Review of Literature
This chapter illustrates a brief literature status and efforts directed towards the bamboo improvement and propagation, through axillary shoot proliferation, somatic embryogenesis, genetic fidelity of *in vitro* regenerants and growth performance of progeny at nursery and field trials.

Bamboo is considered to be economically and industrially important plant species due to its multipurpose uses. Some of the industrially important species are; *Bambusa balcooa, B. bambos, B. pallida, Dendrocalamus brandisii* and *D. giganteus* are mainly used in construction as scaffoldings, handicrafts and substitute of timber. *B. tulda* and *D. asper* are known for their delicious dishes. *B. vulgaris* and *B. wamin* are of in high demand ornamental bamboo. The tuft of bamboo holds the upper layer of soil and hence can be used in conserving soil and holding moisture and certain bamboo species have medicinal value too (Bennet and Gaur, 1990). *D. brandisii* is being a fast growing species used in afforestation, social forestry and agro forestry (Anonymous, 2005).

Bamboos are a group of tall, arborescent, perennial grasses that grow very fast and are evergreen. Bamboos flower at long intervals and in many instances (when gregarious flowering) the plants die after flowering. The length of flowering cycle varies with each bamboo species. It ranges from three years (*Schizostachyum elegantiaium*) to 60 years (*Bambusa polymorpha*), but for most species, it lies between 30-60 years (Varmah and Bahadur, 1980; Watanabe and Hamada, 1981).

Propagation of bamboo is through seed and vegetative methods. Natural regeneration and artificial propagation are mainly by seeds. Propagation through seed is the cheapest and easiest method for production of plants (Banik, 1994).

In bamboo, due to uncertainty of sporadic flowering and long flowering cycle, availability of seeds as when required is uncertain. In addition, viability of seeds is for a short period (3-6 months). Genetic integrity of seed base progenies is also uncertain, due to its segregation traits. Therefore, to exploit full worth of superior genotypes, the best option is to multiply through vegetative propagation.
The plants produced by vegetative propagation, i.e. asexual reproduction are genetically similar copies or ramates of the mother plant. Vegetative propagation of bamboo is mainly by rhizome cuttings, offset cuttings, culm cuttings, branch cuttings and air layering (Adarsh Kumar, 1991). Rhizome cutting and culm cuttings are the most widely used methods of vegetative propagation. Rhizome cuttings seem to be more appropriate for the bamboos that do not form clumps (monopodial). Whereas, culm cuttings are used for the clump forming (sympodial) species (Anonymous, 2003). Noteworthy that, drawback of rhizome cutting is the risk of impairing health and vigor of the mother plant. Other problems are high labour costs involved in the collection and difficulty in transportation of the bulky material (Sharma, 1982). Propagation through culm cuttings (single node, double node, triple node and whole culm cuttings) require large quantity of material and availability of right stage of material is for a short period, which is useful for small scale production. Propagation by branch cuttings is better option, but success rate is generally low and varied with species. Similar to culm cuttings, in branch cuttings also, availability of right stage of material is for a short period. In addition, this method is successful for small scale production of clonal planting material. Classical vegetative propagation methods are inefficient for the production of large quantity of clonal planting material (i.e. > 50,000 plants/year).

Alternatively, micropropagation methods have potential of mass scale production of clonal planting material from a small quantity of source material and can be propagated round the year without depending on season in a small area. About 0.1-0.5 million plants can be produced from single explant in a year (Gielis, 1995).

In the beginning of 1980, significant progress has been made for regeneration of plants in almost all important cereals and grass species, including several species of bamboos (Bright and Jones, 1985; Vasil and Vasil, 1986; Morrish et al., 1987).

Micropropagation involves four stages: Stage I- selection and collection of suitable explant and inoculation thereof in nutrient medium, Stage II- multiplication and growth of explant cultures, Stage III- root formation and Stage IV- transfer of plantlets to green house condition and then acclimatization to field conditions (Gielis et al., 2002).
Because of vast potential, *in vitro* cloning of superior genotypes for rapid and mass production of genetically uniform planting material gained popularity. There are three methods of *in vitro* plant regeneration viz; (i) through axillary shoot proliferation (ii) organogenesis (a) through callus phase (b) direct organogenesis and (iii) somatic embryogenesis; (a) direct somatic embryogenesis without callus phase (b) in-direct somatic embryogenesis through callus phase.

### 2.1 *In vitro* regeneration through axillary shoot proliferation

Micropropagation in bamboo has been dealt with a great extent by earlier reports. Aim of the tissue culture regeneration protocols in bamboo species is to obtain large scale production of clonal plants for operational planting, providing material for breeding programs and conservation of germplasm. First attempt of *in vitro* propagation of bamboo was carried out in 1968 by successful germination of mature embryos on artificial nutrient medium (Alexander and Rao, 1968). Subsequently, several protocols were established for different species of bamboo, especially through axillary shoot proliferation and somatic embryogenesis by using seed/seedling or explants of mature clump as a source material for *in vitro* regeneration.

Axillary shoot proliferation- a universal technique, can be achieved when dormant axillary buds are released from apical dominance either from shoot tip or in nodal shoot segments used as an explant and cultured on medium supplemented with usually a cytokinin, and often with an auxin. The cytokinin stimulates pre-existing shoot buds in the explant to develop into multiple shoots. The individual shoots are excised and subcultured on fresh medium to initiate a new crop of shoots for further multiplication. This method of propagation is preferred for mass scale production of bamboo and is highly efficient ensuring clonal fidelity and true-to-type propagation (Gielis et al., 2002).

#### 2.1a Effect of source of explants (seedling/field grown plant)

Considerable efforts have been made in the past three decades in propagation of bamboo through tissue culture by using seed or seedling based explants. Various authors have been used seed or seedlings as explants to propagate different bamboo species viz;
Dendrocalamus strictus (Nadgir et al., 1984; Shirgurkar et al., 1996; Ravikumar et al., 1998); Bambusa arundinacea and D. brandisii (Nadgauda et al., 1990); B. tulda (Saxena, 1990); D. hamiltonii (Chambers et al., 1991); D. asper (Arya et al., 1999); Thamnocalamus spathiflorus (Bag et al., 2000); B. bambos var. ‘gigantea’ (Kapoor and Rao, 2006); B. oldhamii (Thiruvengadam et al., 2011).

Nadgir et al. (1984) reported micropropagation between seedlings versus explants from mature clumps in D. strictus. Results revealed that, maximum (8-10) shoots obtained from seedling derived explants in MS liquid medium supplemented with BAP (0.2 mg/l) and coconut milk (5%) within 3-4 weeks. Whereas, nodal segments from mature clumps produced 2-3 shoots in MS agar gelled medium containing BAP (0.5 mg/l), Kn (0.2 mg/l) and CM (10%). Furthermore, highest (80%) rooting was observed from seedling derived explants, when treated with IBA (0.1 ppm for 48 hours in dark), followed by transferred to hormone free MS/2 basal liquid medium in light. But, lower rate of (20%) rooting was observed in shoots derived from mature clumps, when treated with IBA (1.0 mg/l for 96 hour in dark), followed by transferring to hormone free MS/2 basal agar gelled medium with 0.25% activated charcoal in light within 4 to 5 weeks.

The use of starting material (seed/adult plants) and the choice of the propagation method are crucial. Two major advantages of using seedlings are (i) it is a new generation and has long cycle before flowering (ii) regeneration is easier, but the disadvantages are very considerable i.e., insufficient or no knowledge of genetic background and restricted availability of seeds and rapid loss of viability.

Major problem associated in propagation of bamboo under in vitro conditions from field grown adult bamboo are: endogenous contamination, hyperhydricity, instability of multiplication rates and lower response rooting in vitro coupled with minimum survival in hardening and acclimatization (Gielis et al., 2002). On the other side, axillary shoot proliferation has proved to be the most applicable and reliable method for production of true-to-type plants of superior genotypes (George and Debergh, 2008).
Clonal propagation through axillary shoot proliferation from field grown mature nodal shoot explants have been reported with varied success in many bamboo species viz; 54 species from 15 genera of bamboo (Prutpongse and Gavinlertvatana, 1992), *Dendrocalamus strictus* (Nadgir et al., 1984; Chaturvedi et al., 1993; Kabade, 2009), *D. longispathus* (Saxena and Bhojwani, 1993), *Bambusa bambos* (Arya and Sharma, 1998; Kabade, 2009), *B. edulis* (Lin and Chang, 1998), *D. giganteus* (Ramanayake and Yakandawala, 1997; Ramanayake et al., 2001), *D. hamiltonii* (Sood et al., 2002; Agnihotri et al., 2009; Agnihotri and Nandi, 2009), *B. nana* (Shirin and Arya, 2003), *B. vulgaris* (Shirin et al., 2003), *B. balcooa* (Das and Pal, 2003a; Mudoi and Borthakur, 2009; Negi and Saxena, 2010), *B. wamin* (Arshad et al., 2005), *Pseudoxytenanthera stocksii* (Sanjaya et al., 2005; Somashekar et al., 2008), *B. vulgaris* ‘Striata’ (Ramanayake et al., 2006), *Guadua angustifolia* (Jiménez et al., 2006), *B. glaucescens* (Shirin and Rana, 2007), *B. oldhamii* (Lin et al., 2007), *B. nutans* (Yasodha et al., 2008; Mehta et al., 2011; Negi and Saxena, 2011) and *Arundinaria callosa* (Devi and Sharma, 2009).

There are four major steps involved in axillary shoot proliferation viz; (i) shoot initiation (ii) shoot multiplication (iii) *in vitro* root rooting and (iv) hardening and acclimatization.

### 2.1.1 Shoot initiation

To achieve rapid shoot initiation, various critical factors which play important role viz; explant diameter, plant growth regulators, liquid Vs agar gelled media, nutrient media, additives, genotype and season or period of explant collection are reviewed.

#### 2.1.1.1 Effect of diameter of the explant

Diameter of the explant play significant role on percentage shoot induction and shoot growth in different bamboo species.

Bag et al. (2000) reported that single node stem segments of 1.5-2.5 mm in diameter and 2.0-2.5 cm in length from 2-year old plants resulted in highest shoot initiation in *Thamnocalamus spathiflorus*. Whereas, single nodal shoot segments of 2-3
mm in diameter with 2.5-3.5 cm in length from branches of elite and mature clump resulted in highest shoot initiation in *Pseudoxytenanthera stocksii* (Sanjaya et al., 2005; Somashekar et al., 2008). Jimenez et al. (2006) also revealed that node shoot segments of 2-3 mm in diameter with 2.5-3.5 cm in length was found suitable for shoot induction in *Guadua angustifolia*. Among different (1-2, 2-3, 3-4 and 4-5 mm) diameter class of explant used, nodal shoot segments of 2-3 mm diameter favoured maximum shoot initiation and shoot growth in *B. bambos* and *D. strictus* within 3 weeks (Kabade, 2009).

### 2.1.1.2 Effect of plant growth regulators (PGRs)

Many authors investigated different plant growth regulator either alone or in combinations at various concentrations for successful shoot initiation in different bamboo species. Plant growth regulators particularly auxins (IAA, IBA and NAA), cytokinins (BA/BAP, Kn and 2-iP and Zeatin) and cytokinin like substances (TDZ) have specific roles in cell elongation, cell division and differentiation (Bonga and Von aderkas, 1992). Endogenous levels of PGR vary with the species and therefore, exogenous requirement also vary with plant species, sometimes between the genotypes and also in different types of explants.

Cytokinins, play multiple roles in the regulation of plant growth and development. However, the mode of their action at the molecular level is uncertain. In some circumstances, cytokininss may activate RNA synthesis, stimulate protein synthesis and activities of some enzymes. Cytokinin treatment also results in an increase of the polyribosome content in cells (van Staden et al., 2008).

Thidiazuron (TDZ) - a substituted phenylurea (N-phenyl-N’-1,2,3-thiadiazol-5-ylurea), which has cytokinin activity (Mok et al., 1982; Huetteman and Preece, 1993; Lu, 1993). Sometimes dual function of auxin and cytokinin are also observed with TDZ. Compared to other cytokinin, at lower concentrations of TDZ can stimulate axillary shoot proliferation in many woody plants. Whereas, higher concentrations of TDZ may result in the formation of both axillary and adventitious shoots (Nieuwkerk et al., 1986; Chalupa, 1988).
The growth and proliferation of axillary shoots in cultures is usually promoted by incorporation of growth regulators (usually cytokinins) into the culture medium. Most often such a treatment effectively removes the apical dominance so that axillary shoots are produced (George and Debergh, 2008).

Nadgir et al. (1984) revealed that, MS agar gelled medium consisted with BAP (0.5 mg/l), Kn (0.2 mg/l) and CM (10%) favoured multiple (2-3) shoot induction per nodal segments from mature clumps in *D. strictus*. In contrary, MS medium with IBA (0.5 mg/l) + adenine sulphate (15.0 mg/l) proved the best for multiple shoot initiation (2-4 shoots/explant) from nodal shoot segments from 10 years old culm of *D. strictus* (Chaturvedi et al., 1993). Whereas, Kabade (2009) reported that, MS liquid medium with additives + NAA (0.1mg/l) + TDZ (0.25 mg/l) proved the best for maximum (100%) percentage shoot initiation with higher (5.97) number of shoots per explant and shoot length (4.19 cm) in 3 weeks period from the explants of the field grown mature clump in *D. strictus*.

Prutpongse and Gavinlertvatana (1992) reported, MS medium with BAP (2.2–44.0 M) alone favoured for multiple shoot induction in 54 species, out of 67 species of bamboo tested within 30 days. Most of the species produced multiple shoots from the axillary buds on medium consisted BA (22 M). Whereas, other cytokinins (2ip and Kn) and CW were inferior to BA.

Saxena and Bhojwani (1993) revealed that, incorporation of BAP in the basal medium improved the incidence of bud-break and produced multiple shoot formation. Maximum (75%) bud-break as well as 6.33 shoots/explant was obtained in MS agar gelled medium supplemented with BAP (12.0 µM) and Kn (3.0 µM) within 2 weeks from the explants of the 4-year old plants of *D. longispathus*. Consequently, in all treatments consisted of either IAA or IBA was inhibitory in frequency of bud-break.

Arya and Sharma (1998) observed 70% bud break from the nodal shoot segments collected from 3 year old plants of *B. bambos* in MS liquid medium supplemented with BAP ranging from 2.0–10.0 mg/l. Concentrations of BAP markedly influenced number of...
shoots/explant. MS liquid medium with BAP (1.0–5.0 mg/l) produced 5-20 shoots/explant in 2-3 weeks period. Furthermore, higher concentration of BAP (>5.0 mg/l) produced dwarf leafy shoots without further development and growth. Incorporation of NAA in the medium did not help in further improvement of shoot number or length and auxin alone did not favour shoot development. Kabade (2009) reported that, TDZ was the best cytokinin for multiple shoot induction as compared to other two purine derivative cytokinins (BAP and Kn). MS liquid medium containing additives + NAA (0.1 mg/l) + TDZ (0.25 mg/l) proved the best in terms of percentage response (100%) and high (6.53 shoots/explants) number of shoots with maximum shoot length (4.39 cm) in 3 weeks period in B. bambos.

Lin and Chang (1998) reported, MS gelrite medium with TDZ (0.1 mg/l) alone favoured for shoot induction from nodal explant of field grown culms. Whereas, medium supplemented with auxin (NAA) alone failed to induce shoots and explant showed senescence and eventually deteriorated. But medium consisted of BA alone or with NAA were also induced shoots in B. edulis.

In D. hamiltonii, highest bud break and shoot proliferation was found in MS liquid medium supplemented with BA (2.5 mg/l) alone (Sood et al., 2002; Agnihotri and Nandi, 2009). Arshad et al. (2005) used various concentrations of BAP (1.0 to 10.0 mg/l) and found medium consisted with BAP (5.0 mg/l) 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. They also observed that bud break was not induced, when medium consisted either NAA or 2, 4-D. Higher concentration of BAP, resulted in less number shoots and were dwarf. Similarly, Arya et al. (2008a) reported that maximum bud break was noticed in MS liquid medium supplemented with high concentration of BAP (5.0 mg/l) in Drepanostachyum falacatum and comparatively low level of BAP (3.0 mg/l) was sufficient enough to induce shoots in Bambusa balcooa. No significant increase in shoot proliferation and shoot number from axillary buds when different auxins (NAA, IAA) were used in MS medium along with BAP. Similarly, MS medium with BAP (3.0 mg/l) alone favoured shoot initiation in G. angustifolia from nodal shoot segment from the field grown as well as greenhouse plants (Jimenez et al., 2006). Devi and Sharma (2009) revealed that, MS
liquid medium consisted only BAP (13.3 µM) favoured high percentage (62%) of bud break with 3 shoots/explant within 15 days in *Arundinaria callosa*.

In *P. stocksii*, Sanjaya *et al.* (2005) used various concentrations of NAA (0.06-5.37 M) and BAP (0.0-11.09 M) in MS liquid medium. Results indicated that, combined use of NAA (2.68 M) and BA (4.44 M) favoured induction of more number of shoots with good shoot length. Similarly, Somashekar *et al.* (2008) also found that MS liquid medium with additives + NAA (0.25 mg/l) + BAP (1.0 mg/l) proved the best for multiple shoot induction (4-6 shoots/explant with shoot length of 4.17 cm) within 3 weeks. Higher concentration of BAP (2.5 mg/l) inhibited shoot growth. Kinetin in the medium at all concentrations induced less number of shoots as compared to BAP in *P. stocksii*. In *B. nutans*, MS gelrite medium with combined use of BA (4.4 M) and Kn (2.32 M) favoured 100% bud-break from nodal shoot segments collected from field-grown clumps (Negi and Saxena, 2011).

### 2.1.1.3 Effect of liquid Vs agar gelled media

Physical status (liquid or agarified) of media also influences on frequency of shoot initiation and shoot growth as documented in many reviews. Some others evaluated the effects of liquid and solidified (agar) media on shoot initiation in different species of bamboo. In accordance to Bonga and Von aderkas (1992), liquid cultures generally grow faster, require less handling and are easier to automate. Whereas, solidified medium was not found suitable due to its high cost and the lack of chemical purity associated with gelling agents.

Nadgir *et al.* (1984) tested, agar gelled and liquid MS media for multiple shoot induction from the explants of seedling as well as in mature plants of *D. strictus*. Maximum (8-10) number of shoots was obtained within 6-7 weeks in liquid shake flasks at 120 rpm under continuous illumination (1000 lux). Whereas, less (2-3) number of shoots was observed in nodal segments from mature plants. However, Chaturvedi *et al.* (1993) reported that MS liquid medium with IBA (0.5 mg/l) + adenine sulphate (15.0 mg/l) proved the best for multiple shoot initiation (2-4 shoots/explant) from nodal shoot
segments from 10 years old culm of *D. strictus*. Similarly, Kabade (2009) revealed that the liquid MS medium was significantly better as compared to agar gelled medium and resulted in maximum (98.47%) shoot initiation and higher (5.98) number of shoots. Whereas, minimum (79.71%) response with less (2.62) number of shoots/explant were observed in agar medium consisted of additives + NAA (0.1 mg/l) and TDZ (0.25 mg/l) within 2 weeks in *D. strictus*.

Saxena and Bhojwani (1993) observed that, MS agar gelled medium supplemented with BAP (12.0 µM) and Kn (3.0 µM) favoured maximum (75%) bud-break as well as 6.33 shoots/explant obtained within 2 weeks in *D. longispathus*. Whereas, Negi and Saxena (2011) reported that maximum (100%) bud break was in 0.2% gelrite MS medium supplemented with BA (4.4 M) and Kn (2.32 M) from nodal shoot segments collected from field-grown clumps of *B. nutans*.

Arya and Sharma (1998) also observed 70% bud break from the nodal shoot segments of *B. bambos* in MS liquid medium, but found poor growth of shoot on the agar gelled medium. In contrary, liquid MS medium favoured maximum (100%) bud-break with highest (4.0 cm) shoot length in *B. bambos*. Whereas, in agar gelled medium shoot initiation frequency was significantly low (76.57%) within 2 weeks (Kabade, 2009).

In *Bambusa wamin* also, highest (83.33%) bud break and more (3.89 ± 0.38) number of shoots were reported in MS liquid medium as compared to agar medium (Arshad et al., 2005). In *P. stocksii* also, MS liquid medium proved the best for high rate of multiple shoot induction (4-6 shoots/explants) with better shoot growth as compared to agar gelled medium within 3 weeks period (Sanjaya et al., 2005). Dwarf and less (2-3 shoots/explant) number of shoots with leaching and browning problems was observed in MS agar gelled medium in *P. stocksii* (Somashhekar et al., 2008). Similarly in *A. callosa*, MS liquid medium supplemented with BAP (13.3 µM) favoured 62% bud-break with 3 shoots/explants within 2 weeks (Devi and Sharma, 2009).
2.1.1.4 Effect of nutrient media

To fulfill nutritional requirement of growing tissues under *in vitro* conditions may vary with the plant species and at different stages of development and morphogenesis (Chang and Ho, 1997).

The regeneration of plantlets through tissue culture is becoming very important for mass propagation of plants. Since single medium cannot be suggested for all types of plant species, tissues and organs. It is necessary to explore suitable nutrient medium for successful establishment of *in vitro* protocol development for each species.

It was revealed from the different literature that nutrient media had significant effect on frequency of shoot initiation, number of shoots/explant and shoot length in different bamboo species.

In *B. bambos*, MS liquid medium was better than Woody Plant Medium (WPM) for successful axillary shoot induction (Arya and Sharma, 1998). Similarly in another report, out of 4 nutrient media (MS, B5, SH and WP) used, MS liquid medium with additives + NAA (0.1 mg/l) + TDZ (0.25 mg/l) proved to be the best and exhibited highest (100%) frequency of shoot initiation with maximum number of shoots/explant (6.45) and shoot length (4.24 cm) in 3 weeks period. WP medium proved least effective in terms of percentage response (56.27%) and number of shoots/explant (3.06) and shoot length (2.55 cm) from nodal shoot segments of *B. bambos* (Kabade, 2009).

Arya *et al.* (2008a) reported high frequency bud break was observed in MS medium as compared to B5 and WP medium in *Drepanostachyum falcatum* and *Bambusa balcooa*. In *P. stocksii*, among the different nutrient media (MS, B5, WP and HE) used for multiple shoot induction, MS medium fortified with additives, NAA (0.25 mg/l) and BAP (1.0 mg/l) induced multiple shoots (4-5 shoots/explant) within 3 weeks, followed by B5 and WP media. Poor multiple shoot induction and growth was observed in HE medium (Somashekar *et al.*, 2008).
MS medium has been used extensively for successful shoot initiation and establishment of cultures in other bamboo species such as; *B. edulis* (Lin and Chang, 1998), *B. wamin* (Arshad *et al.*, 2005), *P. stocksii* (Sanjaya *et al.*, 2005), *G. angustifolia* (Jimenez *et al.*, 2006), *D. strictus* (Kabade, 2009), *A. callosa* (Devi and Sharma, 2009) and *B. nutans* (Mehta *et al.*, 2011; Negi and Saxena, 2011).

### 2.1.1.5 Effect of additives

It is evident from the literature that, explants from mature plants pose problem of leaching and browning lead to poor response on shoot induction and growth. Browning occurs especially in woody plants and is generally considered to result from the oxidation of phenolic compounds released from the cut ends of the explants (Bhat and Chandel, 1991). The oxidized products, e.g. quinines, inhibit enzyme activity, tends to the death of the explants (Hu and Wang, 1983). In order to circumvent this problem different additive were used to over come browning problem and to improve frequency of shoot initiation, shoot number and growth. Various additives were used as a pre-treatment before inoculation or added to the medium.

Auxillary effect of ascorbic acid (50 mg/l) + citric acid (25 mg/l) and adenine sulphate (25 mg/l) on shoot initiation in MS medium with IAA + BAP was observed in *Maytenus emarginata* (Rathore *et al.*, 1992). Similarly, addition of CM (15 %) in the induction medium with BAP (2 mg/l), resulted in maximum (90 %) shoot induction in *Albizia falcataria* (Sinha and Mallick, 1993). In *Picea mariana*, incorporation of ascorbic acid in the medium prevented browning during secondary shoot formation (Rumary and Thorpe, 1984). Anti oxidants viz; ascorbic acid and citric acid in the medium have been used to minimize phenolic exudates and to delay in browning of explants in *Ceratomia silqua* (Romano *et al.*, 2002). Similarly, Annapurna and Rathore (2010) reported the addition of ascorbic acid (568 µM) + citric acid (119 µM) to MS medium and resulted in reduction of browning up to 50% from the nodal shoot segments of mature plants of *Embelia ribes* during shoot initiation.

Chaturvedi *et al.* (1993) incorporated adenine sulphate (15.0 mg/l) in MS medium supplemented with IBA (0.5 mg/l) and favoured multiple shoot initiation (2-4
shoots/explant) from nodal shoot segment from the newly emerged branches from about 10 years old culm of _D. strictus_. Ravikumar _et al._ (1998) used coconut milk (200 ml/l) in MS liquid medium with BAP (0.5 mg/l) + Kn (0.5 mg/l) for multiple shoot induction from nodal shoot segment from seedling and mature culm in _D. strictus_.

Among the different (ascorbic acid, citric acid, cysteine, adenine sulphate, glutamine and coconut milk) additives tested in MS liquid medium supplemented with NAA (0.1 mg/l) + TDZ (0.25 mg/l), medium consisted of ascorbic acid, 50 mg/l + citric acid, 25 mg/l and cysteine, 25 mg/l favoured better multiple shoot induction in _B. bambos_ and _D. strictus_ (Kabade, 2009). Similarly, Somashekar _et al._ (2008) emphasized the use of additives (ascorbic acid, 50mg/l + citric acid, 25 mg/l and cysteine, 25 mg/l) for multiple shoot induction in _P. stocksii_. They also found that incorporation of additives in the medium had an auxiliary effect on shoot number and shoot growth. Sanjaya _et al._ (2005), incorporated different additives (ascorbic acid, 284 µM + citric acid, 118 µM, cysteine, 104 µM and glutamine, 342 µM) in MS liquid medium for culture initiation in _P. stocksii_.

### 2.1.1.6 Effect of genotypes/CPCs

The frequency of axillary shoot proliferation may vary with different genotypes. This may be due to genotypes vary in their natural degree of apical dominance. Selection of elite genotypes in existing populations and subsequent clonal propagation allows a considerable ‘genetic improvement’ particularly refers to better yield and quality (Gielis _et al._, 2002). It was well-documented that the response of bamboo tissues _in vitro_ is highly genotype specific. This type of response is primarily attributed to differences in endogenous PGRs levels and requirement of exogenous PGRs also varied. Genotypic differences in regeneration potential have been reported in woody plant species (Korban _et al._, 1992; Kunze, 1994).

Differential genotypic effect on shoot regeneration was reported in _Populus tremula_ and _P. tremuloides_ (Ahuja, 1983). In _P. deltoides_, out of 16 genotypes, six genotypes were completely non-responsive whereas, genotype number 10 and 175 exhibited maximum 100% and 95.9% response, respectively in WP medium.
supplemented with 0.5 mg/l, Zeatin (Coleman and Ernst, 1989). Similarly, rate of multiple shoot induction varied (0.9-2.4 shoots/explant) among five genotypes of *Robinia pseudoacacia* tested in MS medium with (0.032-3.2 µM) BAP (Davis and Keathley, 1987). Similarly, genotypic variation on multiple shoot induction was observed in 12 clones of *Dalbergia sissoo* belongs to four states (Uttar Pradesh, Uttarakhand, Haryana and Rajasthan) of India (Kalia *et al.*, 2004).

Kabade (2009) revealed that genotype has significant effect on frequency of shoot induction, number of shoots/explant and shoot length. Among the different genotypes; BB1, BB4, BB5, BB12 and BB13 tested in *B. bambos*, percentage response varied from 74.12-100%. Genotype BB13 proved the best for maximum (100%) response with higher (6.52) number of multiple shoots and shoot length (4.51 cm) in MS liquid medium supplemented with NAA (0.1 mg/l) + TDZ (0.25 mg/l) in 3 weeks period. This was followed by BB4, BB5 and BB12. Minimum rate of response (74.12%) with lower number (3.16) of multiple shoots and shoot length (3.88 cm) in BB1. Similarly in *D. strictus* also, percentage of response varied from 77.58-99.34% among the 5 genotypes (DS4, DS36, DS96, DS101 and DS103), tested. Genotype (DS101) proved the best in terms of percentage response (99.34%) with maximum number of multiple shoots (5.76) and shoot length (3.81 cm) in MS liquid medium supplemented with additives + NAA (0.1 mg/l) + TDZ (0.25 mg/l) in 3 weeks. This was followed by genotype DS96, DS36 and DS4. Minimum rate of response (77.58%) with lower number (3.75) of shoots and shoot length (3.23 cm) was observed in genotype DS103.

### 2.1.1.7 Effect of period of explant collection

Saxena and Bhojwani (1993) found that *in vitro* bud break was higher in *Dendrocalamus longispatus* explant collected during monsoon period. Similarly, Chaturvedi *et al.* (1993) reported that nodal explants collected during July-August were suitable for shoot initiation in *D. strictus*. Whereas, in *D. giganteus* peak bud break was observed before the onset of monsoon rains (south-west and north-east monsoons) each year (Ramanayake and Yakandawala, 1997).
Bud break frequency from nodal shoot segments of *Bambusa balcooa* was more in June-October and the highest frequency was recorded in October (Das and Pal, 2005a). In *P. stocksii*, nodal shoot segments collected during April-September, produced a significantly more number of shoots with better shoot growth in MS liquid medium fortified with PGRs (Sanjaya *et al*., 2005). However, Arya *et al.* (2008a) reported that nodal shoot segments collected during February-March and September-October showed maximum bud break response in *Drepanostachyum falcatum* and in *Bambusa balcooa*, respectively on MS medium supplemented with cytokinins.

In *Arundinaria callosa*, explants collected during rainy season (May-July) posed problem of high bacterial contamination and resulted in low percentage of bud-break. However, contamination rate was reduced to 30% with high frequency shoot initiation (62%) after monsoon (September-November) (Devi and Sharma, 2009). Similarly, Mehta *et al.* (2011) reported that February, March and December months were found to be the best for establishment of aseptic cultures with minimum contamination rate (20-30%) in *B. nutans*.

2.1.2 Shoot multiplication

The rate of shoot multiplication is affected by various factors such as plant growth regulators (particularly cytokinin), liquid Vs agar gelled media, nutrient media, additives, sucrose concentrations, pH, subculturing period, inoculum size and genotypes on shoot multiplication and growth. *In vitro* shoot multiplication rate in bamboo varies with the species (Ramanayake and Yakandawala, 1997; Bag *et al*., 2000; Gielis *et al*., 2002).

2.1.2.1. Effect of cytokinins

In order to maintain the shoot multiplication rate and vigor, optimum concentrations of growth regulators vary with the species. Exogenous supply of cytokinins alone in the medium supported enough for optimal shoot multiplication (Thorpe *et al*., 1992).

In support to above statement, BAP alone proved the best for better shoot multiplication in different bamboo species. Prutpongse and Gavinvlertvatana (1992)
reported that, among various cytokinin (BAP, Kn and 2ip) tested, BAP alone in MS medium proved the best for shoot multiplication of 54 species of bamboo. Arya and Sharma (1998) reported that, BAP (3.0 mg/l) alone in MS liquid medium proved the best for shoot multiplication in B. bambos within 4 weeks period. Higher concentrations of BAP (10.0 mg/l) produced dwarf and leafy shoots. Kinetin either alone or in combination did not improve the shoot multiplication rate and incorporation of IBA or NAA in the medium also not favoured improvement of shoot multiplication. Similarly, modified MS medium supplemented with BAP (5.0 mg/l) alone favoured maximum (15.8 ± 1.7) shoots with 4.0 ± 0.8 cm shoot length within 45 days from 3-5 year old plants of D. brandisii (Mukunthakumar et al., 1999). Nadgauda et al. (1990) also revealed that, MS medium supplemented with BAP (0.5 mg/l) alone gave the best response, (15-20 shoots per culture vessel) from seedlings of D. brandisii. Lin and Chang (1998) revealed that among wide range (0.01 – 6.0 mg/l) of TDZ used, TDZ (0.1 mg/l) alone promoted best shoot multiplication but shoots were dwarf and vitrified in the medium consisted of higher concentration TDZ (6.0 mg/l) in B. edulis. Prolific shoot multiplication was observed in static liquid medium supplemented with BA (2.5 mg/l) alone in D. hamiltonii (Sood et al., 2002). Differential rate of shoot multiplication viz; 9-11 fold in Drepanostachyum falcatum and 2-3 fold in Bambusa balcooa were observed on MS medium supplemented with BAP, 3.0 mg/l (Arya et al., 2008a).

Contrary to above, many authors revealed that shoot multiplication rate also favoured when medium supplemented with auxin and cytokinins in different bamboo species. Chaturvedi et al. (1993) observed synergetic effect of NAA + BAP on shoot multiplication from field grown (10 year old) mature clump of D. strictus. Arshad et al. (2005) reported that combined use of BAP (1.0 mg/l) and Kn (0.8 mg/l), exhibited highest multiplication rate (4 fold) with maximum shoot length (4.06 cm). Whereas, lower rate (3.76 fold) of shoot multiplication was observed in BAP (2.0 mg/l) alone within 4 weeks period in B. wamin. In P. stocksii, combined use of NAA (2.68 M) and BA (2.21 M) in MS liquid medium was found suitable for high rate of shoot multiplication with good shoot length. Whereas, shoots were dwarf at higher concentration of BAP. Incorporation of NAA with BAP showed auxillary effect on shoot
multiplication. Kinetin was least effective as compared to BAP (Sanjaya et al., 2005; Somashekar et al., 2008). Similarly, Shirin and Rana (2007) found that, treatment consisted of BAP (5.0 µM) and Kn (15.0 µM) in the medium favoured highest (4 fold) rate of shoot multiplication than BAP alone (3.58 fold) in *Bambusa glaucescens*.

However, in *Bambusa vulgaris* ‘Striata’, different combinations of BA (2.0-6.0 mg/l) and TDZ (0.05-0.5 mg/l) were tested individually. Highest shoot number was observed in the medium consisted of BA (4.0 mg/l). Whereas, number of shoots did not differ significantly in the medium consisted BA (6.0 mg/l) or TDZ (0.1 mg/l). But shoot length was less in medium supplemented with TDZ as compared to BA. Repeated subculture of shoots in the medium with TDZ resulted in stunted shoots (Ramanayake et al., 2006). In *D. strictus*, MS liquid medium supplemented with additives + NAA (0.1 mg/l) + TDZ (0.25 mg/l) proved the best and produced 21.49 shoots/clump within 2 weeks (Kabade, 2009). Devi and Sharma (2009) reported that, significant increase in shoot multiplication rate and shoot growth was observed in the medium consisted of BAP (13.3 µM) + IBA (1.0 µM) in *A. callosa*. Negi and Saxena (2011) also reported that the combined use of IBA (0.98µM) + BA (13.2µM) + Kn (2.32µM) in MS liquid medium and resulted in 3 fold increase in the rate of shoot multiplication in *B. nutans*.

### 2.1.2.2 Effect of liquid Vs agar gelled media

Most of the literature suggests that physical nature (liquid and agar gelled) of the media often varied for different plant species. The physical status of the medium can influence on the rate of shoot multiplication during micropropagation.

Prutpongse and Gavinlertvatana (1992) used 0.6% agar-agar for shoot multiplication of 54 bamboo species. Chaturvedi et al. (1993) used 0.75% agar-agar in the medium for shoot multiplication of *D. strictus*. Arya and Sharma (1998) observed similar rate of shoot multiplication in both liquid and agar gelled media supplemented with BAP (3.0 mg/l) and repeated sub-culturing in liquid medium resulted in vitrification of shoots in *B. bambos*. Arya et al. (1999) used 0.7% agar-agar in MS medium for shoot multiplication in *D. asper*. Similarly, modified MS agar gelled medium supplemented with BAP (5.0 mg/l), favoured maximum (15.8 ± 1.7) shoots with 4.0 ± 0.8 cm shoot
length within 45 days from 3-5 year old plants of *D. brandisii* (Mukunthakumar *et al*., 1999). Lin and Chang (1998) used 0.2% gelrite medium for shoot multiplication in *B. edulis*. Whereas, Bag *et al.* (2000) used phytagel (0.2%) in the medium for shoot multiplication in *Thamnocalamus spatiflorus*. Arshad *et al.* (2005) also reported that rapid shoot multiplication in *B. wamin* was observed by alternate subcultures in liquid and semi solid MS medium supplemented with BAP (2.0 mg/l) + Kn (0.8 mg/l) at a 4-week subculture cycle.

There are disadvantages with the use of semi-solid media. Some agar contains inhibitory substances, which may prevent morphogenesis in certain cultures (Powell and Uhrig, 1987), rates of growth can be slow and toxic exudates from explants do not diffuse away quickly.

Alternatively, use of liquid medium is more economical as compared to agar gelled medium and also helps in faster uptake of nutrients from the medium. In *B. tulda*, 4-5 folds shoot multiplication rate was observed from *in vitro* seedling shoots in MS liquid medium in 3 weeks period and was proved better than agar gelled medium (Saxena, 1990). In *D. hamiltonii*, bud break and shoot multiplication was better in MS liquid medium as compared to agar gelled medium (Sood *et al.*, 2002). Similarly, Somashekar *et al.* (2008) reported that liquid MS medium was better than semisolid medium in terms of number of shoots and shoot length in *P. stocksii*. Kabade (2009) reported that the liquid medium proved best as compared to agar gel medium in terms of number of shoots and shoot length in *B. bambos* and *D. strictus*. Negi and Saxena (2011) revealed that incorporation of different gelling agents (agar, agar-gel and gelrite) lowered the shoot multiplication rate and shoot length as compared liquid medium in *B. nutans*.

Liquid medium proved better than agar gelled medium for shoot multiplication. This may be due to easy availability and faster uptake of nutrients and growth regulators from liquid medium as compared to agar medium. In agar gelled medium, subcultured shoot clump exhibited phenolic exudates, which inhibit shoot multiplication and shoot growth.
2.1.2.3 Effect of nutrient media

Nutritional requirement of in vitro cultures varies with the species and selection of most suitable nutrient medium for subsequent shoot multiplication is important for economic viability of micropropagation protocol.

Among different (MS and B5) nutrient media tested supplemented with BAP, MS liquid medium was better than B5 for better shoot multiplication in B. tulda (Saxena, 1990). Shirin et al. (2003) reported that out of various media (MS, B5 and WP) with BA (0.0-35µM) tested, MS medium significantly proved better than other two media for shoot multiplication. Maximum (4.39 fold) multiplication rate was obtained in MS medium with and BA (15.0 µM). Whereas, shoot multiplication rate was at par with each other on WP and B5 media in B. vulgaris. Similarly, in B. nana, among different nutrient media (MS, WP and B5) with BA (0.0-35 µM) tested, maximum (3.61 fold) multiplication rate was observed in MS medium with BA (5.0 µM), followed by B5 (2.27 fold) and WP (1.72 fold) media (Shirin and Arya, 2003).

Arshad et al. (2005) reported that out of various media (B5, DCR, MS and WP) tested, MS medium was found the best for shoot multiplication and growth in B. wamin. In P. stocksii, MS liquid medium proved the best (21 shoots/clump), followed by WP and B5. HE medium proved the least effective for shoot multiplication (Somashekar et al., 2008). Similarly in D. strictus and B. bambos, out of the various (MS, SH, B5, WP and HE) nutrient media tested, MS medium proved better for the production of maximum number of shoots/clump and shoot length as compared to B5, WP, SH and HE media in 2 weeks period (Kabade, 2009).

MS medium has been extensively used for in vitro shoot multiplication in different bamboo species; D. strictus (Nadgir et al., 1984; Chaturvedi et al., 1993), B. bambos (Arya and Sharma, 1998), B. balcoca (Das and Pal, 2005a; Mudoi and Borthakur, 2009; Negi and Saxena, 2010), G. angustifolia (Jimenez et al., 2006), B. nutans (Yashodha et al., 2008; Mehta et al., 2011; Negi and Saxena, 2011) and A. callosa (Devi and Sharma, 2009).
2.1.2.4 Effect of additives

Incorporation of additives in the shoot multiplication medium favoured auxillary effect on shoot growth and to minimize leaching from the cultures. The promotary effect of coconut milk in the shoot multiplication has been reported in *B. arundinceae* and *D. brandisii* (Nadgauda *et al.*, 1990) and *D. longispatus* (Saxena and Bhojwani, 1993). Use of additives in the medium to overcome leaching problem have been reported in various bamboo species.

Additives like; ascorbic acid, citric acid, cysteine, adenine sulphate, glutamine and coconut milk/coconut water are used to overcome problem of leaching, browning and to improve the rejuvenation and rate of shoot multiplication and growth. Incorporation of additives such as; ascorbic acid (283.93µM), citric acid (118.10 µM), cysteine (104.04 µM), glutamine (342.24 µM) in the shoot multiplication medium favoured overall shoot number and growth with reduced browning problem from the cultures of *P. stocksii* (Sanjaya *et al.*, 2005). Similarly, Somashekar *et al.* (2008) used additives such as; ascorbic acid (50 mg/l), citric acid (25 mg/l) and cysteine (25 mg/l) in the shoot multiplication medium in *P. stocksii*. In *B. bambos* and *D. strictus*, addition of additives (ascorbic acid, 50mg/l + citric acid, 25 mg/l + cysteine, 25 mg/l) in the MS medium proved the best for shoot multiplication and growth (Kabade, 2009).

2.1.2.5 Effect of sucrose concentrations

Carbohydrates play an important role in *in vitro* cultures as an energy and carbon source, as well as an osmotic agent. In addition, plant gene responses to changing carbohydrate status can vary markedly. Some genes are induced, some are repressed, and others minimally affected (Koch, 1996).

Plant tissue culture media provide not only inorganic nutrients, but usually a carbohydrate (sucrose is most common) to replace the carbon, which the plant normally fixes from the atmosphere by photosynthesis. The enhancement of morphogenesis caused by high nitrogen levels may not be apparent unless there is an adequate sucrose concentration in the medium (Margara and Rancillac, 1966; Gamborg *et al.*, 1974).
Normally tissues in culture vessel are photomixotrophic (i.e. the chloroplasts are able to fix part of the carbon that the cells require) and growth is still partly dependent on the incorporation of sucrose into the medium (Vasil and Hildebrandt, 1966). Optimum requirement of sucrose concentration may vary with the plant species (Pontis, 1978).

In accordance to above context, the type of carbohydrate (sucrose/fructose/glucose) source and its concentrations varied with different species of bamboo. Saxena (1990) reported that seedling derived explants of *B. tulda* multiplied the best with better shoot growth in medium consisted 2% sucrose as compared to 3 and 4%. Similarly, Arshad *et al.* (2005) used various concentrations (1-5%) of sucrose and found MS medium with 3% sucrose was the best for shoot multiplication and shoot growth in *B. wamin*. Somashekar *et al.* (2008) reported that among the various concentrations (1.0-6.0%) of sucrose tested, MS medium supplemented with 3% (w/v) sucrose proved the best for the maximum (18.75 shoots/clump) shoot production with better (4.05 cm) shoot length. Medium without sucrose did not support survival of shoots. Whereas, higher concentrations (4.5-6.0%) of sucrose did not improve shoot multiplication rate and growth in *P. stocksii*. In accordance to above reports, Kabade (2009) also revealed that among various concentrations of sucrose (1.5%, 3.0%, 4.5% and 6.0%) tested, medium consisted with 3% sucrose proved the best for shoot multiplication in *B. bambos* and *D. strictus*.

Most of the earlier reports deal with the use of single concentration of sucrose as exogenous energy source. Nadgauda *et al.* (1990) used 2% sucrose in MS medium supplemented with BAP (0.5 mg/l) and CM (5%, v/v) for shoot multiplication from seedling derived cultures of *D. brandisii* and *B. arundinaceae*. Bag *et al.* (2000) also used 2% sucrose in MS medium for shoot multiplication in *T. spathiflorus*. Whereas, sucrose at 3% w/v used extensively in the medium for shoot multiplication of different bamboo species; in *D. asper* (Arya *et al*., 1999), *D. brandisii* (Mukunthakumar *et al*., 1999), *P. stocksii* (Sanjaya *et al*., 2005), *G. angustifolia* (Jimenez *et al*., 2006), *B. balcooa* (Mudoi and Borthakur, 2009; Negi and Saxena, 2010), *A. callosa* (Devi and Sharma, 2009) and *B. nutans* (Yashodha *et al*., 2008; Mehta *et al*., 2011; Negi and Saxena, 2011).
2.1.2.6 Effect of pH

As pH is defined as the negative logarithm of hydrogen ion concentration, acid solutions have low pH values (< 7) and alkaline solutions have high values (> 7). Pure water, without any dissolved gases such as CO₂, has a neutral pH of 7. The pH of a culture medium must be such that it does not disrupt the plant tissue. This means that the effective range of pH for media is restricted. In culture media, detrimental effects of an adverse pH are generally related to ion availability and nutrient uptake rather than cell damage. The pH of the medium has an effect on the availability of many minerals (Scholten and Pierik, 1998). The uptake of ammonium and nitrate ions is markedly affected by pH (George and Sherrington, 1984). The media pH changes rapidly especially, if the cultures are growing fast. The rapid change in pH of the medium is often caused due to depletion of ammonium in the medium and reasonable control of pH is achieved by frequent subculturing (Bonga and Von aderkas, 1992).

In *B. tulda*, shoot multiplications rate significantly affected among different (5.0 and 5.8) pH tested in MS liquid medium supplemented with BAP. Medium with comparative low pH (5.0) proved better for high (4.5 fold) rate of shoot multiplication while, higher the media pH (5.8) resulted in low (3.5 fold) shoot multiplication rate (Saxena, 1990). Similarly, among different (4.0, 5.0, 5.5, 6.0 and 7.0) media pH tested, pH 6.0 was favoured maximum shoot multiplication rate in *B. bambos* and *D. strictus* as compared to lower and higher pH (Kabade, 2009).

Gurel and Gulsen (1998) tested various pH (4.5, 5.0, 5.5, 6.0 and 6.5) in MS medium fortified with GA₃ (0.1 mg/l), IBA (0.1 mg/l) and BAP (1.0 mg/l) and found that pH 5.5 was the best for shoot multiplication of *Amygdalus communis*. Whereas in *Corylus avellana* L., shoot number and shoot length were at par among different pH 4.7, 5.2 and 5.7 tested on DKW medium (Yu and Reed, 1993). A similar result also been obtained in blueberry, which can grow on DKW medium with a pH range from 4.0-6.0 (Wolfe et al., 1986).

But as a rule of thumb, majority of the the published papers on shoot multiplication of bamboo species deals with a single pH viz; pH 5.7 in *B. edulis* (Lin and

### 2.1.2.7 Effect of sub-culturing period

Sub-culture of the tissue on fresh medium is essential to maintain shoot growth and vigor. The interval between sub-cultures depends on the rate at which a culture has grown and with the plant species. Delay in sub-culture results in nutrient depletion, accumulation of toxic gases and change in pH of the medium. Frequent sub-culture carries very little risk of induced genetic irregularity.

Arshad *et al.* (2005) observed that 4 weeks sub-culture interval was ideal and favoured the best results with healthy shoots, whereas longer sub-culture cycles resulted in necrosis of shoots in *B. wamin*. Similarly, among different (7, 10, 14, 21 and 28) subculturing intervals (in days), 14 days of sub-culture proved the best in terms of number of shoots/clump and shoot length. Remarkably, shoot length decreased and medium turned brown when sub-culture period was increased to longer periods (28 days) in *B. bambos* and *D. strictus* (Kabade, 2009).

Most of the published papers deals with single sub-culture period viz; 2 weeks in *D. strictus* (Ravikumar *et al.*, 1998), *P. stocksii* (Sanjaya *et al.*, 2005) and in *B. balcooa* (Arya *et al.*, 2008a), 3 weeks in *B. edulis* (Lin and Chang, 1998), 4 weeks in *B. bambos* (Arya and Sharma, 1998), in *B. glaucescens* (Shirin and Rana, 2007) and in *Drepanostachyum falcatum* (Arya *et al.*, 2008a). Negi and Saxena (2011), suggested that sub-culture at every 3 weeks interval was essential to maintain shoot multiplication vigour. Increased sub-culture time, resulted in desiccation of shoots in *B. nutans*.

### 2.1.2.8 Effect of inoculum size

Inoculum size (unit of propagule) and number of inoculum in each culture container have crucial role to maintain high multiplication rate, better shoot growth, quality of shoots and number of harvestable shoots per culture container. This is very
important aspect from large scale production point of view as well as from cost of
production.

In *B. tulda*, the individual shoots did not survive in sub-culture, but clusters of 3
shoots favoured shoot multiplication (Saxena, 1990). Sood et al. (2002) revealed that, 3-4
shoots/clump responded better than individual shoot for shoot multiplication in *D.
hamiltonii*. Similarly, Sanjaya et al. (2005) observed that, shoot clump (2-3
shoots/clump) proved better than single shoot for shoot multiplication in *P. stocksii*.

Arshad et al., (2005) found that shoot clump of 3-5 shoots favoured best for shoot
multiplication, but rate of shoot multiplication declined when propagules of less than 3-5
shoots/clump in *B. wamin*. In *G. angustifolia*, single shoot resulted in multiplication rate
close to one fold, but shoot clumps of 3-5 shoots produced 2.5 fold multiplication rate
(Jimenez et al., 2006).

Most of the papers deals with studies on the single inoculum size for shoot
multiplication of different bamboo species viz; 3 shoots in *B. bambos* (Arya and Sharma,
1998), 3-4 shoots *D. asper* (Arya et al., 1999), 5-7 shoots in *D. strictus* (Ravikumar et al.,
1998), 3 shoots in *B. glaucescens* (Shirin and Rana, 2007), *A. callosa* (Devi and Sharma,
2009) and 2-3 shoots in *B. nutans* (Negi and Saxena, 2011).

2.1.2.9 Effect of genotypes/CPCs

Clonal forestry based on selecting the plus clumps and mass propagation of these
plus clumps will result in more homogenous populations with higher yields.

Genotype may have differential response on rate of shoot multiplication. Two
genotypes (Nonpareil and Tonda gentile romana) of *Corylus avellana* L. were compared
for their differential response on shoot multiplication rate in DKW medium with BA (2.0
mg/l) + 2ip (2.0 mg/l). It was found that, shoot multiplication rate in Nonpareil was
higher (3.11 folds) as compared to 3.05 folds in Tonda gentile romana (Yu and Reed,
1993). In *Ceratonia siliqua* two genotypes viz; ‘Mulata’ and ‘Galhosa’ have exhibited
differential responses on shoot multiplication. In genotype ‘Mulata’, higher rate of
multiplication (1.5 fold) with maximum shoot length (1.38 cm) was observed in MS medium with Zeatin (4.56 \text{ M}) as compared to 1.3 fold in ‘Galhosa’ genotype with shoot length of 0.92 cm (Romano et al., 2002). Similarly, shoot multiplication and growth was significantly different between genotypes of \textit{B. bambos} (BB1, BB4, BB5, BB12 and BB13) and \textit{D. strictus} (DS4, DS36, DS96, DS101 and DS103) used. Among the genotypes tested, BB13 and DS101 favoured high rate of shoot multiplication in \textit{B. bambos} and \textit{D. strictus} respectively (Kabade, 2009).

Whereas, among the five genotypes (G1, G2, G3, G4 and G5) of \textit{Hagenia abyssinica} tested, slight variation without showing any trend in multiplication rate was observed between genotypes (Feyissa et al., 2005).

There is lack of reports on genotype studies on shoot multiplication, especially in bamboo species.

2.1.3 \textit{In vitro} rooting

Rooting of \textit{in vitro} multiplied shoots is a very important step of any \textit{in vitro} propagation. 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. The induction of rhizogenesis usually requires an adjustment in the levels of auxins and cytokinins or with auxin alone. Rooting frequency also depends on the level of rejuvenation during multiplication stage and quality of the shoots. Frequency and quality/quantity of rooting depends on factors like; species/genotypes, source of auxin and their concentrations, nutrient media, sucrose concentrations and incubation conditions.

2.1.3.1 Effect of auxins

Auxins are known to promote adventitious root formation at higher concentrations. This action is also coupled with stimulation of cell division. Exogenous requirement of auxin and its concentration may vary with the plant species and sometimes between genotypes of the same species.
Mukunthakumar et al. (1999) reported that, multiple shoots pulse treated with IBA (100 mg/l) for 24 h, followed by transfer on MS agar gelled medium with IBA (1.0 mg/l) induced 65% rhizogenesis with 4-5 roots of 4-5 cm length in 60 days in D. brandisii. In B. wamin, in vitro multiplied shoots rooted well on modified MS medium (MS/2) consisted various concentrations of IBA (5.0-10.0 mg/l). Maximum rooting (95%) was obtained at 7.5 mg/l, IBA within 4 weeks (Arshad et al., 2005). Ramanayake et al. (2006) reported that in Bambusa vulgaris var. Striata, highest rooting percentage was observed with a TDZ pre-treatment of 2 or 3 cycles, followed by transferring to medium containing IBA (3.0 mg/l). Shirin and Rana (2007) tested various auxins; IAA, IBA, IPA, NAA, 2,4-D and Coumarin (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 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 MS/2 medium with IBA (50-300 M), followed by sub-culturing on hormone free medium for 8 weeks. Maximum rooting (100%) was obtained in medium supplemented with IBA (150-300 M).

Somashekar et al. (2008) reported that among various auxins (IAA, IBA, NAA, NOA and 2,4-D) tested at 1.0 mg/l in MS/4 medium, 100% rooting was observed in the medium consisted NAA. Medium with IAA proved least effective in P. stocksii. Whereas, Sanjaya et al. (2005) revealed that, addition of BA in the rooting medium along with IBA favoured root induction and normal shoot growth in P. stocksii.

Chaturvedi et al. (1993) reported that none of the auxins viz; IBA, NAA and 2,4-D were effective for root induction in D. strictus, but 30% rooting induced from nodal shoot segments cultured on phloroglucinol (1.0 mg/l) with IBA (1.0 mg/l), NAA (1.0 mg/l) and 2,4-D (0.5 mg/l) in MS medium. Saxena (1990) reported high rate of rooting (90%) on MS medium with IAA (1x10⁻⁵M) and coumarin (6.8x10⁻⁵M) from in vitro shoots (3 shoots/clump) raised from 3 week old seedling of B. tulda. He observed that
IAA was better than IBA, either alone or in combination with coumarin for root induction.

Saxena and Bhojwani (1993) observed 73% of rooting in *D. longispathus* in a medium with combined use of IBA, IAA and Coumarin. In *Dendrocalamus giganteus*, incorporation of IBA (3.0 mg/l) in the shoot proliferation medium for the two sub-cultures before transferring to medium containing IBA + coumarin (10 mg/l) resulted in highest (77.5%) rooting percentage (Ramanayake and Yakandawala, 1997). Lin and Chang (1998) used low concentration of TDZ along with 2,4-D for root induction in *B. edulis*. Sood *et al.* (2002) found the best response on *in vitro* rooting in MS/2 medium with choline chloride (0.9 mg/l) and IBA (1.0 mg/l), followed by coumarin (9.0 mg/l) and NAA (0.5 mg/l) in *D. hamiltonii*.

There are many reports on *in vitro* rooting by using different concentrations of IBA either alone or in combinations in various bamboo species. 84.7% rooting was obtained with IBA (49.0 µM) in *B. nutans* (Yasodha *et al.*, 2008). Whereas, 100% rooting was reported in the medium consisted of IBA (9.8 µM) + IAA (2.85 µM) + NAA (2.68 µM) in *B. nutans* (Negi and Saxena, 2011). About 75% rooting was obtained on MS/2 liquid medium with combined application of IBA (25 µM) and BAP (0.05 µM) in *A. callosa* (Devi and Sharma, 2009).

**2.1.3.2 Effect of nutrient media**

Nutrient media play vital role with auxin on frequency of rooting, root number and root length. Optimum nutritional requirement may vary with the species. Generally, low nutrient media favour early and high frequency rooting.

Saxena (1990) tested different media (MS, modified MS and White’s) supplemented with IAA (1x10^{-5}M) and coumarin (6.8x10^{-5}M) and found that modified MS (half reduced NH_{4}NO_{3}) proved to be the best for high rate (90%) of rooting from *in vitro* shoots of seedling origin explants of *B. tulda*. 
Shirin and Arya (2003) compared effect of various (MS, B5, WP and White’s) liquid media supplemented with different auxins (IAA, IBA and NAA) at 25 µM concentration for root induction in *B. nana*. Maximum (90%) rooting and more (9.33) number of roots were observed in MS medium supplemented with IBA. In *B. vulgaris*, among the various media (MS, B5, WP and White’s) supplemented with auxins (IAA, IBA and NAA) tested to find out their effect on rooting. Maximum (90%) rooting and higher (17.27) number of roots were observed in MS medium supplemented with NAA (25 µM) and was significantly better for rooting than other media (Shirin *et al*., 2003).

Somashekar *et al.* (2008) examined various media (MS, MS3/4, MS/2, MS/4, B5 and WP) supplemented with NAA (1.0 mg/l), maximum (100%) rooting and number of roots (17.75) was observed in MS/2 medium. WP medium was proved better than B5, and HE medium was the least effective for root induction in *P. stocksii*.

Most of the reports on *in vitro* rooting in bamboo species deals with use of single medium viz; MS/2 in *D. giganteus* (Ramanayake and Yakandawala, 1997), MS medium in *D. asper* (Arya *et al*., 1999), MS/2 in *T. spathiflorus* (Bag *et al*., 2000), MS/2 in *D. strictus* (Singh *et al*., 2001), MS/2 in *P. stocksii* (Sanjaya *et al*., 2005), MS/2 in *B. wamin* (Arshad *et al*., 2005) and MS in *B. glaucescens* (Shirin and Rana, 2007).

### 2.1.3.3 Effect of sucrose concentrations

Carbohydrate requirement for rooting in shoots depends upon availability of auxins, nitrogen and light (Bonga and Von aderkas, 1992). Sucrose is often used as the best choice of carbohydrate in cell and tissue culture media, because, it is the main sugar which is translocated in the phloem of many plants (Giaquinta, 1980; Strickland *et al*., 1987).

Most of the reports in bamboo deal with the use of single concentration of sucrose as an exogenous energy source in rooting medium. Sanjaya *et al.* (2005) reported rapid rooting on MS/2 liquid medium with BA (0.44 µM) + IBA (4.90 µM) supplemented with 2% sucrose in *P. stocksii*. Whereas, Somashekar *et al.* (2008) reported that, MS/4 medium consisted 3% sucrose resulted 100% rooting in *P. stocksii*. 
Yasodha et al. (2008) reported that addition of glucose (88 mM) on MS medium along with 49 µM IBA during the root induction and favoured 85% rooting success as compared to sucrose (88 mM) supplemented medium in *B. nutans*. Whereas, in another report, maximum (100%) rooting was observed on MS/2 liquid medium supplemented with 3% sucrose in *B. nutans* (Negi and Saxena, 2011).

### 2.1.3.4 Effect of genotypes/CPCs

The selection and propagation of elite genotypes has major advantages in clonal forestry. There are limited reports on the effect of genotypes on *in vitro* rooting in bamboo species.

Kabade (2009) revealed that, percentage rooting varied significantly with different genotypes evaluated in *B. bambos*. Among the different genotypes (BB4, BB5, BB12 and BB13) tested in MS/4 medium with NAA (2.0 mg/l), maximum (100%) rooting was obtained in CPC BB13 with maximum root number 6.71 and root length 4.27 cm. This was followed by BB4 with 95.20% rooting with root number of 5.80 and root length of 3.96 cm. Minimum root induction frequency (76.15%), root number (4.32) and root length (3.00 cm) was observed in CPC BB12. Similarly, among two genotypes of *Prunus dulcis* (almond), maximum (95%) rooting was reported in genotype (M51) and minimum (58%) in ‘Supernova’ genotype (Caboni and Damiano, 1994).

Differential response was observed on rooting from two genotypes (Mulata and Galhosa) of *Ceratonia siliqua* on MS/2 medium with IBA (4.9 mM). Genotype ‘Galhosa’ exhibited 85% rooting in contrast to 59% rooting in ‘Mulata’ (Romano et al., 2002).

Influence of genotype on rooting also has been reported in *Hagenia abyssinica*. Among five genotypes (G1, G2, G3, G4 and G5) on MS/3 medium with IBA (4.9 µM), maximum (100%) rooting was observed in genotype G2, followed by G3 (93%), G4 and G1 (87%) and minimum (73%) in G5 (Feyissa et al., 2005).

### 2.1.4 Ex vitro rooting

*Ex vitro* rooting reduce one step of *in vitro* rooting, cost of production and improvement in survival rate of plantlets. Auxin type, its concentration and duration of
treatment, rooting medium and incubation conditions are the factors, which influence ex vitro rooting.

2.1.4.1 Effect of auxins

In *D. strictus*, about 85-90% of ex vitro rooting was observed in seedling origin shoots treated with 200 ppm pulse treatment of IBA within 20-25 days period under 85-90% RH at 27-30°C temperatures (Ravikumar *et al*., 1998). Somashekar *et al.* (2008) reported that shoot clumps pulse treated with NAA (1000 ppm) for 10 min, induced 99% rooting in *P. stocksii*. Effect of auxin treatment was significant and increased rooting frequency. Out of various auxins used minimum rooting frequency was observed in NOA. Similarly, Kabade (2009) reported that, among various auxins (IAA, IBA, NAA and NOA) tested at 2000 ppm, shoot clumps (2-3 shoots/clump) pulse treated with NAA and planted in sand medium proved the best for high (89.64%) frequency root induction under ex vitro (green house) conditions in *B. bambos*. This was followed by IBA and IAA. No root induction was observed in shoots pulse treated with NOA.

Rajasekaran (1994) reported, 90% root induction on sterile soil medium (peat moss, red soil and sand; 1:1:2, v/v) upon treatment with concentrated NAA solution in *Grevillea robusta*. Whereas, Das *et al.* (1999) observed 80 % rooting from microshoots of *Litchi chinensis* upon treatment with 25 mg/l, IBA after 4 weeks in vermiculite as rooting medium. In *Embelia ribes*, shoots treated with IBA (4.93 µM) solution for 30 min favoured the highest (95.2%) ex vitro rooting with maximum shoot length (4.5 cm) reported within 4 weeks (Annapurna and Rathore, 2010).

2.2 In vitro regeneration through somatic embryogenesis

Somatic embryogenesis is defined as the initiation and development of embryoids from somatic cells that attain maturity and subsequently germinate into complete plants. The first report on somatic embryogenesis was on *Dacus carota* (Reinert, 1958; Steward *et al*., 1958). The potential of somatic embryogenesis has been shown to be characteristic for a wide range of tissue culture systems in plants. Somatic embryos are used as a model system in embryological studies. However, the greatest interest of somatic embryos is centred in its practical application for large-scale propagation, particularly because of the
possibility to scale up the propagation by using bioreactors. In addition, in most cases, somatic embryos or embryogenic cultures can be cryopreserved, which makes it possible to establish gene banks. Embryogenic cultures are also an attractive target for gene transformation.

Plant regeneration through somatic embryogenesis includes five steps:

1. Initiation of embryogenic cultures by culturing the explant on medium supplemented with PGRs, mainly auxin but often also cytokinin.
2. Proliferation of embryogenic cultures on solidified or in liquid medium supplemented with PGRs, in a similar fashion to callus initiation.
3. Pre-maturation of somatic embryos (usually in the medium lacking PGRs)- this inhibits proliferation and stimulates induction of somatic embryo.
4. Maturation of somatic embryos (usually by culturing on medium supplemented with ABA and/or having reduced osmotic potential).
5. Regeneration of plants (on medium lacking PGRs).

Mehta et al. (1982) for the first time reported somatic embryogenesis in bamboo by using seeds of Bambusa arundinacea cultured on N6 medium. Subsequently, Rao et al. (1985) in Dendrocalamus strictus. Yeh and Chang (1987) in Sinocalamus latiflora are some of the earlier reports have successfully accomplished somatic embryogenesis in different bamboo species.

2.2.1 Callus induction

Somatic embryogenesis can probably be achieved in all plant species provided physic-chemical conditions which triggers embryogenesis. Embryogenic callus induction is influenced by favourable conditions like; explant type and source, PGRs and nutrient media.

2.2.1.1 Effect of explant type

The literature cited in embryogenic callus induction among various species of bamboo has been reported by using different explants. Source of explants like; seeds,
inflorescence, leaf, nodal shoot segments and roots were used for callus initiation in different bamboo species.

Rao et al. (1985) reported callus induction from the seeds of *D. strictus* within 10-12 days in B5 medium supplemented with 2, 4-D (10^{-5}M and 3x10^{-5}M) and observed two types of callus (friable and compact callus). They revealed that, embryogenic callus are of small cell with condensed cytoplasm while, non-embryogenic have long cells with large vacuoles in callus mass. Similarly, Yeh and Chang (1987) observed callus induction from zygotic embryo on MS medium with 2,4-D (6.0 mg/l) + Kn (3.0 mg/l) + PVP (2.50 mg/l) + sucrose (5%) in *Sinocalamus latiflora*.

Woods et al. (1992) observed embryogenic callus induction on MS medium supplemented with 2,4-D (3.0 mg/l) by using seed as a source of explants in *Otaea acuminatea aztecorum*. Yuan et al. (2009) also used seeds of *B. multiplex* for callus induction on NB medium supplemented with 2,4-D (4.0 mg/l). Zhang et al. (2010) used mature zygotic embryo as explant for callus initiation in *D. hamiltonii*. Similarly, mature embryo of the seed were used for callus initiation in *D. farinosus* (Hu et al., 2011).

Huang and Murashige (1983) obtained callus from shoot tips of *B. oldhami*, *B. multiplex*, *Sasa pygmaea* and *Phyllostachys aurea*. Yeh and Chang (1986b) reported embryogenic callus induction from inflorescence of *B. beecheyana* Var. beecheyana on MS medium with 2, 4-D (3.0 mg/l) and Kn (2.0 mg/l). Godbole et al. (2002) used nodal segments for induction of embryogenic callus from field grown *D. hamiltonii*. Lin et al. (2004) reported embryogenic callus induction in *B. edulis* from nodal shoot segment on MS medium with 2, 4-D (13.6 M) + Kn (9.2 M) + coconut (0.1%) + sucrose (6%). Arya et al. (2008b) reported induction of embryogenic callus from explants excised from nodal tissues and basal part of leaves of *in vitro* shoots in *Dendrocalamus asper*.

Hassan and Debergh (1987) reported callus induction from leaf explant on MS medium with 2,4-D (9.0 µM) within 6 weeks period. They observed two types of callus viz; (i) embryogenic callus are compact, opaque and white appearance, shiny globular bodies, comparable to normal zygotic embryos and (ii) the other type was non-
embryogenic which are soft and yellow in colour. Whereas, Jullien and Tran Thanh Van (1994) observed three types of callus from the leaf tissues on MS medium with 2, 4-D (18.0 M) in *B. glaucescens* viz; i) soft, friable, composed of loose and large cells, which was non-embryogenic ii) compact white, organized and embryogenic and iii) white - pale yellow in colour, smooth and inter spread between two types of callus.

2.2.1.2 Effect of plant growth regulators (PGRs)

Requirement of type of PGRs, their combinations and concentrations vary with the plant species. The process of somatic embryogenesis is often initiated in media containing high levels of auxins (especially 2,4-D).

Yeh and Chang (1986b) reported compact embryogenic callus induction on MS medium with 2,4-D (3.0 mg/l) and Kn (2.0 mg/l) within 4 weeks from inflorescence (florets) of *B. beecheyana* Var. beecheyana.

Godbole et al. (2002) obtained induction of embryogenic callus on MS medium supplemented with 2, 4-D and BA (1.0 mg/l, each) within 24 days of incubation in dark from nodal segments collected from field grown plants of *D. hamiltonii*. In case of *D. giganteus*, more than 40% of the explants were induced callus at leaf bases or nodal regions after 4-6 weeks. Among different concentrations of 2,4-D (1.0, 3.0, 7.5 and 15.0 mg/l) and NAA (1.0, 3.0 and 7.5 mg/l) used in MS medium, treatment consisted with 2,4-D (7.5 mg/l) and NAA (3.0 mg/l) proved the best for nodular callus induction (Ramanayake and Wanniarachchi, 2003).

Among different concentrations of 2,4-D (1.0, 1.5, 2.0, 2.5, 5.0 and 7.0 mg/l) used in MS medium, 2,4-D (5.0 mg/l) resulted in response from 30-40% shoot segments responded for callus induction. But no response was recorded at lower or higher concentrations of 2,4-D in *B. nutans* (Mehta et al., 2011). Zhang et al. (2010) suggested that 2,4-D 1.0-3.0 mg/l was suitable range to induce desirable vigorous, granular and compact callus. But at the higher concentration of 2,4-D (10 mg/l) in the medium resulted in less abundant sticky watery mucilage callus from the mature zygotic embryos of *D.*
*hamiltonii*. Maximum (91.6%) callus induction was obtained from nodal shoot segments of *D. asper* on MS medium supplemented with 30 µM of 2,4-D (Arya *et al*., 2008b).

Ogita (2005) tested different PGRs (2,4-D, Picloram and BA) individually in MS/2 medium at various concentrations (1.0, 3.0, 10.0 and 30.0 µM), respectively. Treatments consisted of 2,4-D (3.0 µM) was the most effective for callus induction, followed by Picloram (3.0 µM). But in BA supplemented medium had negative effect on callus induction from the shoots of *P. nigra*. Similarly, embryogenic callus obtained from nodal shoot segments from *in vitro* plantlets of *B. edulis* on MS medium supplemented with Kn (2.0 mg/l) + 2,4-D (3.0 mg/l) + CM (0.1%) consisted of 6% sucrose (Lin *et al*., 2004).

There are very few reports, which deals with the use of auxins other than 2,4-D for the embryogenic callus induction in bamboo species. Hu *et al*. (2011) reported 29.7% callus induction obtained on the 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*. Komatsu *et al*. (2011) found callogenesis from leaf sheath in the medium consisted of Picloram at 8.0 mg/l in *P. bambusoides*.

### 2.2.1.3 Effect of nutrient media

Different nutrient media have differential response on callus induction in 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, differentiation and totipotency (Kirbey *et al*., 1987).

Rao *et al*. (1985) reported callus induction from embryonic end of the seeds in *D. strictus* on B5 medium supplemented with 2,4-D (10⁻⁵M and 3x10⁻⁵M) within 10-12 days. In *B. beecheyana* Var. beecheyana, callus initiation from young florets favoured in MS medium consisted of 2,4-D (2.0 mg/l) + Kn (2.0 mg/l) (Yeh and Chang, 1986b). Similarly, Woods *et al*. (1992) reported that, MS medium was superior as compared to B5 medium for embryogenic callus induction in *Omatea acuminatea aztecorum*. 
Yeh and Chang (1987) observed callus induction and multiplication from zygotic embryo on MS medium with 2,4-D (6.0 mg/l) + Kn (3.0 mg/l) + PVP (2.50 mg/l) + sucrose (5%) in *Sinocalamus latiflora*. Whereas, Hassan and Debergh (1987) observed callus induction from leaf explant on MS medium with 2,4-D (9.0 µM) within 6 weeks period. Lin *et al.* (2004) reported embryogenic callus induction in *B. edulis* from nodal shoot segment on MS medium with 2,4-D (13.6 µM) + Kn (9.2 µM) + coconut (0.1%) + sucrose (6%). Jullien and Tran Thanh Van (1994) observed callus induction from the leaf tissues on MS medium with 2, 4-D (18.0 µM) in *B. glaucescens*. Among MS and B5 media tested for callus induction from nodal segments of *D. asper*, MS medium proved better and induced callus within 4 weeks (Arya *et al.*, 2008b). In *D. hamiltonii*, among the different basal media (MS, MS/2, NB, B5 and HB) evaluated, MS medium was found to be the best for vigorous, granular and compact callus induction from mature zygotic embryos (Zhang *et al.*, 2010). Similarly, callus induction frequency was more (29.7%) on MS medium as compared to WP medium (22.3%) from young shoots of *D. farinosus* (Hu *et al.*, 2011).

Rout and Das (1994) have reported callus induction from zygotic embryo and nodal segment from *in vitro* seedling of *B. vulgaris*, *D. giganteus* and *D. strictus* on MS/2 and medium with 2,4-D (2.0 mg/l) + Kn (0.5 mg/l) + adenine sulphate (10 mg/l) under dark.

### 2.2.2 Callus multiplication

For embryogenic callus multiplication and maintenance of cultures over a long period has been an important aspect for utilizing the phenomenon of somatic embryogenesis.

#### 2.2.2.1 Effect of auxins

Majority of literature revealed that, in embryogenic callus multiplication similar medium conditions were maintained as that of used in callus initiation. But the degree of callus multiplication takes place in the presence of auxin varies in different species.
Yeh and Chang (1986a, b and 1987) maintained embryogenic callus derived from young inflorescence of *B. oldhamii, B. beecheyana* and *S. latifolia* on the same medium used for callus initiation or auxin-free medium for 16 months without loss of totipotency. They also reported that, in the medium consisted 3.0 mg/l 2,4-D and 2.0 mg/l kinetin, yield an abundance of callus within 2 months. They revealed that, induced callus was gelatinous, transluant and smooth at the surface. Rapid growth and nodular callus was observed by subculturing on the medium of same composition.

Higher (70-80%) callus multiplication rates was reported in the MS medium supplemented with 2, 4-D (1 x 10^{-5} M) + KN (5 x 10^{-6} M) + IBA (1x 10^{-5} M) from seeds of *D. stristus*. Whereas, low (20-30%) frequency of callus multiplication was in MS medium consisted of 2,4-D (1 x 10^{-5} M) and BAP (1 x 10^{-5} M) after 5 weeks (Saxena and Dhawan, 1999). Godbole et al., (2002) have also reported that, embryogenic callus obtained from mature nodal segments of elite *D. hamiltonii* could be multiplied efficiently on MS medium supplemented with 2,4-D and BA at 1.0 mg/l each.

Although 2,4-D has proven efficient auxin for callus multiplication, thidiazuron (TDZ) has also been used for this purpose. Lin et al. (2004) tested various concentrations of thidiazuron in MS medium ranging from 0.046 to 0.455 µM in combination with 2,4-D for embryogenic callus proliferation. Maximum (526 mg) fresh weight of callus and better callus proliferation was reported in treatment supplemented with TDZ (0.046 µM) + 2,4-D (13.6 µM) in *B. edulis*. It was observed that TDZ has a positive effect on embryogenic callus proliferation. However, use of TDZ in callus multiplication of bamboo is scanty.

Arya et al. (2008b) tested different phytohormones i.e. 2,4-D, IAA, BAP and Kn in various concentrations and combinations for optimization of embryogenic callus multiplication in *D. asper*. Maximum (100%) embryogenic response, fresh weight (175 mg) and high (3.5 folds) callus multiplication rate was reported in the MS medium supplemented with 2,4-D (9.0 µM), BAP (0.88 µM) and IAA (2.85 µM) in 4 weeks incubation period.
As suggested from above literature, however a number of studies revealed that, 2,4-D alone or in combination with BA/BAP or Kn or NAA was used for efficient multiplication of embryogenic callus of various bamboo species.

### 2.2.2.2 Effect of nutrient media

Optimum nutrient requirement may vary with the plant species. Nitrogen is a major element for \textit{in vitro} morphogenesis (Halperin, 1995). Nutrient media vary from each other in concentrations of macro and micro elements. Nutrient rich media generally favour callus induction and further multiplication.

Rao \textit{et al.} (1985) and Rout and Das (1994) used B5 and MS/2 media, for callus multiplication in seedlings of \textit{D. strictus} and \textit{D. gigantius} respectively. B5 and MS basal media were contrasted for their influence on somatic embryogenesis and concluded that the MS basal medium was superior to maintain embryogenic capacity for more than six passages in \textit{Otaxe acuminata aztecorum} (Woods \textit{et al.}, 1992). Among MS and B5 media used, MS medium found superior to B5 medium for callus multiplication of \textit{D. strictus} (Saxena and Dhawan, 1999).

Most of the earlier reports deal with use of single medium (usually MS medium) for multiplication of embryogenic callus in different bamboo species. MS medium favoured multiplication of callus in \textit{B. beecheyana} (Yeh and Chang, 1986b), \textit{P. viridis} (Hassan and Debergh, 1987), \textit{B. glaucescens} (Jullien and Tran Thanh Van, 1994), \textit{D. hamiltonii} (Godbole \textit{et al.}, 2002; Zhang \textit{et al.}, 2010), \textit{B. edulis} (Lin \textit{et al.}, 2004), \textit{D. asper} (Arya \textit{et al.}, 2008b), \textit{B. nutans} (Mehta \textit{et al.}, 2011) and in \textit{D. farinious} (Hu \textit{et al.}, 2011).

Literatures above cited have suggested that amongst all the media tested by different workers, MS medium widely used for callus proliferation.

### 2.2.2.3 Effect of sucrose concentrations

Nevertheless, for the normal culture of cells, tissues or organs, it is necessary to incorporate a carbon source into the medium. Sucrose is almost universally used for micropropagation purposes.
In most practical situations, cultures should be grown in the presence of an auxin and a carbohydrate (usually sugar) for the induction and multiplication of embryogenic callus. The level of sugar (e.g. sucrose or glucose) in the medium may need to be within critical concentrations.

Generally in somatic embryogenesis of bamboo, different types of callus (embryogenic and non-embryogenic) were observed by different authors. Rao et al. (1985) reported two distinct types of callus (1) Embryogenic callus are small, rounded cells, rich starch granules in cytoplasm and had prominent nucleus (2) Non-embryogenic callus were thin walled highly vacuolated with little cytoplasm. In D. hamiltonii two types of callus was observed (1) fast-growing and friable and (2) slow-growing, compact and nodular. They also reported that only compact, nodular and creamish-white callus was capable to differentiate into somatic embryos, whereas, the friable callus did not show any response (Godbole et al., 2002; Zhang et al., 2010). In Zea mays high concentration of sucrose (12%) in MS medium proved the best for induction of embryogenic callus from immature seed embryos (Lu et al., 1982). Similarly, Ho and Vasil (1983) used 6-10% sucrose in MS medium to promote the formation of pro-embryoids from young leaves of Saccharum officinarum. This shift may be due to increase in osmotic potential of medium at high sucrose concentration.

Osmotic effect of sucrose in culture medium for callus multiplication was well documented by the earlier workers in different bamboo species. High (6% w/v) concentration of sucrose was used in the embryogenic callus multiplication medium in B. oldhamii and B. beecheyana (Yeh and Chang, 1986a and b). In comparision of 2% sucrose Vs 5% sucrose on maintainance of embryogenic callus derived from zygotic embryo of Mexican Weeping Bamboo, 2% sucrose concentration was more condective than in 5% (Woods et al., 1992). In callus proliferation of B. edulis among the sucrose concentrations (1.5, 3.0, 4.5 and 6.0%) used, lower percentage of callus browning and maximum callus fresh weight (707 mg) was reported in MS medium supplemented with 4.5% sucrose (Lin et al., 2004).
However, most of the reports routinely used only 3% sucrose in medium for callus multiplication of different bamboo species i.e. *P. nigra* (Ogita, 2005), *D. asper* (Arya et al., 2008b), *B. nutans* (Mehta et al., 2011), *D. hamiltonii* (Zhang et al., 2010) and in *D. farinosus* (Hu et al., 2011).

### 2.2.3 Somatic embryo induction and maturation

During the recent years, somatic embryogenesis has gained importance in bamboo improvement programmes. Callus induction and regeneration from the callus cultures are two important aspects in *in vitro* studies. Because they serve as a tool for the mass production of plants at large scale and can be used for further genetic improvements. A brief review of work on these aspects is given below.

#### 2.2.3.1 Effect of plant growth regulators (PGRs)

The process of somatic embryogenesis is often initiated in medium consisted high levels of auxins, but embryos usually do not develop further until the auxin concentration is reduced. It was suggested that division of the pro-embryogenic cells and their development into embryos is only resumed at lower auxin concentrations and or incorporation of cytokinins. There are however, many exception reports to this general observation, where somatic embryos are induced even in cultures grown on medium devoid of auxins.

As reported by Raemakers *et al.* (1999), regeneration of plantlets from proliferating embryogenic tissue requires three essential steps, i.e. (i) arrest of division in the embryogenic tissue, (ii) maturation of somatic embryos and (iii) germination thereof.

Woods *et al.* (1992) reported, an average of 10.7 clusters of somatic embryos yielded per culture plate on the MS medium supplemented with BA (0.5 mg/l) + 2,4-D (3.0 mg/l) in Mexican weeping bamboo. Each cluster consisted of at least three somatic embryos. Whereas Rao *et al.* (1985) documented, lower (8.45) embryoids per culture plate in *D. strictus*, which was at least three times less than above studies.
In *D. hamiltonii*, inducation of somatic embryogenesis occurred only by complete elimination of 2,4-D and NAA from the medium. Alternatively, an increase in BA concentration (2.5 mg/l) alone in MS/2 medium resulted in 78% somatic embryo induction, which are prominently visible ivory-white embryoids which turned greenish within 21 days. Whereas, MS medium without PGRs resulted up to 40% callus lumps showed green protuberances. But these did not grow into plantlets despite continuous sub-cultures on the same medium (Godbole *et al*., 2002).

Earlier reviews found abscisic acid (ABA) has been to be suitable for maturation of somatic embryos in many species. Low concentration of ABA is known to influence maturation by accumulation of storage carbohydrates, lipids and proteins (Phillips and Collins, 1981). Chang and Lan (1995) used ABA (0.1-2.0 mg/l) and other osmoticums (PEG, polyamine, mannitol) for further development of embryos in *B. beechyana* Munro var beechyana. In *S. album*, also addition of ABA + PEG in the medium was essential for embryo maturation (Rangaswamy, 2007).

Maturation of somatic embryo was attained when the globular embryos were transferred to medium supplemented with abscisic acid (ABA) or high concentrations of sucrose. In *D. asper* maximum maturation of somatic embryos with scutellar and coleoptillar stages was achieved within 8 weeks of incubation on medium supplemented with ABA (5.0 M) or on 6% sucrose in dark (Arya *et al*., 2008b). They also revealed that, globular and scutellar developmental stages were observed on multiplication medium whereas, scutellar and coleoptillar stages were prominent on maturation medium. However in *B. nutans*, medium supplemented with BAP and 2,4-D (1.0 mg/l each) was found suitable for maturation of somatic embryo (Mehta *et al*., 2011).

### 2.2.3.2 Effect of nutrient media

The success of plant propagation through somatic embryogenesis is greatly influenced by the composition of culture medium. The relative proportion of nitrate and ammonium ions in medium affects the response of cells in terms of both cell division and morphogenesis.
Various reviews are available for successful somatic embryogenesis in many bamboo species by using different types of media. Rao et al. (1985) used B5 medium with 2, 4-D for somatic embryo induction in D. strictus within 4 weeks. Similarly, Woods et al. (1992) found contrast influence between B5 and MS basal media on somatic embryogenesis. They found B5 medium with BAP and 2% sucrose was necessary for optimal production of somatic embryos in Mexican weeping bamboo. In contrast, Godbole et al. (2002) used MS/2 medium with BAP (2.5 mg/l) alone for somatic embryo maturation in D. hamiltonii.

Majority of the literatures used single medium (usually, MS medium) for somatic embryos induction in different bamboo species viz; Yeh and Chang (1986a) maintained for more than 16 months by periodical sub-culturing on MS medium supplemented with 2,4-D (3.0 mg/l) + Kn (2.0 mg/l) in B. beecheyana var beecheyana. Yeh and Chang (1987) reported somatic embryogenesis from zygotic embryo of Sinocalamus latiflora on MS medium fortified with 2,4-D (6.0 mg/l), Kn (3.0 mg/l) + PVP (250 mg/l) + sucrose (5%) under dark condition. Huang et al. (1989) observed embryogenic callus form the explants of axenic plants which underwent organogenesis on MS medium containing BAP (0.5 mg/l) and 2,4-D (3.0 mg/l) in Phyllostachys aurea. Saxena and Dhawan (1999) obtained somatic embryos on MS medium with 2,4-D (3 x 10^{-5} M) in D. strictus. Lin et al. (2004) also used MS medium with TDZ (0.046 M) + 2,4-D (13.6 M) + 3% sucrose for embryogenic callus proliferation. Subsequent sub-cultures, the callus developed into cluster of pale-yellow somatic embryos in B. edulis.

Furthermore, MS medium was widely used for establishment of somatic embryos in D. asper (Arya et al., 2008b), D. hamiltonii (Zhang et al., 2010), B. nutans (Mehta et al., 2011) and in D. farinosus (Hu et al., 2011).

2.2.4 Somatic embryo germination

2.2.4.1 Effect of plant growth regulators (PGRs)

Adjustment of the growth regulators in the culture medium can induce germination of somatic embryos from the embryogenic callus in different bamboo species.
Rao et al. (1985) reported 40% of the embryoids germinate and formed plantlets with roots and shoots. The remaining 25% somatic embryos formed only roots and 35% turned brown and failed to germinate on B5 liquid medium supplemented with IBA (5 x 10^{-7} M) + NAA (10^{-7} M) within 15-20 days in D. strictus. Whereas, Saxena and Dhawan (1999) observed germination of somatic embryos in D. strictus on MS medium with NAA (15 x 10^{-6} M) + Kn (5 x 10^{-6} M) + PVP (250 mg/l). Using this method, than 1,00,000 plants were produced.

In case of B. beeseyana var beeseyana and B. oldhamii, most of the embryoids germinated either on the MS agar gelled medium with 2,4-D (3.0 mg/l) and Kn (2.0 mg/l) or on the hormone-free medium was sufficient for subsequent development of normal plantlets (Yeh and Chang, 1986a and b). While, Hassan and Debergh (1987) reported, MS medium without growth hormones favoured somatic embryo germination and plantlets recovered within 2 weeks in P. viridis.

Woods et al. (1992) reported high efficiency somatic embryogenesis was obtained in the MS medium consisted of 2, 4-D (3.0 mg/l), BA (0.5 mg/l) and 2% sucrose from zygotic embryo explant of Otatea acuminata aztecorum. Whereas, Godbole et al. (2002) used sucrose-enriched (sucrose at 8%) MS medium without PGRs for successful germination of matured somatic embryos into plantlets with a conversion rate of about 80% within 21 days in D. hamiltonii. They also noticed rhizome formation in prolonged incubation in the same medium.

Lin et al. (2004) studied the effect of various cytokinins (BA, BPA, Kn, TDZ and zeatin) on somatic embryo germination. Among cytokinins, TDZ (0.455 M) favoured maximum (84%) somatic embryo germination in MS medium than the other cytokinin treatments. Furthermore they also revealed that, as the NAA concentration increased in the medium supplemented with TDZ (0.046 M), the germination rate was reduced in B. edulis.

In D. asper among different phytohormones (2,4-D, BAP, IAA, GA3 and NAA) used, maximum of 70% normal (intact shoot with roots) germination and 30% of
embryos produced only shoots was observed on MS medium with BAP (4.4 M) + GA3 (2.8 M) within 4 weeks under 16/8 h (light/dark) photoperiod. Somatic embryos also germinated in other treatments but percentage of germination was significantly reduced (Arya et al., 2008b).

In D. hamiltonii among PGRs (BA, Kn and NAA) tested in MS medium, maximum (89.5%) somatic embryos promoted shoot differentiation and development in the treatment consisted of BA (2.0 mg/l), Kn (1.0 mg/l) and NAA (1.0 mg/l) within 3 weeks period (Zhang et al., 2010). In another report, maximum (91.2%) adventitious shoot induction and root formation was observed from callus derived from mature seed embryo of D. farinosus on MS medium supplemented with Kn (2.5 mg/l) + IAA (0.5 mg/l) after 28 days (Hu et al., 2011).

2.2.4.2 Effect of nutrient media

There are several reports in various bamboo species regarding regeneration of plantlets through somatic embryogenesis, but percentage of embryo germination varied among different media used.

Rao et al. (1985) used B5 medium for germination somatic embryos in D. strictus. Woods et al. (1992) used B5 and MS basal media for their influence on somatic embryogenesis. They found that the MS was superior to B5 medium supplemented with BA (0.5 mg/l) and 2,4-D (3.0 mg/l) in Otatea acuminate aztecorum. More than 95% of germinating somatic embryos developed shoots and roots.

Rout and Das (1994) reported somatic embryo germination on half strength MS medium with IBA (0.25 mg/l) + adenine sulphate (0.5 mg/l) + GA3 (0.5 mg/l) in B. vulgaris, D. giganteus and D. strictus. Similarly, Godbole et al. (2002) also reported MS/2 medium was better than MS medium for embryo maturation and germination in D. hamiltonii.

Lin et al. (2004) observed more than 80% somatic embryo germination of B. edulis on MS medium supplemented with TDZ (0.455 M). MS medium with BAP (4.4
µM) + GA3 (2.8 µM) also favoured maximum (70%) somatic embryo germination into plantlets in *D. asper* (Arya *et al.*, 2008b). Zang *et al.* (2010) demonstrated the effect of the strength of MS medium on germination of somatic embryos in *D. hamiltonii*. Among different media (MS, MS/2 and MS3/4) consisted of NAA (1.0 mg/l) + BA (2.0 mg/l) + Kn (1.0 mg/l), MS medium favoured maximum (89.5%) shoot differentiation than compared to other media.

Majority of the reports used single type of medium (usually MS) 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.*, 2011), and in *D. farinous* (Hu *et al.*, 2011).

### 2.3 Hardening and acclimatization

Micropropagated plants, originated either through somatic embryogenesis or axillary shoot proliferation pathway, require a hardening system for acclimitization prior to transplanting *ex vitro* to endure the external environmental stress. *In vitro* raised plantlets are heterotrophic nature in the mode of nutrition and characterized by abnormal leaf morphology with scanty of epicuticular layer, which may leads more susceptible to water loss in an external environment. They may therefore have to be hardened in an atmosphere of slowly decreasing humidity. Therefore, hardening or acclimatization is an essential step before keeping them in open nursery. Hardening conditions and duration may vary with the species for its high rate of survival. Considerable reports are available regarding hardening and acclimatization of micropropagated plants in various bamboo species.

The *in vitro* propagation (either through axillary shoot proliferation or somatic embryogenesis) methods can ultimately succeeded when the plantlets were established in external enviornment (Chandra *et al.*, 2010). Various factors such as potting media, humidity regime and duration are known to influence the transplantation process.

In *B. wamin* *in vitro* rooted plantlets derived from axillary shoot proliferation grown well after transplanted to pots consisted of vermiculite within 6-8 weeks in mist
chamber. Furthermore, acclimatization was made by shifting plants to 50% shade under agronet house (Arshad et al., 2005). Maximum survival rate (92%) was reported during hardening and acclimatization stage of the plants raised through axillary shoot proliferation in *P. stocksii* (Sanjaya et al., 2005). In *D. falcatum* also *in vitro* rooted plantlets were hardened in culture bottles consisted of vermiculite. Later acclimatization of plants was carried out in mist chamber (80-90% RH and 30±2°C temperature) in poly bags containing a mixture of sand, soil and FYM in a ratio of 1:1:1 v/v for 4-5 weeks (Arya et al., 2008a).

In *D. hamiltonii*, plantlets regenerated through axillary shoot proliferation showed 85% survival during hardening stage under green house condition (Agnihotri et al., 2009). But low (78%) survival was reported from plantlets raised through somatic embryogenesis in *D. hamiltonii* (Godbole et al., 2002). Similarly, high rate (96%) of survival was observed from *in vitro* raised plantlets through axillary shoot proliferation in *B. nutans* during hardening and acclimatization stage in nursery (Negi and Saxena, 2011). But, 90% survival was reported from somatic embryo-derived plantlets in *B. nutans* (Mehta et al., 2011). Relatively lower (60-70%) rate of survival was observed during acclimatization phase of *A. callosa* plantlets propagated through axillary shoot proliferation from mature plants (Devi and Sharma, 2009).

Based on the literatures on somatic embryogenesis in bamboo, transplantation aspects of hardening and acclimitization are either totally missing or merely mentioned. In *Ootatea acuminate aztecorum* (Mexican weeping bamboo), plantlets regenerated through somatic embryogenesis were transferred to soil with 85% success in greenhouse (Woods et al., 1992). Similarly in *D. strictus*, rooted plants derived from somatic embryogenesis were successfully transferred to soli in polythane bags with over 80% survival was reported during hardening stage (Saxena and Dhawan, 1999). Plantlets of *D. asper* recovered from germination of embryos were transplanted in polybags consisted of vermiculate under mist chamber for hardening. After 20-25 days the hardened plants were transferred to polybags containing mixture of soil, sand and FYM for acclimitization under agronet shade house (Arya et al., 2008b). In *D. farinosus* the somatic embryo regenerated shoots with roots were transplanted into plastic pots.
consisted of moist autoclaved peat moss, vermiculate and garden soil mixture (2:1:1, ratio) for hardening in greenhouse. After 2 weeks, plantlets in pots were transferred into plastic pots containing autoclaved garden soil under natural light. More than 90% survival was reported during acclimitization (Hu et al., 2011).

2.4 Evaluation of genetic fidelity of in vitro regenerated plants

In recent years, with the advent of recombinant DNA technology and PCR, molecular markers are being used for a variety of studies. More recently, molecular markers have been used for testing the genetic fidelity of micropropagated plants and for characterization of plant genetic resources. In this aspect, the use of molecular markers has received significance in confirmation of clonal trueness in micropropagated plants of elite forestry species, where life span is quite long period.

Micropropagation can be achieved either through axillary shoot proliferation or somatic embryogenesis. These two methods are believed to give rise to genetically uniform and true-to-type plants, since the organized meristems do not undergo genetic changes that might arise during cell division or differentiation from callus cultures (Shenoy and Vasil, 1992).

In commercial industry, where micropropagation technology has been adopted, their foremost concern is the maintenance of true-to-type nature of the micropropagated plants to that of source material. Although, some reports also documented the occurrence of somaclonal variation even among plants derived either through somatic embryogenesis or through enhanced axillary branching cultures. Therefore it has been suggested that, commercial application of tissue culture to perennial crops must await adequate quality check before dispach to consumers irrespective of the method used for micropropagation. Molecular markers have thus become useful tool for genomic investigation.

Molecular markers are routinely used for characterization of genetic diversity, DNA fingerprinting, genome mapping, genome evolution, ecology, taxonomy and plant breeding. DNA-based markers have enormous advantages over classical biochemical
markers due its specificity, abundant, highly polymorphic can detect changes in both coding and non-coding regions of the genome and independent of tissue type.

Most DNA-based markers can be classified into three categories depending on the technique used (Karp and Edwards, 1997): (i) Hybridization-based DNA markers, (ii) Arbitrarily primed Polymerase Chain Reaction (PCR)-based markers and (iii) Sequence targeted/single locus DNA markers. Hybridization-based markers involves digestion of DNA with restriction enzymes followed by blotting and hybridizations with probes (i.e. RFLP- Restriction fragment length polymorphism).

Arbitrarily primed PCR-based markers involve amplification of genomic DNA with primers that are arbitrary, semi- arbitrary or sequence specific. These markers reveal polymorphism based on repetative regions in the genome. These markers include RAPD, ISSR and AFLP. These are employed in organisms for which no genome sequence is available.

Single locus markers are aimed in polymorphism of a single locaus and able to detect the heterozygous state from the homozygous (i.e. homozygous gives one band, while heterozygous gives two bands after electrophoresis). Thus, these markers are Co-dominant in nature. These include Sequence tagged sites (STS), SCARS and Single nucleotide polymorphisms (SNP) markers.

Molecular tools are more reliable than classical phenotypic, cytological and karyotypic observation for evaluating tissue culture induced variations (Leroy et al., 2000). Many authors have reported that plant tissues culture methods lead to genetic modifications (Larkin and Scowcroft, 1981; Negi and Saxena, 2010), but on the contrary, several reports also confirmed genetic integrity of tissue culture derived plants (Gillis et al., 2007; Agnihotri et al., 2009).

In vitro propagation through axillary branching has been used in the micropropagation of several bamboos such as in Dendrocalamus longispathus (Saxena and Bhojwani, 1993), Dendrocalamus asper (Arya et al., 1999), P. stocksii (Sanjaya et al., 2005; Somashekar et al., 2008), Guadua angustifolia (Jimenez et al., 2006) and A.
However, reliable protocols are also available for mass propagation via somatic embryogenesis in mature bamboo species viz; in *D. hamiltonii* (Godbole *et al.*, 2002), *D. giganteus* (Ramanayake and Wanniarachchi, 2003), *B. edulis* (Lin *et al.*, 2004), *P. nigra* (Ogita, 2005), *D. asper* (Arya *et al.*, 2008b) and *P. bambusoides* (Komatsu *et al.*, 2011).

Although, all the tissue cultured plants are expected to be genetically identical yet the possibility of some genetic variation emerging during the *in vitro* process cannot be ruled out. Variations among regenerates mostly occur as a result of the stress induced by the tissue culture process causing alteration in DNA methylation patterns. The lack of reports on ascertaining the genetic fidelity of tissue culture raised plantlets could lead to serious consequences, especially in perennials like bamboo. Therefore, screening for somaclonal variation (if any) among the regenerants is a utmost pre-requisite.

Huang and Huang (1995) at first in time reported on morphological variations in bamboo plants raised through axillary mode of regeneration, where none of the first batch of 100 plants displayed the bulbous intermodal character in just 8 weeks *in vitro* conditions in *Bambusa ventricosa*. Thus, clonal trueness is of major importance in commercial micropropagation and especially in forest trees and other woody plants having long gestration period like bamboos. Gielis *et al.* (2002) highlighted the need of molecular techniques to assess genetic stability in micropropogated bamboo.

### 2.4.1 Genetic fidelity of *in vitro* regenerated plants by using RAPD primers

There are various molecular tools to assess variability, among them RAPD, RFLP, AFLP and ISSR markers are widely 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). RAPD has many advantages being non-radioactive detection, multiple loci detection in a single reaction, requirement of small quantity of DNA, no requirement of prior sequence information, quick, inexpensive and technical simplicity.
RAPD based assessment of genetic stability of micro propagated plants has been reported in many plant species (Rout et al., 1998; Rout and Das, 2002; Martins et al., 2004; Venkatachalam et al., 2007).

There are few published reports in 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 they revealed that, no variation was found in micropropagated plants of both the species. They advocated clonal propagation through axillary shoot proliferation is reliable 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 scoreable amplification products. All amplification products were monomorphic across the micropropagated plants and corresponding mother plant.

Gillis et al. (2007) used flow cytometry to analyse ploidy level for callus, in vivo regenerants and in vivo plants obtained by axillary branching. The histograms shown that all had a dominant peak, indicated all samples examined had the same ploidy level. Furthermore, they used Methylation Sensitive AFLP (MSAP) to assess the epigenetic changes occurred during the process of somatic embryogenesis in B. balcooa. They found that there was no difference in marker pattern among regenerants. Based on the epigenetic and genetic features, they revealed that the method of somatic embryogenesis described allows for true-to-type micropropagation of elite bamboo (i.e. in B. balcooa).

Since the development of the RAPD by Williams et al. (1990), it has been widely employed in many fields, such as studies on taxonomic relationships, gene flow, genetic map constructions, cultivar identifications and in population genetic structure studies. Lai and Hsiao (1997) reported clonal identity and clonal distribution of P. pubescens in Taiwan by using 13 RAPD primers. RAPD technique has the potential use in species identification and genetic relationships between taxa and species of bamboo for breeding program. Nayak et al. (2003) studied identification and genetic relationships between 12 species of bamboo by using thirty 10-mer primers of RAPD. Similarly, Biradar et al. (2005) characterized elite clones of D. strictus and B. bambos from Western ghats of
India by using 80 RAPD markers. The results indicated a genetic similarity range from 61.40% to 84.23% in *D. strictus* while, 51.58% to 93.11% in *B. bambos* and cluster analysis grouped eleven clones of each species into three major groups. Bhattacharya *et al.* (2006) studied morphological characters as well as molecular marker to enable species identification in *B. tulda*. Thirty two key morphological characters were examined along with RAPD fingerprinting patterns between populations of 17 eco-geographical locations.

Many reports are also available regarding genetic fidelity/stability for micropropagated plants in different species by using RAPD markers. Rani and Raina (1998) studied RAPD profiles generated by 20 arbitrary primers for micropropagated plants and their corresponding mother plants in *Eucalyptus tereticornis* and *E. camaldulensis*. Scores produced from the amplification profiles indicated the presence of monomorphic bands between mother plant and the progenies.

Out of the twenty different decamer RAPD primers used, three primers produced good amplification products that were monomorphic across all the micropropagated plants of *Paulownia tomentosa* steud (Rout *et al.*, 2001). Salvi *et al.* (2002) compared the amplified products of 6 control plants against those from 11 micropropagated plants in *Curcuma longa* using 16 arbitrary primers. Control and micropropagated plants showed similar RAPD profile, which confirmed that no polymorphism was observed in the micropropagated plants.

Leva *et al.* (2002) reported genetic stability in the micropropagated plants of Italian olive cultivar (maurino) using RAPD markers. Similarly, Shu *et al.* (2003) compared RAPD profiles of mother plants of *Robinia pseudoacacia* with the micropropagated plants using 25 arbitrary primers and presence of monomorphic bands established the regenerants to be genetically stable. 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 shoot propagation. 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 either within or between the embryogenic lines established.

In *Curcuma longa*, RAPD analysis carried for *in vitro* multiplication cultures at six month interval using 20 arbitrary decamer primers up to 2 years showed monomorphic bands indicating the no variation in the micropropagated plants when compared with control plants (Panda *et al*., 2007). In contrast, Peyvandi *et al*. (2009) analysed long term micropropagated shoots of *Olea europeae* L. (cv. Dezful) with 20 RAPD decamer primers and they revealed that, polymorphism increases as the number of sub-cultures increased.

2.4.2 Genetic fidelity of *in vitro* regenerated plants by using ISSR primers

The major limitations of RAPD and AFLP methods are of its low reproducibility and high cost respectively. Compared with the widely used RAPD markers, ISSR has several advantages particularly in reproducibility, cost effective and informativeness (Yang *et al*., 1996; Nagaoka and Ogihara, 1997). ISSR technique involves amplification of DNA segment present at an amplifiable distance in between two identical microsatellite repeat regions oriented in opposite direction. ISSRs have high reproducibility possibly due to the use of longer primers (16-25 mers) as compared to RAPD primers (10-mers) which permits the subsequent use of high annealing temperature (45-60° C) leading to higher stringency. ISSR primers can be di-nucleotide, tri- nucleotide, tetra- nucleotide or penta- nucleotide. The primers may be either unanchored or more usually anchored at 5′ or 3′ end with 1 to 4 degenerate bases extended into the flanking sequences (Zietkiewicz *et al*., 1994). ISSRs segregate mostly as dominant markers (Gupta *et al*., 1994; Tsumara *et al*., 1996). However, they have also been shown to segregate as co-dominant markers (Wang *et al*., 1998; Sankar and Moore, 2001).

ISSRs have an important role in securing plant variety rights by virtue of its unique efficiency in distinguishing even closely related germplasm (Reddy *et al*., 2002). This technique is also useful in areas of genetic diversity, phylogenetic studies, genome mapping and evolutionary biology in a wide range of crop species.
ISSR markers are highly efficient to ascertain the clonal fidelity of tissue culture raised progenies. Very limited literatures are available regarding genetic fidelity studies particular to bamboo species. Negi and Saxena (2010) employed ISSR markers to validate the clonal fidelity of in vitro raised *B. balcooa* plantlets multiplied by enhanced axillary shoot proliferation up to 33 passages. Fifteen ISSR primers generated a total of 99 amplicons among the tissue cultured progenies. Analysis of ISSR patterns revealed that the bands were shared by both the parent clump and the in vitro raised plants confirmed the genetic stability. Similarly in *B. nutans*, clonal fidelity was conducted by using ISSR technique and authors confirmed clonal uniformity among plantlets regenerated through axillary shoot proliferation up to 27 passages (Negi and Saxena, 2011). In contrast, Mehta *et al.* (2011) reported that, 98.8% genetic stability with 1.2% off-type was confirmed by using AFLP method among plantlets regenerated through somatic embryogenesis in *B. nutans*.

Various reports also cited in view of population genetics and genetic diversity study in bamboo species by using different DNA marker techniques. Nayak and Rout (2005) studied isolation and characterization of microsatellites in *B. arundinacea* and cross species amplification in other bamboos. Das *et al.* (2005) investigated two species-specific SCAR markers, ‘Balco 836’ for *B. balcooa* and ‘Tuldo 609’ for *B. tulda*, were developed by designing primers from sequenced putatively species-specific RAPD bands. To gain a better understanding of gene expression in bamboo (*B. edulis*), Lin *et al.* (2006) used a combination of suppressive subtractive hybridization (SSH), microarray hybridization analysis, sequencing and bioinformatics to identify bamboo genes differentially expressed in a bamboo albino mutant. Genetic similarity among 12 accessions of *Melocanna baccifera* from Mizoram, India was reported by using RAPD and ISSR profiles (Lalhrualiluanga and Prasad, 2009).

ISSR markers also been successfully used to detect somaclonal variation in several plants species. Palombi and Damiano (2002), when analyzing micropropagated plants of kiwi fruit, 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 whereas ISSR markers could detect genetic variation. 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 Robinia ambigua var idahoensis were studied using 32 selected ISSR primers. 24 bands out of the 226 reproducible bands produced 10.62% polymorphism and pointed the occurrence of a low level of genomic variation in the micropropagated plants (Guo et al., 2006).

Joshi and Dhawan (2007) employed ISSR markers to verify the genetic fidelity of micropropagated Swertia chirayita plantlets regenerated by axillary shoot proliferation and multiplied up to 42 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. 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. 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 regenerated plantlets of dessert banana cultivar (Nanjanagudu Rasabale) with 50 RAPD and 12 ISSR primers, no genetic variation observed by Venkatachalam et al. (2007).

The above all literatures clearly demonstrated that use of DNA marker techniques like RAPD, ISSR and AFLP could be used to detect somaclonal variation in different micropropagated plants as well as genetic diversity among the cultivars.

2.5 Growth performance of in vitro regenerated plants at nursery stage

Reports are scanty regarding field performance of axillary derived micropropagated plants and subsequently compared with somatic embryo plants. However, except few preliminary reports without much detail regarding synergistic study between axillary and somatic embryo derived plantlets. In B. balcooa, no significant
differences in number of culms and culm width between somatic embryogenesis and axillary shoot proliferated plants were observed after 18 months (Gillis et al., 2007). Sood et al. (2002) reported growth performance of in vitro and nodal cutting raised plantlets in the field. As evident from report, the in vitro raised plants of D. hamiltonii showed better performance in number and height of culms than cutting raised plantlets after one year.

2.5.1 Field trials

The micropropagation of elite genotypes leads to production of clonal planting stock in mass scale for commercial plantation as well as in afforestation programmes. This has led to a considerable investment in micropropagation industry. The potential benefits of tissue culture plants can be realised by field testing of progenies. However, there are few reports of such field performance tests in bamboo species.

Mascarenhas et al. (1988) reported that the culm formation of 30 month old micropropagated plants was similar to that of 48 month seedling-derived plants of D. strictus. They also observed that height of the culm, number of nodes in each main culm and girth of the internode was nearly double in tissue culture plants than those of seedling-derived plants.

High transplantation and survival rates have also been reported by Saxena (1990) in B. tulda (80-90%) and by Lin and Chang (1998) in B. edulis (100%). Tissue culture raised seedling plants in D. hamiltonii performed relatively better growth than those of vegetatively propagated (nodal cuttings) plants in field trials (Sood et al., 2002). In B. wamin, 4-5 months old plants derived from axillary shoot proliferation were planted in the field. The survival rate of the rooted plant in the field was almost 92% and the micropropagated plants were morphologically uniform and similar to the mother plant with bulbous internodes (Arshad et al., 2005). Similarly, Lin et al. (2007) reported that the axillary shoot proliferated plants were taller and produced more shoots as compared to vegetatively propagated plants of B. oldhamii. Somashekar et al. (2008) conducted field trial for micropropagated plants of P. stocksii at Gottipura, Bangalore. They reported 100% survival after ten months of planting. Agnihotri et al. (2009) revealed that
plant height, number of culms and leaf area significantly increased with almost six-fold between 1 year and 6 months old plants in field raised through axillary shoot proliferation of *D. hamiltonii*. Negi and Saxena (2011) reported that among 12 plants (regenerated through axillary shoot proliferation) were transplanted in field, resulted in 100% survival and the culms attained a height of 202 cm within 9 months in *B. nutans*.

Apart from bamboo species, considerable field trials have been reported in Eucalyptus also. Sreedhar and Rao (1998) developed commercial scale micropropagation technique for *E. citriodora* and introduced in field for evaluation. They recorded growth performance after 13 months of plantation and found that micropropagated clones were distinctly superior and highly uniform. Biswas *et al.* (1999) also reported field trial of tissue culture raised *E. tereticornis* and they revealed that environmental factors are plays key role to the developmental stages in *Eucalyptus*. Similarly, Arya *et al.* (2009) reported both the *Eucalyptus* hybrids FRI-5 and FRI-14 derived from tissue culture technology performed well up to 3 years in terms of height, diameter, clear bole length and self-pruning capability at all three respective plantation sites in Uttarakhand state (Dehradun, Pantnagar and Haldwani).