REVIEW OF LITERATURE
Micropropagation is one of the most important applications of plant biotechnology. It is a billion-dollar industry throughout the world. Practical application of micropropagation in forestry has history of about four decades. The potential usefulness of cell and tissue culture techniques for the propagation of forest trees has long been recognized and discussed. However, it is only during the past two decades or so that concreted efforts have been made to adopt these methods for the propagation of commercially important plant species.

Protocols of about few hundred plant species are known throughout world, but practical use in large scale production is restricted to few dozens, mainly due to lack of reproductively, poor multiplication rate, low rooting frequency, lack of proper hardening procedures, etc. Plant tissue culture based biotechnology has vast potential for propagation of difficult to multiply species, rapid and mass production of clonal planting material of plus trees/elite clones, genetic improvement through anther, pollen, embryo, endosperm culture, development of somaclonal variants, induced flowering, \textit{in vitro} pollination, hybrid embryo rescue, somatic hybridization within and between species and trailer made genetic modification for desired traits.

\subsection*{2.1 \textit{IN VITRO} PLANT REGENERATION:}

There are three methods of \textit{in vitro} plant regeneration viz., (i) axillary shoot proliferation, (ii) somatic embryogenesis which includes a) direct somatic embryogenesis b) indirect somatic embryogenesis and (iii) adventitious shoot induction, which includes a) direct and b) through callus phase.

\subsubsection*{2.1.1 \textit{In vitro} propagation through somatic embryogenesis:}

The first report on somatic embryogenesis was on \textit{Dacus carota} (Reinert, 1958; Steward \textit{et al.}, 1958). Induction of somatic embryo from plant tissues/organ is the most desirable approach in order to avoid somaclonal variation (Aoshima, 2005). Each somatic cell has potential to produce somatic embryos and mature somatic embryo being bipolar in nature are considered to be easy to regenerate complete plant as compared to organogenesis.
Somatic embryogenesis is a very valuable tool for achieving a wide range of objectives; from basic biochemical, physiological and morphological studies, to the development of technologies with a high degree of practical application. The mass propagation of plants through multiplication of embryogenic propagules is the most commercially attractive application of somatic embryogenesis (Merkle et al., 1990). In addition, it permits the culture of large number of reproductive units, and plants derived from somatic embryos are less variable than those derived by the way of organogenesis (Ammirato, 1983).

Plant regeneration through somatic embryogenesis via callus phase includes following different steps:

1. Induction of embryogenic callus cultures.
2. Proliferation of embryogenic callus cultures on solid/liquid medium.
3. Somatic embryo induction and development
4. Germination of somatic embryos into plantlets.

2.1.1.1 Embryogenic callus induction:

The initiation of embryogenic culture is restricted only to certain responsive cells in the primary explant, which have the potential to activate those genes involved in the generation of embryogenic cells. The competence for embryogenic callus induction may be the result of varying auxin sensitivity of these cells (Dudits et al., 1995). Two mechanisms appear to be important for in vitro formation of embryogenic cells; i) asymmetric cell division and ii) control of cell elongation (De Jong et al., 1993). Asymmetric cell division is promoted by PGRs that alter cell polarity by interference with the pH gradient or the electrical field around the cells (Smith and Krikorian, 1990). The ability to control cell expansion is associated with polysaccharides of the cell wall and corresponding hydrolytic enzymes (De Jong et al., 1993).

2.1.1.1.1 Effect of explant types:

The pattern of developmental response of cultured tissue is epigenetically determined and is influenced by the stage of development of plant, the nature and type of the explant tested (Litz and Gray, 1995). The use of different explants for the induction of embryogenic callus has been reported in various species.
Rao et al. (1985) obtained somatic embryos by culturing seeds of *Dendrocalamus strictus* on B5 medium containing 2, 4-D (1.0 x 10^{-5} M and 3.0 x 10^{-5} M). Callus cultures obtained from the embryonal end of the seeds differentiated chlorophyllous embryoids.

Yeh and Chang (1987) reported callus induction from cultured mature zygotic embryos from seeds on MS medium supplemented with 6 mg l^{-1} (27.14 µM) 2, 4-D, 3 mg l^{-1} (13.94 µM) kinetin, 50 g l^{-1} sucrose and 250 mg l^{-1} PVP in *sinocalamus latifolia*.

*In vitro* cultures as a source of explants has several advantages over the field grown plants, viz; better rejuvenated explants, minimum accumulation of phenolics and inhibitors, better uniformity of explants and no carryover effect of sterilant. There are large number of reports in which *in vitro* shoot cultures of mature clump/trees have been used as explants for somatic embryogenesis. Nodal shoot segment as a explants from mature clump were used for callus initiation in *Bambnsa nutans* (Mehta et al., 2010), *Dendrocalamus asper* (Arya et al., 2008), *Phyllostachys stocksi* (Somashekar, 2005), *Bambusa edulis* (Lin et al., 2004), *D. giganteus* (Ramanayake and Wanniarachchi, 2003), *D. hamiltonii*; (Sood et al., 2002) and *B. vulgaris* (Rout and Das, 1997). Using MS medium with 2, 4–D or 2, 4, 5–T as auxin with or without Kinetin/CW.

Rout and Das (1994) reported callus induction from nodal explant of *in vitro* grown seedlings and excised mature zygotic embryos of *Bambusa vulgaris*, *Dendrocalamus giganteus* and *Dendracalamus strictus*. The explants cultured on MS medium with 0.5 mg l^{-1} (2.32 µM) kinetin, 2 mg l^{-1} (9.05 µM) 2, 4-D, 10 mg l^{-1} (54.29 µM) adenine sulphate and 3% sucrose produced embryogenic callus. The rate of callus proliferation was better, when cultures were incubated in the dark.

Hassan and Debergh (1987) used leaf explants for callus induction on MS medium containing 9.0 x10^{-6} M 2, 4-D in *Phyllostachys viridis*. Whereas, Godbole et al. (2002) used new sprouts from nodal segments of field grown bamboo (*D. hamiltonii*) for induction of embryogenic callus. They observed that MS medium with 1 mg l^{-1} each of BA (4.44 µM) and 2, 4-D (4.52 µM) was essential for callusing.
Arya et al. (2008), reported embryogenic callus from explants excised from nodal tissues and basal part of leaves of in vitro shoots of Dendrocalamus asper. Whereas, Godbole et al. (2004) reported callus induction from the new sprouts of in vitro cultures for callus induction, on half strength MS medium containing 1 mg l\(^{-1}\) each of 2, 4-D (4.52 µM), BAP (4.44 µM) and NAA (5.37 µM) in 40 days in D. hamiltonii.

Hung and Murashige (1983) used various explants for callus induction and observed that embryogenic callus induction was from shoot tip of B. oldhamii. B. multiplex, Sesa pygmea and Phyllostachys aurea. Whereas, Lin et al. (2004) reported embryogenic callus induction from nodes and internodes of in vitro raised plantlets on MS medium supplemented with 9.2 µM Kn, 13.6 µM 2, 4-D, 0.1% coconut milk and 6% sucrose.

Yeh and Chang (1986) used young inflorescence of Bambusa beecheyana for embryogenic callus culture establishment on MS medium supplemented with 3 mg l\(^{-1}\) 2, 4-D, 2.0 mg l\(^{-1}\) kinetin and 60 g l\(^{-1}\) sucrose.

Gillis et al., (2007) used pseudospikelets as an explant and reported embryogenic callus induction on MS medium containing 1.0 mg/l 2.4-D and 3 % sucrose under dark condition in B. balcooa.

Ramanayake and Wanniarachchi (2003) used root as an explant for callus induction on medium with 2, 4-D from in vitro established mature shoot clump of D. giganteus within 4-6 weeks. Whereas, Komatsu et al. (2011) reported callus induction from leaf sheaths and young shoots of mature plants of Phyllostachys bambusoides.

### 2.1.1.1.2 Effect of plant growth regulators:

It has been known that, PGRs play a central role in mediating the signal transduction cascade leading to the reprogramming of gene expression. This results in a series of cell divisions that induce either unorganized callus growth or polarized growth leading to somatic embryogenesis (Dudits et al., 1995).

Requirement of type of PGRs, their combination and concentration vary with the plant species. In monocotyledons, 2, 4-D alone is the most commonly used auxin
for embryogenic callus induction, but in dicotyledons, it varied with the species and some times with explant type and source (seedling /mature plant).

In case of *Bambusa oldhamii*, embryogenic callus was initiated on MS medium supplemented with 2,4-D (3.0 mg/l) + Kn (2.0 mg/l) (Yeh and Chang, 1986), whereas, MS medium with 2,4-D and BA 1.0 mg/l, each was found essential for callus establishment in *D. hamiltonii* (Godbole et al., 2002).

Sood et al. (2002) reported induction of compact nodular callus from mature nodal explants of *D. hamiltonii* on MS medium supplemented with 2,4-D (1.0mg/l) and BA (1.0 mg/l). Whereas, in case of *D. giganteus*, MS medium consisted high (7.5 mg/l) concentration of 2, 4-D and NAA (3.0 mg/l) proved the best for nodular callus induction and further proliferation, (Ramanayake and Wanniarachchi, 2003).

Lin et al., (2004) reported embryogenic callus from nodal shoot segments of *in vitro* plantlets of *B. edulis* on MS medium supplemented with + 2,4-D (3.0 mg/l) + Kn (2.0 mg/l) + CW (0.1%) and high concentration (6%) of sucrose. Whereas, Ogita (2005) reported callus induction nodal shoot segment on MS medium consisted of 2, 4-D (3-30 µM) and Picloram (3-30 µM), but medium with 2, 4-D + BA or picloram + BAP had negative effect on callus induction from the shoots in *Phyllostchys nigra*.

Arya et al. (2008) reported maximum (91.6%) callus induction from nodal shoot segments of *D. asper* on MS medium supplemented with 30 μM of 2,4-D. Similarly Mehta et al. (2010) used different concentrations of 2,4-D (1.0, 1.5, 2.0, 2.5, 5.0 and 7.0 mg/l) and observed that 30-40% of shoot segments responded for callus induction on MS medium supplemented with 2,4-D (5.0 mg/l), but no response was observed at lower or higher concentrations of 2,4-D in *B. nutans*.

Zhang et al. (2010) reported that 2, 4-D, 1.0-3.0 mg/l was the best to induce embryogenic vigorous, granular and compact callus, whereas higher concentration of 2,4-D (10 mg/l), in the medium produced sticky watery and mucilage callus from the mature zygotic embryos of *D. hamiltonii*.

Hu et al. (2011) reported, high frequency callus induction on MS medium supplemented with 2, 4, 5-T (2.0 mg/l) + Kn (0.2 mg/l) and IBA (0.4 mg/l) from young shoots of *D. farinosus*. Whereas, Komatsu et al. (2011) reported caulogenesis
from the leaf sheath on the medium consisted of Picloram, (8.0 mg/l) in *P. bambusoides*.

2.1.1.1.3 Effect of nutrient media:

Defferent nutrient media differ from each other in nutrient contents and source of nutrient. Requirement of nutrient media may differ with the plant species. Nitrogen, which is a major component of the different nutrient media, has a key role in plant growth and development as it has direct effects on rate of cell growth and differentiation (Kirbey *et al.*, 1987). Nitrates are good sources of nitrogen supply to plants (Gould *et al.*, 1991; Shanjani, 2003), because it is readily taken up and metabolized by the cells.

Most of the studies on bamboo species are based on single type of medium on embryogenic callus induction. Yeh and Chang (1987) reported callus induction from mature zygotic embryo of *Sinocalamus latifolia* on MS medium with 2,4-D (6.0 mg/l) + Kn (3.0 mg/l) + sucrose 5% and PVP (250 mg/l) under dark condition. Yeh and Chang (1986), also reported callus initiation from young florets of *B. beecheyana* on MS medium with of 2, 4-D (2.0 mg/l) + Kn (2.0 mg/l) and 6% sucrose. Whereas, Rao *et al.* (1985) reported induction of friable and compact callus from the embryogenic end of the seeds (mesocotyl area of the zygotic embryo) of *D. strictus* on B5 medium supplemented with 2, 4-D (3.0 mg/l) + sucrose 2%.

Haung *et al.* (1989) used only one type of medium for callus induction from shoot apices of *Bambusa*, *Phyllostachys* and *Sesa* i.e. MS medium with 2, 4-D. Whereas, Hassan and Debergh (1987), reported callus induction from the explant of *P. viridis* on the medium consisted macronutrients of MS medium and micronutrient of Nitsch medium with 2, 4-D (9 µM) and 2% sucrose within 4-5 weeks period.

Jullien and Tran Thanh Van (1994), reported callus induction in *B. glaucescens* on MS medium with 2, 4-D (18 µM) under dark condition. Similarly, Saxena and Dhawan (1999), used MS medium supplemented with 2, 4-D from seeds of *D. strictus* for embryogenic callus induction.

Rout and Das (1994), reported high frequency callus induction from nodal shoot segments as an explants of *in vitro* grown seedling and excised mature zygotic
embryo of three bamboo species (*B. vulgaris*, *D. strictus* and *D. giganteus*) on MS medium supplemented with 2,4-D (2.0 mg/l) + Kn (0.5 mg/l) + adenine sulphate (10 mg/l).

Whereas, Rao *et al.* (1990) reported callus induction from mature embryo and explants from aseptically grown seedling on B5 medium with 2, 4-D in *D. strictus* and *B. bambos*.

Lin *et al.* (2004) reported use of only MS medium with 2, 4-D (13.6 μM) and Kinetin (9.2 μM) with coconut milk (0.1%) and 6.0% sucrose for the embryogenic callus induction from nodal shoot segments of *in vitro* grown plants of *B. edulis*.

Somashekher (2007) used MS, B5 and WP media and found that MS medium with 2, 4-D (2.5mg/l) alone proved the best for embryogenic callus induction. Whereas, Arya *et al.* (2008) reported use of MS and B5 medium, and found that MS media was the better for callus induction from nodal segments of *D. asper*, within 4 weeks.

Zhang *et al.* (2010), evaluated MS, ½ MS, NB and HB media and reported that MS medium was found the best for vigorous, granular and compact callus induction from mature zygotic embryos in *D. hamiltonii*. Hu *et al.* (2011), used reported that callus induction frequency was more (29.7%) on MS medium using young shoots of *D. farinosus*.

### 2.1.1.1.4 Effect of additives:

Additives like; ascorbic acid, cysteine and citric acid are known to have antioxidant properties and minimize leaching and browning problem of the field grown explants and their effect on callus induction and growth. In addition, in some species activated charcoal and PVP also minimize toxicity of leachants in the medium. Apart from these, glutamine malt extract and coconut milk in the medium have auxillary effect on callus induction.

Yeh and Chang (1987), reported used of PVP (250mg/l) in MS medium with 2, 4-D (6.0 mg/l) + Kn (3 mg/l) + sucrose 5% for callus induction from zygotic embryo of *Sinocalamus latifolia* under dark condition. They encountered exceduation of phenolics problem, which was over come by the addition of PVP in the medium.
Woods et al. (1991) reported use of casein hydrolysate either alone or in combination with BA in the medium and found non-significant effect on embryogenic callus induction in *Oteta acuminata* aztecorum.

Rout and Das (1994), reported that adenine sulphate (10.0mg/l) along with 2,4-D (2.0 mg/l) + Kn (0.5 mg/l) in MS medium favoured embryogenic callus induction from explant of *in vitro* grown seedling and excised zygotic embryo of three bamboo species.

Sha Valli Khan et al., (2002) reported that, in MS medium with NAA (5.0 µM) and BAP (2.5 µM) increased concentration of myo-inositol (200 mg/l) and addition of ascorbic acid (200mg/l) improved callus frequency and growth in *Bixa orelliana*.

Lin et al. (2004), used coconut milk (0.1%, v/v) in MS medium with 2,4-D (13.6 µm), kinetin (9.2 µm) and 6.0% sucrose for embryogenic callus induction in *B. edulis*.

### 2.1.1.1.5 Effect of carbohydrate source:

Plant tissues and organs culture under *in vitro* condition require exogenous supply of energy and carbon source for growth and metabolism. Optimum requirement of different carbohydrates and their concentration may vary with the plant species.

Most of the reports on bamboo species deals with use of sucrose as source of carbohydrate in the medium for callus induction. Yeh and chang (1986) reported that 6% sucrose in the medium favoured high induction of embryogenic callus in *bambusa beecheyana*. Similarly Yeh and Chang (1986) reported high concentration (6%) of sucrose for initiation and maintenance for embryogenic callus in *Bambusa oldhamii*. Lin et al. (2004) also used high concentration (6%) of sucrose for the callus induction in *Bambusa edulis*.

Kochba et al. (1978b, 1982) used various source of carbohydrates in the medium Galactose and lactose in the medium enhanced callus induction over a broad range (8-256mM). Glucose and fructose were ineffective in stimulating nucellus calli of some cultivars. Whereas, Galactose, lactose and raffinose stimulated callus induction considerably in *Citrus*. 
Godbole (2002) reported use of 3% sucrose as a source of carbohydrate for callus induction in *Dendrocalamus hamiltonii*. Most of the report on embryogenic callus induction in bamboo species deals with use of single concentration viz; 2% sucrose in *Otatea acuminata aztecorum* (Woods et al., 1991) and 3% sucrose in the medium in *Bambusa edulis* (Lin et al. 2004) similarly, Somasheker (2007) also reported 3% sucrose is the best for callus induction in *G. angustifolia* and *P. stocksii*

2.1.1.2 Callus multiplication:

Often, embryogenic callus is maintained and proliferated on a medium similar to that is used for callus initiation. Higher the proliferation rate, cultures become more synchronized. Maintenance of cultures in embryogenic form over a long period has been an important aspect for utilizing the phenomenon of somatic embryogenesis in different studies.

2.1.1.2.1 Effect of plant growth regulators:

Yeh and Chang (1987) reported use of IAA, NAA and 2,4-D for callus multiplication and found that MS medium consisted of 2,4-D + Kn favored for embryogenic callus multiplication of *S. latifolia*. They also reported that no other auxins (IAA and NAA) could be the substitute of 2, 4-D.

Saxena and Dhawan (1999), reported that out of the various auxins & their concentration used, MS medium consisted of $3 \times 10^{-5}$ M 2,4-D was the best for multiplication of embryogenic callus cultures of *D. strictus* in 5 weeks period.

Godbole et al. (2002) used different concentrations of cytokinin (BA) and auxins (2,4-D and NAA), and reported that MS medium supplemented with 2,4-D (1.0,mg/l) + BA (1.0, mg/l) proved the best embryogenic callus proliferation in *D. hamiltonii* within four week.

Lin et al. (2004), evaluated various PGRs viz; Kn, TDZ, 2-4-D and coconut milk with different concentration in MS medium and found that maximum (526 mg) fresh weight of callus and better callus multiplication was on medium with 2, 4-D (3.0 mg/l) + TDZ (0.01 mg/l) in *B. edulis*. 
Somashekher (2007) used various concentrations of 2,4-D (1.0-5.0mg/l) alone and or with Kn (0.5mg/l) in medium and found that 2,4-D (1.0mg/l) in MS medium proved the best for embryogenic callus multiplication in *P. stocksii* and *G. angustifolia*.

Arya *et al.*, (2008) used various PGRs; 2,4-D, IAA, BAP and Kn for callus multiplication and reported that MS medium with combined use of 2,4-D (9.0 µM), BAP (0.88 µM) and IAA (2.85 µM) favoured better callus multiplication rate (3.5 fold) with maximum callus fresh weight (175 g) within 4 weeks dark in *D. asper*.

2.1.1.2.2 Effect of nutrient media:

Nutrient rich media generally favor callus induction and further multiplication. Optimum nutrient requirement may vary with the plant species. Nitrogen is a major element for *in vitro* morphogenesis (Halperin, 1995). Different nitrogen balances and sources in the culture medium promoted embryogenic callus multiplication in rice (Ozawa *et al.*, 1996), carrot, alfa-alfa (Lai *et al.*, 1992), white spruce (Barrett *et al.*, 1997) and cotton (Gonzalez-Benito *et al.*, 1997).

Most of the earlier reports deal with use of single type of medium for multiplication of callus. Yeh and Chang (1986) reported use of MS medium with 2,4-D + Kn for multiplication of embryogenic callus of *B. beecheyana*. Hassan and Deberg (1987) also reported use of MS medium with 2, -D for multiplication of callus of *P. viridis*.

Jullien *et al.* (1994) reported multiplicity of compact and nodular callus from the leaf tissues of *B. glaucescens* on MS medium with 2, -D. Rout and Das (1994) also reported use of MS medium with 2, 4-D + Kn for callus multiplication in *B. vulgaris, D. strictus* and *D. giganteus*. Similarly Lin *et al.* (2004) reported that MS medium with 2, 4-D (13.6 µM) + TDZ (0.046 µM) for the successful callus proliferation in *B. edulis*.

Somashekher (2007) used various media like; MS, B5 and WP for embryogenic callus multiplication and found that MS medium was proved the best for multiplication of embryogenic callus in *P. stocksii* and *G. angustifolia*.
2.1.1.2.3 Effect of different carbohydrate sources:

Most of the reports on bamboo species deals with use of single source of carbohydrate (i.e. sucrose) in the medium for embryogenic callus multiplication. Yeh and Chang (1986) reported that 6% sucrose in medium favoured high rate of multiplication of embryogenic callus in *Bambusa beecheyana*. Similarly, Yeh and Chang (1986) used high concentration (6.0%) of sucrose for proliferation for embryogenic callus in *Bambusa oldhamii*. Lin *et al.* (2004) also reported use of 6% sucrose for callus multiplication in *Bambusa edulis*. Whereas, Godbole (2002) used 3% sucrose as a carbohydrate in the medium for embryogenic callus multiplication of *Dendrocalamus hamiltonii*.

Carbohydrate source and concentration has significant effect on callus multiplication. In case of cocoa, embryogenic callus multiplied the best when glucose or fructose was added in the medium in place of sucrose (Elhag *et al.*, 1987, 1988).

2.1.1.2.4 Effect of sucrose concentrations:

Sucrose not only act as a source of carbohydrate, but also as osmoregulator. Osmotic effect of sucrose in culture medium for callus multiplication has been well documented by the earlier workers in different species. Few reports deal with use of high concentration (about 6%) of sucrose in the medium for the callus multiplication viz; *Bambusa oldhamii* (Yeh and Chang, 1986a), *Sinocalamus latifolia* (Yeh and Chang, 1987,) *Oteta acuminata azetecorum* (Woods *et al.*, 1992). Similarly Litz (1988) reported that 6% sucrose in B5 medium favoured multiplication of embryogenic callus of *Euphorbia longan*. In *Zea mays* also high concentration of sucrose (12%) in MS medium proved the best for induction and multiplication of embryogenic callus from immature seed embryos (Liu *et al.*, 1982).

Lin *et al.*, (2004), reported that among the different concentrations (1.5, 3.0, 4.5 and 6.0%) of sucrose tested, lower percentage of callus browning and maximum callus fresh weight (707 mg) on MS medium supplemented with 4.5% sucrose in callus proliferation of *B. edulis*.

2.1.1.3 Somatic Embryo Induction:

Production of normal somatic embryos structurally and functionally is pre-requisite for industrial application. In addition, synchronized embryo differentiation could be valuable in biochemical, genetical and physiological studies.
2.1.1.3.1 Effect of plant growth regulators:

Woods et al. (1991) reported that out of the various PGRs, used, BAP in MS medium proved the best in terms of somatic embryos induction in Mexican weeping bamboo. Saxena and Dhawan (1999) used various combination of 2,4-D, IBA, Kn and BAP and reported that MS medium with 2,4-D (1×10⁻⁵ M), kinetin (Kn) (5×10⁻⁶), 1-indolebutyric acid (IBA) (2×10⁻⁶) and soluble polyvinylpyrrolidon (PVP) (250 mg l⁻¹) found the best for somatic embryo induction in *D. strictus*.

Godbole et al. (2002) used different PGRs (viz. BA, IAA, and GA₃) in ½ MS and MS media and they found that BA (2.5mg/l) + 3% sucrose in MS/2 medium proved the best treatment for somatic embryo induction in *D. hamiltonii*. Whereas, Sood et al. (2002) reported that incorporation of BA (1.0 mg/l) + 2, 4-D (1.0 mg/l) + GA₃ (0.5 mg/l) in MS medium proved the best for somatic embryo induction in *D. hamiltonii*.

Somashekher (2007) used various PGRs 2,4-D (0.1 and 0.25mg/l), NAA (0.25 and 0.5mg/l), BAP (0.25-1.0 mg/l) and CM 10% in MS medium for somatic embryo induction and reported that medium with NAA (0.25-0.5 mg/l) and BAP 1.0mg/l proved the best in terms of somatic embryo induction in *P. stocksii*.

Rangaswamy (2007) used different auxins (2, 4-D, IAA, IBA, NAO, NAA; 1.0 and 2.0mg/l) alone or in combinations with cytokinins (BAP or Kn; 0.1 mg/l) and reported that IAA (2.0 mg/l) in MS medium favoured high (90.90%) frequency somatic embryos induction in *S. album*.

Rugkhla and Jones (1998), used various PGRs and reported that low concentrations of TDZ (1-4μM) was the best for somatic embryo induction in *S. album* and *S. spicatum*. They found that frequency of somatic embryo induction ranged from 64 to 72%, depending on the concentration of TDZ in the medium.

2.1.1.3.2 Effect of nutrient media:

Various workers have reported use of different nutrient media for the high rate of somatic embryo induction. Woods et al. (1991) used MS and B5 media for somatic embryogenesis and found that MS medium with 2% sucrose + 2, 4-D (3.0 mg/l) + BA (0.5 mg/l) was better for somatic embryos induction and multiplication in *Otatea acuminata aztecorum*. Similarly Saxena and Dhawan (1999) compared MS and B5
media for somatic embryo induction and further multiplication in *D. strictus* and that MS medium was better for somatic embryo induction.

Godbole *et al.* (2002) used ½ MS and MS full strength and observed that ½ MS medium favored better response in terms of somatic embryo induction in *D. hamiltonii*.

In *P. stocksii* and *G. angustifolia*, Somasheker (2005) used various nutrient media and found that whitish, compact and slow growing callus after 3 weeks of subculturing induced high rate of somatic embryo induction on the MS medium with 2,4-D 1.0 mg/l.

Patenat *et al.* (2002) used various nutrient media and reported that B5 medium with 2,4-D (0.5 to 2.0 mg/l), coconut milk (10%) and sucrose (6%) was the best for somatic embryo induction in mango (*Mangifera indica*). Shahana and Gupta (2002) conducted studies on the effect of different (White, MS, LS, B5, MT, Nitch, SH and N6) and found that somatic embryo were obtained only of four media Viz; MT, LS, Nitch and MS. Among them, LS medium proved the best differentiated into somatic embryos.

### 2.1.1.3.3 Effect of different carbohydrate sources:

Generally in bamboo most of the reports on bamboo deals with use of sucrose as a source of carbohydrate for somatic embryo induction. Yeh and Chang (1986) reported 6% sucrose for high frequency of somatic embryo induction in *B. beechyana*. Similarly, Yeh and Chang (1986) used high concentration of (6%) of sucrose for somatic embryos initiation in *Bambusa oldhamii*. Lin *et al.* (2004) also reported that 6% sucrose was the best in terms of somatic embryo induction in *Bambusa edulis*. Whereas, Godbole (2002) reported 3% sucrose in medium as a carbohydrate for initiation of somatic embryo in *D. hamiltonii*.

Ho and Vasil (1983) reported 6-10% sucrose in MS medium to promote somatic embryo induction from young leaves of *Saccharum officinarum*. Whereas, Ahloowalia and Maretski (1983) reported somatic embryo induction from callus of *Saccharum officinarum* with 3% sucrose in MS medium, but for further growth of the embryo into complete plantlets, required 6% sucrose in MS medium.
Cheong and Pooler (2004) reported that no somatic embryos induced on medium containing only sorbitol or mannitol as a carbohydrate source in *Prunus incisa*. Whereas, about 50% of callus grown on the medium with sucrose or glucose produced somatic embryos and embryos induced on medium with sucrose 4% or 5% were whiter and more compact than those induced on medium containing lower concentrations of sucrose.

### 2.1.1.3.4 Effect of incubation condition:

Incubation condition particularly temperature, light intensity and photoperiod are known to have influence on somatic embryogenesis. Rao *et al.* (1985), reported somatic embryo induction at 27 ± 2°C temperature under 2500 lux light condition and about 67% of callus cultures induced somatic embryos. Godbole *et al.* (2002) reported that photosynthetic photon flux density (PPFD) of 70±5 μmol m⁻²s⁻¹ from cool, white fluorescent lamps at 25±2°C at 14 h in a 24-h light/dark incubation condition was found the best for induction of somatic embryo in *D. hamiltonii*.

Lin *et al.* (2004) reported that 26°C temperature and 54 μmol m⁻²s⁻¹light intensity for a 16 h photoperiod was the best incubation condition for somatic embryo induction in *B. edulis*.

In *S. album*, Ilah *et al.* (2002) reported that incubation of cultures at 24±2°C temperature, RH of 70 to 80% and illumination at 20μM/m/s light proved ideal for somatic embryo induction. They found that above mentioned incubation conditions increased the percentage of normal embryo induction and reduced the percentage of abnormality. Whereas, Corredoira *et al.*, (2002) observed that incubation of cultures at 25±2°C temperature and dark condition suitable found ideal for somatic embryo induction in *Ulmus minor* and in *Olea europea* (Rugini and Caricato, 1995).

### 2.1.1.4 Somatic Embryo Development and Maturation:

Normal development of somatic embryos requires a fine temporal and spatial regulation of cell division, enlargement and differentiation (Ammirato, 1983). Production of normal somatic embryos synchronized structurally and functionally is necessary for industrial application (Ammirato, 1987).
2.1.1.4.1 Effect of ABA and PEG:

Requirement of growth hormones for the induction and maturation of somatic embryo may be varied. In general, development of somatic embryos is regulated by endogenous and exogenous growth regulators, (Chalupa, 1987) reported that normal somatic embryos was stimulated after transfer of globular somatic embryos development from a nutrient medium containing higher concentration of growth regulators to the medium containing either reduced concentration or devoid of growth regulators in birch.

Chang and Lan (1995) reported that, medium supplemented with ABA (0.1 to 2.0 mg/l) and PEG or polyamines and mannitol favoured development and maturation of somatic embryo in *B. beechyana*.

Muralidharan and Mascarenhas (1995) tested five different concentrations (0.1, 0.5, 1.0, 5.0 and 10.0mg/l) of abscissic acid in MS medium in *Eucalyptus*. They reported that the proportion of mature embryos on ABA containing medium was greater than that on ABA free medium. Growth and total number of embryos decreased with increasing (5.0 to 10.0mg/l) concentrations of ABA in medium.

In *Acacia* spp, presence of ABA in the medium significantly increased the number of embryos that reached more advanced differentiation stages and maturation stages, as compared to the medium without ABA (Ortiz *et al.*, 2000). Similarly, Garcia-Martin *et al.* (2005) reported that addition of ABA (1.0 μM) in the culture medium significantly promoted somatic embryo maturation and increased both fresh and dry matter without affecting the relative water content in *Quercus suber*. They found that endogenous ABA and IAA content showed opposite levels during the development and maturation somatic embryos.

Walker and Parrott (2001) carried that studies on the effect of PEG 4000 (0.0, 5.0, 10.0, 15.0 or 20.0%) on somatic embryo development and maturation in soybean. Globular stage embryos on medium with high concentration of PEG (20%) failed to histodifferentiate, All the media with PEG 5-15% enhanced embryo conversion as compared to medium without PEG (control).They found that increasing concentration of PEG in the medium resulted in reduction in the fresh weight of mature embryos.
Effect of PEG and ABA on maturation of *Panax ginseng* embryos was also reported by Langhansova *et al.* (2004). Cultures were treated with high molecular weight osmoticum, PEG 4000 at 3.75 or 7.50% and ABA (20μM) in the MS medium for 4 weeks. Shoot regeneration increased from 31 to 70%, when treated with PEG and ABA. About 75% of the treated embryos formed roots, while non-treated embryos did not form roots.

In *S. album*, Rangaswamy (2007) used various growth hormones, ABA, GA₃ and PEG in MS medium and found that ABA + PEG in the medium was essential for synchronized embryo maturation.

### 2.1.1.4.2 Effect of nutrient media:

Nutrient media play important role in full filling nutritional requirement for the development and maturation of somatic embryos. Any imbalance in nutrient availability may results in lack of synchronization and abnormality in development.

Rao *et al.* (1985) reported somatic embryo induction and development on B5 medium with 2, 4-D under light of 2500 lux intensity and favoured 67% of embryo development in *D. strictus*. Godbole *et al.* (2002) used MS/2 and MS full strength media for the somatic embryo development and found that MS medium with BAP (2.5mg/l) exhibited the best response for somatic embryo maturation in *D. hamiltonii*. Whereas Ilah *et al.* (2002) compared MS and WP media, for somatic embryo development and found that WP medium favoured development and maturation of somatic embryos in *Santalum album*. They also observed that growth and maturation of embryo arrested unless the embryos were separated and plated on fresh media. Kumar *et al.* (2003) reported use of B5 medium with activated charcoal (0.5g/l) and sucrose (10g/l) for the development and maturation of somatic embryos in *Commiphora wightii* (Arnott.) Bhandari. However, addition of ABA was associated with reduction in cotyledonary stage embryos with high percentage of abnormal germination.

Mehta *et al.* (2010) reported use of only MS medium with BAP and 2,4-D (1.0mg/l each) for somatic embryo maturation in *B. nutans.*
2.1.1.5 Somatic embryo germination:

The quality of somatic embryo is determined by its maturation and germination ability. One of the challenges preventing the wider application of somatic embryogenesis in plant propagation has been the low germination and poor survival during the acclimatization.

2.1.1.5.1 Effect of plant growth regulators:

Plant growth hormones play significant role in germination of somatic embryos in to normal plant. Requirement of growth hormones in the germination medium may depends on the endogenous level of PGRs and plant species.

Yeh and Chang (1987) reported that 2, 4-D (3.0 mg/l) + Kn (2.0 mg/l) in MS medium favoured germination of somatic embryo into plantlets in Sinocalamus latiflora. Whereas, Huang et al. (1989) reported that combination of NAA and BA in the medium was found the best in terms of somatic embryo differentiation and germination of plantlets in Bamboo.

Saxena and Dhawan (1999) reported that among the various treatment of auxins (IBA and NAA) and cytokinins (BAP and Kn) used in MS medium, combined use of NAA + Kn (5.0 µM, each) proved to be the best for somatic embryo germination (90%) in D. strictus.

Sood et al. (2002) used various PGRs for somatic embryo germination and reported that MS medium without phytohormones favoured germination of somatic embryo in D. hamiltonii. Whereas, Godbole et al. (2002) reported that BA (2.5 mg/l) alone in MS/2 medium proved the best for embryo germination into plantlets in D. hamiltonii.

Lin et al. (2004) evaluated various PGRs in the medium for the high rate somatic embryo germination and found that 80 % somatic embryos germination was achieved with only TDZ (0.01 mg/l) in the medium. They found that germination was declined to 28% in the MS medium with NAA (1.0 mg/l) + TDZ (0.01 mg/l) in B. edulis.

Zhang et al. (2010) used various PGRs viz; Kn, BA, TDZ, 2ip and NAA for somatic embryo germination in D. hamiltonni and reported that the treatment
consisted of NAA (1.0 mg/l) with Kn (1.0 mg/l) and BA (2.0 mg/l) was the most
effective combination for somatic embryo germination (89.5%) in D. hamiltonii.

(Hu et al., 2011) conducted studies on the effect of PGRs on the somatic
embryo germination and found that maximum (91.2%) germination on MS medium
with Kn (2.5 mg/l) + IAA (0.5 mg/l) after 28 days in D. fairnosus.

Xie and Hong (2001) have reported that GA3 (5.0mg/l) in MS/2 medium
favoured germination of about 11% of cotyledonary stage embryos into plantlets with
root, elongated hypocotyl and the first pair of leaves in Acacia mangium.

In Quercus robur L, Valladares et al., (2006) reported that somatic embryos of
8-10 mm in length germinated on MS/2 medium supplemented with BA (0.44
μM).They observed that germination and conversion ability of embryos depended
heavily on genotypes and conversion rates varied from 0 to 70%.

Combined use of IAA (1.0mg/l) and GA3 (1.0mg/l) + activated charcoal
(250mg/l) in MS/4 medium proved the best with germination frequency of 50.36%
into normal plantlets with better shoot length (9.49cm) and root length (13.61 cm) in
S. album Rangaswamy (2007). Incorporation of GA3 alone favoured shoots induction
(26.68%) and only 23.44% of normal plants and when IAA was used alone in
medium, it favoured root induction (30.44%) and minimum rate (20.33%) of normal
plants.

2.1.1.5.2 Effect of nutrient media:

Nutrient media have significant effect on germination of somatic embryos and
selection of suitable nutrient medium, which favor high rate of somatic embryo
germination into functional plant is key for success of a protocol.

Yeh and Chang (1986) reported use of MS medium with 2, 4-D (3.0 mg/l) +
Kn (2.0 mg/l) for spontaneous germination of somatic embryos of B. beecheyana.
Woods et al. (1991) used MS and B5 basal media and they reported that more than
95% germination of somatic embryos originated from zygotic embryo as a explant
was observed into shoots and roots on B5 medium in Otatea acuminate aztecorum
(Mexican weeping bamboo).
Godbole et al. (2002) used MS/2 and MS media and found that MS/2 medium was better for embryo maturation and germination in *D. hamiltonii*. Similarly, Rout and Das (1994) also described that MS/2 medium with Kn (0.5 mg/l) favoured germination of somatic embryo, into plantlets in three bamboo species (*B. vulgaris, D. giganteus* and *D. strictus*). In *D. asper*, Arya et al. (2008) used MS medium with BAP (4.4 µM) + GA₃ (2.8 µM) and reported maximum (70%) somatic embryo germination into plantlets and (30%) somatic embryos produced only shoots under 16 h photoperiod within 4 weeks period. Zhang et al. (2010), used MS, NB and HB media for somatic embryo germination of *D. hamiltonii*. They reported that MS medium with NAA (1.0 mg/l) + BA (2.0 mg/l) + Kn (1.0 mg/l), proved the best for maximum (89.5%) germination of somatic embryo as compared with NB and HB media.

Most of the reports on bamboo deals with on use of MS medium for germination of somatic embryos in different bamboo species viz; *D. giganteus* (Ramanayake and Wanniarachchi, 2003), *P. nigra* (Ogita, 2005), *B. balcooa* (Gillis et al., 2007), *B. nutans* (Mehta et al., 2010) and *D. farinosa* (Hu et al., 2011).

2.1.2. *In vitro* propagation through axillary shoot proliferation:

Axillary shoot proliferation occurs when dormant axillary buds are released from apical dominance, mostly by manipulation of the hormones (primarily cytokinins) in the nutrient medium. This method of propagation is preferred for the commercial propagation of hardwoods including bamboo because, it maintains genetic stability better than propagation through adventitious regeneration and somatic embryogenesis (McCown and McCown, 1987).

2.1.2.1 Shoot initiation:

Various critical factors which play important role in shoot initiation are; source of explant (seedling/mature trees), season or period of collection, management of explants source, explant size, plant growth regulators, nutrient medium, additives, incubation condition and genotype.
2.1.2.1.1 Effect of PGRs:

Plant growth regulators particularly auxins and cytokinins have specific roles in cell elongation, cell division and differentiation. Endogenous levels of PGRs may vary with the species and therefore, exogenous requirement also vary with plant species and sometimes between different types of explants. The cytokinins commonly used are BA, kinetin, TDZ, 2-iP and Zeatin. Of these, BA is most commonly used and also cheapest, among the cytokinins (Bonga and Von aderkas, 1992).

TDZ is one of the several substituted ureas such as N-N’-diphenylurea (Mok et al., 1980) and N-(2-chloro-4-pyridyl)-N’-Phenylurea (Fellman et al., 1987) that have been investigated for cytokinin activity (Huetteman and Preece, 1993; Lu, 1993).

Sometimes dual function of auxin and cytokinin are observed with TDZ. Compared to most other compounds with cytokinin activity, lower concentrations of TDZ can stimulate axillary shoot proliferation in many woody plants, whereas higher TDZ concentrations may result in the formation of both axillary and adventitious shoots (Van Nieuwkerk et al., 1986; Chalupa, 1988). Quite often higher concentrations of TDZ induces callusing.

In Rauvolfia tetraphylla, the cultures continuously grown in medium with TDZ (5 μM) resulted in fasciated and distorted shoots (Faisal et al., 2005). The deleterious effect of the continued presence of TDZ on the growth and multiplication of chickpea and pea has been reported (Bohmer et al., 1995; Murthy et al., 1996).

Chambers et al. (1991) reported multiple shoots induction from the epicotyl of 14 days old in vitro seedling of D. hamiltonii on MS medium with 4.4 M BA in 4 weeks. Similarly, Prutpongse and Gavinlertvatana (1992) reported multiple shoot production from 54 species out of 67 bamboo species tested on MS medium with BAP (2.2 – 44.0 M). They recommended 22 M BA in the MS medium for multiple shoot induction from nodal segment with bud from the young shoots.

Arya et al. (2008) reported that maximum bud break response in MS liquid medium supplemented with high BAP (5mg/l) in Drepanostachyum falacatum and comparatively low (3.0mg/l) BAP in Bambusa balcooa. No significant increase in shoot proliferation from cultured axillary buds when different auxins (NAA, IAA) were used in MS medium along with BAP.
In *D. hamiltonii*, highest bud break and shoot proliferation was achieved in MS liquid medium supplemented with BA 2.5mg/l (Sood *et al.*, 2002). Arshad *et al.* (2005) reported that out of various concentrations of BAP tested (1.0 to 10.0 mg/l), medium containing 5.0 mg/l BAP induced highest bud break (83.33%), more number of shoots (3.89±0.38) with good shoot length (3.29±0.31) in *B. wamin*. Bud break was not observed, when NAA and 2, 4-D were used alone for shoot initiation. Higher concentration of BAP resulted in less number of dwarf shoots per nodal explant.

In *P. stocksii*, Sanjaya *et al.* (2005) reported that out of the various concentrations of NAA (0.06-5.37 M) and BAP (0.0-11.09 M) tested in MS liquid medium, combined use of NAA (2.68 M) and BA (4.44 M) resulted in highest number of shoot induction with good shoot length. Similarly, Somashekar *et al.* (2008) found that MS liquid medium with additives + NAA (0.25mg/l) + BAP (1.0mg/l) proved the best in *P. stocksii* and induced 4-6 shoots/explant with shoot length of 4.17cm within 3 weeks. Higher concentrations of BAP (2.5mg/l) inhibited shoot growth. Kinetin in the medium at all concentrations induced less number of shoots as compared to BAP.

Kabade (2009) used various auxins viz. IAA, IBA and NAA with TDZ in MS medium and found the consisted NAA (0.1mg/l) + TDZ (0.25mg/l) proved significantly better than IAA + TDZ and IBA + TDZ in *B. bambos*. Similarly, MS liquid medium containing NAA (0.1 mg/l) + TDZ (0.25mg/l) proved the best for multiple shoot induction in *D. strictus*.

Saxena (1990) reported multiple shoot induction from three weeks old *in vitro* grown seedlings of *B. tulda* in MS liquid medium supplemented with BAP (8 x10-6M) and kinetin (4 x 10-6M). Medium without cytokinin resulted in drying of shoots. Ravikumar *et al.* (1998) reported multiple shoot induction from nodal shoot segment from seedling and mature culm nodal shoot segment of *D. strictus* in MS liquid medium supplemented with BAP (0.5 mg/l) + Kn (0.5 mg/l) + coconut milk (200 ml/l). Seedling explants induced 35-40 shoots within 20-25 days and whereas, field grown plants induced only 3-8 shoots/explant under suspension condition in shaker.

Lin and Chang (1998) reported that MS medium with TDZ (0.01 mg/l) and 2, 4-D (0.5 mg/l) favored shoot induction from the nodal shoot segments of field grown plants of *Bambusa edulis*. They found that auxin (NAA) alone in the
medium failed to induce shoots and explant showed senescence and eventually died, whereas, media containing BA alone or with NAA induced shoots.

### 2.1.2.2 Shoot multiplication:

#### 2.1.2.2.1 Effect of PGRs:

Choice of PGRs, their optimum concentration and combinations requirement very with the plant species, explant source (Seedling/mature plants) and genotypes on shoot multiplication and shoot growth of bamboo species.

In *D. hamiltonii* 1.5 folds shoot multiplication rate has been reported in MS medium supplemented with BA 2.5mg/l alone (Sood *et al.*, 2002). Differential rate of shoot multiplication viz; 9-11 fold in *Drepanostachyum falcatum* and 2-3 folds in *Bambusa balcooa* were observed on MS medium supplemented with BAP 3mg/l (Arya *et al.*, 2008).

Arya and Sharma (1998) reported that, among the various concentrations of BAP (1.0-10mg/l) tested, MS liquid medium with BAP 3.0 mg/l proved the best for shoot multiplication within 4 weeks period in *B. bambos*. Higher concentrations of BAP (10.0 mg/l) produced dwarf and leafy shoots. Better shoot elongation was observed on low BAP (0.5–1.0 mg/l) medium. Kinetin either alone or in combination did not improve shoot multiplication rate. Similarly, incorporation of NAA or IBA in the medium also not favored improvement in shoot multiplication.

In *P. stocksii*, MS liquid medium with combined use of NAA (2.68 M), BA (2.21 M) and additives was found suitable for high rate of shoot multiplication with good shoot length (Sanjaya *et al.*, 2005). Similarly, Somashekar *et al.* (2008) reported maximum (36 shoots/clump) shoots in MS liquid medium with additives, NAA 0.25mg/l and BAP 1.0mg/l in *P. stocksii*. High rate of shoot multiplication was also observed in high concentration of BAP 2.5mg/l, but shoots were dwarf. Incorporation of NAA with BAP showed auxillary effect on shoot multiplication. Kinetin was less effective as compared to BAP.

MS liquid medium supplemented with NAA (0.1mg/l) + BAP (1.0 mg/l) proved the best in terms of good shoot multiplication (15.87 shoots / shoot clump) with maximum (4.22 cm) shoot length with sturdy shoot in *B. bamboos*, whereas MS liquid medium with NAA (0.1 mg/l) + TDZ (0.25 mg/l) resulted in production of
maximum (21.49 shoots per shoot clump) shoots with appreciable shoot length of 3.52 cm in *D. strictus* Kabade (2009).

Arshad *et al.* (2005) reported that in *Bambusa wamin*, MS medium with combined use of 1.0mg/l, BAP and 0.8mg/l, Kn exhibited 4 folds multiplication rate with maximum shoot length of 4.06 cm in 4 weeks as compared to BAP 2.0mg/l (3.76folds) alone. Higher concentration of BAP, although promoted shoot multiplication, but inhibited shoot length. Similarly, Shirin and Rana (2007) found that combined use of BAP (5uM) and Kn (15uM) in the medium favored highest (4 fold) rate of shoot multiplication than BAP alone (3.58 fold) in *Bambusa glaucescens*.

Lin and Chang (1998) reported positive effect of BAP and negative effect of NAA on shoots multiplication in *B. edulis*. They found that the range of TDZ (0.01 – 6.0mg/l) promoted shoot multiplication with an average of 3-4 shoots from one shoot in 21 days, whereas, higher concentration (6.0mg/l) increased shoot number (10 shoots/shoot segment), but shoots were dwarf and vetrified. Similarly, Singh *et al.* (2001) reported that all the concentrations of TDZ (0.01 to 1.0mg/l) tested were effective in shoot proliferation. Maximum shoots (14.8± 1.0) were obtained with 0.5mg/l TDZ in half-strength MS liquid medium in 21 days and subsequently transferred to the same medium devoid of TDZ in *D. strictus*. The longer culture period (i.e. 28 and 35 days) in TDZ medium caused reduction in shoot proliferation.

In *Bambusa vulgaris* Var. ‘Striata’, different concentration of BAP (2-6mg/l) and TDZ (0.05-0.5mg/l) were tested alone and highest mean shoot number was produced in medium consisted 4mg/l BAP. This did not differ significant from shoots in 6mg/l BA or 0.1mg/l TDZ. Shoot length was significantly less in TDZ than BA in the medium. Repeated subculture of shoots in the medium with TDZ resulted in stunted and compact shoot clusters (Ramanayaka *et al.*, 2006).

2.1.2.2.2 Effect of nutrient media:

Saxena (1990) compared MS and B5 liquid and agar gelled media with BAP on shoot multiplication in *B. tulda* and found that MS liquid medium was better than B5 medium for shoot multiplication. Shoot multiplication rate was low and growth was also slow in agar gelled medium.
Shirin et al. (2003) reported that out of various media (MS, B5 and WP) with BA (0.0-35 M) tested in B. vulgaris, MS medium significantly proved better than other two media for shoot multiplication. Shoot multiplication on WP and B5 media were statistically on par with each other. Maximum (4.39 fold) multiplication was obtained on MS medium with and 15 M BA. Similarly, in B. nana, different nutrient media (MS, WP and B5) with BA (0.0-35 M) were tested and MS medium with 5 M BA proved the best with maximum (2.54 fold) multiplication rate. Whereas, shoot multiplication on WP and B5 media were statistically on par with each other (Shirin and Arya, 2003).

Arshad et al. (2005) reported that out of various media (WP, B5, DCR and MS) used, MS medium was found the best for both shoot multiplication and as well as overall growth in B. wamin. In P. stocksii, MS liquid medium with NAA + BAP proved the best (21 shoots/clump), followed by WP and B5 media. Whereas, HE medium proved the least effective for shoot multiplication (Somashekar et al., 2008).

Kabade (2009) found that out of the various nutrient media (MS, SH, B5, WP and HE) tested for shoot multiplication and growth, MS liquid medium with additives + NAA (0.1mg/l) + BAP (1.0 mg/l) proved the best for production of highest (15.90 shoots/clump) shoots per shoot clump with maximum shoot length (4.02 cm) in 2 weeks period, in B. bambos. Similarly, MS liquid medium proved better than all other media (SH, B5, WP and HE). MS liquid medium with additives + NAA 0.1mg/l + TDZ 0.25 mg/l produced maximum (21.41) shoots/clump with maximum shoot length of 3.45 cm in 2 weeks period in D. strictus.

2.1.2.3 *In vitro* rooting:

Success of any micropropagation protocol depends on the rate of shoot multiplication and frequency of rooting. Comparatively, it is easy to induce rooting from the shoots of seedling origin and quite often frequency of rooting reduce with increase in age of the source plants, particularly in tree species and woody plants. Rooting frequency also depends on the level of rejuvenation during multiplication stage and quality of the shoots. Nutrient media, auxin source and their concentrations and incubation conditions play important role in frequency of rooting, root number, root length and shoot length.
2.1.2.3.1 Effect of auxins:

Exogenous requirement of auxin and its concentrations may depend on plant species, source of explant (seedling/mature plant) and sometimes between genotypes of the same species.

Ramanayake et al. (2006) reported that in Bambusa vulgaris var. Striata highest rooting percentage was observed with TDZ pretreatment for 2 or 3 cycles, followed by transferring to medium containing IBA (3 mg/l). Shirin and Rana (2007) tested various auxins; IAA, IBA, IPA, NAA, 2,4-D and Coumarine 25 μM alone for root induction in B. glaucescens. They observed 100 % root induction with an average of 9.67 roots/shoot propagule in MS liquid medium supplemented with IBA in 4 weeks.

Bag et al. (2000) reported in vitro rooting from shoot clumps of 3.5 – 4.0cm in length originated from zygotic embryos excised from germinating seeds and explants (nodal shoot segments) from 2 years old plants of T. spathiflorus in two steps. Initial exposure for 2 weeks on ½ MS medium with IBA (50-300 μM), followed by sub-culture on hormone free medium for 8 weeks. Maximum rooting (100%) was obtained on 150-300 μM IBA ½ MS medium.

Prutpongse and Gavinlertvatana (1992) tested various concentrations of NAA (13.5 μM – 54.0 μM), BA (0.44, and 8.8 μM) and IBA (2.5 – 2.5 μM) in MS, MS/2 and MS/4 media for rooting from micro-shoots. They found that MS medium with NAA (5.4 μM) proved better than IBA for rooting in most of the species.

In B. bambos among the various auxins and their concentrations tested, NAA 3.0 mg/l in MS medium induced 80-85% rooting from in vitro shoots within 20 days period from the 3 year old plants (Arya and Sharma, 1998). Similarly, in D. asper, Arya et al. (1999) have reported root induction (95-98%) from 3-5-shoot clump on MS medium with IBA (10 mg/l) or NAA (3 mg/l) within 30 days period.

Somashekar et al. (2008) reported 100% rooting in MS/4 medium with NAA 1.0mg/l in P. stocksii. Out of the various auxins (IAA, IBA, NAA, NOA and 2,4-D) 1.0mg/l tested in MS/4 medium, IAA proved least effective.
Sood et al. (2002) found the best response on in vitro rooting on ½ MS medium with chlorine chloride (0.9mg/l) and IBA (1.0mg/l), followed by coumarine (9mg/l) and NAA (0.5mg/l) in D. hamiltonii. Whereas, addition of BA in the rooting medium along with IBA favored root induction and normal shoot growth in P. stocksii (Sanjaya et al., 2005). Shirgurkar et al. (1996) reported simultaneous shoot multiplication and rooting from in vitro shoots of D. strictus from seedling origin on MS half strength medium with BAP (0.5 mg/l each). No separate rooting step was involved.

2.1.3 Acclimatization and Hardening:

Ultimate success of micropropagation on commercial scale depends on the ability to transfer plants out of culture room on a large scale at low cost and high survival rates. Heterotrophic mode of nutrition and poor mechanism to control water loss render micropropagated plants vulnerable to transplantation shocks. Transplantation stage continues to be a major bottleneck in the micropropagation of many plants.

2.1.3.1 Hardening of plantlets regenerated through somatic embryogenesis:

Rao et al. (1988) reported hardening of plants raised through somatic embryos. They allowed plants to grow until 8-10 cm tall in soil. The plantlets were removed from the agar medium and potted directly into a soil: fine sand: farmyard manure (1:1:1) as potting mixture in 10cm diameter earthenware pots. The plantlets which have an average height of 9.5 cm with two or three leaves were then maintained in the growth chamber at 90, 80, and 70 percent RH for one week, each (total 3 weeks) under 6000 lux intensity of light. The temperature of the growth chamber was maintained at 29±1°C and percentage of survival was 95%.

Godbole et al. (2000) reported 78 percent of survival of plants raised through somatic embryogenesis during six month of growth in polyhouse and hardened plants were successfully transplanted to the field. Mehta et al., (2010) reported 90% survival of plant raised through somatic embryogenesis in B. nutans.

Lin et al. (2004) reported that regenerated plantlets with well-developed roots were transplanted in to soil (peat:vermiculite:perlite;1:1:1) after three years plant attained three meter high and had produced young shoots in B. edulis.
2.1.3.2 Hardening of plantlets regenerated through axillary shoot proliferation:

Bag et al. (2000) reported hardening of micropropagated plantlets of *T. spathisflorus*. Plantlets were washed with tap water and transferred them in to plastic pots containing soil and sand (3:1, v/v, pH 6.5). Transparent plastic bags were placed on the top of each pot to maintain humidity during hardening and kept them in net house (50% sunlight). Watering was done at 15 days interval with tap water. After one month, the plastic covers were removed and the plants were transferred to earthen pots containing garden soil.

Shirin and Arya (2003) and Shirin et al. (2005) used two steps hardening of micropropagated plants of *B. vulgaris*. The plants were washed under tap water and transferred into screw cap bottles containing 1/4\textsuperscript{th} volume of autoclaved soilrite. The bottles containing plants were kept for two weeks in culture room and supplied with 0.5x MS solution once in a week. Later on the bottles were shifted for 7 days to mist chamber. Finally, the plants were transferred to polybags containing sand: FYM: soil (1:1:1) and kept in shade house for further acclimatization with 80% survival rate.

Whereas, Sanjay et al. (2005) reported maximum survival rate (92%) during hardening and acclimatization stage of the plants raised through axillary shoot proliferation of *P. stocksii*. In *D. hamiltonii* plant regenerated through axillary shoot proliferation showed 85% survival during hardening under green house condition (Agnihotri et al., 2009). High rate (96%) of survival was also reported from *in vitro* raised plantlets through axillary shoot proliferation in *B. nutans* during hardening and acclimatization stage in nursery (Negi and Saxena, 2011).

Growth performance of *in vitro* regenerated plants at nursery stage:

Gillis et al. (2007) reported the culm of *in vitro* plants on comparison with the numbers of culms of *in vivo* plants, which can be seen as ideal markers for growth vigour, were not different in regenerants of the somatic embryos than in plants from axillary branching 18 months after transplanting in somatic embryo regenerants of *B. balcooa*.

Data related to plant height, number of culms and leaf characteristics were recorded by Agnihotri et al. (2009) in axillary proliferated shoots of *D. hamiltonii* showing no morphological variations of field grown plants.
2.2 **BOCHEMICAL STUDIES:**

Many plants undergo changes in their enzyme expression, which in turn regulates different biochemical pathways i.e. metabolism. Infact, these biochemical changes reflect the modification of the flow of carbon and nitrogen along different metabolic pathways (Tsala et al., 1996). Thus, enzymes known as metabolic markers, change during development and differentiation stages (Rout et al., 1999). Chatrah et al. (1996) have opined that the process of differentiation is a consequence of some biochemical and physiological changes induced by plant growth regulators.

Several biochemical variables are known to differentiate between embryogenic and non-embryogenic tissues and somatic embryogenesis process. Biochemical changes associated with micropropagation of plants particularly bamboos have been very poorly investigated.

2.2.1 **Glutamine synthetase** (E.C.6.3.1.2) and other related enzyme:

In addition to L-glutamine, α-alanine or L-glutamic acid strongly stimulates somatic embryo formation in carrot, not only in the number of somatic embryos formed, but also with respect to their development. The effect of the amino acids on somatic embryogenesis is stronger than that of ammonium ion. In particular, L-glutamine strongly stimulated the development of somatic embryos. To understand the different effects of amino acids and ammonium ion, the activity of glutamine synthetase, a key enzyme involved in nitrogen assimilation, was measured. Its activity decreased during the later stages of embryo development (Higashi et al. 1996).

Fransz et al. (1989) carried out isozyme analysis on protein extracts of non-embryogenic and embryogenic callus from *Zea mays* L. using polyacrylamide gel electrophoresis. They examined the isozyme patterns of glutamate dehydrogenase, peroxidase and acid phosphatase for their utility as biochemical markers of maize embryogenic callus cultures. It was observed that the intensity of the GDH isozymes was lower in non-embryogenic callus as compared with embryogenic callus. In contrast with the GDH system, peroxidase and acid phosphatase are not adequate in a cytochemical assay to discriminate between embryogenic and non-embryogenic cells.
Everett et al. (1985) reported GDH as biochemical markers based on the zymogram of isozyme patterns (five bands) from the organogenic callus and (seven bands) in embryogenic callus of maize. They suggested GDH to be a biochemical marker of embryogenesis on account of the similarity in GDH zymograms between embryogenic callus and zygote embryos and between organogenic callus and shoots.

Lai et al. (1992) found that the presence of 2µM ABA decreased total protein accumulation, whereas 50 mM glutamine increased the synthesis of the S-2 relative to S-1 proteins. In the presence of 50 mM glutamine, the quantity of high salt soluble S-2 proteins, consisting of mainly medicagin (11S) storage proteins increased 2-fold in somatic embryos. Sodium dodecyl sulfate polyacrylamide gel electrophoresis showed that glutamine increased the polypeptides of the storage proteins, especially medicagin (11S). This enhancement may represent improved nutrition of the somatic embryo or enhanced transcription or translation of the storage protein genes.

2.2.2 Nitrate reductase (E.C. 1.6.6.1)

Tingey et al. (1974) reported decrease in nitrate reductase activity with increasing leaf age in soybean foliage. Ahmad (1988) reported variation in nitrate reductase activity during the regeneration of adventitious roots at the basal cut end of Pisum sativum seedlings.

Roberts et al. (1996) observed a marked rise in the level of the in vivo nitrate reductase activity of tobacco callus culture just prior to shoot primordium emergence. Cheeseman and Tankou (2005) and John and Tankou (2005) reported that wild type plants of Arabidopsis thaliana during their exponential growth phase had as much as 15 times more nitrate reductase activity than needed to support the recorded growth.

Yadav (2009) reported that during shoot multiplication the nitrate reductase activity increased on day 6, i.e. just before new shoot emergence, while during rooting process the activity of nitrate reductase increased prior to root emergence.

2.2.3 Peroxidase (E.C.1.11.1.7), other enzymes and biochemicals:

Rao et al. (1999) reported two slow migrating cathodal isoperoxidases, specific esterase and malate dehydrogenase present in the embryogenic calli, were absent in non-embryogenic callus. Two isoperoxidase 3 and 4 (Rm 0.11 and 0.14)
observed in embryogenic callus, were absent in non-embryogenic calli indicating a possible association of specific isozyme (2, 10, 11 and 15) were found in embryogenic calli and not detectable in non-embryogenic callus. In the case of MDH isozymes, one fast migrating anodal band (Rm 0.66) was specifically found in embryogenic callus. Even though the embryogenic and non-embryogenic callus derived from the same genotype explant and on the same medium, specific differences in isozyme patterns of peroxidase, esterase and MDH were observed, which may be correlated to their difference(s) in the degree of dedifferentiation. Variable number of isozymes of esterase and malate dehydrogenase callus suggests the differential gene expression.

Godbole et al. (2004) repoeted starch deposition and amylase accumulation during somatic embryogenesis in stem callus of bamboo (Dendrocalamus hamiltonii). They observed that starch grains were clearly visible in the scutellum during the maturation stage of the somatic embryo as the somatic embryo developed further, the scutellum got reduced with corresponding increase in amylase. The amylase activity was absent during callus induction, callus proliferation and globular stages of the somatic embryo development. Tang (2000) observed that the desiccation-tolerent somatic embryos induced by ABA and/or PEG₆₀₀₀ have higher peroxidase activity as compared without treatment with PEG.

Nieves et al. (2003). Reported that embryogenic callus has more soluble proteins, free proline, proteolytic activity, soluble sugars and invertase, and lower putrecine/ (spermidine + spermine ) than non-embryogenic tissue. Non-embryogenic callus has a higher peroxidase and gallic acid level, lower dry matter/fresh matter ratio and more gross fat compared with embryogenic callus. To observed that soluble peroxidase in EC are less than NEC assumed that it can be associated with the restriction of cell wall development in the early stage of embryo formation in sugarcane. Higher metabolic activity in EC than NEC, mainly in nitrogenous components (proteins, free proline, proteolytic activity, and Put/(Spd + Spm) which can be responsible for molecular events conductive to the embryogenesis process.

Jayaseelan and Rao (2005) reported that embryogenic callus had more total carbohydrate and starch contents, total free amino acids, nucleic acids phenols and
ascorbic acid. Non-embryogenic callus exhibited high chlorophyll content, total soluble sugar, protein, ammonia and enzyme like peroxidase (EC 0.07±0.001, NEC 0.14±0.001) and polyphenol oxidase (EC 0.12±0.001 NEC 0.28±0.003), increase in total carbohydrate level (1639µg/FW) and double the amount of starch level (670µg/FW) seemed to be consistent in embryogenic callus.

Jayaseelan and Rao (2005) also reported and specified that the level of total soluble sugar (890 µg/g FW) increased in non-embryogenic callus. Higher total protein levels (440 µg/g FW) were found in non-embryogenic callus compared to embryogenic callus, the levels of total free amino acids doubled (72 µg/g FW ) but proline (850 µg/g FW) and glutamine (100µg/g FW), increased markedly in embryogenic callus. Forest and Weightman (1992) reported that glutamine levels increased, as is one of the preffered amino group donors in transamination reactions, but ammonia level (45µg/g FW) was found elevated in non-embryogenic callus.

Bonfill et al. (2003) reported that peroxidase activity in bud-forming callus was higher than in the other types of callus with the three auxin substances used, suggesting that the endogenous capacity of bud forming callus for organogenesis was stronger than auxinic substance influence. A peroxidase activity higher than 0.8 A430/mg protein per min in soluble fraction seems necessary for organogenesis to begin in Panax ginseng callus. A reductant capacity lower than 0.3 A270/g DW was found in organogenesis of Panax ginseng calluses.

Panigrahi et al. (2007) reported that protein and total soluble sugar contents were maximum during organaogenesis (2.39±0.3µg/g), (20.34±0.4µg/g) and multiple shoot induction phase (2.41±0.2µg/g), (21.36±0.3µg/g) as compared with non-organogenesis callus (0.79±0.32µg/g), (12.46±0.3µg/g), and root induction phase (0.68±0.32µg/g), (11.22±0.3µg/g). Esterase and catalase activity were maximum during organogenic differentiation, while activities were minimum at non-differentiated callus stages. Peroxidase activities were higher during rhizogenesis (8.32±0.2µg/g). Contrary to peroxidase activity, acid phosphatase activity activities were high during organogenesis and declined during rhizogenesis.
Panigrahi et al. (2007) also carried out SDS-PAGE analysis of total soluble proteins revealed expression of non-organogenesis callus (97.9 kDa), organogenic callus (77.2, 74.1, 21.9 kDa), multiple shoot induction phase (106.6, 26.9, 11.6 kDa) and root induction phase (15.9kDa), Esterase zymogram revealed one band (Rm 0.204) in both organogenic callus and multiple shoot induction. Peroxidase zymogram detected two stage specific bands, one band (Rm0.42) was specific to root induction, while another (Rm0.761) was specific to multiple shoot induction. Catalase and acid phosphatase zymogram resolved one band (Rm 0.752 and 0.435, respectively) in different stages including both multiple shoot induction phase and root induction phase, but absent in undifferentiated callus.

Kouakou (2009) reported that PPO activity was increased from primary culture to third subculture, and the highest PPO activity was obtained in third subculture. PPO activity of Coker 312 was strongest than that of R405-2000. SDS-PAGE analysis of the molecular weight and purity of the PPO revealed only one major protein band detected under the staining condition, which corresponds to a molecular weight of 55 kDa for Coker 312 and 42 kDa for R405-2000.


Sharma et al., (1999) reported that during somatic embryogenesis, sequential shift in total soluble protein content, peroxidase, IAA-oxidase, amylase (α, β) and G-6-pase enzyme activity were noticed. Linear rise in activity of peroxidase, IAA-oxidase, α, β amylase was quite evident with the appearance of proembryogenic sectors in saffron. High activity of peroxidase with decline in level of IAA-oxidase was clearly observed during subsequent stages of embryogenesis and indicates the possible role of anti-cytokinins, which induce changes in the cell oxidative status of the tissue to give an embryogenic response (Somaleva et al., 2000).

Sanchez et al. (1989) reported increase in peroxidase activities in cell wall fraction during the growth of epicotyl of Cicer arietinum. Yoshizawa et al. (1989)
observed lignification activity to be histochemically associated with the growth of bamboo shoot and high activities of water-soluble peroxidase were evident around the mid parts of the bamboo shoot, the site of active elongation.

Gaspar et al., (1992) found a peak of peroxidase prior to root formation. Kevers and Gaspar (1992) reported increase in peroxidase activity in *Kalmia latifolia* cultures, when shoots were transferred from multiplication to rooting medium. Zheng and Van Huystee (1992) demonstrated that peroxidase was directly involved in growth modulation of peanut hypocotyls.

Booj et al. (1993) established a positive relationship between peroxidase activity and cyclic budding phenomenon in date palm cultured *in vitro*. The changes in soluble peroxidases correlated well with budding. The modification of peroxidase activities and expression of isoperoxidases always preceded the morphological appearance of buds. They concluded that elevation in peroxidase activity to be an efficient marker for the budding process.

Calderon-Baltierra (1994) studied changes in peroxidase activity in root induced from *in vitro* shoots of *Eucalyptus globulus*. When 100% rooting was produced, three peaks were obtained on 1st, 7th and 10th days of culture. The maximum peak was obtained on day 10, when white compact globular masses were formed at the shoot base, and 5-8 days later root development was clearly visible. When roots were not produced, no changes in peroxidase activity were observed.

Ansari et al. (1995) studied peroxidase activity in branch cuttings of *Populus deltoides* on 0, 7, 12 and 15 days after planting. They reported that peroxidase activity in the rooting zone and immediate above 1-3 buds, significantly declined to a minimum level during root initiation stage. Peroxidase activity followed a definite pattern with respect to position of tissues and stages of rhizogenesis.

Ansari et al. (1996) reported that the peroxidase activity of *in vitro* proliferated shoots of *Bambusa arudinacea* transferred to rooting medium exhibited a definite pattern attaining significant minimum values prior to appearance of root or flower primordia, whereas, it elevated until formation of roots and flowers. Thus, the low peroxidase activity reflected early events of cell division and cell elongation during organogenesis rather than differentiation of roots or flowers.
Cortelazzo et al. (1996) studied the growth parameters of a cell suspension culture of *Rubus fruticosus* over a culture period including exponential growth, stationary phase and glucose starvation period at the end of the normal culture cycle. They observed changes in peroxidase during the culture growth and obtained two peaks of peroxidase activity in callus cultures of *Ruta fruticosus*. The first peak coincided with exponential growth phase and the second peak with the elevation of total dry weight of culture.

Tsala et al. (1996) observed that peroxidase activity in soluble fraction decreased with plant regeneration and, in bud forming condition, it decreased up to day 30 and then increased. In case of ionically bound fraction, peroxidase activity increased with plant regeneration, whereas, in bud forming condition, it initially increased for 10 days, then decreased up to day 30 and then again increased. Electrophoretic patterns of soluble peroxidase isozyme showed five bands for leaves from plantlets and three bands for leaves from bud forming shoots.

Agnihotri (1997) found that application of auxins, non-auxins and their interactions promoted chlorophyll content in *B. vulgaris* and *D. strictus*. The plausibility was raised that the enhanced formation of root primordia due to application of auxins could have favored the synthesis of kinetin that, in turn, promoted the elevation of chlorophylls in the leaves of auxin treated nodal segments. She concluded that (i) biochemical analysis during the rooting process triggers a cascade of anabolic metabolism at the expense of soluble sugars, phenols and available reduced nitrogen, and (ii) low activity of peroxidase marks the beginning of rooting in bamboo. Besides, elevation in chlorophyll content and nitrate reductase might also act as the indicator of adventitious rhizogenesis in bamboo.

Gunes (2000) investigated peroxidase enzyme activity during rooting in cuttings of *Populus* species. Enzyme activity started to increase in the early stages of root primordium formation and reached the highest level before root emergence. However, no apparent correlation was found between the activity of peroxidase and the ability of the cuttings to form roots.

Goleniowski et al. (2001) studied the relationship between *in vitro* bulbification and peroxidase activities in garlic. Two stages were distinguished during
in vitrō bulb formation characterized by peroxidase activity and isoenzymatic patterns especially of the soluble fractions. The first stage called the morphogenic stage, started after planting until 30 days of culture with a maximum soluble peroxidase activity. At that time axillary buds preformed at the base of the leaves grew and the in vitrō bulb was generated. The second stage was of bulb maturation. They reported high soluble peroxidase activity during morphogenesis stage and also observed high intensity bands on zymogram for the same stage. The second stage presented anodic ionic peroxidases exhibiting substantial increase in staining of anodic covalent peroxidase fraction.

Kanmegne and Omokolo (2003) studied changes in peroxidase activity during in vitrō organogenesis in Xanthosoma sagittifolium. Each form of morphogenesis, induced with a growth regulator, was preceded by an increase in total peroxidase activity. On hormone free medium, organogenesis occurred, but there was no increase in total peroxidase activity. The appearance of isoperoxidase A2 was associated with root initiation, while the disappearance of isoperoxidase A5 and appearance of isoperoxidase A6 preceded multiple shoot formation.

Tang and Newton (2005) estimated peroxidase and catalase activity during direct shoot induction and differentiation in easternwhite pine. They demonstrated the lowest peroxidase activity in 5-6 week old culture on medium with thidiazuron (TDZ). No such change was observed on medium without TDZ. These results demonstrated that peroxidase activity was involved in direct adventitious shoot formation induced by TDZ.

2.3 GENETIC FIDELITY STUDIES:

In micro-propagation, the most crucial concern is to retain genetic integrity of the regenereted plants as domains in vitrō culture phase somaclonal variation may be induce (Larkin and Scowcroft, 1981). Several strategies are known for detecting genetic variation, including phenotypic identification and DNA analysis techniques. Phenotypic identification based on description of the morphological and physiological traits can be used, but this method requires an extensive observation from the induction of somatic embryos to hardened plants.
Clonal truefulness is one of the major concerns in commercial micropropagation and especially in forest trees and woody plants having long rotation periods. The economic consequence of unrecognized somaclonal variation could be enormous. Therefore, genetic analysis of micropropagated plants is essential prior to use in operational planting programs (Leena and Tuija, 2005). Geilis et al. (2002) highlighted the need for assessing genetic stability in bamboo tissue culture using molecular techniques.

2.3.1 Molecular markers:

There are various molecular tools to assess variability and among them RAPD, RFLP, AFLP and ISSR markers are used to assess genetic fidelity. Of several molecular markers used for such assessment, RAPD is the simplest, cheapest and appears to be a useful tool for analysis of genetic fidelity of in vitro propagated plants (Williams et al., 1990; Rout and Das, 2002; Singh et al., 2002; Martins et al., 2004; Leena and Tuija, 2005). Compared with the widely used RAPD markers, ISSR has several advantages particularly in reproducibility and informativeness (Yang et al., 1996; Nagaoka and Ogihara, 1997).

2.3.1.1 Evaluation of genetic fidelity using RAPD markers:

RAPD based assessment of genetic stability of micropropagated plants has been reported in many plant species (Rout et al., 1998; Rout and Das, 2002; Martins et al., 2004; Venkatachalam et al., 2007). Molecular and physiological studies of tissue culture regenerated plants in different species have shown the presence of genetic variation between the mother and regenerated plants (Hashmi et al., 1997; Zucchi et al., 2002; Modgil et al., 2005; Peredo et al., 2006).

There are few published reports on genetic fidelity of bamboo species. Das and Pal (2005b) evaluated genetic fidelity of in vitro raised plants of B. balcooa and B. tulda by RAPD analysis and no variation was found in micropropagated plants of both the species. They advocated clonal propagation through axillary shoot proliferation as a reliable method for commercial exploitation. Agnihotri et al. (2009) used RAPD markers to assess genetic fidelity of 10 micropropagated plants of D. hamitonii. Out of the eighty different decamer RAPD primers tested, six primers produced clear and score able amplification products. All
the amplified products were monomorphic across the micropropagated plants and corresponding mother plant.

Martins et al. (2004) did not observe variation between the mother plants and micropropagated plantlets of *Prunus dulcis*, when fidelity was tested using 64 RAPD primers. The results strongly suggested that axillary branching strategy ensures maintenance of genomic integrity in almond propagation.

Ryynänen and Aronen (2005) reported genetic fidelity of the tissue culture raised plants of five genotypes of *Betula pendula* (E1987, E5201, E5382, E5387 and E5398) by comparing their RAPD profiles with the markers amplified with the original donor trees. A polymorphic amplification pattern was observed in 18 of the tested 20 primers separating all the tested clones from each other. No reproducible intraclonal variation could be observed in the RAPD profiles.

Valladares et al. (2006) conducted RAPD analysis on embryogenic lines derived from 3 genotypes of *Quercus robur* using 40 arbitrary 10-mer primers using Kits A and S. No evidence of genetic variation was observed either within or between the embryogenic lines established from three of these trees, or between these lines and trees of origin, or between somatic embryos derived plantlets and the trees of origin were found.

In *Curcuma longa*, RAPD analysis carried for in vitro multiplication cultures at six month interval using 20 arbitrary decamer primers up to 2 years and showed monomrophic bands indicating no variation in the micropropagated plants, when compared with control plants (Panda et al., 2007).

While analyzing the long term micropropagated shoots of *Olea europaeae* L. with 20 RAPD decamer primers, it was observed that increasing the number of subcultures, polymorphism increases (Peyvandi et al., 2009).

### 2.3.1.2 Evaluation of genetic fidelity using ISSR markers:

ISSR markers have been successfully used to detect somaclonal variation in several plants including potato (Albani and Wilkinson, 1998), horseradish (Rostiana et al., 1999), cauliflower (Leroy et al., 2001), Camellia sinensis (Devarumath et al., 2002), Prunus dulcis (Martins et al., 2004) and Robinia ambigua (Wanli et al., 2006).
Leroy et al. (2001) assessed genetic stability of cauliflower (*Brassica oleracea* var. botrytis L.) calli using ISSR primers and detected the genetic instability at very early stage. Whereas, Martins et al. (2004) observed monomorphic banding in 22 plantlets of almond (*Prunus dulcis*) using 10 ISSR primers indicating no variation in micropropagated plantlets.

Genetic fidelity of a sample of 41 morphologically normal plants of *Robinia ambigua var idahoensis* were studied using 32 selected ISSR primers. 24 bands out of the 226 reproducible bands produced were found to be polymorphic representing 10.62 % polymorphism and pointed occurrence of a low level of genomic variation in the micropropagated plants (Guo et al., 2006).

Joshi and Dhawan (2007) used ISSR markers to verify the genetic fidelity of micropropagated plants of *Swertia chirayita* regenerated by axillary shoot proliferation and multiplied up to forty-two passages. Sixteen ISSR primers generated a total of 102 amplicons, among the tissue cultured plants. A homogenous amplification profile was observed for all the micropropagated and the donor plant conformed genetic stability in plants and reliability of protocol for large scale production of plants.

### 2.3.1.3 Evaluation of genetic fidelity using RAPD and ISSR markers:

Palombi and Damiano (2002) suggested the use of more than one DNA amplification techniques as advantageous in evaluating somaclonal variation. In Kiwi fruit, analysis of micropropagated plants, through RAPD markers did not able to detect changes that occurred in the repetitive region of the genome, where as ISSR markers could detect genetic variation.

Martins et al. (2004) adopted the use of two PCR based techniques; RAPD and ISSR for somaclonal evaluation of almond plantlets. And they opinioned that the use of 48 primers of two types of markers, which amplify different regions of the genome, allows better analysis of genetic stability/ variation of the plantlets.

Three elite banana cultivars (Robusta, Giant Governor and Martaman) were raised from shoot tip meristems. 21 RAPD and 12 ISSR markers were used to assess the genetic relationships and fidelity among the cultivars and the micropropagated
plants. The percentage of polymorphic loci by RAPD and ISSR were found to be 1.75, 5.08 in Robusta and 0.83, 5.0 in Giant Governor. More polymorphism was detected by the ISSR than RAPD in Robusta and Giant Governor with most of the primers showing similar fingerprinting profiles, whereas, Martaman revealed complete genetic stability in the studies done by Ray et al. (2006). While analyzing the micropropagated and mother plants of dessert banana cultivar (Nanjanagudu Rasabale) with 50 RAPD and 12 ISSR primers, no genetic variation was observed by Venkatachalam et al. (2007).

Kabade (2009) reported use of genotype BB 13 in B. bambos and DS101 in case of D. strictus for genetic fidelity studies of the micropropagated plants using RAPD and ISSR markers and observed that both the species have not shown any morphological and genetic variation. Amplified products exhibited monomorphic banding pattern across the progenies raised through axillary shoot proliferation and conformed true-to-type mother plants in both the species.