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In Vitro Regeneration of Bamboo Species

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Abstract – This review paper discussed about publications related to micropropagation of bamboo species. In recent years, the application of tissue culture technique like *in vitro* micropropagation has been used to meet the demands for bamboo planting materials. In the past 30 years, protocols for micropropagation of various bamboo species have been established by researchers from all over the world. The controlling factors for cultures such as the explants, culture medium, carbon sources, combination and concentration of plant growth regulators and other additional additives are varied. The controlling factors are crucial in developing successful regeneration protocols for various bamboo species. This paper attempts to review and summarize the available and up-to-date information regarding *in vitro* micropropagation of bamboos.

Keywords: Callus, organogenesis, plant growth regulators, somatic embryogenesis

Introduction

Bamboo, a woody perennial plant, is one of the largest members of the subfamily Bambusoideae (Chaowana, 2013). Over 1,200 species of bamboo are distributed mainly in tropical and subtropical countries (Lobovikov et al., 2007; Singh et al., 2013a). They are fast growing, renewable and annually harvestable, which makes it a good wood alternative for building material, furniture, handicraft, pulp and paper, biomass energy, food sources, as well as for erosion control (Wong, 1995; Chaowana, 2013). In the global market report by FMI (2017) bamboo is expected to be valued at US$ 3.6 billion by 2017 and projected to be reaching US$ 10 billion by 2027(FMI 2017). To fulfill the huge global demands, bamboo plantations are now in the need.

Propagation through seeds is not the preferred route because the majority of bamboo species have long and unpredictable flowering cycle, and due to their monocarpic nature, they exhibit poor seed setting and low seed viability (Singh et al., 2013a). Propagation through clump divisions, rhizomes, offsets and culm cuttings are vegetative techniques being practiced but these methods suffer from serious drawbacks such as difficulties in handling the bulky materials (Sharma & Sarma, 2013) when large-scale propagation is the main aim. Most of the conventional techniques are efficient for smaller scale production (10,000 plants per year), for large-scale propagation (100,000 – 500,000 plants per year), classical technique is greatly insufficient and inefficient, thus tissue culture is the only viable method (Gielis & Oprins, 2002).

Starting from the 1980's, there are many research papers documenting *in vitro* micropropagation technique for bamboo species, especially from India and China. This paper is to review and summarize the available and up-to-date information regarding *in vitro* micropropagation of bamboo

species. The focus will be on the parameters such as types of explants used, sterilization procedure, types of growth medium, and plant growth regulators used for different stages.

Organogenesis

Surface sterilization

Among the common sterilants used for surface sterilization, mercuric chloride ($HgCl_2$) has shown better effect over sodium hypochlorite (NaOCl) and hydrogen peroxide (H_2O_2). Aseptic cultures were obtained as high as 90-95% in *Dendrocalamus asper* (Singh et al., 2012a), 85% in *Bambusa balcooa* (Negi & Saxena, 2011a), and 80% in *Bambusa nutans* (Negi & Saxena, 2012b) with $HgCl_2$. $HgCl_2$ was superior to NaOCl in *Bambusa tulda* (Mishra et al., 2008), however there was no significant variations among concentrations of 0.05, 0.1 and 0.2%. Wei et al. (2015) reported 0.1% $HgCl_2$ was effective over 2% NaOCl and 10% H_2O_2 in *Bambusa ventricosa*. The results showed shorter exposure time to $HgCl_2$ (5 minutes) was effective with lower microbial infection and higher survival rate. However, exposure duration extended from 5 to 10 minutes in 0.1% $HgCl_2$ was effective to eliminate bacterial and fungal contaminant in *Dendrocalamus strictus* (Goyal et al., 2015).

Explants

Various parts of the bamboo can be used as the explants for culturing, however the most frequently used are the nodal segment from axillary branch, and some were using aseptically germinated seedling (Kapoor & Rao, 2006; Venkatachalam et al., 2015). Mudoi et al. (2014) reported that the 5th to 7th node having active axillary bud showed maximum regeneration potential in *Bambusa nutans*; while Saxena and Bhojwani (1993) reported that the mid culm nodes of secondary branches are the most suitable explants for *Dendrocalamus longispatus*. They also found that bud-break frequency in *D. longispatus* was greatly influenced by the juvenility of lateral shoots, position of axillary bud on the branch, and the season in which explants were collected. Explants collected during spring (February-April) gave better response in terms of lower contamination, early bud break with higher number of shoots in *D. asper* (Singh et al., 2012a, 2012b). Pre-monsoon (May-June) was recorded best for *D. asper* in terms of response to culture condition but with higher contamination rate (Banerjee et al., 2011). Explant collection and culture establishment on early summer (April-June) was found to be the best period for *D. hamiltonii* (Singh et al., 2012c). Mudoi et al. (2014) reported that early autumn (Sept-Oct) was the best timing for explant collection of *B. nutans*. Ramanayake and Yakandawala (1997) recorded peak bud break of explants in the month just before the onset of the two monsoon rains (south-west and north-east monsoons). The explants collected during July-August showed far better rooting response (Chaturvedi et al., 1993).

Medium

Murashige and Skoog (MS) medium (1962) has been widely used for both direct and indirect organogenesis of bamboo species. Singh et al., (2012c) compared the effect of different media such as MS, SH (Schenk & Hildebrandt, 1972), B5 (Gamborg, 1968) and NN (Nitsch & Nitsch, 1969) on axillary bud break for *D. hamiltonii* and found better response in MS medium. Similarly, *D. aspe*r and *D. hamiltonii* shoots cultured on half-strength MS medium gave better rooting response compared to the other nutrient strength tested (Singh et al., 2012a). However, half-strength MS medium was less efficient for *in vitro* rooting of *D. strictus* compared to full-strength MS medium (Goyal et al., 2015).

In general, agar solidified medium gave better growth response for tissue culture of bamboo species. However, several researchers have reported better shoot multiplication and growth rate in liquid medium compared to semi-solid medium (Saxena, 1990; Bhojwani, 1993; Sood et al., 2002; Das & Pal, 2005; Shirin & Rana, 2007). Addition of gelling agent in nutrient medium resulted in stunted shoots of *B. balcooa* (Negi & Saxena, 2011a) and reduced multiplication rate and the shoot length of *B. nutans* (Negi & Saxena, 2011b). The poorer shoot multiplication on semi-solid medium over liquid medium may be due to the fact that solubilized agar tends to binds water, absorbs nutrients and plant growth regulators (PGRs) from the medium which will results in the reduced uptake of nutrients, PGRs and other essential constituents by cultured tissues. On the other hand, reduced multiplication rates of shoots have also been recorded in liquid medium due to vitrification (Saxena & Bhojwani,

1993). Gantait et al. (2016) reported that establishment of explants was best observed in semi-solid medium whereas liquid medium has superior result than semi-solid for proliferation and rooting.

Plant Growth Regulators

6-Benzylaminopurine (BAP) is the most common cytokinin used in nutrient medium to induce bud break, axillary bud release and to enhance shoot multiplication of most bamboo species. However, higher concentration of BAP had reverse effects on *in vitro* shoots such as lowering the bud sprouting, shoot multiplication rate and formation of stunted shoots (Sanjaya et al., 2005; Jimenez et al., 2006; Ramanayake et al., 2006; Banerjee et al., 2011; Mehta et al., 2011; Negi & Saxena, 2011b; Kalaiarasi et al., 2014; Waikhom & Louis, 2014; Goyal et al., 2015; Gantait et al., 2016). Kalaiarasi et al. (2014) reported that BAP was more effective than Kinetin (KIN) for bud initiation in *B. arundinacea*. Same results were obtained in culture initiation and *in vitro* shoot multiplication of *D. asper* (Singh et al., 2012a), *D. strictus* (Goyal et al., 2015) and *B. balcooa* (Gantait et al., 2016).

Synergistic effect of two cytokinins i.e. BAP & KIN were proven to be effective in bud break of *B. balcooa* and *B. nutans* (Negi & Saxena, 2011a, b) and increased shoot multiplication in *B. glaucescens* (Shirin & Rana, 2007). By incorporating cytokinins in combination, Waikhom and Louis (2014) obtained better shoot multiplication as well as better quality of shoot texture in culture initiation of both *B. nutans* and *M. baccifera*. Similar synergistic effects were reported by Negi and Saxena (2011b) when shoot induction medium of *B. nutans* were fortified with combination of cytokinin and auxin. They found that incorporation of auxins such as Indole-3-acetic acid (IAA) or 1-Naphthaleneacetic acid (NAA) with cytokinins BAP or KIN increased the length of shoots but lowered their multiplication rate. Instead the use of Indole-3-butyric acid (IBA) showed better response in increasing the shoot multiplication rate.

In general, IBA is the most commonly used auxin for *in vitro* rooting of bamboo shoots. Nutrient medium supplemented with IBA alone was effective in root induction of *B. vulgaris* (Ramanayake et al., 2006) and *D. asper* (Banerjee et al., 2011). Even though auxin IAA was essential in root development of *B. balcooa* and *B. nutans* (Negi & Saxena, 2011a, b), Banerjee et al. (2011) found that IAA was inept for root initiation and elongation of *D. asper* shoots. On the other hand, Mudoi et al. (2014) reported both IAA and IBA had no effect on enhancing the rooting ability of *B. nutans* shoots. IBA was found to be superior in terms of root development compared to NAA and IAA in *B. arundinacea* (Venkatachalam et al., 2015), in *B. balcooa* (Das & Pal, 2005) and in *Pseudoxytenanthera stockii* (Sanjaya et al., 2005). Increased level of NAA (5 mg/L) resulted in limited root development as well as reduction of root length and number in *B. nutans* (Mudoi et al., 2014). The use of single auxin had no positive effect in root development of *D. asper* shoots (Singh et al., 2012a). Thus, maximum rooting response was only obtained after incorporation of IBA and NAA in combination. Even though Goyal et al. (2015) reported that NAA induced higher number of roots in *D. strictus*, shoots the combination of 1 mg/L IBA + 3 mg/L NAA further enhanced their root length.

Pretreatment of *B. vulgaris* var. striata *in vitro* shoots with 0.5 mg/L Thidiazuron (TDZ) for 3 subculture cycles before being transferred to rooting medium supplemented with 3 mg/L of IBA, successfully increased rooting response from 40% to 100% (Ramanayake et al., 2006). Coumarin had crucial effect in rooting ability of *B. tulda* (Saxena, 1990), *D. longispathus* (Saxena & Bhojwani, 1993) and *D. giganteus* (Ramanayake & Yakandawala, 1997). Coumarin was used to stimulate IAA synthesis and further increase the endogenous-free IAA level during the induction phase of rooting to initiate more roots. However, it also inhibited the root length in higher concentration. Two step rooting methods had been introduced in some studies. Agnihottri and Nandi (2009) initially introduced the propagated shoots into MS medium with IBA for 10 days and transferred to an IBA-free medium for 2 weeks which resulted in more than 90% of rooting. Bag et al. (2000) recorded 100% of rooting in *Thamnocalamus spathiflorus*, after culturing the shoots on medium supplemented with 30.5 mg/L of IBA for 2 weeks, followed by PGR-free medium for 8 weeks. Some common techniques involved in *in vitro* propagation of bamboo species were summarized in Figure 1.

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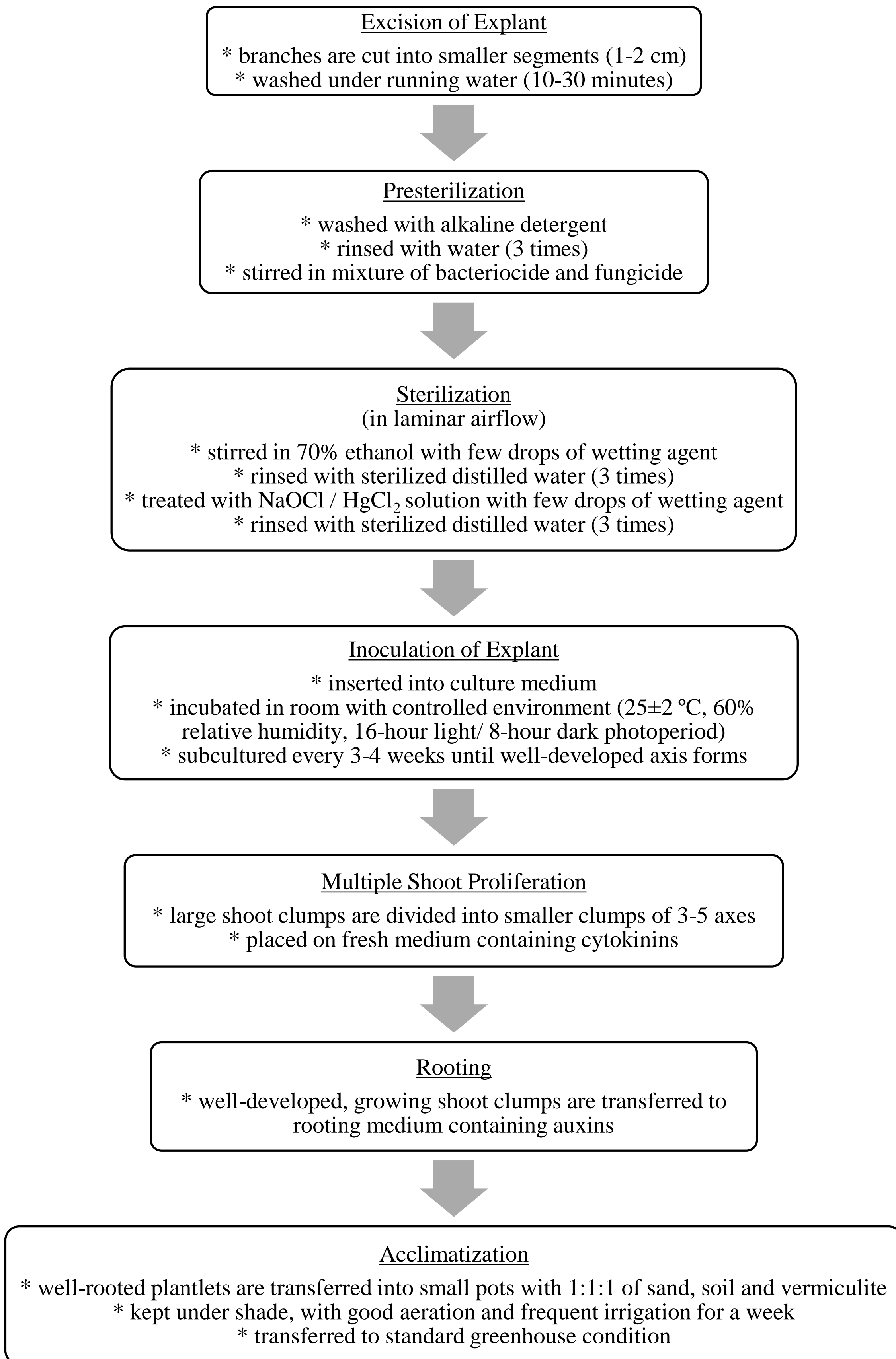

Figure 1. Standard procedures of *in vitro* propagation of bamboo species.
Note: NaOCl – sodium hypochlorite; $HgCl_2$ – mercuric chloride

Other additives

Significant control of browning with enhanced shoot multiplication was achieved using ascorbic acid in *D. hamiltonii* while polyvinylpyrrolidone (PVP) and activated charcoal were ineffective in doing so (Singh et al., 2012c). On the contrary, PVP improved shoot health in *D. strictus* cultures (Saxena & Dhawan, 1999) and control phenol production from axillary buds of *B. glaucescens* in liquid medium (Shirin & Rana, 2007). Addition of coconut water in *B. balcooa* medium had a promoting effect on cultures (Negi & Saxena, 2011a), however higher level of more than 2.5% (v/v) promoted shoot vitrification and reduced shoot proliferation. Addition of adenine sulfate (AdS) in shoot induction medium together with BAP enhanced shoot proliferation and increased the number of *D. asper* shoots (Banerjee et al., 2011). Similarly, a significant increment in the shoot multiplication rate with healthier culture was noticed by Singh et al. (2012a) when AdS was added into *D. asper* culture. Glutamine had been incorporated into shoot multiplication medium as a source of reduced nitrogen for *in vitro* cultures of bamboo species (Sanjaya et al., 2005; Mishra et al., 2008). The use of glutamine in the medium promoted axillary growth and minimized leaching problem in *Dendrocalamus giganteus in vitro* cultures (Sanjaya et al., 2005). Significance of the effects of silver nitrate ($AgNO_3$) in enhancing shoot multiplication was also observed in shoot multiplication of *B. arundinacea* cultures (Venkatachalam et al., 2015). However, the incorporation of $AgNO_3$ in shoot induction media consisting of BAP and KIN inhibited shoot multiplication of *B. arundinacea* (Venkatachalam et al., 2015).

Carbon Source

Sucrose is the most widely used carbon source in the bamboo cultures, with varying concentration of 2 to 6% and 3% being the most commonly used concentration for optimum growth in most bamboo species. Saxena (1990) found that 2% of sucrose was ideal for optimum shoot multiplication of *B. tulda*. In contrast, 1% of sucrose was found to be effective for better culture growth of *B. balcooa* (Brar et al., 2014) and 4% for *B. arundinacea* (Venkatachalam et al., 2015). Sucrose replaced with cheaper table sugar had imperceptible effect on shoot multiplication rate of *D. asper* cultures (Singh et al., 2012a).

Somatic Embryogenesis

Medium

MS medium was employed in most of the callus induction and regeneration procedures. Yuan et al. (2013) tested callus induction of *P. heterocycla* in 5 different media and found that MS yielded the highest relative numbers of callus and embryogenic callus. The other media resulted in reduced growth of calluses which easily turned brown. Zhang et al. (2010) also reported that MS was the best medium that resulted in best culture growth compared to NB (Wu & Chen, 1987) and HB (Sun et al., 1999) in propagation of *D. hamiltonii*. MS medium was found to be distinctively superior to B5 for multiplication and germination of embryos (Saxena & Dhawan, 1999). Godbole et al. (2002) used half-strength MS throughout the experiment, whereas Zhang et al. (2010) reported that half-strength MS improved the callus formation but the number of vigorous, granular and compact calluses which generate plantlets was reduced. Mehta et al. (2011) preferred full-strength MS in development of proliferated somatic embryos into plantlets. In germination phase, agar lowered the germination frequency drastically (30-50%) and retarded plant growth (Saxena & Dhawan, 1999).

Plant Growth Regulators

In somatic embryogenesis, 2,4-Dichlorophenoxyacetic acid (2,4-D) is the most common PGR used for callus induction and formation of embryogenic callus. Bag et al. (2012) incorporated the same amount of PGRs into medium for culturing both 10-year-old and 45-year-old of *D. hamiltonii* explants; both reacted to the hormone with 80% and 73% of callus induction rate, respectively. Mehta et al. (2011) reported that the explants showed no response to induce any callus at any concentration below or higher than 5 mg/L. Concentration of 2,4-D lower than 4 mg/L showed higher response in zygotic germination of *Phyllostachys heterocycla* seed but resulted lower callus induction (Yuan et al., 2013). Yuan et al. (2013) found that KIN and BAP inhibited induction of callus and completely blocked the

induction in concentration higher than 0.5 mg/L. TDZ was found to be the main factor influencing the callus formation compared to 2,4-D and NAA for *B. ventricosa* (Wei et al., 2015).

Zeatin was found to turn calluses completely green in 7-10 days and formation of embryos (35-37%) within 30 days, while BAP and KIN were both ineffective in differentiation and regeneration of *P. heterocycla* (Yuan et al., 2013). Elimination of 2,4-D and NAA from callus induction medium, while with corresponding increase of BAP concentration (up to 2.5 mg/L) elevated vigorous formation of somatic embryos in *D. hamiltonii* (Godbole et al., 2002), and further elimination of all PGR led embryos to mature and further germinated into plantlets. Prolonged sub-culturing on static MS liquid medium without PGRs resulted in rhizome induction after 8 weeks. Calluses transferred to PGR-free basal MS medium developed 89.6% somatic embryos of *B. balcooa* within 4-6 weeks' time (Gillis et al., 2007). PVP control phenolic oxidation in multiplication phase (Saxena & Dhawan, 1999). Incorporation of PVP into the germination medium also enhanced the frequency of somatic embryos.

Carbon Sources

Commonly sucrose has been incorporated in the medium to enhance the callus formation, regeneration as well as differentiation. In regeneration phase, maltose promoted greening of embryogenic calluses better than sucrose, however further incubation in maltose caused death of callus. While sucrose supplementation showed bud differentiation in 20 days and plant regeneration in 30 days (Yuan et al., 2013). In comparison, fructose, glucose and lactose showed vigorous growth of calluses, but with no differentiation or regeneration. Godbole et al. (2002) tested various sucrose levels in embryo maturation and germination and reported the optimal concentration of 8%. Any relatively lower concentration of sucrose of 2-6% caused albino plantlets. For the same maturation and germination phase, Mehta et al. (2011) recorded that long-term maintenance of the culture in medium with 3% of sucrose resulted in browning of somatic embryos. However, the problem of browning was not observed under the medium supplemented with 2% of glucose.

Discussion

The crucial challenge in micropropagation of forest trees is to maintain the elite selected characteristics, especially propagation via somatic embryogenesis, as somaclonal variation can occur during callus induction. In addition, micropropagation via axillary bud too had the same risk of losing the elite characteristics of the mother plant. For example, propagation of *B. ventricosa* via axillary buds caused loss of the characteristics of bulbous internode (Wei et al., 2015). Recently, the research papers of *in vitro* propagation protocol of bamboo species included genetic fidelity analysis as the researchers looking into producing true-to-type tissue culture plants.

The analysis of *D. strictus* with RAPD and ISSR showed nearly no variability among the micropropagated plantlets and conclusion made that the *in vitro* propagated plants bypass the genomic aberrations and did not promote any somaclonal variation (Goyal et al., 2015). Similar results found in clonal plants of *B. balcooa* (Brar et al., 2014) and *B. arundinacea* (Kalaiarasi et al., 2014). Via ISSR analysis, Negi and Saxena (2011b) had proven the genetic uniformity of clonal plants reached 27 passages. Singh et al., (2013b) did an assessment on the genetic stability of *in vitro* raised plants from Singh et al. (2012a) by analyzing the regenerated plantlets and mother plant of *D. asper* with DNA-based markers. Genetic stability has been confirmed (at least up to 30 passages/ 2 years), and protocol developed was approved for commercial scale utilization. Similar result was reported in micropropagated plants of *D. hamiltonii* (Singh et al., 2013a) and *B. balcooa* (Negi & Saxena, 2010).

Micropropagation via somatic embryogenesis has higher risk of having aberrations of regenerated plantlets, because somaclonal variation occurs during callus induction, multiplication of somatic embryos as well as in regeneration phase. The use of seed/ seedlings for induction of somatic embryo has higher disadvantages due to the unknown genetic background since seedling populations of bamboo are highly variable. The most common aberrations are albinism in plantlets that happens during regeneration phase of *D. strictus* (Saxena & Dhawan, 1999); occurrence of albinism and *in vitro* flowering in regeneration phase of *B. balcooa* (Gillis et al., 2007); and mosaic leaf, albinism, etiolated shoots, early *in vitro* flowering in *D. hamiltonii* (Zhang et al., 2010). After Gillis et al. (2007)

did a few fidelity analyses, they concluded that the tissue cultured plants through somatic embryogenesis are true-to-type. They recommended to use explants from mature bamboo plants rather than using seeds or seedlings to minimize the aberration. Furthermore, the subculture of callus should be limited to three times, due to longer period of maintaining callus cultures will increase the chances of somaclonal variation (Gillis et al., 2007).

Conclusion

It can be concluded that the factors controlling *in vitro* cultures vary with the species. As the exact mechanism of tissue culture remains widely unknown, various combinations of PGRs are essential to be tested in order to determine the optimal culture conditions. Moreover, the optimal concentrations of nutrients to be used rely not only on the feasibility or availability but also the cost incurred. Hence choosing the right composition of media could reduce the cost of production. Somaclonal variations that occur during *in vitro* culture could be avoided by choosing the suitable explants. However, the variation may provide a resource by creating new cultivars with certain favourable trait which then generate marketable value.

In terms of mass-producing clonal plants for commercial plantation, agroforestry, or industry usage, gaining true-to-type propagules is the main aim. In order to secure the elite characteristics of the donor plant, earlier assessment of clonal fidelity before field testing could greatly curtail the risk and cost of maintaining bamboo plants with genetic instability. Most of the reviewed papers were for experimental purposes with the lack of establishment in the field. Moreover, most of these techniques haven't been tested for clonal propagation for establishment of plantation. Therefore, the protocols described should be adapted and modified to establish and develop efficient protocol for *in vitro* micropropagation of other bamboo species.

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