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In vitro clonal propagation of 10- year-old clumps of *Bambusa nutans*

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Abstract: Plantlets of *Bambusa nutans* were produced through *in vitro* axillary shoot proliferation followed by rooting, using nodal segments collected from ten-year-old field-grown clumps. Different seasons strongly influenced aseptic culture establishment, axillary bud break and culture initiation. Variation among the three field clumps (genotypes) existed for aseptic culture establishment, but not for bud break. MS liquid medium (Murashige and Skoog) supplemented with 31.06 µM BA (N^6-benzyladenine) and 2.85 µM IAA (indole-3-acetic acid) recorded maximum shoot multiplication rate of 3.18 fold and that with 20-25 µM IBA (indole-3-butyric acid) induced 66.7-77.8 per cent *in vitro* rooting. Different auxins took variable number of days for root initiation with IBA initiating roots in the shortest period of two and a half weeks. After successful hardening, the *in vitro* raised plantlets exhibited 90 per cent survival in polybags filled with a mixture (v/v) of soil, sand and farmyard manure (1:1:1) and more than 2000 plantlets were produced for field planting.

Key words: Adventitious rooting, axillary bud proliferation, hardening, seasonal variation, shoot multiplication.

INTRODUCTION

Propagation of bamboo through seeds is difficult due to unreliable flowering habit at an interval of 30-100 years and short period of seed viability. The traditional propagation method by offsets limits the number of propagules and the use of cuttings for propagation is cumbersome and labour-intensive for large-scale establishment of bamboo plantations. During the last few decades, bamboo tissue culture techniques emerged as a viable alternative for large-scale propagation (Mehta *et al.*, 1982; Chaturvedi *et al.*, 1993; Arya and Arya, 1997; Mishra *et al.*, 2001; Shirin *et al.*, 2005). The investigations have established that seasonal variations influence explant infestation and bud break (Saxena and Bhojwani, 1993; Ramanayake and Yakandawala, 1997; Das and Pal, 2005), whereas different basal media and growth regulators affect shoot multiplication and rooting in bamboos (Saxena, 1990; Shirin *et al.*, 2003).

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Bambusa nutans Wall. a medium sized bamboo, is commonly used in Indian paper industry. The culms are strong and straight and locally used for poles (Tewari, 1992). It is a very reluctant-to-root species (McClure and Kennard, 1955; Rao *et al.*, 1992). Yashoda *et al.* (1997) have developed an *in vitro* procedure for mass propagation of *B. nutans* through nodal segments of seedlings. Thus, we conducted experiments for determination of suitable season and concentration of sterilant for aseptic culture establishment and culture media and growth regulators for *in vitro* regeneration of plantlets via axillary shoot multiplication and adventitious rhizogenesis from nodal explants of 10-year-old field-grown culms of *B. nutans*.

MATERIALS AND METHODS

Three 10-year-old vigorously growing healthy field clumps (genotypes) of *B. nutans* were selected (Fig. 1a). Secondary shoots of current year growth were collected in four different seasons of the year, *i.e.* rainy (July-August), autumn (October-November), winter (January-February) and summer (April-May). The shoots were cut into 4-5 cm long nodal segments with a single preformed axillary bud. The basal sheaths of the nodal segments were removed. The explants were immersed for 15-20 min in 2 per cent Cetrimide® solution (ICI Ltd., India), and made free from detergent by 3-4 washings with distilled water. The washed explants were treated for 30 min with 0.2 per cent (w/v) Bavistin® solution, a systemic carbendazim fungicide (BASF India Ltd., Mumbai, India) , followed by rinsing with distilled water. Subsequently, the fungicide treated explants received a quick dip for 30 seconds in 70 per cent alcohol before administration for 10 min of 0.05 per cent, 0.1 per cent and 0.2 per cent (w/v) aqueous mercuric chloride solution in a complete randomized block design under aseptic conditions. The explants obtained so were thoroughly rinsed three times with sterilized distilled water and inoculated in culture tubes (25 mm x 150 mm) containing semisolid MS medium (Murashige and Skoog, 1962) supplemented with 10 µM BA and 3 µM IAA (Fig. 1b). The nodal explants produced multiple axillary shoots, which were detached and proliferated further over several subculture cycles of 15 days each.

One experiment on shoot multiplication and two experiments on root induction were conducted using propagules of 3-4 microshoot (2-3 cm in height). The shoot multiplication experiment was carried out in a two-way factorial design testing five concentrations of BA (0, 4.44, 13.32, 22.19 and 31.06 µM) and four concentrations of IAA (0, 0.57, 2.85 and 5.71 µM) alone and in all possible combinations. Auxin sources [IAA, IBA, NAA (napthalene acetic acid) or IPA (indole-3-propionic acid) at 15 µM] in the first experiment and doses of IBA (0, 5 10, 15, 20 and 25 µM) in the second experiment were tested on rooting percentage, root number per propagule and root length. Duration for *in vitro* root initiation denoted by 25 per cent rooting in all treatments in the first experiment was also determined. The rooting medium was changed after an interval of four weeks and observations were noted till nine weeks.

For acclimatization to environmental conditions, the plantlets thus obtained were removed from the flasks, washed thoroughly with 0.2 per cent (w/v) Bavistin® and tap water. Then, they were planted in root trainers consisting of 25 cells each of 150 cc (Neevedita Plastic Industries, Nagpur, India) filled with a mixture of autoclaved soilrite and compost (1:1) and soaked in 0.5x MS inorganic salts. The root trainers were placed for two weeks in a plastic tray filled with 0.5x MS inorganic salts and covered with perforated transparent polythene sheet in culture room for two weeks and then shifted to room temperature for one week followed by shade-house for one week before transfer to polybags containing soil, sand, and FYM in 1:1:1 proportion. The plantlets in polybags were irrigated with tap water on alternate days and sprayed with 0.5x MS inorganic salt solution once in 15 days.

Culture conditions

MS liquid or semisolid (0.8 % (w/v) agar (Qualigens Ltd., India)) medium was uniformly supplemented with 3 per cent (w/v) sucrose and 0.01 per cent (w/v) myo-inositol in all experiments. The pH of the medium was adjusted to 5.8, prior to autoclaving for 15 min at 1.06 kg cm^{-2} (121°C). Each explant was cultured in a 25 x 150 mm culture tube (Borosil, India) containing 15 ml semi-solid medium for initiation of cultures and 150 ml Erlenmeyer flasks containing 20 ml liquid medium for *in vitro* shoot multiplication or rooting. Filter paper bridges were used to provide support to the shoots in static liquid media. The cultures were incubated at a temperature of 25 ± 2°C under 16 h daily illumination with white fluorescent light (~ 45 μ mol m^{-2} s^{-1}) in the culture room.

Statistical analysis

Experiments had three replications each of 10 culture tubes/flasks. The data were subjected to one-way and two-way analysis of variance as per design of experiments, using SX statistical package. The arc sine transformations were made for the data expressed in percentage. The significance of the data was ascertained by F-test and the Least Significant Difference values at $P = 0.05$ ($LSD_{0.05}$) computed for comparing means of various treatments (Gomez and Gomez, 1984).

RESULTS

Culture establishment

Nodal segments exhibited axillary bud break 7-10 days after inoculation (Fig. 1c). The multiple axillary shoots of around 2 cm were excised from the nodal segments and transferred to the fresh medium for further multiplication (Fig. 1d). Highly significant effect of season was noted on both percentage of aseptic culture and bud break (Table 1). The aseptic cultures obtained in the seasons of rain, winter and summer were at par and maximum; but were significantly less in autumn season. The bud

Table 1. Effect of season and mercuric chloride individually and their interactions on aseptic culture and axillary bud break in *B. nutans*

Season	Mercuric chloride concentration (%)			
	0.05	0.1	0.2	Mean
	A. Aseptic culture			
Rain	58.33 (49.76)	53.98 (47.07)	73.22 (60.56)	61.84 (52.47)
Autumn	33.33 (34.62)	38.33 (37.10)	26.66 (30.00)	32.75 (33.91)
Winter	71.66 (59.04)	80.00 (65.02)	91.66 (73.40)	81.10 (65.82)
Summer	65.00 (59.04)	68.33 (60.97)	85.00 (72.62)	72.77 (64.21)
Mean	57.08 (50.62)	60.16 (52.54)	69.12 (59.14)	
	B. Bud break			
Rain	43.33 (40.96)	27.97 (31.01)	48.82 (44.45)	40.04 (38.81)
Autumn	16.66 (22.97)	25.00 (28.16)	20.00 (21.15)	15.22 (24.09)
Winter	48.33 (44.04)	55.00 (47.89)	55.00 (48.97)	52.77 (46.99)
Summer	63.33 (54.74)	66.66 (56.66)	85.00 (72.62)	71.66 (61.34)
Mean	42.91 (40.68)	43.65 (40.93)	48.20 (46.79)	

*Values expressed in parenthesis are arc sine transformed

break was maximum in summer season, which was at par with that in winter season. Effect of mercuric chloride treatment and its interaction with season was non-significant on aseptic culture establishment and bud break.

The three field clumps exhibited significant differences for percentage of aseptic culture establishment but had no significant variation for percentage of bud break. The highest percentage of aseptic culture was obtained from nodal segments collected from Clump II (Table 2).

Table 2. Effect of genotypic variation on aseptic culture and axillary bud break in *B. nutans*

Field clump	Response (%)	
	Aseptic culture	Bud break
I	48.75 (44.50)	45.42 (42.77)
II	68.92 (60.60)	53.26 (48.25)
III	69.94 (57.21)	39.26 (37.38)
LSD (0.05)	12.96	N.S.

*Values expressed in parenthesis are arc sine transformed.

***In vitro* shoot multiplication**

BA individually and its interaction with IAA had significant effect on rate of shoot multiplication; 31.06 µM BA had the highest shoot multiplication rate registering an increase of 56 per cent over the control. For shoot multiplication, 31.06 µM BA and 2.85 µM IAA proved to be the best interaction resulting in 167 per cent increase over the control. On the contrary, BA and IAA alone and their interactions had non-significant effect on shoot length (Table 3, Fig. 1e).

Table 3. Effect of BA (B), IAA (I) and their interactions on shoot multiplication and shoot length in *B. nutans*

BA (mg l^{-1})	IAA (mg l^{-1})									
	Shoot multiplication (fold)					Shoot length (cm)				
	0	0.57	2.85	5.71	Mean	0	0.57	2.85	5.71	Mean
0	1.08	1.20	1.28	1.15	1.18	4.45	4.16	3.53	4.18	4.08
4.44	2.03	1.92	1.94	1.45	1.83	4.28	4.13	4.55	3.88	4.21
13.32	2.31	2.73	2.39	2.38	2.45	3.26	3.25	4.3	3.03	3.46
22.19	2.50	2.47	2.68	2.41	2.51	3.85	3.41	4.03	3.60	3.72
31.06	2.77	2.85	3.18	2.40	2.80	3.88	3.66	4.58	3.25	3.84
Mean	2.14	2. 32	2.30	1.96		3.94	3.72	4.20	3.59	

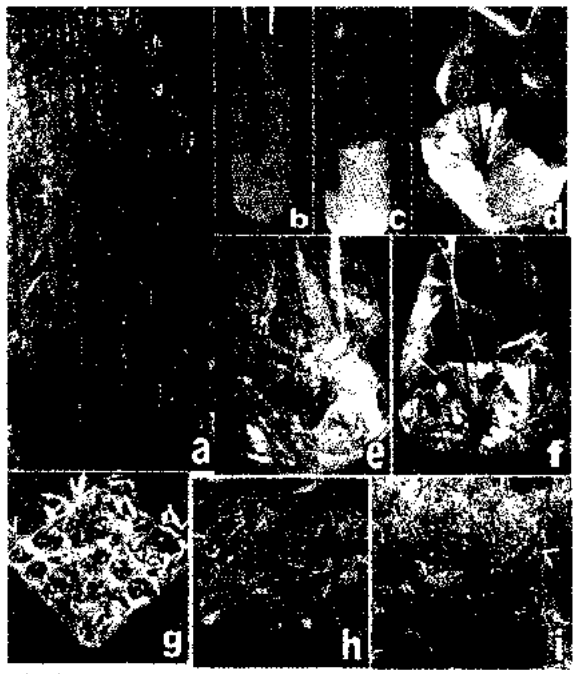

Figure 1. Micropropagation and hardening of *B. nutans* plantlets. a: mother clump; b: inocultion of explants on MS semi-solid medium supplemented with 10 μ M BA + 0.1 μ M IAA; c: bud break ; d: excision and transfer of sprouted axillary buds on MS liquid medium + 10 μM BA +0.1 μM IAA; e: shoot multiplication on MS liquid medium + 31.06 μM BA + 2.85 μM IAA; f: rooting on MS liquid medium + 25 μM IBA after nine weeks; g: transfer of plantlets of root trainers; h: shadehouse acclimatized plantlets in polybags; i: plantlets after six months growth in polybags.

In vitro rooting

On liquid rooting medium root initiation started in 10-15 days after transfer of shoots and completed in nine weeks. Auxins exhibited variation in time lapse for 25 per cent *in vitro* rooting, with IBA initiating roots in the shortest duration of two and a half week

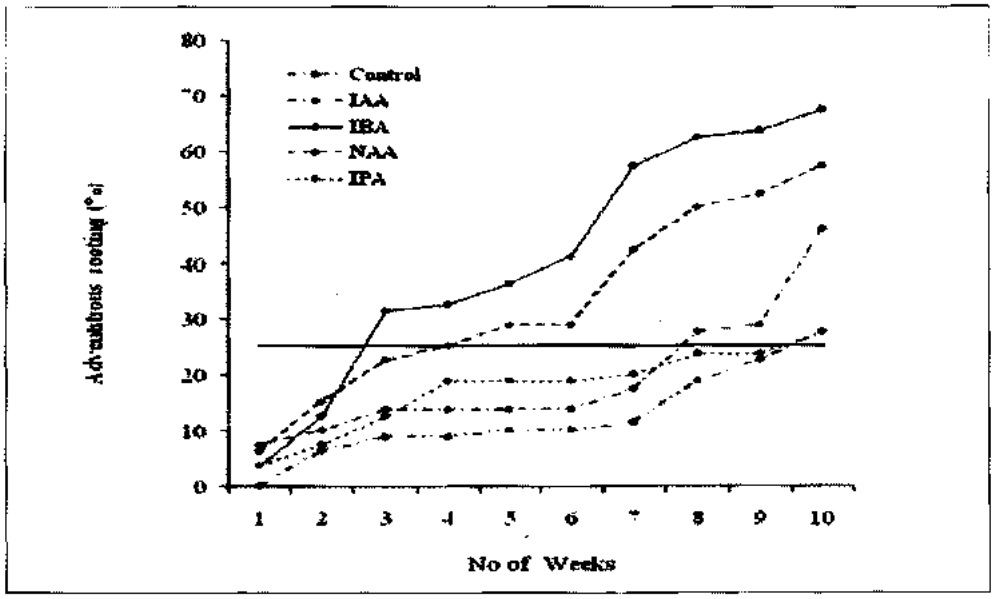

Figure 2. Time variable response for initiation of *in vitro* adventitious rooting as influenced by different auxins at a uniform dose (15 μM) in *B. nutans*. The horizontal baseline represents 25% rooting.

followed by NAA which initiated roots in four weeks (Fig. 2). Three auxins (IAA, IBA and NAA) induced significantly maximum, but at par rooting but IBA produced significantly more number of roots per propagule than IAA and NAA (Table 4, Fig. 1f). Among the various concentrations of IBA, 10-25 µM induced at par but maximum rooting percentage, which was significantly higher than that of the control (Table 5).

Hardening and acclimatization

The gradual process of hardening and acclimatization of plantlets in the controlled conditions of the culture room in root trainers (Fig. 1g) and shade house resulted in around 90 per cent survival of plantlets (Fig. 1 h,i).

Table 4. Effect of 15 µM concentration of different auxins on various parameters of *in vitro* rhizogenesis of *B. nutans*

Treatment (15 µM)	Rooting percentage	Number of roots per propagule	Root length (cm)
Control	26.25 (22.50)	0.60	10.30
IAA	46.25 (43.50)	1.16	12.27
IBA	63.75 (56.25)	2.22	15.55
NAA	60.00 (51.75)	1.56	12.22
IPA	28.75 (28.50)	0.71	11.02
$LSD_{(0.05)}$	15.76	0.63	N.S.

*Values in parenthesis are arc sine transformed.

Table 5. Effect of different concentrations of IBA on various parameters of *in vitro* rhizogenesis in *B. nutans*

TreatmentIBA (μM)	Rooting percentage	Number of roots per propagule	Root length (cm)
0	22.22 (27.77)	0.33	12.27
5	38.89 (38.49)	1.66	14.73
10	50.00 (45.00)	1.77	16.30
15	55.55 (48.23)	2.61	13.40
20	66.66 (55.19)	3.05	10.33
25	77.77 (62.15)	3.27	15.00
$LSD_{(0.05)}$	11.81	1.10	N.S.

*Values in parenthesis are arc sine transformed.

DISCUSSION

The *in vitro* propagation method using explants taken from established and selected field-grown culms offers a desirable alternative for large-scale multiplication of 'plus' or 'elite' bamboo and eliminates the variation inherent in seed-raised populations.

Culture establishment

Aseptic culture establishment was similar in summer, winter and rainy seasons except in autumn season. The bud break was the highest in summer season followed by winter season. Thus, summer and winter seasons are suitable for culture establishment in *B. nutans*. The influence of seasons on axillary bud break and contamination has earlier been reported on *D. giganteus* (Ramanayake and Yakandawala, 1997) and *D. longispathus* (Saxena and Bhojwani, 1993). However, low aseptic culture establishment and bud break during autumn does not match with the findings of Das and Pal (2005), who obtained maximum bud break in the month of October.

The three concentrations of mercuric chloride did not differ in their effect on aseptic culture establishment and bud break. The effectiveness of mercuric chloride as a sterilizing agent for *in vitro* explants of various plant species is well documented (Mascarenhas *et al.*, 1987; Joshi and Nadguada, 1997; Shirin *et al.*, 2000). Variable production of aseptic cultures in different clumps of *B. nutans* indicates towards different population density and kind of micro-flora on their surface which depend upon locality and genotype of collected explants (Jamaluddin, 1999).

In vitro shoot multiplication

A high rate of shoot multiplication in *B. nutans* was obtained due to BA in the medium, which is also in agreement with Nadgir *et al.* (1984) on *D. strictus* seedlings and Nadgauda *et al.* (1990) on *D. brandisii* and *B. bambos*. Many other workers have also recommended BA for bamboo shoot multiplication (Das and Rout, 1991; Prutpongse

and Gavinlertvatana. 1992; Ansari *et al.*, 1996; Arya and Arya, 1997; Huang and Huang, 1995; Yasodha *et al.*, 1997; Arya and Sharma, 1998; Sood *et al.*, 2002).

Rooting of *in vitro* shoots

Different auxins exhibited time variation for initiation of *in vitro* rooting. Maximum and earliest rooting was obtained in IBA followed by NAA. In contrast, rooting in control, IAA and IPA was delayed by several weeks. Both IBA and NAA are less quickly destroyed by autoclaving or light than IAA (Nissen and Sutter, 1988), and exhibit long tissue half-life because of their synthetic nature (Zaerr and Mapes, 1988). In comparison to IAA, IBA also produces less ethylene which is an inhibitor of rooting (Jacobs, 1979). Poor effect of IPA on *in vitro* rooting may be attributed to the odd number of carbon atoms in the side chain of the molecule (Devlin, 1975)

Of the various auxin treatments, 10-25 µM IBA emerges to be the most suitable auxin and corresponds in its effect to rooting of several bamboo species like *D. strictus* (Nadgir *et al.*, 1984), *D. brandisii* and *B. bambos* (Nadgauda *et al.*, 1990), *D. giganteus* and *D. strictus* (Das and Rout, 1991), *Thamnocalamus spathiflorus* (Bag *et al.*, 2000) and *B. balcoou* (Das and Pal, 2005).

Hardening and acclimatization

High rate of transplantation success (90%) can be attributed to the gradual procedure of hardening and acclimatization of the plantlets. High multiplication efficiency, good rooting, easy establishment in the soil and normal growth performance of micropropagated plants obtained in this study are features necessary for adoption of *in vitro* propagation procedure for large-scale multiplication of *B. nutans* plantlets (Fig.1i).

CONCLUSION

The *in vitro* clonal propagation system was developed for nodal segments of 10-year-old field-grown clumps of *B. nutans*. Standardized aseptic culture establishment in winter and summer seasons using MS medium supplemented with 31.06 µM BA and 2.85 µM IAA for high shoot multiplication rate, with 20-25 µM IBA for >77 per cent adventitious root formation, and two step gradual hardening of plantlets for 90 per cent survival in the natural open environment. The regeneration system reported here has been successfully employed for the production of > 2000 hardened plants of *B. nutans* for field planting.

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