In vitro cloning of *Bambusa pallida* Munro through axillary shoot proliferation and evaluation of genetic fidelity by random amplified polymorphic DNA markers

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Abstract

Multiple shoots emerged from the nodal shoot segments of the field-grown candidate plus clump explants of *Bambusa pallida* Munro when cultured on Murashige and Skoog (MS) liquid medium with additives (ascorbic acid 50 mg/L + citric acid 25 mg/L + cysteine 25 mg/L) and combined use of α-naphthalene acetic acid (NAA) 1.34 µM + thiodiozuron 1.125 µM in a 2-week period. Further shoot multiplication was achieved in MS liquid medium with additives + NAA 1.34 µM + 6-benzylaminopurine 4.4 µM at 25±2°C and 33.78 µmol photons m^{-2} s^{-1} light illumination for a 12-h photoperiod. These shoots were rooted within four weeks in MS/2 basal salt medium with additives + 2% sucrose +1% glucose, and 0.6% agar by pulse treatment of shoots with indole 3 butyric acid 0.5 mg/mL for 30 min prior to inoculation. Rooted plants were successfully hardened in the mist chamber. Survival rate during hardening was more than 95%. Micropropagated plants achieved a height of 25-30 cm with 3-4 tillers (shoots) with miniature rhizome in a 4-month period. Genetic stability was observed in the micropropagated plants.

Introduction

Bambusa pallida Munro is one of the most industrially important bamboo species. It is distributed in India and Burma. In India, it is found in Arunachal Pradesh, Nagaland, Mizoram, Tripura, Assam, North Bengal, Meghalaya, Sikkim and Myanmar.[1] It naturally occurs on gentle slope at altitudes up to 2000 m and in the plains. It is cultivated in the planes, mostly in North-Eastern India. Culms are green and are covered with white powder. They are 13-20 m height with a diameter of 5.0-8.0 cm. *Bambusa pallida* Munro is mainly used to build homes, and make baskets, mats, toys, wall plates, screens and wall hangers. In addition, it is one of the most important bamboos used in the pulp and paper industry.[2] From a commercial point of view, it is one of the 14 most important bamboo species selected by the National Mission on Bamboo Applications because of its high tensile strength and it is considered an important vehicle for widespread and sustainable development. The Food and Agricultural Organization has reported that its young juvenile shoots are rich in calcium, vitamin, cellulose, amino acids and other trace elements, and it is commonly eaten as a vegetable in the local community. Charcoal from *B. pallida* provides effective adsorbents to remove iron from water.[3] Leaf and leaf sheath litter decay of these species are a good source of carbon, nitrogen and lignin.[4] It has a wide range of applications, and ever increasing demand and unsuccessful attempts to replenish cultivation following indiscriminate overexploitation are resulting in severe depletion of wild stock. A long flowering cycle (40 years) and the low success rate of propagation through culm cuttings are the main problems associated with conventional methods of propagation of the species.[5,6] Since the gap between demand and supply of bamboo is widening, plant tissue culture-based biotechnological tools provide scope for rapid and mass production of clonal planting material of candidate plus clumps, quickly improving rejuvenation. The main disadvantages of the use of seedling material are insufficient or no knowledge of genetic background, restricted availability, and rapid loss of germination. The problems associated with adult bamboos are endogenous contamination, hyperhydricity, instability of multiplication rate at initial stage, poor rejuvenation, and low rate of rooting.[7] Although management of adult tissues is difficult, micropropagation through axillary shoot proliferation is considered safer and a preferred method for the commercial propagation of hardwoods because it maintains genetic stability better than propagation by organogenesis.[8] Superior genotypes (Candidate Plus clumps) of *B. pallida* have been selected by the Arunachal Pradesh Forest Research Institue, Itanagar, North Eastern India, based on the morphological traits as well as by the Rain Forest Research Institute, Jorhat.[9] *In vitro* propagation of bamboo through axillary shoot proliferation from mature clumps has been reported in *Dendrocalamus strictus*, *Bambusa edulis*, *D.brandisii*, *D.giganteus, Pseudoxyte - nanthera stocksii*, *Bambusa balcoa, Guadua angustifolia* and *B.glaucescens*.[10-17] However, use of tissue culture as a continuous source of disease-free planting material for commercial purposes and periodic monitoring of the degree of genetic stability among *in svitro* grown plantlets are of utmost importance. The chances of somaclonal variation at early or late phase of culture can be minimized by regular assessment of genetic stability.Various molecular tools, such as Random Amplified Polymorphic DNA (RAPD), Restricted Fragment Length Polymorphism (RFLP), Amplified Fragment Length Polymorphism (AFLP) and Inter Simple Sequence Repeats (ISSR) markers, are available to assess variability. Among these, RAPD is the simplest and cheapest for analysis of genetic fidelity of *in vitro* propagated plants.[18-20] There are no reports of micropropagation of *B. pallida*. This is the first report on *in vitro* cloning of *B. pallida* through axillary shoot proliferation from the explants of superior genotypes of mature clumps and evaluation of genetic fidelity of micropropagated plants through RAPD analysis.

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Materials and Methods

Collection, processing and surface sterilization

Offset cuttings of superior genotypes (Candidate Plus Clumps) of *Bambusa pallida*

were collected from the germplasm bank of the State Forest Research Institute, Itanagar, and the Rain Forest Research Institute, Jorhat (Figure 1A). These genotype offset cuttings were planted in gunny bags with sand, soil and compost (6:1:3, v/v) and kept in a greenhouse at 85% RH for new shoot growth and root development. After proper hardening, the plants were transplanted to the bamboo germplasm bank, Gottipura, Bangalore, while a few plants were kept at the Institute of Wood Science and Technology (IWST) nursery (Figure 1B) as explant for micropropagation studies. The shoots of newly grown culm branches were collected from the germplasm bank and the IWST nursery. Culm sheath was removed after swabbing the surface of shoots with 70% (v/v) ethanol. Explants were excised into single nodal segments 2.5-3.5 cm long and 3-4 mm wide with dormant buds and were immersed in 0.01% (v/v) liquid detergent (Tween 80, Himedia, India) for 5 min to remove dust and dirt particles. This was followed by 5-6 washes of distilled water to remove the traces of detergent. Explants were later dipped in 0.2% (w/v) bavistin (systemic fungicide) for 5 min to reduce the chances of fungal contamination, followed by washing 4-5 times in sterile distilled water. Surface sterilization of the explants was carried out under aseptic conditions in a laminar air flow. The first step of the surface sterilization was carried out using 70% ethanol (v/v) for 30 s, followed by washing 4-5 times with sterile distilled water. Subsequently, surface sterilization was carried out using 0.075-0.1% (w/v) mercuric chloride (Himedia, India) for 5 min followed by thorough washing (6-7 times) with sterile distilled water.

Culture media and culture conditions

Murashige and Skoog (MS) liquid medium supplemented with growth regulators,[21] additives (ascorbic acid 50 mg/L + citric acid 25 mg/L + cysteine 25 mg/L) and 3% sucrose was used for shoot induction and shoot multiplication. Modified MS (MS/4) agar gel medium supplemented with sucrose (3%) and glucose (1%) was used for *in vitro* rooting. Agar (Himedia, India) 0.6% was used as a solidifying agent. pH of the medium was adjusted to 6.2 for the medium containing additives and 5.8 for the rooting medium before autoclaving at 15 lb per square inch (15 psi) for 20 min. Cultures were maintained at 25±2°C with 33.78 µmol photons m^{-2} s^{-1} light illumination for a 12-h photoperiod and 55±5% relative humidity.

Shoot initiation

Surface sterilized explants were inoculated vertically in the Borosil culture tubes (25×150 mm) containing 10 mL of MS liquid medium

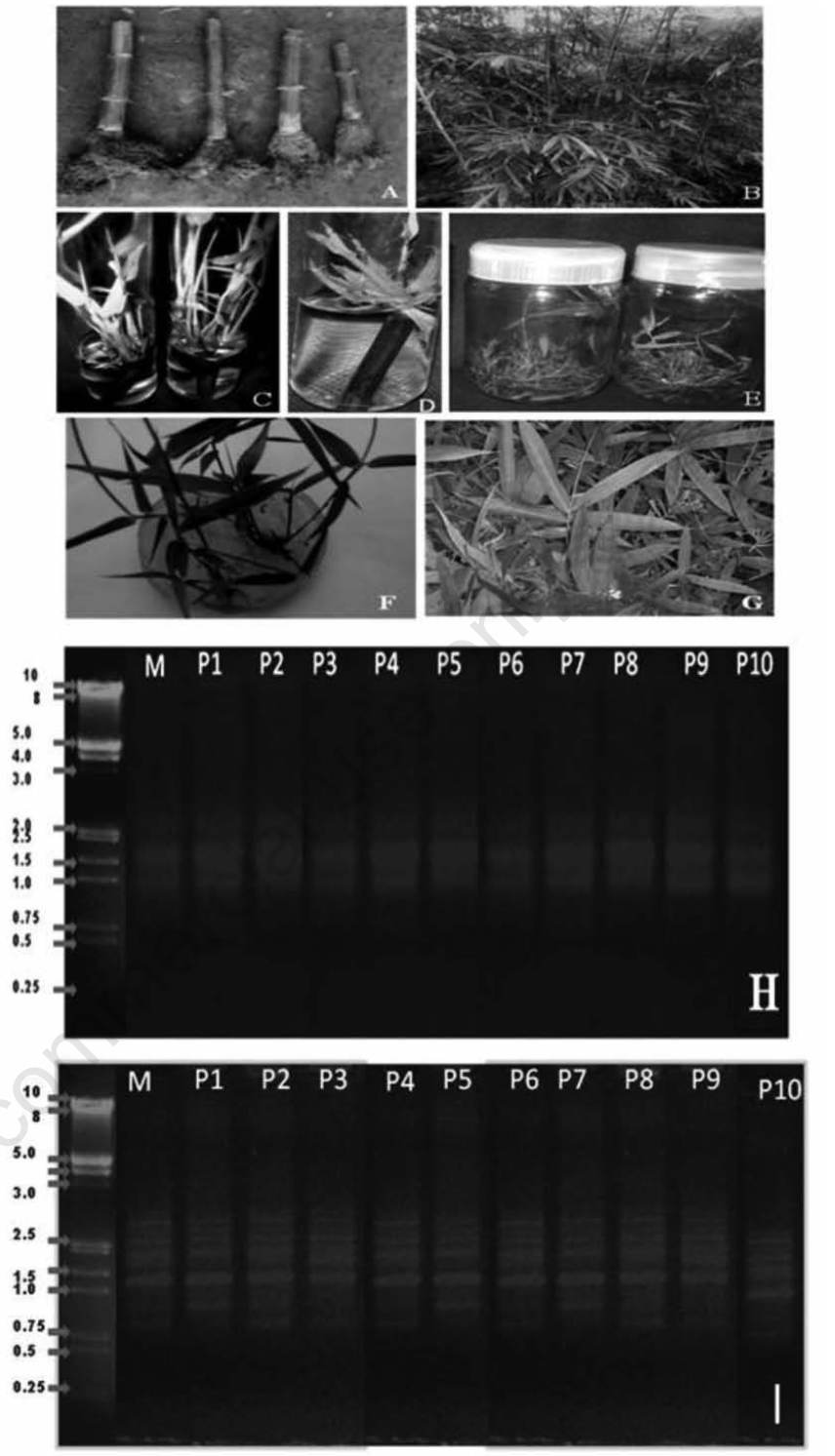

Figure 1. A) Offset cuttings of *B. pallida* collected from the germplasm bank of Rain Forest Research Institute, Jorhat.; B) Plants raised through offset cuttings in gunny bags after 30 months at IWST nursery, Bangalore; C) *In vitro* shoot induction in MS liquid medium supplemented with additives, NAA 1.34µM and TDZ 1.125 µM; D) dwarf shoots induced in the MS liquid medium supplemented with additives, NAA 0.54 µM and TDZ 2.27 µM; E) *In vitro* shoot multiplication in the MS liquid medium supplemented with additives, NAA 0.54 µM and BAP 4.44 µM; F) *In vitro* root induction from the clumps pulse treated with IBA 0.5 mg/mL for 30 minutes on MS/2 medium consisting sucrose (2%) and glucose (1%); G) Four months old hardened plantlets of *B. pallid*; H,I) RAPD banding pattern with primers (H: OPA-02 and I: OPB-12) in both micropropagated and field grown mother plant of *B. pallida* (lane 1- marker, M- mother plant and P1-P10 progenies).

fortified with additives and various concentrations of auxins [0.52, 1.425 µM indole acetic acid (IAA) and 0.54, 1.34 µM α-naphthalene acetic acid (NAA)] and cytokinins [benzylaminopurine (BAP) 2.22-22.2 µM; Kinetin 2.325 µM and 11.625 µM, and thiodiozuron (TDZ) 1.125-4.54 µM]. Observations were recorded at the end of two weeks, and various parameters were taken into consideration such as: percentages of response on shoot induction, number of shoots/explant and shoot length.

Shoot multiplication

In order to optimize the growth regulators for high frequency shoot multiplication, 2.5-3.5 cm long *in vitro* proliferated shoot clumps (3-4 shoots/clump) were transferred to MS liquid medium supplemented with additives and cytokinins; BAP 2.22-22.2 µM and TDZ 1.125-4.54 µM in combination with auxin; NAA 0.54 and 1.34 µM. The average number of propagule obtained from the culture after 15 days was recorded as the rate of multiplication.

In vitro rooting

For rooting, the *in vitro* multiplied shoots (2-3 shoot clumps, shoot length 3.0-4.0 cm) were excised and pulse treated with various concentrations (0.25-2.5 mg/mL) of IBA for 30 min. The pulse-treated shoots were then transferred to half-strength MS basal salts agar gel medium consisting of sucrose (2% w/v) and glucose (1% w/v). Data were recorded after four weeks taking into consideration the parameters of rooting percentage, root number and root length.

Transplantation and hardening

In vitro rooted shoots (2-3 tillers) with shoot length 5.0-7.0 cm were carefully removed from the bottle and washed thoroughly under running tap water to remove adhered medium to the surface. Plants were then kept in 0.1% (w/v) fungicide (bavistin) for 5-10 min before being transferred to the polybags (600 cc) containing sand, soil and compost in 4:2:4 (v/v) ratios fortified with 10 kg/cum neem cake, 2.4 kg/cum single super phosphate and 0.4 kg/cum phorate + 0.4 kg/cum bavistin as a prophylactic measure. Plants transplanted in polybags were kept in a polytunnel (an iron frame 2.0 m long and 0.6 m high covered with a polythene sheet) at 90% relative humidity and internal temperature 26±3°C for three weeks in a mist chamber. The polythene sheet was gradually removed and plants were kept in the mist chamber for one more week. Before transferring to open nursery conditions, plantlets were kept in shade (50% in an agroshade net house) for two weeks.

Genetic fidelity

Young leaves were collected from 10 randomly selected plants regenerated through axillary shoot proliferation from the 1-year old shoot multiplication cultures and their respective mother plants for DNA extraction. Genomic DNA was extracted using the phenol chloroform method. Fresh leaf tissue (0.2 g) was grounded into a fine powder using liquid nitrogen in a mortar and pestle, and transferred to 5 mL of pre-heated extraction buffer containing 1,000 mM Tris HCl (pH 8.0), 1.4 mM NaCl, 20 mM EDTA (pH 8.0), 3% CTAB (w/v), 2% β-mercaptoethanol (w/v) and 2% PVP (w/v). After proper mixing, the slurry was incubated at 65°C for 1 h by mixing thoroughly at regular time intervals and cooled to room temperature. It was then extracted with equal (1:1) volumes of phenol: chloroform and centrifuged at 12,000 rpm for 10 min (Eltek Refrigerated Centrifuge RC 4100). The supernatant was transferred to a fresh tube and the phenol chloroform step was repeated until the white interphase disappeared. The aqueous phase was extracted with a chloroform: isoamyl alcohol (24:1) mixture and centrifuged at 12,000 rpm for 10 min. The supernatant was precipitated with 1.5-2.0 volumes of cold ethanol by incubating at -20°C for 1 h. The DNA was pelleted by centrifugation (10,000 rpm for 8 min). The recovered DNA was washed with 70% (v/v) ethanol, air dried and resuspended in 100 mL of TE buffer (10 mM Tris HCl, pH 8.0; 0.1 mM EDTA, pH 8.0) and stored at 4°C until use. The concentration of DNA samples was determined by an UV absorbance at 260 nm in a spectrophotometer (Hitachi) and also by visual observation using 0.8% agarose gel. Purity of the DNA samples was estimated by calculating the ratio of percent absorption at 260 and 280 nm. DNA samples were adjusted to an appropriate dilution of 50 ng/mL with TE buffer. DNA sample preparation was tested for molecular weight size by agarose gel electrophoresis. Genomic DNA (10 mg) was electrophoresed on horizontal 0.8% (w/v) agarose gel in 1 x TAE buffer (0.04 M Tris acetate, 0.001 M EDTA (pH 8.0) at 75V for 2 h, gel was stained with ethidium bromide, and photographed in a gel documentation system (Herolab).

Random amplified polymorphic DNA amplification

RAPD fingerprinting was obtained by using randomly chosen arbitrary, 10-mer primers (A, B, E, J and K series) obtained from Operon Technologies Incorporation, Almeda, USA. Polymerase chain reaction (PCR) was performed according to the protocol of Williams *et al.*[18] with minor modifications. Amplification was carried out in 25 mL of reaction mixture containing 2.5 mL of 10 X assay buffer containing 20 mM Tris-Cl (pH 9.0), 50 mM KCl (Bangalore Genei), 220 µM each of dATP, dCTP, dGTP, dTTP Bangalore Genie), 0.3 µM primer, 2.5 mM $MgCl_2$ (Bangalore Genei), 0.75 U of Taq polymerase (Bangalore Genie) and 50 ng of template DNA. DNA amplification was carried out in an Eppendorf (Master) cycler gradient PCR programmed for: 1 cycle at 94°C for 3 min, followed by 30 cycles of denaturation at 92°C for 30 s, annealing at 52°C for 30 s, extension at 72°C for 1 min, and a final extension of 7 min at 72°C.

After completion of PCR, 4 mL of 6 X loading dye was added to each of the samples. The amplification products were separated according to size by electrophoresis in 1.5% agarose gel containing 0.05 mL/mL ethidium bromide (Bangalore Genei, Bangalore) in 1 X TAE buffer. EcoR I + Hind III double digest DNA (Fermentas) was used as the standard DNA ladder for reference. Gel was photographed under UV light. Fingerprinting with each primer was repeated at least three times. Only consistently reproducible bands were considered for data analysis. Out of 25 primers tested, 12 produced quality reproducible amplification products and these primers were selected for RAPD amplification of the mother plant and 10 randomly selected plantlets derived from axillary shoot proliferation.

Experimental design and data analysis

For shoot initiation, a minimum of 12 culture tubes were used per treatment in 3 replicates. For shoot multiplication, 3 clumps each of 3-4 shoots/clump, and for *in vitro* rooting, 4 clumps each with 2-3 shoots/clump, were used in 5 bottles. The experiment was repeated three times. Data were recorded after 45 days for rooting. Experiments were set up according to a completely randomized design. Variance (one way or single factor analysis) in treatment means and standard errors was determined, followed by the least significant difference (LSD) test at P=0.05 to compare means.

DNA fingerprinting with RAPD markers was performed three times and only quality reproducible bands in the range of 350-2500 bp were scored. DNA banding pattern generated by RAPDs was recorded as *1* for presence of band and *0* for its absence. Genetic difference between the parents and their progeny was calculated according to the RAPD marker data set as described by Ward. RAPD markers were identified by the source of primer (OP Operon), kit letter, primer number and the appropriate size in base pairs.

Results

Shoot initiation

Nodal explants cultured on MS basal medium did not induce multiple shoots even after four weeks. Media supplemented with auxins (NAA and IAA) and cytokinins (BAP, TDZ and Kinetin) induced bud break within a week. Among the various concentrations of auxins and cytokinins tested, combination of NAA and TDZ was found to be more effective for multiple shoot induction. Maximum (4.90) shoots/responding explant with 98.52% shoot induction was obtained in the medium fortified with NAA 1.34 µM along with TDZ 1.125 µM (Figure 1C). The shoots initiated at a higher concentration of BAP (22.2 µM) and TDZ (2.27 µM) along with NAA 1.34 µM (Figure 1D) were dwarf and were not suitable for further shoot multiplication or rooting. In general, shoot length was longer in medium with BAP/TDZ + NAA than with BAP/TDZ + IAA. The highest (5.11 cm) shoot length was obtained in hormone-free medium (Table 1).

Shoot multiplication

Among the two cytokinins (BAP and TDZ) tested, BAP was found to be ideal for ensuring quality of shoot multiplication. NAA with BAP/TDZ in the medium had a significant effect on shoot multiplication. A higher concentration (1.34 µM) of NAA along with BAP proved to be the best in terms of shoot number and shoot length. Shoot number increased along with increasing concentrations of BAP (2.22-22.2 µM) and TDZ (1.125-4.54 µM). Medium consisting of NAA 1.34 µM and BAP 22.2 µM produced the highest number of shoots: 8.05 shoots/clump. But these shoots were dwarf and vitrified with condensed nodes that were not suitable for further multiplication. Therefore, NAA (1.34 µM) with BAP (4.44 µM) was found to be ideal for shoot multiplication, with an average 6.34 shoots/clump with shoot length of 4.5 cm in the 2-week period (Table 2 and Figure 1E).

In vitro rooting

Lower concentration (0.25 mg/mL and 0.5 mg/mL) of IBA enhanced rooting percentage, root number and root length.

Maximum (67.5%) rooting with highest (5.36) root number and root length (5.97 cm) was obtained from the plants pulse-treated with IBA (0.5 mg/mL) for 30 min (Figures 2A,B,F) followed by those pulse-treated with IBA 0.25 mg/mL. Increased concentrations (1 mg/mL and 2.5 mg/mL) not only decreased the rooting percentage, but also decreased root number and root length.

The plants with reduced root length did not survive hardening.

Transplantation and hardening

The method that involved keeping plants in a polytunnel in a greenhouse for an initial three weeks followed by one week in a greenhouse and two weeks in shade (50%) proved the most suitable with a high rate (>95%) of plant survival. Within four months after hardening, the plants produced 3-4 tillers growing to a height of 25-30 cm with miniature rhizome (Figure 2G). During hardening, leaves turned green with a healthy appearance and expansion of the leaf lamina.

Table 1. Effect of plant growth regulators on high frequency shoot induction from the nodal shoot segments of *B. pallida* in Murashige and Skoog liquid medium supplemented with additives.

Treatments	Plant growth regulators (µM)	% of response	No. of shoots/explant*	Shoot length (cm)
T1	Control	21.77q	1.01kl	5.11a
T2	NAA 0.54+BAP 2.22	42.13n	1.35j	5.00a
T3	IAA 0.52+BAP 2.22	38.34o	1.12k	4.81b
T4	NAA 0.54+BAP 4.44	95.5d	1.40j	4.61b
T5	IAA 0.52+BAP 4.44	43.56m	1.19k	3.92e
T6	NAA 0.54+BAP 11.1	98.10b	3.79d	4.53c
T7	IAA 0.52+BAP 11.1	80.32i	1.95i	3.47f
T8	NAA 1.34+BAP 4.44	96.89c	2.79e	4.66b
T9	IAA 1.425+BAP 4.44	82.17h	2.30h	3.69e
T10	NAA 1.34+BAP 11.1	98.11b	2.50g	4.22d
T11	IAA 1.425+BAP 11.1	87.42g	2.56g	3.58f
T12	NAA 1.34+BAP 22.2	98.57a	4.01c	2.37i
T13	IAA 1.425+BAP 22.2	87.64f	2.71f	1.23i
T14	NAA 0.54+BAP 2.22+Kin 2.325	60.36j	1.52j	1.02k
T15	NAA 0.54+Kin 11.625	41.91n	1.81i	2.11j
T16	NAA 0.54+TDZ 4.54	32.33p	2.91e	1.91j
T17	NAA 0.54+TDZ 2.27	50.31k	4.59b	2.25i
T18	IAA 0.52+TDZ 2.27	49.52l	2.78ef	2.15i
T19	NAA 0.54+TDZ 1.125	95.00e	4.61b	3.07g
T20	IAA 0.52+TDZ 1.125	82.33h	2.85e	2.80h
T21	NAA 1.34+TDZ 1.125	98.52a	4.90a	3.11g
T22	IAA 1.425+TDZ 1.125	87.70f	2.96e	4.08d
	SE (0.05)	0.16	0.10	0.34
	CD (0.05)	0.26	0.17	0.23

Treatments followed by different letters are significantly different from each other. SE, standard error of the mean; CD, Critical difference at ∞ =0.05; BAP, benzylaminopurine; IAA, indole acetic acid; NAA, α-naphthalene acetic acid; TDZ thiodiozuron. *mean number of 12 replicates.

Genetic fidelity

No morphological variation was seen in the micropropagated plants of the *B. pallida*. Out of the 25 decamer RAPD primers initially screened, 12 oligonucleotides produced clear and discernable amplification products. The products were also monomorphic across all the micro-propagated plants. The number of bands produced by a single primer ranged from 3 (OPA-02) to 9 (OPB-12) (Figures 2H, I). A total of 181 bands were produced with an average frequency of 6 bands per primer. The amplified products ranged in size from 250 bp to 4200 bp. RAPD fingerprints of all primers used revealed no variation in micro-propagated plants of *B. pallida.*

Discussion

A long flowering cycle and sporadic flowering lead to non-availability of seed as and when required for afforestation and establishment of bamboo plantations. Propagation of bamboo through offset cuttings, rhizome splitting, air layering, culm cuttings and culm branch cuttings are the traditional methods of vegetative propagation. These methods are limited by the short period of availability of cuttings at the appropriate stage, the low rate of rooting from culm and branch, the need for bulk quantities of material and also the potentially small-scale production. For large scale production of clonal planting material, tissue culture is the only reliable method.[7] Micropropagation of bamboo species from the explants of field grown clumps have been reported in a few species but there have been no reports on the evaluation of genetic fidelity of micro-propagated bamboo plants by molecular markers. MS liquid medium supplemented with additives, TDZ 1.125 µM and NAA 1.34 µM proved to be the best combination for high-frequency multiple shoot induction. Superiority of TDZ over BAP for multiple shoot induction was also reported in *B. edulis*.[22] Higher concentrations of cytokinins resulted in a greater number of shoots. However, shoots were dwarf with more leaves which is found to agree with the result obtained by Arya and Sharma in *B. bambos,*[23] whereas in *B. wamin,* a higher concentration (22.2 µM) of BAP in MS medium favored high-frequency (83.33%) bud breaks.[24] The problem of phenolic exudation at the cut ends of explants which leads to the eventual death of initiated shoots is the main problem associated with adult bamboo tissues. This problem was overcome in *Arundinaria callosa* by correct removal of brown leaf sheaths and quick transfer to fresh medium whenever browning appeared.[25] The present protocol differs significantly in that addition of additives in the cul-

Table 2. Effect of plant growth regulators on shoot multiplication and growth from shoot clumps of *B. pallida* in Murashige and Skoog liquid medium supplemented with additives.

T. No.	PGRs (µM)	No. of shoots/Clump	Shoot length (cm)
1	HF	3.10h	5.32a
2	NAA 0.54+BAP 2.22	3.30h	4.56b
3	NAA 0.54+BAP 4.44	4.20g	4.47b
4	NAA 0.54+BAP 11.1	4.50f	3.82c
5	NAA 0.54+BAP 22.2	4.80e	3.57d
6	NAA 1.34+BAP 2.22	5.00e	4.63b
7	NAA 1.34+BAP 4.44	6.34c	4.50b
8	NAA 1.34+ BAP 11.1	7.00b	4.10c
9	NAA 1.34+BAP 22.2	8.05a	3.78c
10	NAA 0.54+TDZ 1.125	4.18g	4.51b
11	NAA 0.54+TDZ 2.27	4.46f	3.72c
12	NAA 0.54+TDZ 4.54	5.37d	2.43e
	SE (0.05)	0.12	0.26
	CD (0.05)	0.22	0.43

Treatments followed by different letters are significantly different from each other. SE, standard error of the mean; CD, Critical difference at ∞ =0.05; BAP, benzylaminopurine; IAA, indole acetic acid; NAA, α-naphthalene acetic acid; TDZ thiodiozuron.

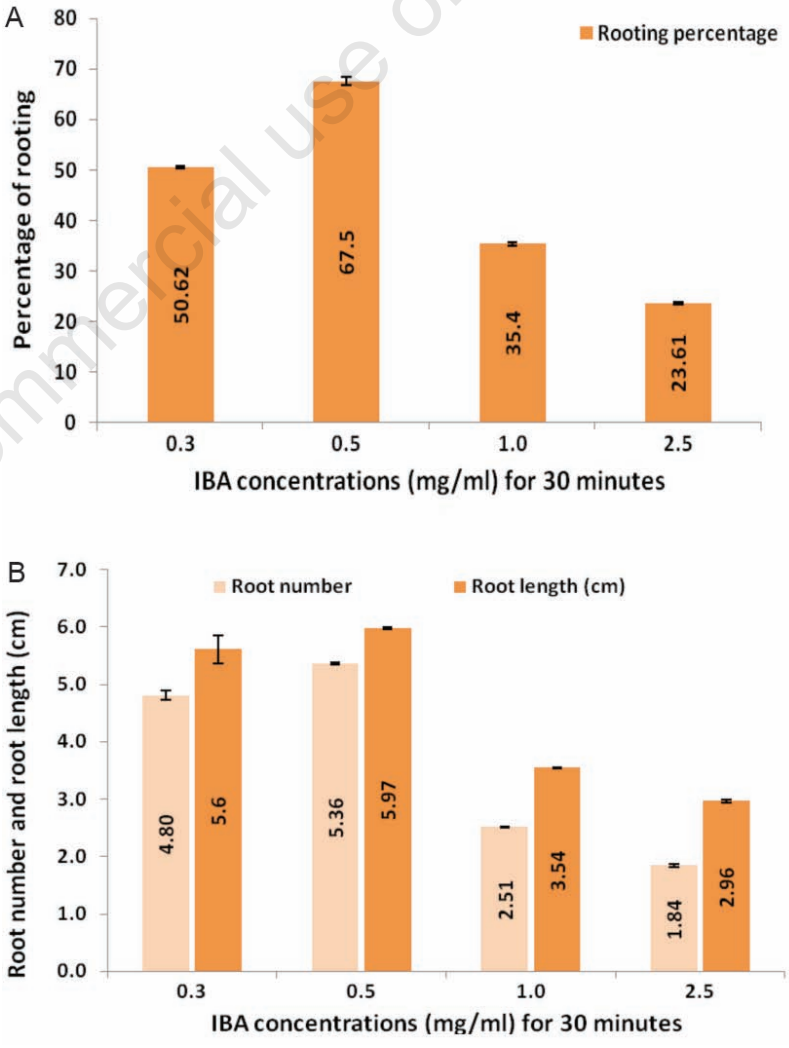

Figure 2. A) Effect of various concentrations of IBA pulse treatment for 30 minutes on *in vitro* rooting percentage from shoot clumps of *B.pallida*; B) Root Effect of various concentrations of IBA pulse treatment for 30 minutes on *in vitro* rooting (root number and root length) from shoot clumps of *B. pallida*.

ture medium helped to overcome leaching.

Research carried out on micropropagation of bamboo showed variation in species in response to levels of BAP for shoot multiplication.[25,26] MS liquid medium consisting of additives, NAA 1.34 µM and BAP 4.44 µM helped to further multiplicate *B.pallida*. Similary, the synergetic effect of NAA 1.34 µM and BAP 4.44 µM in MS liquid medium favored better shoot multiplication in *P. stocksii.*[13,14] This clearly indicates that, though the growth regulators are the same, their endogenous concentrations and exogenous requirement varies with the species. A serious problem that prevents the successful micropropagation of bamboo and other woody species is difficulty in rooting that was also observed in *B. pallida.*[27,28] The induction of rooting in many bamboo species requires many additives, such as coumarin, phloroglucinol and TDZ, particularly when the explant is taken from the nodal segments of adult plants.[26,29] In *B. wamin, B. nutan* and *T. spathiflorus*, rooting was achieved by IBA treatments of shoots for three days, four weeks and two weeks, respectively, before transferring to the MS/2 medium-consisting sucrose.[24,30,31] But in *B. pallida*, well developed shoots when pulse-treated with IBA 0.5 mg/mL for 30 min were rooted on the MS half-strength plant growth hormone medium consisting of both sucrose (2%) and glucose (1%) in 4-5 weeks at 25±1°C and 33.78 µmol photons m^{-2} s^{-1} light for a 12-h photoperiod. The two-step protocol followed in the present study resulted in excellent rooting in a basal medium without any additives. It is not advisable to let the roots grow too long as this increases the probability of root damage during transplantation. Also, roots frequently die after transplantation and new roots must then develop *in vivo* if the plants are to survive.[32,33] A maximum 100% survivability was achieved in micro-propagated plant of *Thamnocalamus spathiflorus* with root length 3.14 cm and survivability reduced to 60% when root length decreased to 1.84 cm.[34] In the present study, plants with root length less than 2 cm did not survive after transplantation.

The initial 3-week period in which plants were kept in a polytunnel in a mist chamber followed by two weeks in 50% shade proved the most suitable for a high rate (>95%) of plant survival. RAPD markers have been used to study the genetic variation of micro-propagated plants in many other plant species.[35,36] Distinct polymorphism was observed in the micro-propagated plants of 2 clones of *Populus deltoids*,[37] whereas in *B. pallida* the present investigation based on RAPD markers revealed no genetic variation in the micro-propagated plants of *B. pallida* compared to the mother plant.

Conclusions

This investigation presents a detailed protocol for successful rapid clonal propagation of *B. pallida*, an economically important and edible bamboo from nodal explants of adult plant. High multiplication efficiency, good rooting, excellent establishment in the soil and normal growth performance of micro-propagated plants without genetic variation, as reported in this study, are the necessary requisites for the industrial adoption of *in vitro* propagation technology for large-scale production.

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