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In vitro Shoot Cut: A High Frequency Multiplication and Rooting Method in the Bamboo *Dendrocalamus hamiltonii*

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Abstract: A rapid and high frequency reproducible *in vitro* regeneration protocol of a multipurpose bamboo species *Dendrocalamus hamiltonii* Nees et Arn. Ex Munro has been developed through single node cutting taken from the lateral branches of a 20 year old field grown elite bush. Axillary buds on the nodal explants sprouted within a fortnight of culture on Murashige and Skoog (MS) medium without any Plant Growth Substance (PGS). After 3-4 weeks of incubation, the sprouted buds were excised from the mother stumps and placed on MS medium supplemented with different concentrations of 6-benzylaminopurine (BAP; 2.0-12.0 μM) and 1.0 μM α-naphthaleneacetic acid (NAA). Enhanced proliferation was induced on the propagules (small clusters with 3-5 multiple shoots and rhizomatous part) on medium supplemented with 8.0 μM BAP and 1.0 μM NAA; subsequent removal of the shoots (about 1.5 cm) from the rhizomatous portion (shoot cut) and placing them on the same media combination influenced multiplication capacity. A multiplication of 20 folds was achieved on MS medium supplemented with 8.0 μM BAP and 1.0 μM NAA at the end of the 2nd subculture. Enhanced root formation (>90%) occurred when the propagules following shoot cut were placed on to MS medium supplemented with 100 μM indole-3-butyric acid (IBA) for 10 days and then transferred to IBA-free medium. This is the first report from this species where 20-fold multiplication was obtained and subsequent enhanced rooting (>90%). The hardened plants, established in the field, exhibited normal growth even after 2 years.

Key words: Bamboo, micropropagation, nodal segment, propagules

INTRODUCTION

Bamboo is the most diverse group of plants in the Poaceae family and the most primitive sub-family. They are distinguished by having woody culms and complex branching, a complex and generally robust rhizome system and infrequent flowering. It has a cosmopolitan distribution, reaching elevations as high as 4000 m in the Himalayas and parts of China (Anonymous, 1978). There is high demand of bamboo for commercial purposes; however, productivity of bamboo forest in India is far below compared to China and can be attributed to over exploitation, lack of scientific management, recurrent fire, poor natural regeneration and damage by cattle (INBAR, 1991). Cultivation of bamboo in India is still at infancy stage. Almost 99% of annual bamboo production in the country comes from the natural stands in the forests and only 1% is derived from plantations. Nevertheless, increased productivity can be achieved following selection of the right species, elite clones, genotype match with site characteristics and proper management practices (Gielis *et al*., 2001). In Indian Central Himalaya and particularly in Uttarakhand, bamboo forests are very much limited and only few stands can be seen here and there; these are also man-made (human plantations). In view of the importance, the need for bamboo cultivation has been realized.

Dendrocalamus hamiltonii Nees et Arn. Ex Munro, a multipurpose, fast growing bamboo species with strong culms is commonly used in the Western Himalayan Region for its leaves as good quality cattle fodder particularly during winter months holds considerable promise in Central Himalaya also as a green fodder (Negi *et al*., 1980). Moreover, its use in paper production, house building, making furniture, agricultural implements, mat boards, baskets, handicrafts and numerous traditional uses is well known. Thus, this bamboo has a great potential for hilly regions of the Indian Central Himalaya. Use of tissue culture for plant multiplication and subsequent field plantation has also been reported for

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this species (Chambers *et al*., 1991; Godbole *et al*., 2002; Sood *et al*., 2002). Although, multiple shoot formation occurs routinely, rooting has been inconsistent with up to only 30% rooting only (Sood *et al*., 2002), which limits large scale plant production.

Use of the cytokinin 6-benzylaminopurine (BAP), either alone or in combination with kinetin (cytokinin) or the auxin α-naphthaleneacetic acid (NAA) in basal medium has resulted in higher rate of shoot multiplication in several species of bamboo (Arya *et al*., 1999; Bag *et al*., 2000; Bag, 2001; Sood *et al*., 2002; Arshad *et al*., 2005; Kapoor and Rao, 2006; Ramanayake *et al*., 2006; Jimenez *et al*., 2006). Although, the auxin indole-3-butyric acid (IBA) has been generally effective in *in vitro* rooting (Bag *et al*., 2000; Bag, 2001; Sood *et al*., 2002; Arshad *et al*., 2005; Ramanayake *et al*., 2008), the phenolic compound coumarin (Saxena, 1990; Ramanayake and Yakandawala, 1997; Mishra *et al*., 2007; Ramanayake *et al*., 2008) and glucose (Yashodha *et al*., 2007) have also been successful in bamboo.

In the present study, an efficient and high frequency *in vitro* multiplication protocol for *D. hamiltonii*, using nodal explants taken from a 20 year old field grown elite mother bush to enhance multiplication following shoot cut method has been described.

MATERIALS AND METHODS

Single node cutting, taken from one year old lower and lateral branches of a 20 year old vegetatively propagated plant (mother bush) of *D. hamiltonii* growing at the Institute nursery at Kosi, District Almora (79°38'10"E and 29°28'15"N; 1150 m altitude) were collected during May 2004 and used for developing *in vitro* cultures. The cutting (about 3.0 cm in length) was soaked in distilled water containing Labolene (Liquid detergent; 0.2% v/v; Qualigens, India) for about 2-3 min, washed with distilled and sterilized water 3-4 times, disinfected with 0.2% mercuric chloride (w/v, BDH, India; 5 min) and again rinsed with distilled and sterilized water (x4). Subsequently both the ends were trimmed and segments cultured on Murashige and Skoog's (1962) medium (20 mL) with out any Plant Growth Substances (PGSs) by placing them vertically in test tubes (15×1.5 cm). The pH of the medium was adjusted to 5.8 and gelled with 0.2% phytagel (w/v, Sigma) before autoclaving (1.05 kg cm^{-2}, 121°C, 20 min). All the cultures were maintained at 25±1°C in a culture room with 14/10 h day/night cycle. All laboratory experiments and hardening of *in vitro* raised plants were carried out at the Institute and its nursery located at Kosi-Katarmal, District Almora (1200 m altitude).

After 3-4 weeks of incubation the axillary buds sprouted from the nodal explants; these buds (2.0-2.5 cm long) were excised from the mother stumps and cultured on MS medium supplemented with different concentrations of BAP (2.0-12.0 μM) and 1.0 μM NAA. The buds produced small clusters of 3-5 multiple shoots (2.5-3.0 cm) with a rhizomatous portion, hereafter called propagules. The shoots were individually cut about 1.5 cm above the base and discarded, while the propagules, now referred to as shoot cut was separated and further cultured for multiplication. For rooting these propagules (with shoot cut) were separated again and placed in 1/2 MS media containing IBA at different concentrations (10.0-200.00 μM) for 7 days followed by placing in medium without IBA for 2 weeks. The plantlets were removed from the flasks, thoroughly washed with water, transferred to plastic cups (6×6×7 cm) containing 250 g of autoclaved soil and placed under greenhouse conditions (25°C air temperature, relative humidity 65%; 18/6 h day/night cycle) for 15 days. Following another 15 days, they were shifted to polythene bags (16×10 cm) containing equal proportion of soil and farm yard manure (1:1) and transferred to a polyhouse/nethouse for hardening. Following one month of hardening, the plants were planted in the field and their growth performance was monitored.

SD was calculated following the method of Snedecor and Cochran (1967).

RESULTS AND DISCUSSION

The sprouted axillary buds on the nodal explants (Fig. 1A) were excised and transferred on MS medium containing different concentrations of BAP (2.0-12.0 μM) and 1.0 μM NAA. Subsequently within the next 3-4 weeks, differentiation was observed and multiple shoots were formed. Out of the various concentrations of BAP used, the medium supplemented with 8.0 μM BAP and 1.0 μM NAA resulted in profuse shoot formation with increased rhizomatous portion. Shoot portions were removed and the propagules were separated and multiplied on media containing different concentrations of BAP (2.0-12.0 μM) and 1.0 μM NAA (Fig. 1B, Table 1). The medium containing 8.0 μM BAP and 1.0 μM NAA further enhanced multiplication, for example, starting with 1 explant, more than 20 propagules could be obtained; the average shoot length and leaf size were also highest when compared to other treatments of BAP (Table 1). This is the first report from these species where adopting this method higher number of propagules and multiple shoots were obtained by the end of the 2nd subculture (Table 1, Fig. 1C). Data on multiplication of propagules and shoots

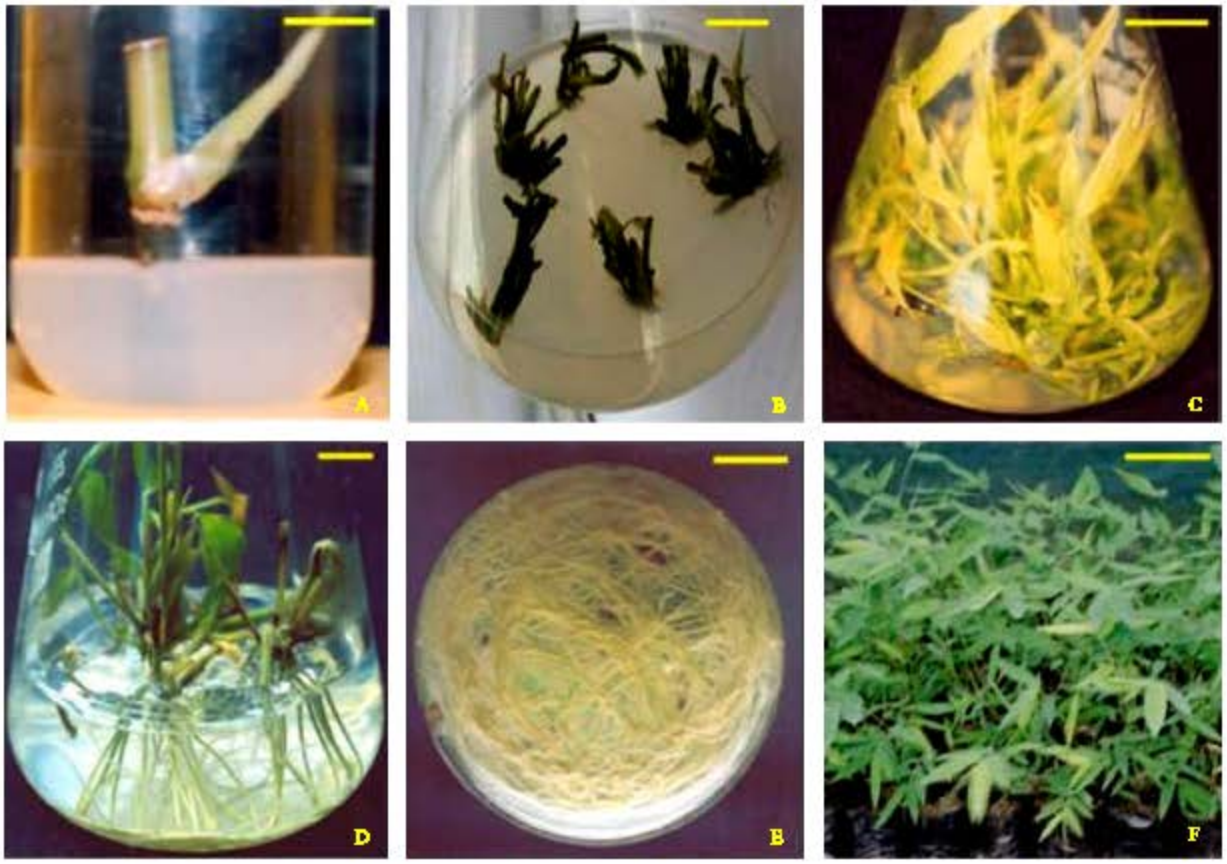

Fig. 1: Various stages during the development of *in vitro* propagation protocol of *D. hamiltonii*. (A) establishment of explant in MS medium without PGSs; bar = 1 cm, (B) a representative photo depicting culture of propagules following *in vitro* shoot cut method on MS medium supplemented with 8.0 μM BAP and 1.0 μM NAA; bar = 1.5 cm, (C) profuse shoot multiplication after *in vitro* shoot cut on medium containing BAP and NAA as in Fig. B, during 2nd subculture; bar = 1.5 cm, (D) and (E) profuse rooting in propagules after shoot cut and following culture on MS medium containing 100 μM IBA and subsequently placing in IBA-free medium; bar = 1.5 cm and (F) transfer of *in vitro* raised plants to soil and hardening in a polyhouse; bar = 8 cm

Table 1: Effect of BAP and *in vitro* shoot cut on multiplication of propagules and shoots of *D. hamiltonii*

BAP concentration[a] (μM)	Multiplication[b] (No. of propagules)	Shoot length (cm)	Leaf width (mm)
0 (Control)	5.20±1.09	1.62±0.50	2.48±0.32
2.0	7.33±0.58	1.62±0.52	2.10±0.29
4.0	8.67±0.58	1.42±0.31	2.20±0.75
6.0	11.33±5.86	2.56±0.46	3.74±0.97
8.0	26.33±4.04	3.70±0.35	6.34±0.44
10.0	7.67±1.15	1.98±0.34	2.40±0.64
12.0	5.33±0.58	1.86±0.36	2.44±0.44

A propagule consisted of 3 shoots, each treatment consisted of 5 propagules cultured per flask and conducted in triplicate, data were recorded after 3 weeks, all results were obtained using propagules during 2nd subculture, [a]all concentrations of BAP in the medium contained 1.0 μM NAA, [b]Mean±SD

Table 2: Effect of shoot cut on multiplication of propagules and shoots of *D. hamiltonii*

		Without shoot cut (control)		After *in vitro* shoot cut	
Treatment (μM)	Set No.[a]	Multiplication[a,b] (No. of propagules)	Average shoot length (cm)	Multiplication[a,b] (No. of propagules)	Average shoot length(cm)
8.0 BAP+	1	3.6±1.34	0.97±0.31	16.6±2.07	3.10±0.26
1.0 NAA	2	3.8±0.84	1.53±0.45	16.4±1.95	2.87±0.40
	3	3.2±0.84	1.33±0.55	14.8±2.77	2.57±0.45
	4	3.4±0.89	1.47±0.64	17.2±0.84	2.77±0.67
	5	3.8±0.84	1.43±0.72	16.4±1.95	2.77±1.03

A propagule consisted of 3 shoots, [a]each set represents treatment given to various batches using 3 propagules per flask and conducted in triplicate, data were recorded after 3 weeks during 2nd subculture, [b]Mean±SD

(Table 2) indicates that by following *in vitro* shoot cut method there was a 5-fold increase when compared to control (without shoot cut); moreover, length of shoots was also found to nearly double following shoot cut. Compared to an earlier study (Bag 2001) wherein a combination of 5.0 μM BAP and 2.0 μM NAA supplemented to MS medium was used for multiplication,

this study resulted in generation of 20% higher number of multiple shoots (Table 1, Fig. 1C). Studies using bunches, each containing 3-5 shoots, for shoot proliferation in bamboo have been reported earlier, e.g., *Thamnocalamus spathiflorus* (Bag *et al*., 2000) and *D. hamiltonii* (Bag, 2001; Sood *et al*., 2002).

The roles of the cytokinin, particularly BAP in *in vitro* bud break and shoot multiplication is well known. Although, BAP has been used to induce bud break in nodal explants of *Bambusa wamin* (Arshad *et al*., 2005) and *Guadua angustifolia* (Jimenez *et al*., 2006), it was not required in this study as reported earlier by Bag (2001) and Sood *et al*. (2002). Like in several plant species, BAP alone has been very effective for shoot multiplication in bamboos *Dendrocalamus asper* (Arya *et al*., 1999), *Bambusa bambos* (Kapoor and Rao, 2006), *B. vulgaris* (Ramanayake *et al*., 2006) and *G. angustifolia* (Jimenez *et al*., 2006). However, in this investigation, BAP in combination with NAA was found to be effective; similar results were reported for this species earlier (Bag, 2001) and in the temperate bamboo *T. spathiflorus* (Bag *et al*., 2000). BAP in conjunction with kinetin was also demonstrated effective in *B. wamin* (Arshad *et al*., 2005). It must be mentioned that BAP and its metabolites have been identified as naturally occurring cytokinins in plant tissues (Nandi *et al*., 1989a, b).

Pulse (short) treatment of the propagules with IBA for 7 days resulted in maximum rooting at 100 μM concentration and using this method more than 90% success could be achieved (Table 3, Fig. 1D). The number of roots per plant was more than 6 and the length of longest root was about 5.0 cm (results not shown). In an previous study using IBA (2.1 or 4.2 μM) or NAA (2.7 or 5.4 μM) up to 30% rooting was observed (Sood *et al*., 2002). Well-rooted plantlets (Fig. 1E) following proper hardening (Fig. 1F) formed profuse root system with a survival rate of over 85% (Table 3) under polyhouse/nethouse conditions. The performance of the plants transferred in the field was satisfactory and exhibited normal growth even after 2 years. A two-step rooting procedure using IBA treatment followed by transfer to auxin-free media had been reported by Bag *et al*. (2000).

The auxin IBA is being widely used as an effective PGS for root induction in various plant species. In several species of bamboo, medium supplemented with IBA alone was used for successful rooting; *B. wamin* (Arshad *et al*., 2005), *B. vulgaris* (Ramanayake *et al*., 2006) and *Dendrocalamus hookeri* (Ramanayake *et al*., 2008) are some examples. In this investigation, a short treatment with IBA alone was sufficient enough to induce rooting as reported earlier in this species (Bag, 2001) and in *T. spathiflorus* (Bag *et al*., 2000). Phenolic compounds, which act as auxin protectors, have also been reported to promote rooting. While, in *Bambusa tulda* use of coumarin alone resulted in maximum rooting response of 98% (Mishra *et al*., 2007), in *Dendrocalaamus giganteus* IBA together with coumarin was necessary (Ramanayake *et al*., 2008). In *Bambusa nutans*, addition of glucose along with IBA supported 85% rooting (Yashodha *et al*., 2007). These results indicate a close interaction amongst various PGSs and/or with different compounds to evoke a physiological response.

Table 3: Effect of different concentrations of IBA on rooting of propagules following *in vitro* shoot cut

Treatment (IBA μM)	Average rooting (%)[a]	Plantlet survival (%)
0	0	-
10	0	-
20	8	100
40	8	90
60	13	100
80	32	90
100	93	100
150	26	70
200	4	60

-: Not applicable. A propagule consisted of 3 shoots, each treatment consisted of 10 propagules per flask and in replicates of 10, data were recorded following 7 days treatment in IBA and then placing in medium without IBA, [a]calculated on per flask basis

Nodal segments taken from mature bushes (Ramanayake and Yakandawala, 1997; Bag, 2001; Sood *et al*., 2002; Arshad *et al*., 2005; Ramanayake *et al*., 2006; Jimenez *et al*., 2006) were also used in this study; this approach ensures propagation of true-to-type plants. The shoot cut method used in this study has enhanced *in vitro* multiplication by 5 fold (when compared to control) followed by high rooting efficiency (>90%). In an earlier report only up to 30% rooting was demonstrated in this species (Sood *et al*., 2002). Hardening and field performance of micropropagated plants are crucial aspects that need utmost attention. Substantial numbers of micropropagated plants do not survive after transfer from controlled *in vitro* conditions to *ex vitro* environment of the greenhouse and later in the open field. Present results demonstrated more than 70% plant survival in the field even after two years of plantation and are comparable with a previous investigation (Sood *et al*., 2002).

This study clearly demonstrates high frequency multiplication and enhanced rooting method for *in vitro* propagation of *D. hamiltonii*. These features are necessary for increased production of true-to-type plants for large-scale multiplication of this species. Tissue culture method allows the production of a large number of plantlets identical to the mother plant, is less labour intensive and cheaper once the protocols has been standardized and hence offers distinct advantages over conventional methods of multiplication of different species of elite bamboo clones (Saxena and Dhawan, 1999; Godbole *et al*., 2002; Sood *et al*., 2002; Das and Pal, 2005; Jimenez *et al*., 2006; Ramanayake *et al*., 2006, 2008; Yashodha *et al*., 2007). Therefore, this study will not only help in large scale multiplication of this useful

multipurpose bamboo species for restoration of degraded land but also result in economic benefits of the inhabitants of Indian Central Himalaya.

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