin for shoot multiplication in shoot bud culture of trees is BAP and its effectiveness has been demonstrated in the seedling and mature tissues of \textit{Betula pendula} (Besendorfer and Kolevska-

Use of BAP and 2ip has been reported to promote shoot multiplication in shoot bud culture of *Lagerstroemia speciosa* (Lim-Ho and Lee 1985). BAP and Kn (1.0 mg/l each) enhanced shoot multiplication in the culture of axillary buds from root suckers of 60-80-year-old elite tree of *Dalbergia latifolia* (Lakshmi Sita and Raghavaswamy 1992).

Gupta et al. (1981) obtained shoot induction and multiplication in shoot bud culture of *Eucalyptus citriodora* on MS medium supplemented with BAP (0.3 mg/l), Kn (0.2 mg/l), biotin (0.1 mg/l) and calcium pantothenate (0.1 mg/l).

Auxin and cytokinin have been included in nutrient medium for shoot multiplication. Rao and Venkateswaran (1985) reported proliferation of shoots in shoot bud culture of mature *Eucalyptus grandis* on MS medium supplemented with BAP (1-2x10^-4 M) and NAA (5x10^-5 M).

Mac Rae and Staden (1990) reported that the jelling agent can influence the rate of shoot multiplication in vitro. They obtained shoot bud induction from nodal ex-
plants of 3-month-old seedlings of Eucalyptus grandis on MS + BAP (0.1 mg l\(^{-1}\)) + NAA (0.01 mg l\(^{-1}\)) and shoot multiplication on MS medium supplemented with BAP (0.2 mg l\(^{-1}\)) + NAA (0.01 mg l\(^{-1}\)) and jelled with different jelling agents. The shoot multiplication was 7 fold on gelrite (0.2%), 6 fold on agarose (0.6%) and 2 fold on Agar (0.8%).

In some cases, the shoots remain stunted and give bushy appearance. Gibberellic acid and activated charcoal have been reported to be useful for overcoming the problem of stunting of shoots in shoot bud cultures of Eucalyptus grandis (Rao and Venkateswara, 1985; Das and Mitra, 1990).

iii) Rooting and Hardening:

Rooting is a difficult stage of plantlet regeneration in vitro (Hu and Wang, 1983). There are 3 phases in root development: induction, initiation and elongation. Usually induction is combined with initiation, as it is difficult to isolate induction in most experiments. Generally an auxin is included in the culture medium to induce rooting. The commonly used auxins are IAA, IBA, IPA and NAA. Strength of the medium, and auxin concentration
are important factors for induction and development of roots.

The kind of auxin has been reported to be most important factor for rhizogenesis IBA was found better than IAA for rooting the microshoots of *Quercus suber* (Manzanera and Pardos, 1990). IAA stimulated rooting in *in vitro* raised shoots of *Leucaena leucocephala* (Datta and Datta 1985). IBA promoted rooting in microshoots of *Betula pandulata* (Besendorfer and Kolevska-Pletikapic 1991), *Faronia limonia* (Purohit and Tak 1992), *Eucalyptus grandis* (Mac Rae and Staden, 1990), *Leucosceptrum canum* (Pal 1983) and *Tilia amurensis* (Youn and Ohba 1990). NAA induced rooting in microshoots of *Prosopis juliflora* (Nandwani and Ramawat, 1991).

Combination of auxins have been reported to be useful for root induction. Combination of IAA and IBA promoted rooting in *Eucalyptus tereticornis* (Das and Mitra 1990), and IBA and NAA in *Terminalia bellerica* (Roy et al., 1987).

In some cases combination of more than two auxins have been used for rooting of microshoots. Roy et al., (1988) reported rooting in micropropagated shoots of
*Mitragyna parvifolia* on 1/2 MS medium supplemented with IAA + IBA + NAA (1.0 mg/l each) and containing 1.5% sucrose. Raddy and Srivasuki (1992) used White’s nutrient medium supplemented with IAA + IBA + NAA (1.0 mg/l each) for stimulating rhizogenesis in *in vitro* raised shoots of *Pterocarpus santalinus*. In this study, they found November to January as best period for rooting of microshoots. They achieved ex-vitro rooting in shoots derived from juvenile explants, whereas shoots derived from mature plants did not respond to ex-vitro rooting.

Pulse treatment of excised shoots with high concentration of auxin followed by root growth in a hormone-free medium has proved more efficient than a longer treatment in a low concentration, for many hardwoods. Vieitez *et al.* (1989) induced rooting in microshoots of *Camellia japonica* by dipping of shoot base in 1.0 mg/l IBA for 15 min. followed by 9 days dark treatment.

An immediate post-dip dark period is considered essential for rooting. Light inhibits root formation in many species (Bassuk and Maynard 1987). Mato and Vieitez (1986) found that IBA promotes the transport of auxin protectors from the upper to the lower parts of chestnut shoots during rooting. Probably light reduces the synthesis of
auxin protectors and/or blocks their basipetal transport.

Zhang and Davies (1986) achieved ex-vitro rooting of in vitro propagated shoots of *Lagerstroemia indica* by dipping their base into 49.7 μM IBA.

Lim-Ho and Lee (1985) excised in vitro grown shoot of *Lagerstroemia speciosa* and rooted in agar medium or sterilized sand supplemented with MS medium and IBA. The agar medium proved superior than sterilized sand.

Passage of subcultureing for shoot proliferation influenced rooting of in vitro raised shoots from mature tree of *Artocarpus heterophyllus* (Amin and Jaiswal 1993). In this case, 80% rooting was observed after 10th passage, whereas 4th subculture showed 40% rooting on 1/2 MS medium supplemented with 10 μM IBA or NAA.

The rooting ability of in vitro cultured shoots is influenced by juvenility and genotype of the parent stock. San-Jose et al., (1990) regenerated shoots in vitro from 6 clones of juvenile and 1 clone of adult tree of *Quercus petraea* and rooted them on half-strength Grasshoff and Doy medium after their bases had been dipped in 0.5 gl⁻¹ IBA for 6 min. or 1 gl⁻¹ for 2 min. Juvenile explants showed 59-100% rooting, whereas, adult tree explants pro-
duced roots in 38% shoots

B. REGENERATION FROM ADVENTITIOUS MERISTEMS:

(For plantlet regeneration in tissue culture via shoot bud differentiation (organogenesis), the explants are placed on suitable medium to initiate callus cultures which on differentiation yield shoot buds. Shoots are excised and placed on rooting medium to obtain plantlet.

Adventitious buds can also be formed directly on the explant without an intervening callus stage. Directly differentiated shoot buds produce true to type plants without any risk of change in chromosome number (Von Arnold and Wallin 1988). Chromosomal aberrations have been observed in the callus (Bayliss, 1973).

Organogenic differentiation is controlled by a delicate balance of growth regulators, particularly cytokinins and auxins (Skong and Miller 1957). In general, cytokinins stimulate shoot bud formation; whereas auxins favour root differentiation in cultures undergoing organogenesis. The explant type, culture medium and growth regulators are main factors that influence callus formation and organogenesis in cultures.

Explant:
Plant regeneration via organogenesis has been accomplished from several types of explants of dicotyledonous tree species. A list of some of these species with explant and reference is given in appendix B. In general, the seedling explants have been reported to be better for organogenic differentiation than explants of physiologically mature plants.

Plantlet regeneration has been reported from hypocotyl explants of Aegle marmelos (Arya et al., 1981), Albizia amara, A. lucida (Tomar and Gupta 1988b); from cotyledon explants of Albizia falcataria (Sinha and Mallick 1993), Seshania grandiflora (Khattar and Mohan Ram 1983), and Tamarindus indica (Jaiswal and Gulati 1991); from stem segments of Acacia nilotica (Mathur and Chandra 1983) and Dalbergia latifolia (Sudha Devi and Nataraja 1987); from roots of Dalbergia sissou (Mukhopadhyay and Mohan Ram 1981) and Prosopis cineraria (Kackar et al., 1992); from lignotubers of Eucalyptus citriodora (Aneja and Atal 1969); and from leaf disc of mature tree of Azadirachta indica (Ramesh and Padhya 1990) and Morus indica (Mhatre et al. 1985).

Triploid plantlets have been regenerated from endosperm tissue of Putranjiva roxburghii (Shrivastava
1973), and haploid plantlets from anther culture of *Albizia lebbeck* (Charyal et al., 1983).

**Culture Medium:**

(MS medium is most frequently used medium for organogenesis. Other commonly used media are WPM, BS and Wood and Braun (1961) medium. Addition of growth regulators in culture medium is essential for induction of callus and differentiation. In general, two different media are used for callus induction and shoot bud differentiation. Callus induction medium is supplemented with auxin(s), whereas shoot bud differentiation takes place in the same medium [*Acacia nilotica* (Mathur and Chandra, 1983), *Dalbergia latifolia* (Lakshmi Sita and Raghavaswamy, 1992; Rai and Jagadishchandra, 1989)].

Most frequently used auxins are 2,4-D, NAA and IAA; and most frequently used cytokinins are BAP, KIN and zeatin.)

Kumar (1992) reported callus induction from stem explants of 15-18-year-old *Bauhinia purpurea* trees on MS medium supplemented with 10 uM 2,4-D and shoot bud differentiation on MS medium supplemented with 0.5-10 uM Kinetin.
Sudha Devi and Nataraja (1987) obtained callus induction from stem segments of *Dalbergia latifolia* on MS medium supplemented with IAA (1.0 mg l\(^{-1}\)) and shoot bud differentiation on MS medium supplemented with 0.25-2.0 mg l\(^{-1}\) BAP.

Anand and Bir (1984) reported segments of seedling of *Dalbergia lancemaria* on MS + NAA (4.0 mg l\(^{-1}\)) + KIN (1.0 mg l\(^{-1}\)) + yeast extract (600 mg l\(^{-1}\)) and differentiation of adventitious shoot buds on MS + BAP (1-4 mg l\(^{-1}\)).

Han *et al.*, (1990) reported callus induction and shoot bud differentiation from internodal segments of mature trees of *Robinia pseudoacaia* on MS medium containing different concentrations with BAP. They obtained callus on MS medium supplemented with 10 μM BAP and differentiation on MS medium supplemented with 0.3 μM BAP.

Gharyal and Maheshwari (1990) reported callus induction and shoot bud differentiation from petiole and stem explants of *Albizia lebbeck* on B5 medium supplemented with IAA (0.5 mg l\(^{-1}\)) and BAP (1.0 mg l\(^{-1}\)). Similarly Ramesh and Padhya (1990) reported callus induction and shoot bud differentiation from leaf disc of elite trees of *Azadirachta indica* on Wood and Braun (1961) medium supplemented with adenine sulphate (16 μM), BAP (4 μM) and KIN (4 μM).
Patri et al., (1988) have shown that the callus induction and shoot bud differentiation require different temperature. They induced callus from cotyledon explant of Pterocarpus santalinus on MS + BAP (3x10^{-6} M) at 25°C and stimulated shoot bud differentiation on the same medium at 28°C.

C. SOMATIC EMBRYOGENESIS:

Somatic embryogenesis in a forest tree was first reported by Rao (1985) in Santalum album. Since then it has been investigated in large number of woody forest trees. A short list of these species is given in appendix C. Somatic embryos obtained in culture are bipolar structures which germinate to produce plantlet. Thus the problem of rooting dose not arise in this technique. Moreover, they are identical to zygotic embryos with respect to development, structure and biochemical constitution. Major factors which influence initiation and development of somatic embryos in culture are explant, culture medium and plant growth regulators.

Explant:

Embryogenic cultures have been initiated from
zygotic embryos (mature & immature), hypocotyls, cotyledons, leaves and stems. Rapidly dividing, undifferentiated embryogenic cells grow easily in culture and generate adventitious embryos.


Embryogenic response was reported from cotyledon explants of *Feijoa sellowiana* (Cruz et al. 1990), and *Juglans regia* (Tulecke and McGranahan 1985).

In forest trees, economically useful traits
appear at maturity. Embryogenic cultures have been initiated from explants having mature morphology. Michler and Bauer (1991) reported high frequency somatic embryogenesis (32 globular embryos per ml) in cell suspension cultures derived from leaf tissue of hybrid popular (Populas alba × P. grandidentata NC 5339). Leaf explants from adult tree have shown embryogenic response in Leucosceptrum canum (Pal et al. 1985), Quercus ilex (Feruad-Keller and Espagnac 1989) and Sapindus trifoliatus (Desai et al. 1986). Cultures derived from shoot tips of Crataeva nurvala (Inamdar et al. 1990), Santalum album (Lakshmi Sita et al. 1979), Dalbergia latifolia (Lakshmi Sita and Raghavaswamy 1992), Populus ciliata (Cheema 1989) and Tectona grandis (Lakshmi Sita and Chattopadhyay 1987) have shown differentiation of somatic embryos.

Culture Medium:

'A wide range of media have been used for growing somatic embryos of woody angiospermic species in tissue culture.' MS medium (1962) has been used for the formation of somatic embryos in culture of Crataeva nurvala (Inamdar et al. 1990), Dalbergia latifolia (Lakshmi Sita and Raghavaswamy 1992), Populairs (Michler and Bauer 1991),
Populus ciliata (Cheema 1989), P. nigra × P. maximowiczii (Park and Son 1988), Quercus ilex (Feruad-Keller and Espagnac 1989), Santalum album (Lakshmi Sita et al. 1979) and Theobroma cacao (Pence et al., al. 1979, 1980); WPM for Aesculus hippocastanum (Joergensen 1989 & Radojevic 1988) and Juglans nigra (Neuman et al. 1993); BS for Albizia lebbeck (Gharyal and Maheshwari 1981) and A. richardiana (Tomar and Gupta 1988a); Blaydes medium for Liquidambar styraciflua (Sommer and Brown 1980) and Liriodendron tulipifera (Merkle and Sommer 1987); DKW medium for Juglans regia (Tulecke and McGranahan 1985); IS medium Ilex aquifolium (Hu and Sussex 1971); N7 medium for Ratula pendula (Nautila et al. 1991). The key elements of the MS medium are the presence of high level of reduced nitrogen in the form of ammonium nitrate and chelated iron with the EDTA. The source of reduced nitrogen in the medium is ammonium ions. The importance of reduced nitrogen for initiation and maturation of somatic embryos in well established (Reinert et al. 1967).

The other sources of reduced nitrogen can be in the form of complex additives such as casein hydrolysate or a single amino acid such as asparagine, L-alanine, L-glutamine, L-proline etc.

- Casein hydrolysate has been supplemented with
basal medium for the production of somatic embryos in *Betula pendula* (Nuutila et al. 1991), in *Juglans nigra* (Neuman et al. 1993) and *Iririodendron tulipifera* (Merkle and Sommer 1987).

Muridharan et al. (1989) reported somatic embryogenesis in *Eucalyptus nitriodora* on MS medium containing 5.0 mg l\(^{-1}\) NAA, 500 mg l\(^{-1}\) casein hydrolysate and 500 mg l\(^{-1}\) glutamine.

Michler and Bauer (1991) observed that the nitrogen provided by MS salts was optimum for embryogenic callus formation from vein region of young leaf explants of *Populus alba x P. grandidentata*. In this case, presence of L-glutamine promoted direct embryogenesis, but inhibited embryogenic callus formation.

**Carbohydrate Source:**

Sucrose is an important ingredient of culture media used for initiation and development of somatic embryos. It serves as a carbohydrate source and as an osmoticum. The optimum concentration of sucrose in culture medium differs from species to species. Bapat and Rao (1984) found 4% sucrose concentration to be most effective for somatic embryo production from hypocotyl callus of *Santalum album*.  

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In this case, the callus turned brown at 6-10% concentration of sucrose. Nautila et al. (1991) obtained maximum production of embryos in callus culture of *Betula pendula* at 20.8 g l\(^{-1}\) concentration of sucrose. 2% concentration of sucrose has been reported to be optimum for somatic embryogenesis in callus cultures derived from hypocotyl explants of *Albizia richardiana* (Tomar and Gupta 1988a).

**Growth Regulators:**

Exogenous auxin is required for induction and growth of callus and initiation of somatic embryos. The most frequently used auxin for initiation of somatic embryos is 2,4-D, followed by NAA, IAA and IBA. In most of the cases, it has been found that reduction or complete removal of auxin from the medium is essential for development of somatic embryos. However, in some cases, presence of auxin has been found useful for development of somatic embryos. Cytokinin is added in low amount for development of somatic embryos or for initiation and development of somatic embryos. RAP, and Kn are most frequently used cytokinins.

Michler and Bauer (1991) reported direct embryogenesis on leaf explants and indirect embryogenesis in cultured cells of hybrid poplar (*Populus alba* x *P. grandidentata*). They induced embryogenic callus from leaf explants.
in darkness on MS medium with \(5.0 \text{ mg}^{-1}\) 2,4-D, 0.5 \(\text{ mg}^{-1}\) BAP and 30 \(\text{ g}^{-1}\) sucrose. They achieved initiation of globular-shaped embryos from cell suspension callus on \(5.0 \text{ mg}^{-1}\) 2,4-D and 0.05 \(\text{ mg}^{-1}\) zeatin, heart-shaped and torpedo-shaped embryos on 2,4-D free medium, and germination of torpedo-shaped embryos on MS medium containing 5.0 \(\text{ mg}^{-1}\) IAA and 0.05 \(\text{ mg}^{-1}\) BAP.

Jorgensen (1989) reported induction of callus from filaments of 10-100-year-old trees on modified WPM medium containing 2.5 \(\mu\text{M}\) BAP and 5.0 \(\mu\text{M}\) 2,4-D, development of embryos on the same medium without 2,4-D and transformation of whole callus into embryos on the medium containing lower level (0.75 \(\mu\text{M}\)) of BAP.

Inamdar et al. (1990) reported initiation and development of somatic embryos from stem-apex callus of adult tree of *Crataeva nurvala* on MS medium with 2.0 \(\text{ mg}^{-1}\) 2,4-D and 0.4 \(\text{ mg}^{-1}\) Kn.

Jha (1988) reported induction of callus and initiation of somatic embryos from zygotic embryos of *Anacardium occidentale* on MS medium supplemented with 4.0 \(\text{ mg}^{-1}\) 2,4-D, 4.0 \(\text{ mg}^{-1}\) NAA and 2.0 \(\text{ mg}^{-1}\) Kn. The development of embryos was reported on the medium without 2,4-D and reduced
levels of NAA (0.5 -0.005 mgl⁻¹) and Kn (0.5 mgl⁻¹).

Retention of Morphogenic Potential:

Variation in retention of capability of embryo differentiation in callus culture has been reported in various species. Embryo differentiation has been reported in 2-year-old callus culture of Albizia lebbeck (Gharyal and Maheshwari 1981), 4-year-old culture of Camellia japonica (Vieitez and Barciela, 1990), 5-year-old culture of Crataeva nurvala (Inamdar et al. 1990), and 1-year-old culture of Populus ciliata (Cheema 1989).

Abnormalities in Somatic Embryos:

The development of somatic embryos is similar to that of zygotic embryos. They traverse through the same stages by which the zygotic embryos develop. However, sometimes the development of somatic embryo, is arrested in the intermediate stage or alteration in the organization events occur due to nutritional deficiency. Many abnormal form of somatic embryos have been reported (Michler and Bauer 1991, Goebel-Tourand et al. 1993).

D. Variation in Isoenzyme Pattern:

Isoenzymes are defined as multimolecular forms of an enzyme occurring within a single species, as a result
of more than one structural gene. The multiple genes may be
due to the presence of multiple gene loci or of multiple
alleles (IUPAC - IUR, 1977). Isoenzymes can be employed as
effective markers particularly in studies on differentiation
and genetics (Scandalios, 1974; Jacobs, 1975).

The isoenzyme changes that occur in initial
stages of differentiation have been reported. Thorpe and
Gaspar (1978) reported change in peroxidase isozyme pattern
in tobacco callus induced to form shoots.

Coppens and Dewitte (1990) analyzed esterase
and peroxidase isozyme patterns in 3 types of visually
differentiable callus of Hordeum vulgare. The organogenic
and embryogenic calli indicated differences in peroxidase
isoenzyme pattern much before the appearance of any visual
sign of differentiation.

Cell lines from citrus callus have been re-
ported to exhibit increased activity of oxidase and appear-
ance of new isozyme bands during embryogenesis (Chen and

Rao et al. (1990) found association of two
slow migrating cathodic bands of peroxidase, specific ester-
ase and melate dehydrogenase with embryogenic calli of Zea
Variation in isoenzyme pattern during dedifferentiation and differentiation has been observed in several studies (Das and Das 1991; Shen 1990; Rao et al. 1983) and variation in protein has been observed in rice (Chen & Luthe 1987) and sorghum (Wozniak & Partidge 1988).