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SEASONAL INFLUENCES ON *IN VITRO* BUD BREAK IN *DENDROCALAMUS HAMILTONII* ARN. EX MUNRO NODAL EXPLANTS AND EFFECT OF CULTURE MICROENVIRONMENT ON LARGE SCALE SHOOT MULTIPLICATION AND PLANTLET REGENERATION

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SUMMARY

A highly efficient and cost effective protocol for rapid *in vitro* propagation of *Dendrocalamus hamiltonii* Arn. ex Munro through multiple shoot formation from nodal explants, followed by mass scale production and field evaluation, has been developed after examining the effect of season, media type, carbon source, growth regulators and transplanting media on micropropagation. Early summer (April-June) was the best period for explant collection. Among the different media (B_5, MS, NN and SH) tested, MS was found to be the best for micropropagation. A multiplication rate of about 5.6-folds with healthy cultures was achieved by the 3rd subculture, when shoots were transferred every 3 weeks to fresh MS medium supplemented with 1.5 µM TDZ and 56.0 µM ascorbic acid. TDZ was found superior to BAP and kinetin for both axillary buds sprouting, as well as, shoot multiplication. Replacement of sucrose with table sugar during shoot multiplication did not affect the multiplication frequency. Optimal rooting of 89% was achieved on half MS medium supplemented with 25.0 µM IBA and 36.0 µM choline chloride. Regenerated plantlets were acclimatized and hardened in green house using dune sand and vermi-compost (3:1) with 79.76% success, and successfully transferred to the field with ~85% survival rate. More than 3000 tissue culture raised plantlets have been successfully transferred to approximately 7.5 hectares of land. A cost effective method of clonal propagation of *D. hamiltonii* with a better field survival rate has been developed.

Key words: **Ascorbic acid, axillary bud proliferation, choline chloride, commercial propagation, *Dendrocalamus hamiltonii*, micropropagation, thidiazuron**

INTRODUCTION

Bamboos belonging to grass family Poaceae subfamily Bambusoideae include approximately 80–90 genera and 1000–1500 species (Lin *et al.* 2006). These have been resolved as a monophyletic group, consisting of herbaceous and woody bamboos (Clark *et al.* 1995). However, phylogenetic analyses have implied that woody bamboos are not monophyletic and could be geographically divided into temperate and tropical woody bamboos, the latter having a sister relationship to herbaceous bamboos (Sungkaew *et al.* 2009). The bamboos are distinguished from other species by their woody stem and infrequent sexual reproduction, with long flowering intervals, as long as 120 years (Janzen 1976). This group is of a notable economic significance

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and form the backbone of rural economy of many Southeast Asian countries. Recently, bamboos have become an important component of social forestry programs also due to their fast growth, and ability to be harvested periodically after 4-5 years of growth on sustainable basis.

Dendrocalamus is a tropical genus of giant clumping bamboo which includes many species and varieties mainly confined to the Indian subcontinent throughout Southeast Asia. *Dendrocalamus hamiltonii* (Maggar bamboo) is native to the Northeastern Himalayas and is distributed in the North-Western Himalayas, Sikkim, Arunachal Pradesh, Assam, Manipur, Meghalaya, Mizoram, Nagaland and Tripura in India, and also neighbouring parts of Bhutan and Bangladesh (Rao and Ramakrishnan 1988). The distinguishing features of this species are brown pubescent culm with bent top in mature culms, root verticils that are seen in almost all the nodes of the culm, largely broad ovate branch buds and zig-zag internodes in some culms (Alam 1982).

Enormous reduction in natural stocks of *D. hamiltonii* due to over-exploitation and increased demand beyond availability has been observed. Harvesting of the culms before seed formation, irregular and poor seed production, long flowering cycle, seed sterility and short viability of seeds has limited the propagation of *D. hamiltonii* through seeds. Limited availability of offsets and rhizomes is a major bottleneck in vegetative propagation of this species through conventional methods. Therefore, alternative methods are required for its mass multiplication, conservation and survival. Reports are available on the *in vitro* multiplication of *D. hamiltonii* through axillary shoot proliferation using juvenile (Chambers *et al.* 1991) and mature explants (Sood *et al.* 2002a and 2002b, Agnihotri and Nandi 2009, Agnihotri *et al.* 2009) as well as somatic embryogenesis (Godbole *et al.* 2002, Sood *et al.* 2002a, Zhang *et al.* 2010). However, limited number of plantlet production has been achieved in all the above studies due to inconsistency in rooting of shoots (Sood *et al.* 2002a and 2002b). Hardening and acclimatization of *in vitro* raised plants to *ex-vitro* conditions remains another limiting step in lab to land transition of tissue culture technology in this species. These studies have demonstrated the feasibility of micropropagation technology in *D. hamiltonii,* however, more efforts are required to develop a cost effective protocol for mass multiplication so that the plants may be produced for large scale plantations. Therefore, the present study was conducted to develop a cost effective and highly efficient protocol for mass multiplication of *D. hamiltonii* on commercial scale followed by field evaluation of *in vitro* raised plants.

MATERIAL AND METHODS

Young branches collected from 6 year-old *D. hamiltonii* clumps (Fig. 1a), established at our centre, using vegetative propagules from 30-35 year-old clumps were used in the present study. Shoots were cut into small pieces (2.5 to 3 cm long) each with a single node and used as an explant. These nodal segments were rinsed with 3% (v/v) teepol for 10 min followed by 15 min wash with 0.2% antibacterial agents (90% streptomycin sulphate + 10% tetracycline hydrochloride) and with 0.2% fungicide (Bavistin) solution. Thereafter, the explants were thoroughly washed with sterile distilled water and treated with 0.1% $HgCl_2$ for 5 min followed by rinsing with 70% alcohol (2 min) and sterile distilled water (5 times) under aseptic conditions in the laminar air flow.

To determine the most suitable hormonal combination for explant establishment and bud proliferation, sterilized explants were cultured on MS medium supplemented with BAP, KIN (5-20 µM) and TDZ (0.75-4.5 µM), singly as well as, in combination with NAA (1-5 µM). Similarly, for the selection of most suitable basal medium, sterilized explants were cultured on MS (Murashige and Skoog 1962), SH (Schenk and Hildebrandt 1972), B_5 (Gamborg *et al.* 1968) and NN (Nitsch and Nitsch 1969) media supplemented with 3.0 µM TDZ. To study the impact of season of explant collection, nodal segments were collected in the first week of each month of the year and cultured on MS medium supplemented with 3.0 µM TDZ. For multiplication, clumps of shoots initiated from nodal explants were cultured on MS medium supplemented with different concentrations of BAP (5.0-15.0 µM) and TDZ (0.75-3.0 µM) alone and TDZ (1.5 µM) in combination with ascorbic acid (28.0-84.0 µM), PVP (0.1%) and activated charcoal (0.02%). The effect of carbon source on shoot multiplication was also studied by culturing shoot

propagules on MS medium supplemented with either 3% sucrose or table sugar or glucose.

For rooting, 3 to 5 cm long shoots obtained from the cultures multiplied on MS + 1.5 µM TDZ + 56.0 µM ascorbic acid were cultured on MS medium supplemented with various auxins viz. IAA, IBA and NAA (5.0-50.0 µM) singly, as well as, IBA (25.0 µM) in combination with choline chloride (7.2-50.4 µM), a growth inhibitor and coumarin (6.8-34.0 µM), a phenol. Shoots cultured on MS medium without growth regulators served as control. Shoots were also cultured on ¼, ½, ¾ and full strength MS medium supplemented with 25.0 µM IBA and 36.0 µM choline chloride each to determine the most suitable strength of MS medium for rooting.

The rooted shoots (5 to 8 cm long) were washed thoroughly in running tap water and transferred to seedling trays containing different types of transplanting media viz. soil, river bed sand, dune sand and dune sand with farm yard manure (FYM)/vermi-compost. These were irrigated with half strength MS minerals and maintained in the mist chamber for 2 to 3 weeks. The plants were then transferred to perforated poly bags containing dune sand and vermi-compost (3:1 ratio) for further growth till field transfer. During field transfer, the plants were subjected to various treatments of manures viz: FYM, urea and FYM + urea to find out their impact on the growth of plants under natural conditions.

All the media used in the present study were fortified with 3% sucrose and 0.8% agar. The pH of the media was adjusted to 5.8 prior to autoclaving at 121ºC for 20 min. All the cultures were maintained at 25 ± 2°C under 16 h light photoperiod. For each experiment, 18 explants per treatment were used and each experiment was repeated twice. Observations were made up to four weeks of inoculation and data on percent explant response, days required for axillary bud proliferation, shoot number/explant and shoot length were recorded. For rooting experiments, percent rooting, root number and root length were measured. Similarly, various growth parameters like number and height of culms, number of internodes/culm and culm diameter were recorded for the field transferred plants. Data were subjected to statistical analysis for standard error using SPSS software.

RESULTS AND DISCUSSION

Culture initiation: The morphogenetic potential of explants derived from mature trees holds great commercial value as it can be applied directly for multiplication of elite genotypes and cultivar improvement in silviculturally important species. Progressive specialization of the tissues with age reduces the plasticity and capability of the cells to dedifferentiate, therefore, establishment of *in vitro* cultures from mature tissues remains a major challenge to plant biologists. Plant regeneration from pre-existing axillary meristems usually results in the formation of genetically identical and stable populations free from somaclonal variations. Presence of genetic fidelity among *in vitro* raised plants of *D. hamiltonii, Bambusa balcooa* and *B. tulda* raised from axillary buds has been confirmed using RAPD markers (Agnihotri *et al.* 2009, Das and Pal 2005). Therefore, axillary buds collected from mature culms formed an ideal material for *in vitro* propagation and field evaluation of *D. hamiltonii* in the present study.

Surface sterilization of the explants with 0.1% $HgCl_2$ for 5 min was most suitable for obtaining aseptic (90 to 95%) and viable cultures. Nodal explants cultured on MS basal medium without any growth regulators failed to respond, however, addition of cytokinins led to sprouting of multiple shoots from nodal buds (Fig. 1b-e). KIN (5-20 µM) was least effective towards axillary bud proliferation and supported very poor or no response (Fig. 1b) followed by BAP which showed 93.2% bud break with 2.76 shoots/explant after 7 days of inoculation at 15 µM concentration (Fig. 1d, Table 1). However, MS medium supplemented with 3.0 µM TDZ was most effective for bud break (98.8%) with 5.23 shoots/explant after 4.9 days of inoculation (Fig. 1e). The bud break response declined with increased (4.5 µM), as well as, decreased (0.75-2.5 µM) concentrations of TDZ. High concentration of BAP and TDZ reduced the percent bud break, as well as the number of shoots per explants, while the number of days required for bud break increased (Table 1). A high level of cytokinins is known to induce programmed cell death in cell cultures, and yellowing of leaves and reduced root mass in intact plants (Carimi *et al.* 2003). Moreover, cytokinins often promote ethylene biosynthesis (Abeles *et al.* 1992) thereby

adversely affecting growth. The results obtained in the present study are contrary to the earlier findings of Sood *et al.* (2002a) and Agnihotri *et al.* (2009) who reported axillary bud break from nodal explants of *D. hamiltonii* on hormone free MS medium. TDZ is a potent bioregulant of *in vitro* morphogenesis (Murthy *et al.* 1998) which exhibits strong cytokinin like activity and promotes the proliferation of axillary shoots (Lin *et al.* 2007), adventitious organ regeneration (Lin *et al.* 2006), as well as, somatic embryogenesis (Lin *et al.* 2004). Addition of NAA along with cytokinins did not improve the response of explants towards *in vitro* conditions (Fig. 1c) contrary to the report of Agnihotri and Nandi (2009) and Agnihotri *et al.* (2009) in *D. hamiltonii* and Vadawale *et al.* (2006) in *Vitex negundo*. The variable response of different species and genotypes to growth regulator supplemented media may be due to different endogenous levels of respective growth regulators. The inhibition of shoot formation may be due to action of auxins accumulated at the basal end of the explants (Marks and Simpson 1994).

In the present study, nodal explants cultured on MS medium produced an average of 5.23 shoots per explant with 97.3% bud break within 5.7 days, which was significantly higher as compared to B_5, SH or NN media (Table 1). Similar preference for specific media formulations was also reported in *D. asper* (Singh *et al.* 2011). Poor response in B_5 medium was attributed to the presence of high ammonium and nitrate content which inhibit explant establishment and shoot multiplication (Constabel 1984). McCown and Sellmer (1987) reported that weaker salt formulations like WPM, GD, NN etc. can promote axillary bud development in forest trees however the same was not true for *D. hamiltonii*. Replacement of the agar solidified medium with liquid medium during axillary bud break from nodal explants was found more effective as also reported in the earlier studies of Nurul Islam and Rahman (2005), Diab and Mohamed (2008), Ogita *et al.* (2008) and Bisht *et al.* (2010) wherein liquid medium was used during explant establishment in *Bambusa balcooa*, *B. nutans*, *B. salarkhanii*, *B. vulgaris*, *Oxytenanthera abyssinica, Phyllostachys meyerii* and *Thyrsostachys oliveri*. However, replacement of the agar solidified medium with liquid medium during axillary bud break was not effective in *D. asper* (Singh *et al.* 2011).

Percent contamination as well as bud break and number of shoots per explant were significantly affected

Table 1. Axillary bud proliferation from nodal explants of *D. hamiltonii* in different basal media (with 3.0 µM TDZ) and MS media (with different cytokinins). Data recorded after four weeks of inoculation (±SE)

Basal medium/ Growth regulator	Days required for bud break	Bud break (%)	No. of shoots	Shoot length (cm)
Basal medium				
MS	5.66 ± 0.23	97.33 ± 1.03	5.23 ± 0.49	3.63 ± 0.29
B_5	9.23 ± 0.39	79.76 ± 3.13	2.34 ± 0.49	2.50 ± 0.40
NN	9.13 ± 0.21	76.90 ± 2.77	1.86 ± 0.22	2.30 ± 0.28
SH	10.43 ± 0.59	63.90 ± 3.08	1.70 ± 0.17	1.23 ± 0.34
Growth regulator (µM)				
None	-	0	0	0
BAP (5.0)	16.10 ± 2.06	57.20 ± 2.61	0.67 ± 0.33	1.50 ± 0.76
(10.0)	6.30 ± 0.51	73.60 ± 3.19	1.50 ± 0.29	2.47 ± 0.82
(15.0)	7.07 ± 0.23	93.20 ± 0.49	2.76 ± 0.27	3.10 ± 0.74
(20.0)	17.40 ± 0.67	81.63 ± 1.56	2.67 ± 0.21	2.60 ± 0.52
TDZ (0.75)	12.30 ± 0.98	81.66 ± 2.60	1.86 ± 0.30	1.33 ± 0.33
(1.5)	7.50 ± 0.25	93.87 ± 1.16	3.03 ± 0.38	2.06 ± 0.18
(3.0)	4.93 ± 0.33	98.76 ± 0.91	5.23 ± 0.29	2.17 ± 0.38
(4.5)	8.20 ± 0.59	98.07 ± 0.29	4.97 ± 0.12	3.03 ± 0.16

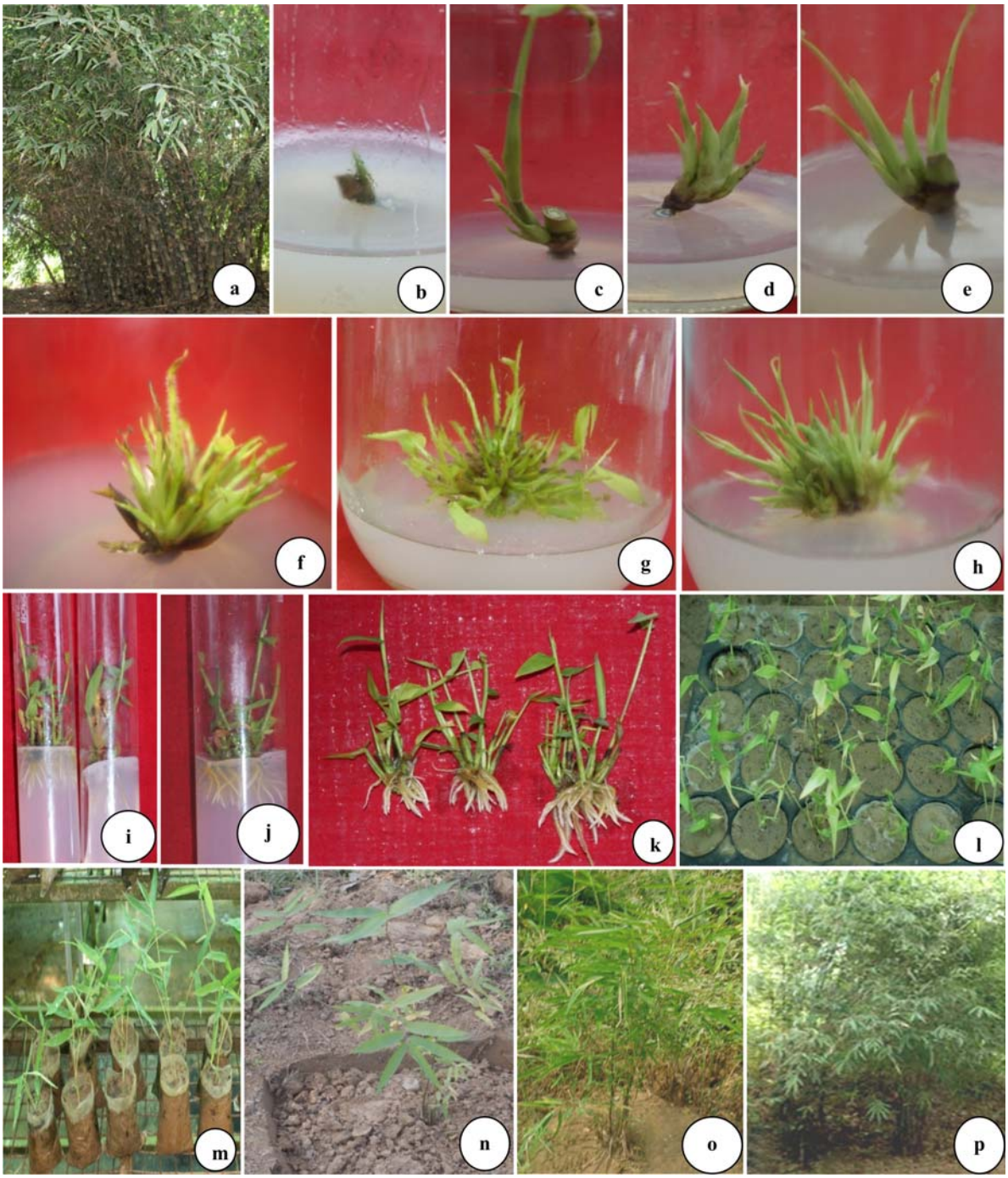

Fig. 1. *In vitro* propagation of *Dendrocalamus hamiltonii* using nodal explants. (a) a 6-year-old mother bush of *D. hamiltonii*, the source of explants, b - e axillary bud break on, **(b)** MS + 15 µM KIN, **(c)** MS + 5 µM BAP + 2.5 µM NAA, **(d)** MS + 10 µM BAP, **(e)** MS + 3.0 µM TDZ, f - h Multiplication of shoots on, **(f)** MS + 10 µM BAP, **(g)** MS + 1.5 µM TDZ, **(h)** MS + 1.5 µM TDZ + 56.0 µM ascorbic acid, i - k rooting of shoots on, **(i)** ½ MS + 25.0 µM IBA, **(j)** ½ MS + 25.0 µM IBA + 36.0 µM choline chloride, **(k)** rooted plantlets, **(l)** *in vitro* raised plants transferred to seedling trays for acclimatization in green house, **(m)** six-month-old acclimatized and hardened plants in the green house, **(n)** *in vitro* raised plant 6 months after field plantation, **(o–p)** tissue culture raised plants in the field after 1 and 3 years of plantation respectively.

by the time of explant collection during the year (Table 2). Collection of explants during early summer (April to June) gave better response in terms of lesser contamination (8.9 to 18.7%), early shoot initiation (5.0 to 7.6 days) and better percent bud break (94.5 to 98.9%) with higher number of shoots per explant (4.5 to 5.3). Young buds are known to produce more auxins during spring season and stimulate cell division in the cambium thus supporting increased cell division and growth (Funada *et al.* 2001). The months of October to December were highly unsuitable for *in vitro* establishment of nodal explants. Time of collection of explants has been reported to have significant influence on culture establishment and similar seasonal effects were also studied by Ramanayake *et al.* (1995) in *Dendrocalamus giganteus* and *Bambusa vulgaris*, Saxena and Dhawan (1994) in *D. longispathus* and Singh *et al.* (2011) in *D. asper*. Explants collected in the actively growing season were more responsive towards bud break (Yadav *et al.* 2008, Bisht *et al.* 2010, Singh *et al.* 2011). Fungal and bacterial pathogens surviving beneath the leaf sheath may lead to 40-50% contamination with moderate bud break during rainy season (July-September), and poor response during winter (October-December) can be attributed mainly to dormant and slow growth of the plant, as was also reported by Singh *et al.* (2011) in *D. asper*. These results revealed that the type of culture medium used, type and concentration of growth regulators supplemented in the medium as well as the season of explant collection were critical factors determining the regenerative response of *D. hamiltonii* nodal buds.

Shoot proliferation: The axillary shoots which sprouted on the nodal segments after 4-weeks of growth on initiation medium were excised and cultured on MS medium supplemented with different concentrations of BAP and TDZ. No shoot multiplication was observed on MS basal medium without growth regulators and the shoots gradually died within 2 to 3 weeks. Although, good shoot multiplication was observed in MS medium supplemented with BAP (Figs. 1f and 2) but the cultures started to turn brown which eventually died after 2-3 subcultures. However, Sood *et al.* (2002a) and Agnihotri *et al.* (2009) have reported shoot proliferation in *D. hamiltonii* on BAP (8.8 µM) and BAP (8 µM) + NAA (1 µM) supplemented media respectively without browning of shoots. Browning of cultures as observed in this study has also been reported in cultures of *D. latiflorus*, *B. oldhamii* and *B. multiplex* (Rao *et al.* 1990, Liang 1996). Addition of 1.5 µM TDZ to MS medium showed an increased rate of shoot multiplication (3.8 folds) compared to BAP with an average shoot number of 30.9 and shoot length of 2.1 cm after 3-weeks

Table 2. Effect of time of explant collection on axillary bud proliferation from nodal explants of *D. hamiltonii* cultured on MS medium supplemented with 3.0 µM TDZ. Data recorded after four weeks of inoculation (±SE)

Month of collection	Days required for bud break	Contamination (%)	Bud break (%)	No. of shoots	Shoot length (cm)
January	11.07 ± 1.18	27.34 ± 0.85	37.66 ± 0.51	1.66 ± 0.32	6.26 ± 0.62
February	9.33 ± 0.38	21.90 ± 1.16	63.73 ± 0.29	2.13 ± 0.21	4.06 ± 0.96
March	7.76 ± 0.23	19.43 ± 1.44	81.66 ± 2.53	3.63 ± 0.38	2.90 ± 0.68
April	7.56 ± 0.29	18.67 ± 1.69	94.46 ± 1.36	4.53 ± 0.12	2.76 ± 0.32
May	5.02 ± 0.10	8.86 ± 0.22	98.66 ± 0.78	5.33 ± 0.32	2.26 ± 0.91
June	6.76 ± 0.19	15.10 ± 0.58	96.86 ± 1.94	4.66 ± 0.56	2.27 ± 0.41
July	9.07 ± 0.25	36.07 ± 1.38	86.67 ± 1.04	3.86 ± 0.42	3.13 ± 1.20
August	8.54 ± 0.12	37.43 ± 1.45	77.43 ± 1.16	3.23 ± 0.92	3.23 ± 1.92
September	8.43 ± 0.49	53.96 ± 1.59	69.76 ± 1.87	2.86 ± 0.21	3.50 ± 0.15
October	11.33 ± 0.68	33.34 ± 3.41	60.20 ± 2.99	2.34 ± 0.23	4.96 ± 1.02
November	11.56 ± 0.21	31.63 ± 0.84	50.90 ± 0.41	1.43 ± 0.30	3.56 ± 1.40
December	15.20 ± 0.75	30.00 ± 0.75	41.53 ± 1.85	1.23 ± 2.06	4.73 ± 0.35

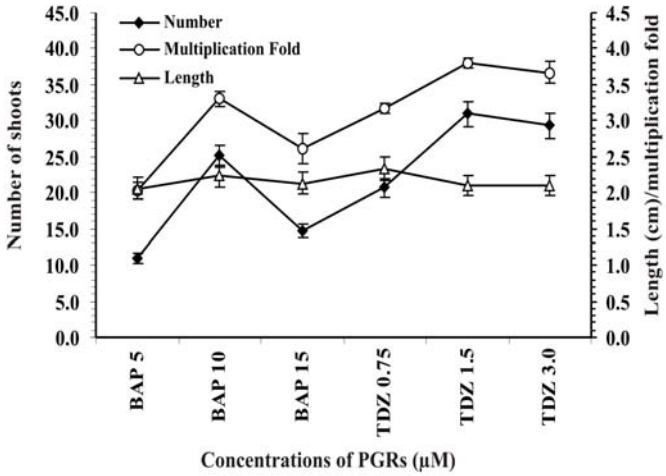

Fig. 2. Effect of BAP and TDZ supplemented in MS medium on shoot multiplication in *D. hamiltonii.* Bars represent ± SE.

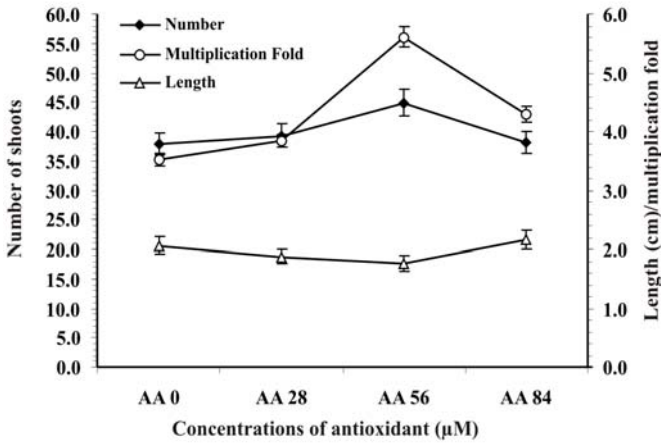

Fig. 3. Effect of different concentrations of ascorbic acid added to MS + 1.5 µM TDZ on multiplication of shoots in *D. hamiltonii.* Bars represent ± SE.

and lesser browning of shoots (Figs. 1g and 2). A lower TDZ concentration (0.45 µM) was also found effective in controlling browning of callus and sufficed for embryo formation in *Bambusa edulis* as compared to KIN (Lin *et al.* 2004).

Several authors have suggested solutions to minimize the lethal browning or blackening of explants caused by phenolic compounds in plant tissue culture. These include addition of polyphenol adsorbents like activated charcoal (Arditti and Ernst 1993), antioxidants such as cysteine (Sanyal *et al.* 2005), ascorbic acid (Arditti and Ernst 1993), PVP (Laine and David 1994) or silver nitrate (Sanyal *et al.* 2005) to the culture medium. Effect of addition of activated charcoal, ascorbic acid and PVP to minimize the lethal browning of the cultures during multiplication was studied. A significant control of browning with enhanced shoots multiplication was achieved with ascorbic acid (28 to 84 µM). MS medium supplemented with 1.5 µM TDZ + 56.0 µM ascorbic acid gave a maximum rate of multiplication (5.61 folds) with 44.90 shoots which were 1.76 cm long (Figs. 1h and 3) with negligible browning. The use of PVP (0.01 to 0.1%) and activated charcoal (0.05 to 0.2%) completely failed to suppress the browning of *D. hamiltonii* shoot cultures. These observations confirmed the earlier reports documenting the effect of PVP in *Phyllostachys nigra* (Ogita 2005) and effect of activated charcoal in *D. latiflorus* (Zamora *et al.* 1988). However, these results are contrary to the findings of Saxena and Dhawan (1999) wherein use of PVP improved shoot health in the cultures of *D. strictus*. The use of ascorbic acid has been restricted to *in vitro* cultures of *Pseudoxytenanthera stocksii* Munro (Sanjaya *et al.* 2005) in bamboos. A multiplication rate of 5.61 folds was achieved in this study compared to 3.8 fold multiplication reported by Agnihotri *et al.* (2009) in *D. hamiltonii*. They reported a fivefold increase in multiplication rate over controls (intact shoots) when the shoots were cut individually to a length of 1.5 cm above the base which were referred to as 'shoot cuts'. However, this method increased the labour inputs making the process cost-intensive and also prone to contamination.

The effect of number of shoots (i.e. 4, 6, 8 and 10) per propagule, used for sub-culturing, on multiplication was evaluated by culturing the clumps on MS medium supplemented with 1.5 µM TDZ + 56.0 µM ascorbic acid. Propagules with 8-10 shoots were found most effective for multiplication of shoots, ~5 folds with 39 shoots of 1.5 cm length (Fig. 4). Better results were also reported in the previous studies when shoot clumps rather than single shoots were used for multiplication of bamboo plants through axillary shoot proliferation (Arya *et al.* 1999, Bag *et al.* 2000, Ramanayake *et al.* 2001, Singh *et al.* 2011). To make the protocol cost effective,

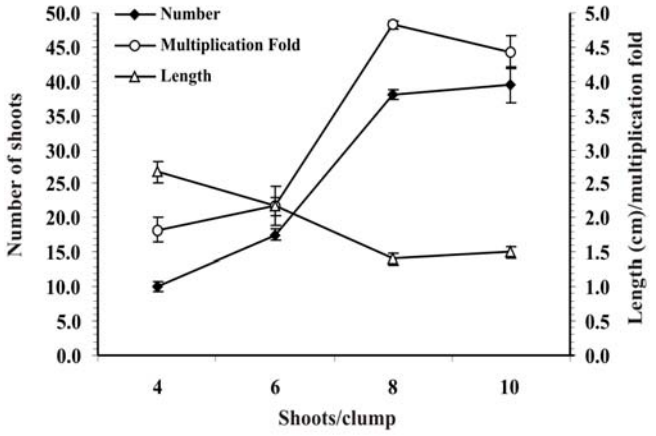

Fig. 4. Effect of number of shoots/clump on shoot multiplication in *D. hamiltonii*. Shoots were cultured on MS medium supplemented with 1.5 µM TDZ + 56.0 µM ascorbic acid. Bars represent ± SE.

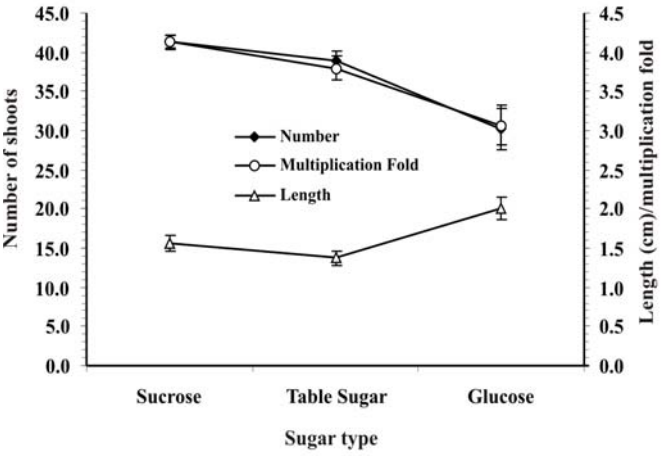

Fig. 5. Effect of different carbon source (3%) added to MS + 1.5 µM TDZ + 56.0 µM ascorbic acid on multiplication of shoots in *D. hamiltonii*. Bars represent ± SE.

8 to 10 shoots per propagule were cultured on MS medium supplemented with 1.5 µM TDZ, 56.0 µM ascorbic acid and containing different carbon sources (3% sucrose or glucose or table sugar). Replacement of sucrose with less expensive table sugar had negligible effect on rate of shoot multiplication (Fig. 5), however, reduced the cost of plant production considerably. The use of glucose instead of sucrose showed deleterious effects on shoots multiplication. Table sugar has also been used as an alternative low cost carbon source during *in vitro* micropropagation of *Pogostemon cablin* (Swamy *et al.* 2010) and *D. asper* (Singh *et al.* 2011). Therefore, MS medium supplemented with 3% table sugar, 1.5 µM TDZ and 56.0 µM ascorbic acid was standardized as multiplication medium and propagules with 8-10 shoots were inoculated for further multiplication. Induced shoots were sub-cultured on multiplication medium for 5 to 6 cycles followed by rooting. Sub-culturing of shoots was done at periodic interval of 3 weeks so as to maintain healthy cultures. Longer sub-culture durations of 4-5 weeks led to longer and pale shoots which gradually turned brown to black instead of enhancing the multiplication rate further. The available nutrients in the culture medium become a limiting factor hampering the health of shoots. Similar observations have also been recorded by Bisht *et al.* (2010) and Mudoi and Borthakur (2009) in *Gigantochloa atroviolaceae* and *Bambusa balcooa*, respectively.

Rooting of shoots: Clumps of 4 to 6 shoots (>3.0 cm) were used for root induction rather than single shoots due to better response. The shoot clumps were inoculated on MS medium adjuvated with different auxins (IAA, IBA and NAA) singly and also in combination with coumarin or choline chloride. The shoots failed to root even after four weeks of culture on MS basal medium without auxins and also when IAA and NAA were added singly. However, IBA alone at a concentration of 25.0 µM gave 3-4 roots after 4-5 weeks (Fig. 1i). Better performance of IBA towards root induction was also reported by Bag *et al.* (2000) and Agnihotri *et al.* (2009) in *Thamnocalamus spathiflorus* and *D. hamiltonii*, respectively. Combination of IBA (25.0 µM) and choline chloride (36.0 µM) gave better rooting response with 89% rooting with an average of 12.2 roots per propagule with mean root length of 2.0 cm after 4 weeks of inoculation (Table 3, Figs. 1j-k). Varied rooting responses have also been reported by other workers when different auxins were used. Saxena (1990) accomplished rooting of *B. tulda* shoots in the presence of coumarin as supported later by Ramanayake and Yakandawala (1997) in *D. giganteus*. Better rooting was reported in *D. hamiltonii* when IBA was used in combination with coumarin or choline chloride (Sood *et al.* 2002a). A single step procedure for rooting of shoots was used in the present investigation resulting in 89%

Table 3. Effect of different concentrations of choline chloride supplemented in MS + 25.0 µM IBA on rooting efficiency of *in vitro* raised shoots of *D. hamiltonii*. Data recorded after four weeks of inoculation (±SE)

Concentration (µM)	Days required for root induction	Rooting (%)	No. of roots/ propagule	Shoot length (cm)
0.0	26.83 ± 0.76	58.96 ± 0.84	5.23 ± 1.12	0.68 ± 0.34
7.2	26.20 ± 0.45	65.23 ± 0.78	6.90 ± 0.32	0.73 ± 0.91
21.6	22.46 ± 0.88	77.34 ± 2.40	9.10 ± 0.45	1.46 ± 0.15
36.0	17.23 ± 0.78	89.00 ± 0.17	12.20 ± 0.59	2.00 ± 0.11
50.4	17.56 ± 1.34	89.10 ± 0.90	12.00 ± 0.35	2.03 ± 0.22

rooting response compared to 90% rooting achieved by Agnihotri *et al.* (2009) using a two step procedure for rooting of *D. hamiltonii* shoots. They cultured the shoots initially on 100 µM IBA supplemented medium for 7 days followed by transfer to auxin free MS medium. In an earlier study, only 30% rooting was reported in 4-6 weeks in *D. hamiltonii* shoots cultured on IBA or NAA supplemented media (Sood *et al.* 2002a and 2002b). The strength of the basal medium was also found to affect the rooting of shoots. Among the four tested strengths (¼, ½, ¾ and full), ½ MS was found better for rooting (data not shown) as has also been reported in *D. asper* (Singh *et al.* 2011). This may be due to the need for only a small amount of total nitrogen for rooting (Ajithkumar and Seeni 1998). Use of reduced salt concentrations to one half during rooting of shoots has been also described by Das and Pal (2005), Sanjaya *et al.* (2005), Ogita *et al.* (2008) and Agnihotri *et al.* (2009).

Hardening, acclimatization and field transfer: Healthy rooted plantlets were transferred to seedling trays containing different types of transplanting media, like soil, river bed sand, dune sand and dune sand with farm yard manure (FYM)/vermi-compost. These were irrigated with ½ MS minerals and maintained in the mist chamber for 2 to 3 weeks. During hardening, use of medium with reduced mineral salts forces the regenerates to rely on their own photosynthetic apparatus for nutrition (Kozai *et al.* 1988). In the present study, use of this strategy resulted in better survival of plantlets when transferred to *ex vitro* conditions. Combination of dune sand and vermi-compost (3:1) was found optimal for good survival (79.76 %) and vigorous growth of plantlets (Figs. 1l and 6). Mishra *et al.* (2011) used soilrite, perlite, vermiculite and compost as potting mix in seedling trays, and found best response in perlite followed by soilrite, for hardening of *B. tulda* plantlets. Initial application of ½ MS minerals to the plantlets was found essential for better acclimatization (Arya *et al.* 1999). After 2 to 3 weeks, plants from the trays were transferred to poly bags containing dune sand and vermi-compost (3:1) in the green house followed by transfer to net house after another 2 to 3 weeks of hardening in green house (Fig 1m). Addition of vermi-compost to the sand increases the porosity resulting in better aeration of roots and thus better growth of the plantlets (Singh *et al.* 2011). However, Mishra *et al.* (2011) used sand, soil and FYM in 1:1:1 ratio in polybags for transfer of

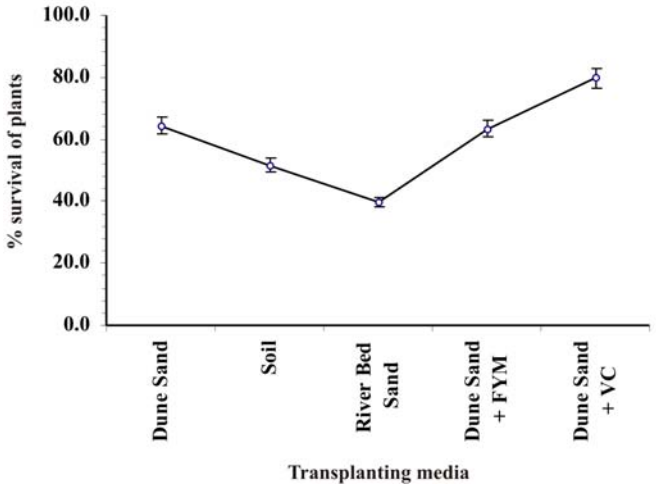

Fig. 6. Effect of transplanting media on survival percentage of *in vitro* raised plants of *D. hamiltonii* during acclimatization and hardening to *ex vitro* conditions. Bars represent ± SE.

Table 4. Field performance of *in vitro* raised plants of *D. hamiltonii* transferred to soil under natural conditions with different manure treatments. Data recorded after one year of transplantation (±SE)

Treatment (g/plant)	Survival (%)	Number of culms/clump	Tallest culm height/clump (cm)	Culm dia at 5th internode (mm)	Number of internodes
Control	81.00 ± 2.08	8.30 ± 1.00	79.43 ± 2.94	8.43 ± 0.54	10.30 ± 0.58
FYM (300)	79.33 ± 1.76	10.23 ± 0.79	112.10 ± 1.48	11.13 ± 0.43	13.17 ± 0.62
Urea (3.0)	82.00 ± 1.15	13.34 ± 0.52	191.13 ± 3.29	13.60 ± 1.31	17.33 ± 1.71
FYM (300) + Urea (3.0)	85.33 ± 2.81	17.00 ± 0.68	179.43 ± 2.17	16.80 ± 0.86	26.67 ± 1.69

hardened and acclimatized *B. tulda* plants. Finally the acclimatized and hardened plants (1 to 2 feet height) were transferred to the field under natural conditions. The plants resumed growth after field transfer (Figs. 1n-p) and the season was found to influence the survival rate and growth of the plantlets in the field. Plantlets transferred in the rainy season (July to August) showed higher survival rate with sprouting of more culms than those transferred in other months. High humidity in the environment during the rainy season provides optimum conditions for the survival of delicate tissue culture raised plants in the field (Singh *et al.* 2011).

Plants derived from axillary buds are usually free from somaclonal variations and are true to the mother plant. However, morphological, physiological and molecular evaluation of *in vitro* raised plants is essential for commercial application of tissue culture technology. The tissue culture raised plants transferred to the field after hardening and acclimatization reached a height of approximately 1.9 m within 1 year. An average of 17 culms per clump with a culm diameter of 16.8 mm at the fifth internode was recorded in the present study (Table 4). The leaf length and width showed growth corresponding to the clump growth and increase in plant height. Agnihotri *et al.* (2009) evaluated the field performance of 20 *in vitro* raised plants over a period of 1.5 years and made similar observations in morphological growth parameters in *D. hamiltonii*.

The *in vitro* raised hardened and acclimatized plants showed 80-85% survival in the field depending upon the fertilizer treatment given at the time of field transfer. Application of FYM + urea was found optimal for healthy growth of plants and production of new culms and lateral branches with ~ 85% survival followed by urea. However, Mishra *et al.* (2011) reported that application of 100 ppm urea supported better growth of *B. tulda* plants. The plants treated with only FYM behaved more or less similar to the control plants (without any treatment). So far more than 3000 *in vitro* raised plants have been successfully transferred to the fields which have not shown any morphological growth variations over three years of growth (data not shown). Previously Sood *et al.* (2002a) and Agnihotri *et al.* (2009) also reported successful field transfer of *in vitro* raised plants of *D. hamiltonii*, demonstrating the potential of tissue culture technique for micropropagation of this giant bamboo. The survival percentage recorded in the present study (85%) was more as compared to 70% reported earlier by Sood *et al.* (2002a) and Agnihotri *et al.* (2009) in *D. hamiltonii*. This is the first report documenting production of *D. hamiltonii* plants through tissue culture on a commercial scale. The *in vitro* raised plants have been transferred to approximately 7.5 hectares of land under the Bamboo Mission Program of Government of India where they are contributing in increasing the forest cover in the state.

CONCLUSIONS

This study describes a simple, efficient and cost effective procedure for *in vitro* propagation of *D. hamiltonii* on a large scale through proliferation of nodal buds collected from mature culms. Direct proliferation of pre-existing axillary meristems ensures true-to-type nature of the plants produced. High multiplication efficiency, good rooting percentage, easy establishment in the soil and normal growth performance of micropropagated plants in the field are the major accomplishments of this study. Use of table sugar instead of highly purified sucrose has considerably reduced the

production cost. The protocol developed involves less labour and is cost intensive compared to the protocol developed by Agnihotri *et al.* (2009) due to omission of 'shoot cut' method of shoot multiplication and one step procedure for rooting of shoots. Successful rooting was achieved in 89% shoots comparable to earlier report of Agnihotri *et al.* (2009), while quite high compared to 30% rooting reported by Sood *et al.* (2002a, 2002b). In addition, the hurdles usually encountered in lab to land transition of tissue culture raised plants have been successfully addressed and a better field survival rate of 85% was achieved compared to 70% reported previously in *D. hamiltonii*. This study has demonstrated the potential of tissue culture and *in vitro* propagation technology for large scale multiplication of this important bamboo species. The outcome of the present study would further help in conservation and reforestation of *D. hamiltonii* in various parts of the country. Moreover, the plantations raised will improve the social status of local communities who depend on bamboo based cottage industries for their living by enhancing economic benefits.

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