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ORIGINAL ARTICLE

In vitro propagation of *Bambusa nutans* Wall. *ex* Munro through axillary shoot proliferation

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Abstract This communication describes for the first time an efficient and reproducible protocol for large-scale multiplication of *Bambusa nutans*. Nodal segments collected from field-grown clumps and cultured on Murashige and Skoog (MS) medium supplemented with 4.4 μM benzylaminopurine (BA) and 2.32 μM kinetin (Kin) gelled with 0.2% gelrite yielded 80% aseptic cultures with 100% bud-break. The in vitro-formed shoots obtained after bud-break were successfully multiplied in MS liquid medium supplemented with 13.2 μM BA, 2.32 μM Kin, and 0.98 μM indole-3-butyric acid (IBA). Sub-culturing of shoots every 3 weeks on fresh multiplication medium yielded a consistent proliferation rate of 3.5-fold. Shoot clusters containing three to five shoots were successfully rooted with 100% success on half-strength MS liquid medium supplemented with 9.8 μM IBA, 2.85 μM indole-3-acetic acid (IAA), 2.68 μM naphthaleneacetic acid (NAA), and 3% sucrose. Plantlets grown in vitro were acclimatized and subsequently transferred to the field. Inter-simple sequence repeat analysis has confirmed the genetic uniformity of the tissue-cultured plants up to 27 passages.

Keywords *Bambusa nutans* · Axillary shoot proliferation · Tissue culture · Rhizome · ISSR

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Introduction

Bamboos are one of the world's most important renewable natural resources. In East and Southeast Asia in particular, bamboos are closely linked to the social and economic well-being of rural communities, being used in house construction and as agricultural tools as well as supporting traditional cottage industries, such as furniture-making, basket-making, handicrafts, among others. Bamboos are also an important source of cellulose for paper and rayon.

Bambusa nutans Wall. ex Munro is a medium-sized woody bamboo which is naturally distributed throughout various states of India, such as Himachal Pradesh, Arunachal Pradesh, and Meghalaya up to an altitude of 1500 m a.s.l. *B. nutans* is one of the priority species of bamboos identified by the National Bamboo Mission (NBM) formulated by the Ministry of Agriculture, Government of India, for commercial cultivation and is exploited at the present time for the pulp and paper industry in India. Culms can measure 6–15 m in height and 5–10 cm in diameter and are smooth, straight, and thick-walled; they are used for various purposes, including timber, house construction, scaffolding, paper mats, etc. (Tewari 1992). The leaves of *B. nutans* are used as fodder for livestock.

In nature, the species regenerates through vegetative means—culm and branch cuttings, rhizomes, and offsets. However, this is a slow process on account of season specificity, relatively small number of propagules, and low rooting ability. Singh et al. (2002) could only achieve 46.7% and 10% rooting in culm and branch cuttings of *B. nutans* respectively, during the month of April. Propagation through seeds, although possible, is not practical because of a long flowering cycle of 35 years (Tewari 1992). In contrast, in vitro techniques for propagation purposes have an immense potential to meet the demand of plant material of this species.

Kalia et al. (2004) raised plantlets of *B. nutans* from organogenic calli obtained from internode and leaf explants. However, this method of in vitro regeneration carries a higher risk of genetic instability (somaclonal variation), thereby defeating the very purpose of micropropagation. In contrast, the axillary branching method is considered to be fairly reliable from the point of view of clonal uniformity among the regenerants. Earlier reports on the micropropagation of *B. nutans* by the axillary branching method used seeds or seedlings, which are rarely available due to long flowering cycle and short viability (Yasodha et al. 1997) or are associated with specific shortcomings, such as low multiplication rates, low rooting frequencies, among others, that restrict the micropropagation of this species on a mass scale. Moreover, when seed is used as the explant in developing the micropropagation protocol, due to segregation of genes it becomes impossible to predict the phenotype and the passing of desired traits from the mother plant to the progenies obtained from them. The unknown genetic background and the heterogeneity in seedling populations are the major constraints associated with seed-based micropropagation protocols. In addition, none of these studies attempted to analyze the clonal fidelity of the tissue-cultured plants of *B. nutans*, which is an essential step for determining the practicality of any micropropagation protocol.

Here we describe an efficient micropropagation protocol suitable for the production of clonally uniform plants through axillary branch proliferation using vegetative tissue derived from field-grown clumps of *B. nutans*.

Materials and methods

Culture initiation

Single-node segments (2–2.5 cm) were excised from actively growing branches of a *B. nutans* clump growing at the bambusetum of the Tropical Forest Research Institute (TFRI), Jabalpur in July. Prior to surface sterilization, the leaf sheath that envelops the axillary bud and a part of the upper internode was removed. The explants were given a quick rinse in 70% ethanol, washed with Teepol (4–5 drops; Reckitt Benckiser, Gurgaon, India) and four to five drops of a germicide called Savlon (Johnson and Johnson, India) for 15 min followed by thorough washing under running tap water for 20 min. The nodal segments were then surface sterilized with 0.1% mercuric chloride for 10 min. After three washings in sterile distilled water, the cut ends of the segments were trimmed and the nodal segments cultured on MS (Murashige and Skoog 1962) medium supplemented with the cytokinins (Sigma Chemicals, St. Louis, MO) 4.4 μM benzylaminopurine (BA) and 2.32 μM kinetin (Kin), and 3% sucrose (commercial grade; DCM Sriram Industries, Meerut, India) and gelled with 0.2% gelrite (Aceto Corp, Lake Success, NY) in 25 × 100-mm glass tubes (Borosil, Mumbai, India). The pH of the medium was adjusted to 5.8 with 1 N NaOH (Qualigens, Mumbai, India) and 1 N HCl (Qualigens, India) prior to autoclaving at 121°C for 20 min.

Shoot multiplication

The in vitro-formed shoots (2–3) obtained from 3-week-old cultures on initiation medium were excised from the nodal segments and cultured in liquid MS medium containing 3% sucrose. Filter paper strips obtained after passing Whatman filter paper No. 1 (Whatman, New York, NY) through a paper shredder were used to provide support to shoots in the liquid medium. Since these shoots remained dormant and eventually turned necrotic on MS basal medium, the MS medium was supplemented with different concentrations of the cytokinins BA (4.4–22 μM) and Kin (2.32–6.97 μM), either individually or in various combinations. Once the best combination of cytokinins for shoot multiplication had been determined (13.2 μM BA, 2.32 μM Kin), the cytokinin-supplemented medium was tested with the addition of different auxins (Sigma Chemicals), such as indole-3-acetic acid (IAA; 1.14–7.13 μM), indole-3-butyric acid (IBA; 0.98–6.12 μM), and naphthaleneacetic acid (NAA; 1.07–5.37 μM). After the combination/concentration of phytohormones had been optimized for shoot multiplication, the effect of various gelling agents, such as agar (0.8%, commercial grade; Supreme Marine Chemicals, Mumbai, India), agar-gel (0.4%; commercial grade, Sigma), and gelrite (0.2%) was also tested.

Shoot multiplication was carried out in 400-ml ordinary glass jars (Hindustan National Glass and Industries, Konnagar, India) with each jar containing one cluster of at least three to four shoots (propagule) in 40 ml of medium. Since the use of plain caps in preliminary experiments had resulted in hyperhydration of shoots, in all subsequent experiments, as a matter of routine the jars were capped with transparent polypropylene lids bearing a hole plugged with non-absorbent cotton wrapped in muslin cloth.

Shoot clusters were sub-cultured at a regular interval of 3 weeks by dividing the shoot cluster obtained at the end of each multiplication cycle into smaller clusters of three to four shoots (a propagule). Multiplication rates were calculated on the basis of number of propagules derived from one shoot cluster after the completion of each cycle of 3 weeks. Necrotic and decayed shoots or leaf sheaths, if any, were removed from the clusters before transfer to fresh medium.

Rooting

Clusters of three to four shoots, each measuring at least 3.5 cm in length, were obtained after completion of the shoot multiplication cycle and transferred to various rooting media. Initially, in a broad range experiment, the shoot clusters were cultured on MS and modified MS with major salts reduced to half-strength (1/2 MS) and one-fourth-strength. These basal media were supplemented with various auxins (IAA, 2.85–17.13 μM; IBA, 2.45–14.7 μM; NAA, 2.68–16.12 μM) either individually or in combinations. Since in all the media combinations involving 1/2 MS, the results were better than full-strength MS or 1/4 MS, all of the rooting experiments described here were performed only with 1/2 MS (Table 3). Among the various experiments related to the optimization of phytohormones, the highest rooting frequency was obtained on 1/2 MS + 2.85 μM IAA + 9.8 μM IBA + 2.68 μM NAA. To further enhance the rooting frequency, additional experiments were performed using media containing different concentrations of sucrose (1–4%), gelling agent, and closure caps.

Culture conditions

All of the cultures were maintained at 25 ± 2°C, under a 16/8-h (light/dark) photoperiod with a light intensity of 47.29 μmol m^{-2} s^{-1} provided by cool-white fluorescent tubes (40 W; Phillips, India).

Hardening

The in vitro-raised plantlets obtained after 3 weeks of inoculation on rooting medium were taken out of the culture jars and washed thoroughly under running tap water to remove shredded Whatman filter paper and traces of medium attached to the roots. In total, 24 plants in triplicates were transferred to polythene bags (16 × 8 cm), with each of the potting mixes containing soil and agropeat (Varsha Enterprises, India) in different ratios (v/v; 1:0; 1:1; 2:1; 3:1; 0:1).

The plants were reared in a greenhouse maintained at 28 ± 2°C. The plants were initially placed close to the cooling pads (relative humidity 80–85%), gradually being shifted away from the pads towards the exhaust fans over a period of 1 week. Thereafter, the plants were nurtured inside a polyhouse for 15–20 days and then under a shade net (50% light cut-off) for 20–30 days, following which they were transferred to an open nursery where they were retained until use.

Field transplantation

On an experimental basis, 12 fully hardened tissue-cultured plants of *B. nutans* were transplanted from the nursery to the field (4 m × 4 m) at our campus in Gual Pahari, Haryana, India, during the monsoons.

Statistical analysis

All of the experiments were carried out with 24 samples in three replicates. The effect of different treatments was quantified, and the level of significance was determined by analysis of variance (ANOVA) using CoStat software (Tempus Software, Birmingham, UK). Significant differences between the means were assessed by Duncan's Multiple Range Test (DMRT) at $p = 0.05$.

DNA extraction and PCR amplification

The leaves from the *B. nutans* mother plant, in vitro-grown shoots in the 11th, 17th, and 27th passages, and the hardened plant growing in the polyhouse and field as well as from an outlier (*Melocanna baccifera*) were used for inter-simple sequence repeat (ISSR) analysis. These were labeled and lyophilized in the Virtis freezemobile G lyophilizer (SP Industries, Warminster, PA) for 48 h at −70°C. Total DNA was extracted following a modified cetyl trimethyl ammonium bromide (CTAB) DNA extraction procedure based on the protocol by Doyle and Doyle (1990). Qualitative and quantitative assessment of total genomic DNA was carried out by agarose (U.S. Biochemicals, USA) gel electrophoresis. Each DNA sample was diluted to a concentration of 25 ng/μl with sterile distilled water and stored at 4°C.

Twenty-four University of British Columbia (Vancouver, BC, Canada) ISSR primers were tested, and on the basis of their suitability, 15 were selected for detailed study. PCR analyses were performed in a volume of 20 μl containing 25 ng DNA, 10× PCR buffer (Biotools, Granada, Spain), 50 mM $MgCl_2$ (Biotools), 10 mM dNTPs (Promega, Madison, WI), 1 U *Taq* DNA polymerase (Biotools), and 10 mM primer. Optimized PCR conditions used for amplification consisted of an initial denaturation step at 94°C for 2 min, 35 cycles of 30 s at 94°C (denaturation), 30 s at 42°C (annealing), and 1 min at 72°C (extension), followed by one final extension of 5 min at 72°C, with a soak temperature of 4°C performed in a 'Bio-Rad DNA Engine' (Peltier Thermal Cycler, MJ Research, Watertown, MA). The amplified DNA was separated by electrophoresis on a 2% agarose gel (voltage 80 mV, time 3–4 h). The size of the amplification products was estimated using a 1-kbp ladder (New England Biolabs, Ipswich, MA). The gels were visualized and photographed using a gel documentation system (AlphaImager EC; Alpha Innotech Corp, San Leandro, CA). All PCR reactions were repeated at least twice to check their reproducibility.

Only consistently reproducible, well-resolved fragments, ranging from 100 to 1600 bp, were scored as present or absent for the ISSR markers in each sample.

Results

Culture initiation

Using the sterilization procedure described in the "Materials and methods", we obtained 80% aseptic cultures with 100% bud break (Fig. 1a). To study the effect of season, cultures were also initiated in different months. However, the best results, as described here, were obtained with cultures initiated in July.

Shoot multiplication

Preliminary experiments in liquid MS basal medium in the presence of 3% sucrose led to necrosis and death of shoots in the first passage. This necessitated the inclusion of cytokinins in the shoot multiplication medium, either alone or in combination (Table 1). Of the different concentrations of BA (4.4–22 μM) tested, 13.2 μM BA gave the best proliferation rate: 2.16-fold. Higher concentrations of BA not only lowered the multiplication rate but also resulted in stunted shoots. Kin did not result in shoot proliferation when added alone at concentrations ranging from 2.32 to 6.97 μM: the shoots remained dormant for some time and ultimately died. However, 2.32 μM kin when used with the optimal concentration of BA (13.2 μM) increased the multiplication rate up to 2.33-fold; Kin at concentrations higher than 2.32 μM proved inhibitory.

To study the synergistic effect (if any) of auxins and cytokinins, the optimal combination of BA (13.2 μM) and Kin (2.32 μM) was tested with different auxins IAA (1.14–7.13 μM), IBA (0.98–6.12 μM) and NAA (1.07–5.37 μM) (Table 2). Of the various combinations tried, 0.98 μM IBA in conjunction with 13.2 μM BA, 2.32 μM Kin, and 3% sucrose proved to be the best shoot multiplication medium, resulting in a threefold increase in the multiplication rate and an increase in shoot length of 3.67 cm (Fig. 1b). Thereafter, the shoots could be multiplied consistently for 27 passages without any abnormality or decline in vigor. The rate of shoot proliferation on this 'optimized' medium was further enhanced to 3.5-fold by the fifth passage. In the eighth passage, about 15% cultures formed in vitro rhizomes on this medium (Fig. 1c). The percentage of cultures exhibiting in vitro rhizome formation increased with increasing number of passages.

The use of IAA (3.99–7.13 μM) and NAA (1.07–5.37 μM) in conjunction with BA (13.2 μM) and Kin (2.32 μM) increased the length of the shoots but lowered the multiplication rate.

After standardizing the concentrations of the hormones supplemented to the MS basal medium for shoot

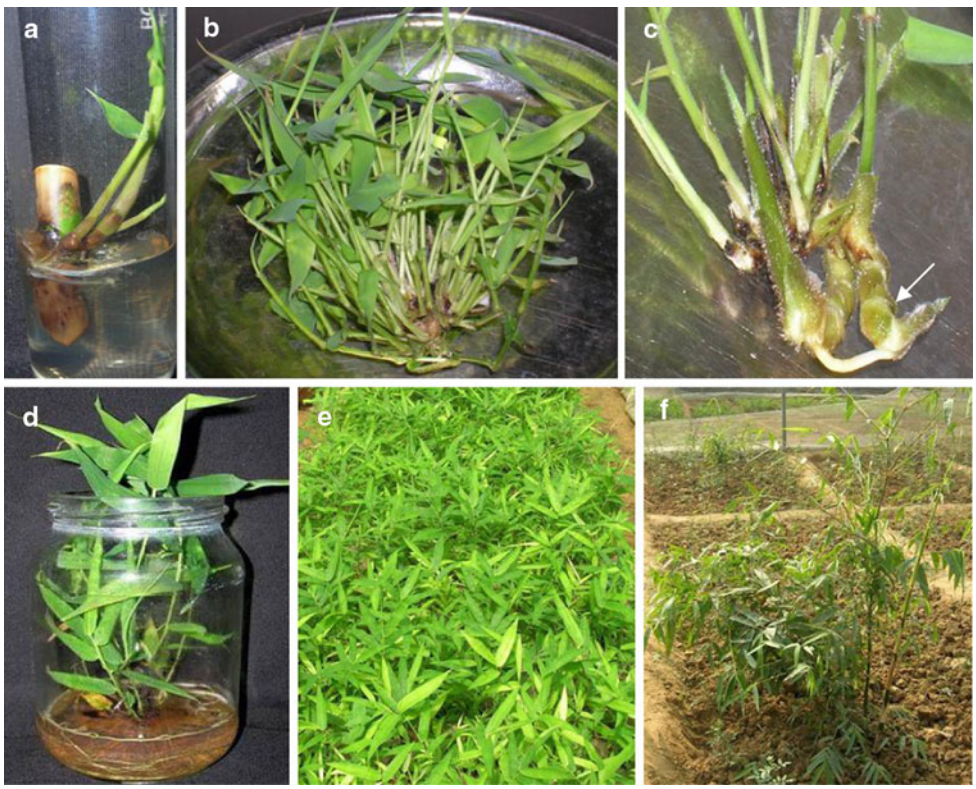

Fig. 1 Micropropagation of *Bambusa nutans*. **a** Nodal explant from field-grown clump, **b** shoot proliferation in Murashige and Skoog basic medium (MS) + 13.2 μM benzylaminopurine (BA) + 2.32 μM kinetin (Kin) + 0.98 μM indole-3-butyric acid (IBA) in liquid medium after 3 weeks, **c** in vitro rhizome formation on MS + 13.2 μM BA + 2.32 μM Kin + 0.98 μM IBA in liquid medium after 3 weeks, **d** rooting on 1/2-strength MS + 9.8 μM IBA + 2.85 μM indoleacetic-3-acid + 2.68 μM naphthaleneacetic acid and 3% sucrose after 3 weeks, **e** hardening in nursery, **f** 9 months after field transfer

Table 1 Effect of different concentrations of the cytokinins benzylaminopurine and kinetin supplemented individually and in combination to MS basic medium on in vitro shoot multiplication after 3 weeks

Hormone concentration (μM)	Shoot length (cm)	Multiplication fold
BA (4.4)	3.58 ± 0.12^{a}	1.75 ± 0.04^{abc}
BA (8.8)	3.34 ± 0.08^{b}	1.85 ± 0.02^{abc}
BA (13.2)	3.23 ± 0.07^{c}	2.16 ± 0.02^{ab}
BA (17.6)	2.56 ± 0.13^{l}	1.68 ± 0.1^{abc}
BA (22)	2.89 ± 0.07^{f}	1.73 ± 0.08^{abc}
Kin (2.32)	2.78 ± 0.42^{g}	0.66 ± 0.11^{d}
Kin (4.65)	2.58 ± 0.39^{hi}	0.66 ± 0.11^{d}
Kin (6.97)	2.60 ± 0.38^{h}	0.66 ± 0.11^{d}
BA (8.8) × Kin (2.32)	3.08 ± 0.05^{d}	2.27 ± 0.02^{ab}
BA (8.8) × Kin (4.65)	2.92 ± 0.06^{e}	1.94 ± 0.07^{abc}
BA (13.2) × Kin (2.32)	3.09 ± 0.08^{d}	2.33 ± 0.03^{a}
BA (13.2) × Kin (4.65)	2.75 ± 0.06^{g}	1.73 ± 0.63^{abc}
BA (17.6) × Kin (2.32)	2.76 ± 0.06^{g}	1.60 ± 0.03^{bc}
BA (17.6) × Kin (4.65)	2.46 ± 0.06^{j}	1.43 ± 0.01^{c}

BA benzylaminopurine, *Kin* kinetin

Values are given as the mean ± standard error (SE). Values followed by the same letters in superscript within the column are not significantly different at the 5% level (Duncan's multiple range test)

Table 2 Effect of different concentrations of auxins (IAA, IBA and NAA) in combination with the optimized combination of cytokinins (BA, 13.2 μM; Kin, 2.32 μM) on in vitro shoot multiplication after 3 weeks

Hormone concentration (μM)	Shoot length (cm)	Multiplication fold
0	3.54 ± 0.08^{de}	2.33 ± 0.03^{bc}
NAA (1.07)	3.72 ± 0.09^{cde}	1.6 ± 0^{cde}
NAA (2.68)	4.38 ± 0.14^{bc}	1.4 ± 0.02^{de}
NAA (3.76)	4.39 ± 0.12^{bc}	1.4 ± 0.02^{de}
NAA (5.37)	5.07 ± 0.08^{a}	1.00 ± 0.00^{e}
IBA (0.98)	3.67 ± 0.05^{cde}	3.00 ± 0.1^{a}
IBA (2.45)	4.06 ± 0.09^{cde}	2.66 ± 0.02^{ab}
IBA (3.43)	3.98 ± 0.06^{cde}	1.72 ± 0.01^{cd}
IBA (4.9)	4.18 ± 0.1^{bcd}	1.83 ± 0.02^{cd}
IBA (6.12)	4.00 ± 0.12^{cde}	1.66 ± 0.05^{cd}
IAA (1.14)	3.36 ± 0.07^{e}	2.16 ± 0.05^{bc}
IAA (2.85)	3.50 ± 0.04^{de}	1.83 ± 0.11^{cd}
IAA (3.99)	4.35 ± 0.01^{bc}	1.72 ± 0.13^{cd}
IAA (5.71)	4.75 ± 0.18^{ab}	1.55 ± 0.07^{cde}
IAA (7.13)	4.04 ± 0.12^{cde}	1.55 ± 0.07^{cde}

IBA indole-3-butyric acid, *IAA* indoleacetic-3-acid, *NAA* naphthaleneacetic acid

Values are given as the mean ± SE. Values followed by the same letters in superscript within the column are not significantly different at the 5% level (Duncan's multiple range test)

multiplication medium, the effect of different gelling agents, such as agar (0.8%), agar-gel (0.4%), and gelrite (0.2%), was compared with that of liquid medium. The use of any gelling agent lowered the multiplication rate and reduced the length of the shoots as compared to the control (liquid culture), clearly indicating that the liquid medium was the best suited for shoot multiplication. However, if proliferating shoots were left submerged in the liquid medium, they became hyperhydrated and died over a period of time. To solve the problem of submergence (hyperhydration), the shoot clusters were supported by shredded Whatman filter paper strips.

The proliferating clusters were sub-cultured every 3 weeks. Increasing the subculture time resulted in dessication and necrosis of the leaves and leaf sheath.

Rooting

When added individually to 1/2 MS basal medium, IAA (17.13 μM), IBA (9.8 μM), and NAA (10.75 μM) yielded a maximum rooting percentage of 12.5, 58.33, and 37.5%. However, when used in combination, these auxins significantly enhanced the rooting frequency (Table 3). 1/2MS liquid medium supplemented with IBA (9.8 μM), IAA (2.85 μM), NAA (2.68 μM), and sucrose (3%) proved to be the best combination, resulting in 100% rooting within 3 weeks (Fig. 1d). There was no alteration in the rooting frequency and the quality of the shoots when the same medium was used but gelled with 0.2% gelrite; however, the time required for rooting increased significantly (40–45 days).

Hardening and field transplantation

To optimize the hardening procedure, the plants were transferred to various potting mixes, as described in the "Materials and methods". After 4 weeks of transplantation, the hardening survival was 98% in the 2:1 soil:agropeat mixture, 92% in the 1:1 soil:agropeat mixture, 90% in agropeat, 88% in the 3:1 soil:agropeat mixture, and 80% in soil alone. Plants potted in polybags containing the 2:1 (soil: agropeat) potting mix showed a successful hardening rate of 95.83% up to the nursery stage (Fig. 1e). On an experimental basis, 12 plants transplanted to the field at Gual Pahari with 100% success showed active growth and the culms reached to a height of 2.02 m within 9 months of transplantation to the field (Fig. 1f). In total, more than 2,500 plantlets have been produced using this protocol.

Clonal fidelity

DNA samples from in vitro-grown shoots in the 11th, 17th, and 27th cycle of shoot multiplication, hardened plants

Table 3 Effect of different concentrations of IAA, IBA and NAA on in vitro root induction after 3 weeks

Hormone concentration	Rooting percentage (%)	Number of roots	Root length (cm)
IAA (5.71 μM)	0	0.00 ± 0.00^{k}	0.00 ± 0.00^{w}
IAA (11.42 μM)	8.33	0.12 ± 0.06^{k}	0.18 ± 0.1^{u}
IAA (17.13 μM)	12.5	0.58 ± 0.23^{jk}	0.16 ± 0.05^{v}
IBA (4.9 μM)	33.33	3.12 ± 0.74^{ij}	0.46 ± 0.08^{r}
IBA (9.8 μM)	58.33	3.24 ± 0.3^{e}	0.76 ± 0.14^{m}
IBA (14.7 μM)	45.83	3.33 ± 0.37^{e}	0.65 ± 0.05^{o}
NAA (5.37 μM)	29.1	1.41 ± 0.4^{ghi}	0.25 ± 0.06^{s}
NAA (10.75 μM)	37.5	1.58 ± 0.4^{ghi}	0.22 ± 0.05^{t}
NAA (16.12 μM)	29.1	3.58 ± 0.47^{de}	0.25 ± 0.03^{s}
IAA (2.85 μM) × IBA (4.9 μM)	41.66	1.2 ± 0.37^{hi}	0.8 ± 0.21^{l}
IAA (2.85 μM) × IBA (7.35 μM)	66.66	2.41 ± 0.5^{f}	0.91 ± 0.16^{k}
IAA (2.85 μM) × IBA (9.8 μM)	62.5	3.2 ± 0.72^{e}	1.33 ± 0.24^{I}
IAA (5.71 μM) × IBA (4.9 μM)	50	1.75 ± 0.5^{ghi}	0.7 ± 0.2^{n}
IAA (5.71 μM) × IBA (7.35 μM)	79.16	6.00 ± 0.82^{a}	3.47 ± 0.39^{a}
IAA (5.71 μM) × IBA (9.8 μM)	83.33	4.83 ± 0.63^{b}	3.37 ± 0.39^{b}
IAA (8.56 μM) × IBA (4.9 μM)	58.33	1.83 ± 0.55^{fgh}	1.75 ± 0.43^{g}
IAA (8.56 μM) × IBA (7.35 μM)	41.66	3.66 ± 0.82^{de}	2.66 ± 0.62^{c}
IAA (2.85 μM) × IBA (2.45 μM) × NAA (2.68 μM)	75	3.29 ± 0.52^{e}	1.81 ± 0.27^{f}
IAA (2.85 μM) × IBA (7.35 μM) × NAA (2.68 μM)	91.66	4.16 ± 0.51^{cd}	1.33 ± 0.21^{j}
IAA (2.85 μM) × IBA (9.8 μM) × NAA (2.68 μM)	100	4.29 ± 0.44^{bc}	2.23 ± 0.16^{d}
IAA (5.71 μM) × IBA (2.45 μM) × NAA (2.68 μM)	54.16	1.66 ± 0.38^{ghi}	0.57 ± 0.16^{q}
IAA (5.71 μM) × IBA (4.9 μM) × NAA (2.68 μM)	75	2.04 ± 0.29^{fg}	1.44 ± 0.29^{h}
IAA (5.71 μM) × IBA (7.35 μM) × NAA (2.68 μM)	95.83	6.08 ± 0.58^{a}	2.13 ± 0.14^{e}
IAA (5.71 μM) × IBA (9.8 μM) × NAA (2.68 μM)	83.33	4.66 ± 0.63^{bc}	0.63 ± 0.11^{p}

Values are given as the mean ± SE. Values followed by the same letters in superscript within the column are not significantly different at the 5% level (Duncan's multiple range test)

growing in the polyhouse, plants growing in the field, the mother plant, and an outlier (*M. baccifera*) were subjected to ISSR analysis. The rationale for using an outlier was to ensure the suitability of the primers for their ability to detect polymorphism in the species under consideration. Of a total of 24 primers screened, only 15 were amplified; the remaining nine primers did not work. The assay with these 15 ISSR primers generated 93 scorable band classes (Table 4), with the number of bands for each primer varying from 2 to 13 and an average of 6.2 bands per ISSR primer. A total of 1,581 bands (no. of plantlets analyzed × no. of band classes with all ISSR primers) were generated, giving rise to monomorphic patterns across all *B. nutans* plantlets analyzed. No polymorphic bands were observed in the parent plant and the tissue-culture raised progenies, whereas a polymorphic banding pattern was obtained with the outlier *M. baccifera* (Fig. 2). Furthermore, the polymorphic bands scored for the outlier proved that the primers employed were competent enough to distinguish the plantlets based on genetic variations. The visual analysis of *B. nutans* plants also did not reveal any morphological variations. These results confirm that *B. nutans* plants obtained by the axillary branching method retained their genetic fidelity for a prolonged period (27 passages) under in vitro conditions.

Discussion

The aim of this study was to develop an efficient micropropagation protocol for *B. nutans* by the axillary branching method using vegetative tissue of a field-grown bamboo clump. Yasodha et al. (1997) used seed as the starting material, but the availability of *B. nutans* seed is highly restricted due to its long vegetative cycle of 35 years. Moreover, in order to accrue both quantitative and qualitative gains in this cross-pollinating species, explants derived from adult plants growing in the field are always preferred over seeds. The only other reported protocol for the micropropagation of *B. nutans* using nodal segments obtained from a field-grown bamboo clump incorporates a combination of antibiotics (streptomycin and kanamycin) during the culture initiation stage (Yasodha et al. 2008). However, it is always desirable to obtain aseptic cultures on antibiotic-free media because antimicrobial agents may retard the growth of the explant.

Table 4 The PCR amplicons obtained with 15 inter-simple sequence repeat primers ascertaining the clonal fidelity in micropropagated plants of *Bambusa nutans*

ISSR primer	Primer sequence (5′–3′)[a]	Monomorphic bands in tissue culture-raised plant + outlier	Number of bands amplified only in the outlier (*Melocanna baccifera*)	Total number of bands amplified
UBC 810	$(GA)_8$ AT	10	7	17
UBC 812	$(GA)_8$ A	5	0	5
UBC 813	$(CT)_8$ T	2	0	2
UBC 814	$(CT)_8$ A	5	3	8
UBC 818	$(CA)_8$ G	5	4	9
UBC 830	$(TG)_8$ G	3	5	8
UBC 840	$(GA)_8$ YT	9	4	13
UBC 841	$(GA)_8$ YC	6	4	10
UBC 842	$(GA)_8$ YG	7	6	13
UBC 843	$(CT)_8$ RA	7	2	9
UBC 848	$(CA)_8$ RG	7	5	12
UBC 852	$(TC)_8$ RA	4	5	9
UBC 857	$(AC)_8$ YG	13	11	24
UBC 860	$(TG)_8$ G	4	3	7
UBC 868	$(GAA)_6$	6	4	10
	Total number of bands produced	93	63	156

ISSR, Inter-simple sequence repeat

[a] Key to base compositions: R = A, G; Y = C, T

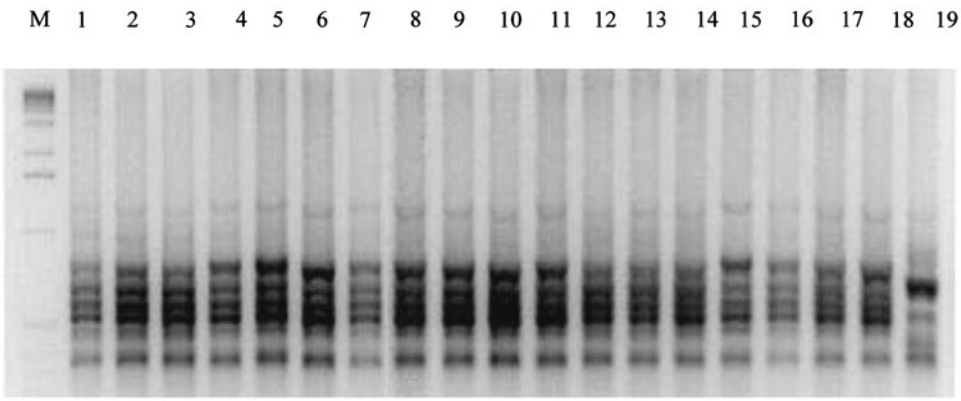

Fig. 2 DNA amplification obtained with primer UBC 848. *Lanes*: *M* 1-kbp DNA ladder, *1* mother plant, *2–6* micropropagated plants at 11th passage, *7–11* micropropagated plants at 17th passage, *2–16* micropropagated plants at 27th passage, *17* plant at polyhouse stage, *18* plant at field stage, *19* outlier (*Melocanna baccifera*)

Additionally, the entire process of working with antibiotics is time consuming and costly. Using our protocol, we obtained an 80% rate of aseptic cultures with 100% bud-break without incorporating any antibiotic in the sterilization procedure, in contrast to the protocol reported by Yasodha et al. (2008).

Our results show that the combined presence of BA and Kin in the MS medium stimulated the growth and multiplication of shoots. While BA alone has been reported to be an important cytokinin for multiple shoot formation in several bamboo species, such as *Guadua angustifolia* (Jimenez et al. 2006), *Pseudoxytenanthera stocksii* (Sanjaya et al. 2005), and *Dendrocalamus giganteus* (Ramanayake et al. 2001), the synergistic effect of BA and Kin on the shoot multiplication rate has been demonstrated in *Bambusa balcooa* (Chowdhury et al. 2004; Das and Pal 2005; Negi and Saxena, unpublished), *B. tulda* (Saxena 1990), and *D. giganteus* (Arya et al. 2006). Yasodha et al. (2008) used BA alone and obtained only a threefold increase in shoot multiplication after 6 months.

Cytokinins can, at times, also act synergistically with auxins. In our study, the cytokinins BA and Kin, in conjunction with the auxin IBA, enhanced the multiplication rate, increased the shoot length, and improved the overall health of the shoots. A similar synergistic effect of the cytokinin–auxin combination has been reported in *Bambusa bambos* (Kapoor and Rao 2006), *B. tulda* (Mishra et al. 2008), and *Dendrocalamus longispathus* (Saxena and Bhojwani 1993) as well.

For successful establishment, a bamboo clump must possess healthy shoots and a well-developed root and rhizome system. In nature, *B. nutans* clumps produce

sympodial rhizomes (Tewari 1992). The thickenings of the pachymorph (sympodial) rhizome allow a greater storage potential and serve as an important adaptation to dry conditions (with hard soils and periodic drought) (Stapleton 1998). In our study, rhizomes were induced in vitro in the optimized shoot multiplication medium (MS + 13.2 μM BA + 2.32 μM Kin + 0.98 μM IBA), possibly due to the interaction between IBA and the cytokinins. Kapoor and Rao (2006) reported in vitro rhizome formation in 100% of the cultures of *B. bambos* in medium containing the optimal concentration of both BA and NAA. Sanjaya et al. (2005) obtained in vitro rhizomes in *P. stocksii* with continued sub-culturing of rooted plantlets on medium containing 1/2-strength major salts, 4.9 μM IBA, 0.44 μM BA, 283.93 μM ascorbic acid, 118.1 μM citric acid, 104.04 μM cysteine, and 342.24 μM glutamine. Occasional in vitro rhizome formation has been reported after 30 days of culture of *Dendrocalamus strictus* in a rooting medium that contained 1/2-strength major salts and 0.2 mg/l IBA (Chowdhury et al. 2004). However, in our study, the formation of in vitro rhizomes was confined to the shoot multiplication stage only.

We found that cultures in liquid medium performed better than those cultured on solid medium following the addition of a gelling agent (agar, agar-gel, or gelrite). This poorer performance on solid medium can be attributed to the binding of water and the absorption of minerals and growth hormones by the gelling agents, resulting in a restricted supply of the former to the growing shoots (Debergh 1983). In comparison, liquid medium facilitates the easier uptake and better absorption of medium components as the growing shoots are in direct contact with the medium. Saxena (1990) also obtained the highest shoot multiplication rates and growth in liquid medium in *B. tulda*. In addition, the shoots were greener and healthier in liquid medium than on semi-solid medium: the lamina of the leaf, which was almost inconspicuous or under-developed on agar medium, was green and well developed in the liquid medium. We successfully solved the problem of hyperhydration of the shoots by growing the shoot clusters of *B. nutans* in liquid medium in culture jars capped with polypropylene caps plugged with cotton wrapped in muslin cloth instead of airtight polypropylene caps. This improvement may be due to a lowering of the relative humidity, as high relative humidity is known to cause hyperhydricity (Zimmerman et al. 1991; Ziv 1991; Tornero et al. 2001), or to better aeration which allowed the removal of C_2H_4 (if any) from the culture vessel.

Conventional cuttings of *B. nutans* are reported to yield a low rooting frequency of 46.7% (Singh et al. 2002) and, therefore, the 100% rooting rate achieved in our study assumes a great significance in mass multiplication of this species. In comparison to the two studies of Yasodha et al. (1997, 2008) in which a rooting percentage of 84.7 and 68%, respectively, was achieved in a two-step rooting procedure, the 100% rooting frequency on a single medium in our study represents a significant improvement. The rationale behind the favorable effect of a reduced macronutrient concentration is that the concentration of nitrogen ions required for root formation is much lower than that for shoot formation and growth (Driver and Suttle 1987).

The survival rate of in vitro-raised plantlets of *B. nutans* during the hardening stage was high. There is no mention of hardening and acclimatization data in previous studies. This high hardening success can be attributed to the induction of in vitro rhizomes, as these structures not only improve the establishment of plants in the field but also early culm production (Kapoor and Rao 2006).

Of the various methods of in vitro propagation, axillary branching is the least susceptible to somaclonal variations that might occur under in vitro conditions (Shenoy and Vasil 1992). Consequently, this method has been used for the micropropagation of several economically important plant species, including bamboos, such as *B. tulda* (Mishra et al. 2008), *D. giganteus* (Arya et al. 2006), *D. longispathus* (Saxena and Bhojwani 1993), and *G. angustifolia* (Jimenez et al. 2006). Heterogeneity in tissue-cultured plants can seriously limit the purpose of in vitro propagation systems. Therefore, it is advisable to test the clonal fidelity of tissue culture-raised plants at regular intervals. In our study, the ISSR analysis was performed to screen for the occurrence of somaclonal variants in tissue culture-raised plantlets of *B. nutans* in various passages of shoot multiplication as well as the hardening stage prior to the dispatch of plants. ISSR markers are universal, quick to use, and easy to apply; they have the reproducibility of SSR markers because of the longer length of their primers (Bornet and Branchard 2001). Amplification by ISSR markers does not require prior information of the genome sequence and leads to multilocus and highly polymorphic patterns (Tsumara et al. 1996; Nagaoke and Ogihara 1997). Prior to this study, the authors used ISSR markers to establish the clonal uniformity of tissue culture plants in another bamboo species, *Bambusa balcooa* (Negi and Saxena 2009). Even though we conducted clonal fidelity studies up to the 27th passage, it was purely an academic exercise as it is always advisable stop cultures in the production line after 15–16 passages because the risk of genetic variation increases with every passage.

This is the first report of a complete micropropagation protocol for the production of clonally uniform plants of *B. nutans* using single-node segments from a field-grown clump as explant material. With the production of over 2500 plants, we suggest that the suitability of our protocol for large-scale propagation of species has been successfully demonstrated.

Conclusions

A long flowering cycle (35 years) and a slow vegetative method of propagation are the major difficulties confronted in meeting the rising demand for *B. nutans*. We have established an efficient method for the in vitro propagation of clonally uniform *B. nutans* plants through axillary shoot proliferation. The high rates of shoot multiplication, rooting, and plant survival in the field obtained using this method suggest that this protocol will contribute to meeting this growing demand for true-to-type, disease-free, and high-quality plant material.

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