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Effect of nodal positions, seasonal variations, shoot clump and growth regulators on micropropagation of commercially important bamboo, *Bambusa nutans* Wall. ex. Munro

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An efficient protocol for *in vitro* micropropagation of *Bambusa nutans* Wall. ex. Munro has been described. Nodal explants obtained from 1½-year-old field-grown culms of *B. nutans* produced up to 7.0 multiple shoots per explant on Murashige and Skoog (MS) basal medium supplemented with 6-benzylaminopurine (BAP, 1.0 mg/L). Continuous shoot proliferation up to 11.33 shoots was achieved by sub-culturing shoot clumps (4 shoots/cluster) in BAP (0.5 mg/L) and 0.1 mg/l α-naphthalene acetic acid (NAA) fortified medium every 4 weeks. 85% rooting was recorded on 2.0 mg/L NAA supplemented medium after 30 to 35 days of culture period. Micropropagated plantlets of *B. nutans* showed 70% survivability during the hardening stage. After hardening, rooted plantlets were successfully transferred to the soil and exhibited 80% survivability and normal growth. Plantlets cultivated in field condition achieved 95% survivability. Seed explants were also used for *in vitro* culture establishment of *B. nutans* on different combination of MS medium.

Key words: *Bambusa nutans*, micropropagation, nodal explants, seed explants.

INTRODUCTION

Bambusa nutans Wall. ex. Munro (local name Mokal bah) is naturally occurring in sub-Himalayan tracts from Yamuna eastwards to Arunachal Pradesh between 600 to 1500 m of altitude. It is very common in India and Nepal and widely cultivated in the villages of Bangladesh (Banik, 1987) and also reported as an important commercial species of Thailand (Anantachote, 1987). It is a graceful medium-sized, thick-walled bamboo with 6 to 15 m culms height, 5 to 10 cm in stem girth, and usually with 25 to 45 cm long internodes. Among the sympodial bamboos, *B. nutans* produced highest number of culms per clump. This bamboo is reported to be useful for

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Abbreviations: BAP, 6-Benzylaminopurine; **GA_3,** gibberellic acid; **IAA,** indole-3-acetic acid; **IBA,** indole-3-butyric acid; **Kin,** kinetin; **MS,** Murashige and Skoog medium; **NAA,** α-Naphthalene acetic acid; **2ip,** 2-isopentenyladenine; **PGR,** plant growth regulators.

cellulose (paper) manufacture and for various purposes, including pulping, timber, handicrafts, furniture, house construction etc (Anonymous, 1988). In India, it is one of the species prioritized by National Mission of Bamboo Application (NMBA), which is popular for utilization in cottage industries as well as for construction purposes. The seed availability of this bamboo is restricted due to its long flowering cycle of 35 years (Seethalakshmi and Kumar, 1998).

Various attempts have been made in recent past to micropropagate this particular species of bamboo. Yasodha et al. (1997; 2008); Kalia et al. (2004); Islam and Rahman (2005); Negi and Saxena (2011) reported establishment of *in vitro* culture of *B. nutans* by using seed, leaf, internodal and nodal explants. However, until now, no report has been available regarding effect of various disinfecting agents, nodal positions, and seasonal variations on *in vitro* derived clones of *B. nutans*. Moreover, field performance and survivability of this species has not also been studied yet. Therefore, it is proposed to exploit the *in vitro* micropropagation technique to develop an efficient and reproducible protocol of *B. nutans*.

MATERIALS AND METHODS

Explant preparation

Commercially, important *B. nutans* was obtained from experimental farm of CSIR-North East Institute of Science and Technology, Jorhat, Assam, India (Figure 1a). Tender nodes (12 to 18 mm in length, due to variation in sizes) obtained from minor branches of 1½-year-old culms of *B. nutans* regenerated from approximately 40 year elite plants having single axillary buds were used as explants (Figure 1b to 1c). Explants were collected from the farm at the middle of every month from the same donor plant throughout the year for culture establishment. Seeds of *B. nutans* were collected from M/S Shidh Seeds Sales Corporation, Dehradun, India. After removal of leaf sheath, individual node containing axillary buds (explants without a visible bud were discarded at this point) of *B. nutans* were washed with running tap water for 10 min. Explants were then cut into 12 to 18 mm in length and node containing axillary bud was dipped in 5% (v/v) Tween 20 solution for 3 h for tissue softening followed by thorough washing under running tap water for 20 min (Figure 1d).

Establishment of *in vitro* culture

A set of 25 nodal explants was inoculated (one explant/culture vessel) each month into the MS (Murashige and Skoog, 1962) basal medium containing 1.0 mg/L of BAP (6-benzylaminopurine) for shoot induction and proliferation. The inoculated culture materials were kept in culture room, maintained at 25±2°C temperature and 60 to 70% relative humidity. All cultures were maintained under 16 h photoperiod with light intensity of 10 µmol m^{-2} S^{-1} (Cool white fluorescent light).

Effect of disinfecting agents on axenic culture establishment

Nodal explants were decontaminated with mixtures of disinfecting agents (for 5 min) prior to surface sterilization for optimal recovery of *in vitro* culture. Thereafter, surface sterilization was done with 0.1% mercuric chloride solution for 5 to 7 min and rinsed thoroughly with sterile distilled water prior to culture *in vitro* and for initial axenic establishment; cut ends of nodal explants were trimmed and placed vertically on 25 ml of culture medium in culture tubes. Numbers of nodal explants exhibiting bud-break and percentage of contaminants were recorded each day for observing efficiency of tested agents in contaminant reduction.

Effect of node positions on *in vitro* shoot culture establishment

Collected explants were cut into appropriate sizes (12 to 18 mm) due to variability of nodal explants and arranged them according to their nodal position from 1st to 11th (that is, from apex to base). After proper sterilization, explants were cultured accordingly. The bud break frequency of the nodal explants in comparison to their size and position was scored.

Influence of seasons on *in vitro* shoot culture establishment

As per the Indian noted six seasons, we have collected experimental materials in six different seasons in the middle of every month throughout the year to determine the most suitable time for culture establishment from the same donor plant in three successive years. Bud break frequencies of the axillary buds towards the six different seasons were recorded.

In vitro seed germination

After dehusking, healthy seeds of *B. nutans* were selected for culture establishment. They were given a quick rinse in 70% ethanol and then washed with 5% Tween 20 for 10 min. The seeds were further surface sterilized by treating them with 0.1% mercuric chloride for 10 min. After three washings in sterile double distilled water, the seeds were cultured on different combination of MS medium and observed their responses towards germination at 16 h light and 8 h dark condition and continuous dark condition within 15 days duration. Seed germination of *B. nutans* was carried out on both ½ strength of MS and MS basal medium. Different combination of BAP (1.0 to 5.0 mg/l) with one concentration of GA_3 (0.5 mg/L) was also tested.

In vitro shoot multiplication

Proliferated shoots (2 shoots/clump) from the axillary buds of *B. nutans* were excised and cultured into MS and B_5 (Gamborg et al., 1968) medium by supplementation of various concentration of cytokinin, auxin and GA_3 (gibberellic acid). Different concentrations (1.0 to 3.0 mg/L) of BAP, Kin (Kinetin) and 2ip (2-isopentenyladenine) with or without supplementation of GA_3 (0.5 mg/L) and NAA (0.1 and 0.5 mg/L) were also tested.

Effect of shoot clump size on shoot multiplication and growth

The *in vitro* regenerated shoots of *B. nutans* were sub-cultured every 20 to 25 days. After selecting the best treatment, clusters having more or less 2 shoots of *B. nutans* were sub-cultured into the responsive optimal media for observing their effect on shoot multiplication.

In vitro root induction

Different concentrations (0.5 to 5.0 mg/L) of auxins, that is, NAA (α-

Figure 1. a. Mother plant. **b.** Newly emerging minor secondary branch. **c.** Tender branches of *B. nutans*. **d.** Nodal explants. **e.** Axillary shoot bud initiation. **f.** Shoot initiation from shoot bud culture. **g.** Optimum shoot multiplication. **h.** Root induction. **i.** Rooted shoot. **j.** Hardening stage. **k.** Emergence of white coloured new secondary roots. **l.** Acclimatization. **m.** 1-year old plant in field condition.

naphthalene acetic acid), IBA (Indole-3-butyric acid) and IAA (Indole-3-acetic acid) were tested for the root induction frequency of *B. nutans*. Data were recorded after 30 to 35 days of culture period. The steps involved in hardening procedure of *B. nutans* were transfer of rooted shoot to MS basal liquid medium for 15 to 20 days, followed by exposing the rooted shoots in half strength of MS basal liquid medium (without sucrose) for another 15 days. After that, plantlets were kept in unsterilized filtered water for a total of 30 days that is, 15 days in culture room followed by another 15 days in ambient room temperature (28±2°C) conditions. During that stage, rooted shoots were washed thoroughly in running tap water to remove adhered agar from the roots. Caps of the flasks were also removed. Hardened plantlets of *B. nutans* were transferred to netted green house for acclimatization before transferring to the field.

Statistical analysis

The experiments were conducted in completely randomized design consisted of three replications. Experimental results were analyzed statistically using the techniques of analysis of variance for single factor experiments. The significance of the treatment means differences were tested by the procedure of Duncan's Multiple Range Test (Duncan, 1955).

RESULTS AND DISCUSSION

Morphogenetic responses of surface sterilized explants of *B. nutans* were assayed for optimization of the following factors for establishment of *in vitro* shoot culture.

Effect of different disinfecting agents on optimal recovery of *in vitro* culture

In our experiment, contamination was observed within the

Table 1. Effect of different pre-treating agents on *in vitro* culture establishment of *B. nutans.*

Treatment	Contaminant (%)	Survivability (%)
Tween 20 (5%)	85	15
Savlon (2%)	83	17
Tween 20 (5%) + Alcohol (70%)	78	22
Savlon (2%) + Alcohol (70%)	75	25
Tween 20 (5%) + Savlon (2%)	70	30
Tween 20 (5%) + Gentamicin (0.1%)	68	32
Tween 20 (5%) + Mancozeb (0.1%)	65	35
Tween 20 (5%) + Savlon (2%) + Alcohol (70%)	62	38
Tween 20 (5%) + Mancozeb (0.1%) + Gentamicin (0.1%)	58	42
Tween 20 (5%) + Mancozeb (0.1%) + Gentamicin (0.1%) + Alcohol (70%)	55	45

first 10 days in culture. Both bacterial and fungal contaminants appeared at the cut ends of the node or near the axillary buds of *B. nutans*. This fact suggests that majority of the contaminants were enclosed within the sheaths that cover the bud, and due to which disinfection agents do not reach the surface area. Sometimes contaminants appeared even after 3rd or 4th sub-cultures, when the shoot had prolific growth. Hence, various mixtures of pretreatments containing Mancozeb and Gentamicin etc were used to study their effectiveness towards *in vitro* culture establishment of this species. Among the ten different mixtures used, Tween 20 (5%) + 0.1% solution of Mancozeb (fungicide) + Gentamicin (antibiotic) + alcohol (70%) containing treatment was the best for axenic culture establishment of *B. nutans* (Table 1). In this case, 45% culture was recovered. However, single use of Tween 20 and Savlon did not show good response towards culture establishment and 85% infection was recorded. Like our study, Jimenez et al. (2006) also followed a disinfection procedure that comprised the sequential use of an alkaline detergent, a mixture of Benomyl and Agri-mycin, followed by immersion in sodium hypochlorite (1.5% w/v); but observed microbial contaminants within the first 10 days in the culture. In contrast to our work, Ramanayake and Yakandawala (1997) did not observe contamination for first 3 weeks of culture in *Dendrocalamus giganteus*.

In vitro nodal culture of *Dendrocalamus strictus* was also obstructed by microbial culture contaminants (Mascarenhas et al., 1988). Dalsaso and Guevara (1989) used different disinfection pretreatments for the explants of *Persea americana*. Ramanayake and Yakandawala (1997) used Benlate as a disinfectant for reducing the contaminants of *D. giganteus* and incorporate into the media. However, Yasodha et al. (2008) applied Streptomycin and Kanamycin as a disinfectant for *B. nutans* with 30% culture establishment. But, we had recorded maximum 45% recovery in our study on *B. nutans*. The morphogenetic competence of nodal explants of *B. nutans* was adversely affected by the phenolic exudates release from the excised explants, which caused browning of the medium and ultimately resulted in necrotic appearance of the shoots. These brown colour phenolic exudates were also released from the basal portion of the nodal explants from 4th d onwards from this species. In this case, frequent transfer to the fresh medium led to overcome this problem. Similarly, Das and Pal (2005) also recorded same type of exudates in *Bambusa balcooa* during culture initiation from nodal explants.

Effect of various node explants on *in vitro* culture initiation

It is an established fact that different parts of a plant vary in their regenerative potentiality (Evans et al., 1981; Chaturvedi, 1984). In case of *B. nutans* 5th to 7th node containing axillary bud showed maximum regeneration potentiality (Figure 1e and 1f). In this case, up to 7.0 shoot buds were initiated (Table 2). Significant difference was recorded among the 1st, 2nd, 3rd and 4th node position. No significant differences were recorded between 8th and 9th node. No bud breaking occurred beyond 9th node onwards. Hence, for further study, only selected node position, that is, 5th to 7th nodes were taken for *B. nutans*. The above result indicated that the variation of new shoot emergence may be due to size, age or other associated conditions of explants. As per report of McClure (1966), nature dormancy and breaking dormancy in buds of bamboo varied with their position in the plant, the season of the year and the species. Similarly, Saxena and Bhojwani (1993) reported the mid culm nodes of secondary branches as the best explant for axillary shoot initiation.

Influence of different seasons on *in vitro* culture establishment

The season of explant collection is a critical factor in the establishment and growth of *in vitro* cultures (Hu and Wang, 1983; Tisserat, 1985). In certain cases, the season

Table 2. Effect of various node explants on *in vitro* culture initiation of *B. nutans*.

Node position	Girth of node (cm)	Axillary bud break/explant	Shoot length (cm)
1st	0.8	2.1[f]	1.0[f]
2nd	0.9	2.9[e]	1.1[e]
3rd	1.0	3.5[d]	1.3[d]
4th	1.0	5.2[b]	1.4[cd]
5th	1.3	**6.7[a]**	**1.8[a]**
6th	1.4	**7.0[a]**	**1.8[a]**
7th	1.4	**6.9[a]**	1.6[b]
8th	1.5	4.2[c]	1.5[bc]
9th	1.5	3.7[c]	1.2[de]
10th	1.6	---	---
11th	1.6	---	---

Means are from four repeated experiments. Each treatment consisted of three replicates. Means within a row followed by the same letters are not significant at $P = 0.05$ according to DMRT.

Table 3. Effect of different seasons on *in vitro* culture establishment of *B. nutans*

Different season	Months of collection	Percentage (%) Bud break	Shoot number/explant
Winter	January–February	35.02[c]	1.93[d]
Spring	March-April	25.00[d]	3.02[c]
Summer	May-June	20.00[e]	2.60[c]
Monsoon	July-August	15.04[f]	5.50[b]
Early Autumn	September-October	**45.00[a]**	**6.30[a]**
Late Autumn	November-December	40.00[b]	5.90[a]

Means are from four repeated experiments. Each treatment consisted of three replicates. Means within a row followed by the same letters are not significant at $P = 0.05$ according to DMRT.

of explants collection is more important than the selection of a right kind of media (Chaturvedi, 1984). In *B. nutans,* maximum bud break (45%) and number of proliferating shoots (5.90) took place during early autumn (Table 3). In winter, 35.02% of bud breaking was recorded. In this season, minimum number of sprouted shoot bud was recorded for the species. During this period, phenolic exudation was more and shoot growth was recorded as slow. This result supported the findings of Sahoo and Chand (1998) and Andersone and Ievinsh (2002) in *Tridax procumbens* and *Pinus sylvestris*. In spring and summer, bud breaking and shoot growth of the species decreased. During these seasons, microbial contamination and browning of sprouted axillary buds was more. These are the major constrains for axenic establishment of culture.

In monsoon, minimum bud breaking percentage of the species was recorded. Explants collected during this season showed reduced phenolic exudation. Sprouted axillary buds were heavily contaminated in this season even after 3 to 4 weeks of culture period. The influence of seasonal rainfall pattern on the rate of axillary bud-break and fungal contamination in bamboos was reported previously by (Ramanayake and Yakandawala 1997; Saxena and Bhojwani, 1993).

In vitro seed germination

Seed germination started from the 2nd to 3rd day onwards in both 8 h dark and continuous dark condition. 30% fungal contamination was observed after 8 days culture period. Moreover, contaminants appeared together with the emergence of the radicle. This indicates the presence of endogenous microorganisms within the seed. Arce-Montoya et al. (2006) reported the presence of endogenous fungus from the seeds of *Yucca valida*. A varied germination rate and shoot growth was recorded in respect of exposure to different cultural condition. Moreover, results also varied as per the additional supplements of various hormones. Between the two cultural conditions tested for germination, maximum germination rate up to 63% was recorded from the treatment of ½ strength of MS basal, from the cultures kept in 16 h light condition (Table 4). Keeping the same treatment in total dark condition resulted lower germination percentage

Table 4. Effect of photoperiod and growth regulators on seed germination of *B. nutans*.

Basal Medium	BAP (mg L^{-1})	GA_3 (mg L^{-1})	Seed germination (%)		Number of shoot emergence		Shoot length (cm)	
			Dark	16 h light	Dark	16-h light	Dark	16 h light
½ strength of MS	-	-	**50.33^{a}**	**63.00^{a}**	0.64^{e}	0.90^{e}	**1.50^{ab}**	**1.07^{ab}**
½ strength of MS	1.0	-	47.80^{a}	45.00^{d}	1.00^{cd}	1.00^{de}	1.07^{abc}	0.80^{b}
½ strength of MS	1.0	0.5	33.33^{c}	41.33^{e}	1.12^{cd}	1.10^{de}	0.93^{abc}	0.83^{b}
½ strength of MS	2.0	-	39.67^{b}	40.33^{e}	**2.33^{abc}**	**2.00^{cd}**	0.60^{bc}	0.57^{b}
½ strength of MS	3.0	-	32.77^{d}	39.00^{f}	2.00^{abcd}	1.15^{de}	0.60^{bc}	0.37^{b}
½ strength of MS	4.0	-	30.00^{cd}	41.00^{e}	1.00^{cd}	1.12^{de}	0.60^{bc}	0.47^{b}
½ strength of MS	5.0	-	25.07^{d}	18.67^{h}	1.69^{bcd}	1.13^{de}	0.60^{bc}	0.33^{b}
MS	-	-	**49.00^{a}**	**55.00^{b}**	0.72^{d}	1.12^{de}	**1.77^{a}**	**1.30^{a}**
MS	1.0	-	45.33^{ab}	51.67^{c}	1.33^{bcd}	2.00^{cd}	1.51^{ab}	0.90^{b}
MS	1.0	0.5	31.67^{c}	46.00^{d}	1.67^{bcd}	2.67^{c}	1.19^{abc}	0.83^{b}
MS	2.0	-	14.33^{ef}	23.00^{g}	**3.33^{a}**	**6.00^{a}**	0.93^{abc}	0.40^{b}
MS	3.0	-	11.67^{f}	29.00^{f}	2.67^{ab}	4.67^{b}	0.60^{bc}	0.36^{b}
MS	4.0	-	17.67^{e}	15.67^{i}	2.00^{abcd}	2.00^{cd}	0.45^{c}	0.40^{b}
MS	5.0	-	14.77^{ef}	15.67^{i}	1.33^{bcd}	1.20^{de}	0.60^{bc}	0.30^{b}

Means are from four repeated experiments. Each treatment consisted of three replicates. Means within a row followed by the same letters are not significant at $P = 0.05$ according to DMRT. BAP = 6-Benzylaminopurine; GA_3 = Gibberellic acid; MS = Murashige and Skoog medium.

of 50.33. Treatment containing MS basal also showed satisfactory results regarding seed germination at both the cultural conditions (that is, 49.00 and 55.00, respectively). Addition of BAP (1.0 and 5.0 mg/L) to the above basal medium could not enhance seed germination. BAP (2.0 mg/L) enriched MS basal media, showed maximum number of shoot emergence (6.00) from the culture kept in 16 h light condition. However, treatment with increase concentrations of BAP (3.0 to 5.0 mg/L) resulted in gradual reduction of shoot numbers from both the basal medium as well as in both the cultural condition. Considering the shoot length, it can be said that there is a relation with the photoperiod. In this experiment, we observed maximum shoot length (1.77 cm) from the MS basal medium without any PGR and in total dark condition. In this case regenerated shoots were whitish in colour and thin. The same treatment with light condition generated healthy and normal growth with shoot length 1.07 cm. From the above study, it can be said that minimal nutritional support, that is, ½ strength of MS is enough to obtain optimal germination of *B. nutans*. To enhance multiple shoot regeneration, BAP (2.0 mg/L) can be added to the basal medium. Moreover, 16 h light condition was the best to get normal and healthy seedlings. Similar studies were conducted on seed germination of three bamboo species, viz. *Dendrocalamus membranaceus*, *D. strictus* and *B. nutans* to determine the best cultural combination. In this case, the presence of light was found to be the ideal condition for seed germination (Rawat, 2005). When the seedlings attained a growth of 6 to 7 cm, these were transferred to the MS basal liquid medium followed by soil for further growth. One-year-old seedlings of *B. nutans* showed various morphological differences in respect of their growth, shoot length, tiller numbers, leaf numbers and leaf sizes etc. Different leaf morphology, that is, either with alternate leaf pattern or opposite leaf pattern was observed. Variation in tiller numbers and shoot numbers were reflected, the heterozygous characteristics of the seed raised plants (Figure 2a, b, c, d, e, f, g, h).

In vitro shoot multiplication

Among the single concentration of BAP (1.0, 2.0, 3.0 mg/L), 1.0 mg/L BAP induced more number of shoots, with increase shoot length and leaf numbers in comparison to the other two concentrations. These shoots no longer attained its greenish colour. Therefore, single combination of BAP was not applied in further experimental work. Combined effect of BAP (0.5 and 1.0 mg/L) with NAA (0.1 and 0.5 mg/L) was also tested. Under this investigation, treatment containing BAP (0.5 mg/L) and NAA (0.1 mg/L) enhanced the maximum morphogenetic potential of *B. nutans* (Figure 1g). In this case, shoot numbers and shoot length was recorded as 11.33 and 4.40 cm, respectively. Here, shoots were greenish and healthy in nature. In this treatment, a 4 fold increase of shoot length was obtained. Similarly, combination of BAP (1.0 mg/L) with NAA (0.1 mg/L) resulted in maximum leaf numbers (18.30). In this regard, shoot length and shoot number was not encouraging with 8.33 shoot numbers and 2.83 cm shoot length. Moreover, BAP (1.0 mg/L) and NAA (0.5 mg/L) combination was

Figure 2. a. Seeds. **b.** Germinated seedlings. **c – h.** Various morphological differences obtained from *in vitro* germinated seedlings.

also not suitable for shoot regeneration resulting 6.33 shoot numbers, 2.73 cm shoot length and 10.67 leaf numbers. Addition of Kin (0.5 mg/L), BAP (1.0 mg/L) and NAA (0.1 mg/L) did not show any significant result. Likewise, single supplementation of Kin (1.0, 2.0, 3.0 mg/L each) did not enhance the shoot regeneration capacity of *B. nutans*. Addition of 2 ip alone had not affected the shoot multiplication of *B. nutans*. Moreover, GA_3 (0.5 mg/L) along with various concentration of BAP (viz. 1.0, 2.0, 3.0 mg/L) also did not show any significant result. Yasodha et al. (2008) used BAP alone and obtained only a 3 fold increase in shoot multiplication. However, Negi and Saxena (2011) obtained 3.5 fold shoot multiplication in MS liquid medium supplemented with 13.2 IM BAP, 2.32 IM Kin, and 0.98 IM indole-3-butyric acid (IBA).

When the sprouted shoots of *B. nutans* were transferred to the B_5 medium with BAP (1.0 mg/L) recorded less number of shoots (6.00) and shoots length (1.93 cm) and leaf numbers (5.33). MS basal and ½ strength of MS basal media either alone or supplemented with BAP (1.0 to 3.0 mg/L) were not suitable for shoot proliferation. Addition of GA_3 (0.5 mg/L) in combination with BAP did not show any significant result (Table 5a). Hence, the use of basal media without PGRs resulted in gradual reduction in shoot and leaf numbers and shoot length, although regeneration of shoots was recorded immediately after placing to these media. Under this study, the shoot multiplication rate could be maintained up to 6th sub-culture cycle and then gradually declined. Similar to our findings, Arya et al. (2008) also reported same rate of shoot multiplication for *Dendrocalamus asper*. Similar results was demonstrated by various workers on many bamboo species viz. in *B. balcooa* (Dutta-Mudoi and Borthakur, 2009) and *Drosera hamiltonii* (Agnihotri and Nandi, 2009; Agnihotri et al., 2009). The multiplication cycles of shoots did not involve a callus phase in this species.

Effect of shoot clump size on shoot multiplication and growth

Four shoots/clump was the best propagule size for *in vitro* shoot multiplication of *B. nutans*. In this case, maximum shoot numbers (11.40), shoot length (4.66 cm) and leaf numbers (18.60) were recorded (Table 5b). Three shoots/clump showed same significant result with the four shoots/ clump size in relation with shoot numbers and shoot length. In this case the nature of the shoots was not so satisfactory. Similarly, 5 shoots/clump also showed same result as above. In this case, leaves and shoots gradually turned pale green in colour. Similarly, 1 shoot/ clump and 2 shoots/clump were also not appropriate for shoot multiplication. It was observed that apart from optimal medium composition towards production of healthy

Table 5a. Morphogenetic response of *B. nutans* at different concentration and combination of growth regulators.

Basal medium	Kin (mg L^{-1})	2ip (mg L^{-1})	BAP (mg L^{-1})	NAA (mg L^{-1})	GA_3 (mg L^{-1})	Number of shoots	Shoot length (cm)	Number of leaves
½ MS	-	-	-	-	-	2.33^{j}	1.00^{d}	4.69^{gh}
½ MS	-	-	1.0	-	-	4.67^{defghi}	1.33^{cd}	6.67^{defg}
½ MS	-	-	1.0	-	0.5	5.00^{cdefgh}	2.83^{b}	7.33^{defg}
½ MS	-	-	2.0	-	-	4.67^{defghi}	1.00^{d}	5.68^{efg}
½ MS	-	-	2.0	-	0.5	3.67^{fghij}	1.00^{d}	4.67^{gh}
½ MS	-	-	3.0	-	-	5.33^{cdefg}	1.17^{d}	8.10^{cdef}
½ MS	-	-	3.0	-	0.5	5.33^{cdefg}	1.13^{d}	8.00^{cdef}
MS basal	-	-	-	-	-	2.33^{j}	1.03^{d}	3.39^{h}
MS	1.0	-	-	-	-	2.67^{ij}	2.23^{bcd}	7.67^{cdefg}
MS	2.0	-	-	-	-	3.33^{ghij}	2.17^{bcd}	8.12^{cdef}
MS	3.0	-	-	-	-	3.00^{hij}	1.83^{bcd}	5.67^{efgh}
MS	-	1.0	-	-	-	2.67^{ij}	1.57^{bcd}	6.34^{efgh}
MS	-	2.0	-	-	-	4.00^{efghij}	2.07^{bc}	9.67^{cd}
MS	-	3.0	-	-	-	2.67^{ij}	1.57^{bcd}	6.68^{defg}
MS	-	-	1.0	-	-	7.00^{bc}	2.67^{bc}	9.67^{cd}
MS	-	-	2.0	-	-	6.00^{cde}	1.50^{bcd}	8.00^{cdef}
MS	-	-	3.0	-	-	5.67^{cdef}	1.27^{d}	8.67^{cde}
MS	-	-	0.5	0.1	-	11.33^{a}	4.40^{a}	12.00^{b}
MS	-	-	1.0	0.1	-	8.33^{b}	2.83^{b}	18.00^{a}
MS	-	-	1.0	0.5	-	6.33^{bcd}	2.73^{b}	10.67^{bc}
MS	0.5	-	1.0	0.1	-	6.00^{cde}	2.23^{bcd}	8.67^{cde}
MS	-	-	1.0	-	0.5	4.33^{defgh}	1.13^{d}	6.33^{efgh}
MS	-	-	2.0	-	0.5	4.00^{efgh}	1.17^{d}	5.00^{fgh}
MS	-	-	3.0	-	0.5	4.00^{efgh}	1.00^{d}	3.33^{h}
B_5	-	-	1.0	-	-	6.00^{cde}	1.93^{bcd}	5.33^{fgh}

Means are from four repeated experiments. Each treatment consisted of three replicates. Means within a row followed by the same letters are not significant at $P = 0.05$ according to DMRT. BAP = 6-Benzylaminopurine; GA_3 = Gibberellic acid; MS = Murashige and Skoog medium; Kin = Kinetin; 2ip = 2-isopentenyladenine; NAA = α-Naphthalene acetic acid.

Table 5b. Effect of shoot clump size on shoot multiplication and growth of *B. nutans*.

Number of shoots/clump	Shoot number	Shoot length (cm)	Leaf number
1	4.20^{c}	3.08^{c}	6.00^{c}
2	7.40^{b}	3.68^{b}	9.20^{c}
3	10.20^{a}	4.54^{a}	14.00^{b}
4	**11.40^{a}**	**4.66^{a}**	**18.60^{a}**
5	9.80^{a}	3.32^{b}	15.60^{a}

Means are from four repeated experiments. Each treatment consisted of three replicates. Means within a row followed by the same letters are not significant at $P = 0.05$ according to DMRT.

shoots, regeneration capacity of the *in vitro* shoot was found to be dependent upon (1) size and number of shoots/clump and (2) time of sub-culturing. The shoot multiplication rate declined sharply if propagule of sub optimal size was taken for sub-culturing. Arya et al. (1999; 2002) studied in detail of the effectiveness of different propagule size on shoot multiplication of *D. asper*. According to their observation, 3 shoots/clump was the best propagule size for inducing shoot multiplication of *D. asper*.

In vitro root induction

Among the different treatments, highest adventitious rooting frequency was obtained on NAA (2.0 mg/L) supplemented medium. In this treatment, maximum root length (1.66 cm) and root numbers (2.60) was achieved after 30 to 35 days of culture (Table 6). Increased level of

Table 6. Effect of different auxins on rooting of *in vitro* shoots of *B. nutans*.

NAA (mg L^{-1})	IBA (mg L^{-1})	IAA (mg L^{-1})	Number of roots	Root length (cm)
0.50	-	-	0.96^{bc}	0.98^{bc}
1.00	-	-	1.30^{b}	1.20^{ab}
2.00	-	-	2.60^{a}	1.66^{a}
3.00	-	-	1.28^{b}	1.12^{b}
4.00	-	-	1.00^{bc}	1.05^{bc}
5.00	-	-	0.60^{c}	0.66^{c}
-	0.50	-	0.00	0.00
-	1.00	-	0.00	0.00
-	2.00	-	0.00	0.00
-	3.00	-	0.00	0.00
-	4.00	-	0.00	0.00
-	5.00	-	0.00	0.00
-	-	0.50	0.00	0.00
-	-	1.00	0.00	0.00
-	-	2.00	0.00	0.00
-	-	3.00	0.00	0.00
-	-	4.00	0.00	0.00
-	-	5.00	0.00	0.00

Means are from four repeated experiments. Each treatment consisted of three replicates. Means within a row followed by the same letters are not significant at P = 0.05 according to DMRT. NAA = α-Naphthalene acetic acid; IBA = indole-3-butyric acid; IAA = indole-3-acetic acid.

NAA (5.0 mg/L) resulted in limited root induction as well as reduction of root length and numbers. In this treatment, roots were thick and stout. Survivability percentage of these rooted shoots were also very less. The addition of NAA at 3.0 and 4.0 mg/L produced roots of a good quality, but root induction frequency was low (1.28 and 1.00). Similarly, lower concentration of NAA (0.5 and 1.0 mg/L) also resulted less number of root induction (0.96 and 1.30), respectively. In this case, the roots were thin. Treatments with IBA and IAA did not induce root induction at all. Unlike our study, Yasodha et al. (1997; 2008) obtained optimal root induction of *B. nutans* on IBA fortified medium. Hence, medium containing 2.0 mg/L NAA was the best for high frequency root induction of *B. nutans* (Figure 1h and i). During our study, we had recorded 85% rooting after 30 to 35 days of culture. Yasodha et al. (2008) recorded only 68% rooting in this bamboo species. Similarly to our study, the effect of single concentration of NAA was reported as optimal for root induction of *Berberis vulgaris, D. asper* and *D. membranaceus* (Arya et al., 2002) and *Dendrocalamus latiflorus* (Lin et al., 2007).

Hardening of *in vitro* plantlets

After root initiation, when the roots of *B. nutans* attained a length of 0.5 to 1.0 cm, then the rooted shoots were transferred to MS basal liquid medium for 15 to 20 days for both shoot and root elongation, which was followed by exposing the rooted shoots in half strength of MS basal liquid medium for another 15 days. Plantlets were then kept in unsterilized filtered water for 30 days; 15 days in culture room followed by another 15 days in ambient room temperature (28±2°C) conditions. During this phase, white-colour new secondary roots developed. In the hardening stage of *B. nutans*, we had lost 10 to 15% plantlets and 70% survivability was recorded (Table 7; Figure 1j and k). To overcome this problem, ideal shoot selection for rooting (20 to 25 days old; 1.5 to 2.5 cm in length) was the most important factor. It allowed the minimizing of the mortality rate during the hardening period.

Acclimatization and field response of tissue culture raised plantlets

The transplantation stage continues to be major bottle neck in the micro propagation of many plants (Hazarika, 2003). Plantlets that were grown *in vitro* had continuously exposed to a unique microenvironment with high level of humidity, aseptic conditions, on a medium containing ample sugar and nutrients. Thereafter, *in vitro* raised plantlets of the species should be gradually acclimatized to the environment of the poly house or green house with lower relative humidity, higher light level, septic environment that was stressful to *in vitro* raised plantlets. Successful transfer of plantlets from tissue-culture vessels

Table 7. General description of hardening stage in *B. nutans*.

Factor	Standard
General appearance	Healthy, greenish
Height of plantlets	3.0-6.0 cm
Tillers/ plantlets	2-4
Leaves/ plantlets	5-10
Visible Nodes/ plantlets	2-5
Nature of roots	Fibrous type
Number of roots	2-6 with initiation of white coloured secondary roots
Length of roots	5-10 cm

Table 8. General description of tissue culture raised *B. nutans* plantlets during acclimatization stage.

Factor	Standard (1 to 4 months old)
General appearance	Healthy, greenish
Height of plantlets	4 - 10 cm
Tillers/ plantlets	1 - 3
Leaves/ plantlets	3 - 10
Visible Nodes/ plantlets	1 - 3
Insect/ Disease Pest infestation	Less than 10%

Table 9. Performance of tissue culture raised *B. nutans* plantlets after field transfer.

Factor	Standard	
	6 months old	12 months old
General appearance	Healthy, greenish	Healthy, greenish
Height of Plantlets	15-20 cm	22-25 cm
Tillers/ plantlets	1- 5	1-7
Leaves/ plantlets	10 -15	20-30
Visible Nodes/ plantlets	2 - 6	6-8
Insect/ Disease Pest infestation	Less than 10%	Less than 10%

to the ambient *ex vitro* condition can determine the significance of any micro propagation system.

In this study, micro propagated plantlets of *B. nutans* were potted in polythene sleeves containing 1:1:2 soil : sand : cow-dung mixture and kept in netted poly house for acclimatization process. Plantlets were healthy and greenish and grew well in poly house. In this stage, we had recorded 80% survivability in *B. nutans* and morphological characteristics were satisfactory (Table 8; Figure 1l). Satisfactory results obtained here in the transfer and acclimatization could be related to the easy adaptation of the bamboo in marginal ecological conditions (Crouzet, 1981). After acclimatization, *in vitro* raised plantlets of *B. nutans* were transferred to the field condition. In field condition, 95% survivability was recorded from *in vitro* derived plantlets evaluated after two years old *B. nutans* plantlets (Table 9; Figure 1m). In this case, field performance experiment of *B. nutans* was conducted with 6 to 24 months old plants. Micro propagated plantlets of studied bamboo species recorded well morphological characteristics in field condition. No morphological variation was observed in the species. Plantlets were grown very uniformly.

Gupta et al. (1991) also recorded positive response of tissue culture raised plantlets, that is, mainly forestry species in field condition. Similarly, Wei and Tien (1995) successfully transferred *in vitro* regenerated plants of *Bambusa beecheyana* Munro var *beecheyana* into the field.

Conclusion

The present study describes an effective regeneration and multiplication protocol for *in vitro* propagation of *B.*

nutans. Micropropagated plantlets and seedlings of this species showed considerable differences during hardening stage. As seedling raised plants proved the heterogeneous characters of the seeds; hence, *in vitro* raised shoots of *B. nutans* could be effectively used in maintaining the clonal fidelity of elite genotypes. High multiplication efficiency, good rooting, easy establishment in the soil and normal growth performance of micropropagated plants, as reported in this study, are features necessary for the adoption of *in vitro* propagation technology for large scale multiplication of this species. Thus, standardized protocol of *B. nutans* can be said as easy to raise, economic to adopt and convenient to transport, thus serving commercial interest.

Conflict of Interests

The author(s) have not declared any conflict of interests.

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