



Research article

Development of efficient micropropagation protocol through axillary shoot proliferation for *Bambusa vulgaris* ‘wamin’ and *Bambusa bambos* and assessment of clonal fidelity of the micropropagated plants through Random Amplified Polymorphic DNA markers

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Abstract

Micropropagation protocols were described for two important bamboo species (*Bambusa vulgaris* ‘wamin’ and *Bambusa bambos*) through axillary shoot proliferation. Explants were surface sterilized by sequential application of antifungal (Carbandazim-50%), various antibacterial (Cefotaxime, Kanamycin and Streptomycin) agents and mercuric chloride. The axenic cultures established using *B. vulgaris* and *B. bambos* were 78% and 94%, respectively. Initiation and bud break were obtained on Murashige and Skoog (1962) medium supplemented with 3% sucrose. *B. vulgaris* shoots were best multiplied (6.20 ± 0.32 shoots/explant) on MS containing $8.87 \mu\text{M}$ 6-benzylaminopurine (BAP) + $0.54 \mu\text{M}$ naphthaleneacetic acid (NAA) while *B. bambos* shoots were best multiplied (7.10 ± 0.26 shoots/explant) on MS containing $8.87 \mu\text{M}$ BAP. Shoot clumps, containing three to four shoots, were rooted on half strength MS supplemented with varying concentrations of indole-3-butyric acid (IBA). *B. vulgaris* shoots were best rooted (4.4 ± 0.18 roots/clump) on $\frac{1}{2}$ MS supplemented with $21.48 \mu\text{M}$ IBA while *B. bambos* shoots were best rooted (2.5 ± 0.12 roots/clump) on $\frac{1}{2}$ MS supplemented with $10.74 \mu\text{M}$ IBA. Plantlets regenerated in this manner were primary hardened in the greenhouse and acclimatized in the polyhouse under a shade net. The overall survival rates recorded were 85% and 80% for *B. vulgaris* and *B. bambos*, respectively. The clonal fidelity of *in vitro* regenerated plantlets was assessed using 10 random amplified polymorphic DNA (RAPD) markers. All the bands generated using the 10 RAPD decamers were monomorphic and produced more than six scorable bands within the band size of 240–2,505 base pairs.

Introduction

India is the second highest bamboo producer in the world after China (Pathak, 1989). Bamboo is a perennial, giant and woody grass which belongs to the subfamily Bambusoideae and comprises 60–70 genera and 1,200–1,500 species (Wang and Shen, 1987). According to Suri and Chauhan (1984), 136 species in 30 genera occur in India. Bamboos have a number of applications from cradle to coffin (Yeasmin et al., 2015).

Bambusa vulgaris ‘wamin’ is a tropical as well as subtropical clumping bamboo native to south China and is also known as dwarf ‘Buddha’s Belly’ bamboo (Chung et al., 2008). *B. vulgaris* is a hexaploid species and the chromosome number ranges from

$2n = 52$ to $2n = 72$ (Dransfield and Widjaja, 1995; Li et al., 2001). *B. vulgaris* ‘wamin’ is mainly cultivated in north-eastern parts of India such as Orissa, Arunachal Pradesh and Manipur and has high value as an ornamental plant due to the presence of prominent swollen internodes (Rai and Chauhan, 1999). It is widely used in making miniature landscapes and decorative materials and is also reported to be associated with the control of soil erosion (Baskaran et al., 2014). This bamboo has multifarious uses, but production is much less than global demand. The vegetative propagation method using offshoots and culm cuttings has proved rigorous and produces a very small number of plants (Muralidharan, 1995). Propagation of *B. vulgaris* ‘wamin’ through seeds is also limited as there is no report on its flowering (Arshad et al., 2005). Thus, micropropagation is the method

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of choice for the production of large numbers of genetically identical plants of *B. vulgaris* in a relatively shorter time.

Another bamboo often called ‘Indian giant thorny bamboo’ (*B. bambos*) has extensive application in the pulp and paper industry due to its near solid culms (Kapoor and Rao, 2006). The chromosome number reported for *B. bambos* varies from $2n = 70$ to $2n = 72$ (Duriyaprapan and Jansen, 1995). *B. bambos* is a densely tufted bamboo and grows up to a height of 25 m (Anand et al., 2013). India’s National Bamboo Mission (2017) has recognized *B. bambos* as one of the important plants for the production of quality timber material. Recently, the International Union for Conservation of Nature has categorized *B. bambos* in the red list of endangered bamboo species due to overconsumption of the forest-based material by pulp and paper mills (Bystriakova et al., 2003). There were only 17 paper mills in India in 1950 but at present more than 759 such mills exist with an installed capacity of 12.7 million t of wood which produce 10.11 million t of paper and paper boards annually (Kulkarni, 2013). In addition, every year India imports another 1.04 million t of wood to fulfill the demand for the paper and by 2025, the demand is expected to increase to 24 million t (Kulkarni, 2013). Traditional propagation methods have several limitations such as the use of bulkier offshoots and limited numbers of branch cuttings (Muralidharan, 1995) together with the issue of unpredictable and long flowering cycles of 40–60 yr (Thapliyal et al., 2015). Furthermore, bamboos have the peculiar phenomenon of gregarious flowering followed by massive seeding which is threatened by seed predators and rodent population that greatly reduces the chance of seed germination (Kapoor and Rao, 2006). Bamboo seeds also have no dormancy period and also have short viability (Nadgir et al., 1984). Therefore, to meet the ever-increasing demand for raw material by the pulp and paper industry and to alleviate the limitations of traditional propagation methods, micropropagation is the best alternative for large-scale multiplication of *B. bambos*.

Evaluation of the clonal fidelity of the large number of micropropagated plants is of utmost importance as it validates that all the plants produced through tissue culture are true-to-type in nature. Molecular markers such as random amplified polymorphic DNA (RAPD), inter-simple sequence repeat (ISSR) and simple sequence repeat (SSR) provide a quick and easy assessment of clonal fidelity. Goyal et al. (2015) used 10 RAPD markers to confirm clonal propagation of *Dendrocalamus strictus* (Roxb.). Anand et al. (2013) assessed the clonal fidelity of an edible bamboo *B. bambos* using 15 RAPD and 10 ISSR markers.

Therefore, the present investigation was mainly focused on the development of an efficient micropropagation protocol for large scale production of plantlets of two important bamboo species using nodal segments as explants and assessment of clonal fidelity of the micropropagated plants using RAPD markers.

Materials and Methods

Surface sterilization of explants and culture establishment

Single nodal explants were collected from *B. vulgaris* and *B. Bambos* plants grown at the Anand Agricultural University farm, Gujarat, India during July to September 2013. The nodes were excised using sharp scissors and the size was reduced to a length of 3–4 cm. The explants were excised very carefully as the size of the explants can result in varying response (Buchsbbaum, 1932). Explants were then thoroughly washed with Tween-20 solution. Carbendazim-50% at 2000 parts per million (25 min), Cefotaxime at 1000 parts per million (18 min), Kanamycin at 1000 parts per million (18 min) and

Streptocycline at 1000 parts per million (15 min) were used for surface sterilization of the explants. Final sterilization was achieved using 0.1% HgCl_2 (18 min). The explants were given a fresh cut under a laminar air flow and inoculated in liquid Murashige and Skoog (1962) (MS) medium supplemented with 3% sucrose (Qualigens; USA). All the cultures were maintained at $25 \pm 2^\circ\text{C}$ under a photoperiod of 16 hr with a light intensity of $36.8 \mu\text{mol}/\text{m}^2/\text{s}$ provided by cool, white fluorescent tubes (36 W; Phillips; India) followed by 8 hr of dark.

Preparation of the culture medium

MS medium was prepared and dispensed into 25×100 mm glass tubes (Borosil; India) and autoclaved at 121°C and 15 pounds per square inch pressure for 15 min. The pH of the medium was adjusted to 5.7 ± 0.01 prior to autoclaving.

Shoot multiplication

Each *in vitro*-formed shoot produced after bud break, was excised from the respective node and subcultured in liquid MS medium supplemented with different combinations of 6-benzylaminopurine (BAP; Sigma Chemicals; Germany) and naphthaleneacetic acid (NAA; Sigma Chemicals; Germany) as shown in Tables 1 and 2. The shoots were subcultured in the same medium for more than four subcultures for identification of the best shoot multiplication medium. As bamboo is a very fast growing crop, shoot clusters were subcultured at regular interval of 10 d; however, observations of each subculture were noted down after 21 d. The number of shoots, length of the shoots and number of leaves formed were recorded for both bamboo species. Multiplication rates were calculated on the basis of the number of propagules derived from one shoot cluster after the completion of each 3-week cycle. Necrotic and decayed shoots or leaf sheaths, if any, were removed from the clusters before transferring to the fresh medium.

Rooting

Shoot clumps containing three to four shoots, measuring around 3–4 cm in length, derived after completion of each 3-week (21 d) multiplication cycle, were transferred to rooting media composed of MS supplemented with a fixed $53.71 \mu\text{M}$ choline chloride concentration and varying concentrations of indole-3-butyric acid (IBA) along with 1.5% sucrose (Table 3). Agar-Agar type-I at 0.9% weight per volume (HIMEDIA; India) was used as the gelling agent. The number of roots and length of roots were recorded for both bamboo species.

Hardening

The *in vitro* rooted plantlets were removed from the culture tubes and washed thoroughly with water to remove traces of the medium. Plantlets were treated with Mancozeb, Metalaxyl-M and copper oxychloride each for 2 min followed by rinsing in distilled water. The plantlets were then transferred to separate 10×9 cm plastic cups containing autoclaved cocopeat and covered with polyethylene material to maintain high relative humidity. The plants were kept in the dark for the initial 2 d and watered daily. Primary hardened plantlets were then transferred to a polyhouse and maintained in soil bags until transplanted to the field. The plantlets were fertigated with nutrient mixtures at least twice a week. Damage due to fungi and bacteria was reduced by using an aerial spray of antifungal (Carbendazim 50% at 500 parts per million) and antibacterial (Streptocycline at 500 parts per million) agents.

Table 1 Effect of various combinations of cytokinins and auxins on *in vitro* shoot induction and multiplication of *Bambusa vulgaris* 'wamin' and *Bambusa bambos*

Treatment	Plant growth regulator (μM)				<i>B. vulgaris</i> 'wamin'						<i>B. bambos</i>			
	BAP	NAA	SpROUTING (%)	Number of shoots	Number of leaves	Length of shoot (cm)	SpROUTING (%)	Number of shoots	Number of leaves	Length of shoot (cm)	SpROUTING (%)	Number of shoots	Number of leaves	Length of shoot (cm)
T1	0	0	20.00 $\pm$ 0.00 ^d	3.30 $\pm$ 0.30 ^d	4.30 $\pm$ 0.36 ^{bc}	4.83 $\pm$ 0.28 ^{bc}	80.00 $\pm$ 0.00 ^a	2.30 $\pm$ 0.15 ^{bc}	4.85 $\pm$ 0.49 ^{bed}	5.36 $\pm$ 0.38 ^{bed}	80.00 $\pm$ 0.00 ^a	2.30 $\pm$ 0.15 ^{bc}	4.85 $\pm$ 0.49 ^{bed}	5.36 $\pm$ 0.38 ^{bed}
T2	0	0.54	90.00 $\pm$ 0.14 ^a	4.00 $\pm$ 0.29 ^{cd}	5.40 $\pm$ 0.52 ^{bed}	5.23 $\pm$ 0.22 ^b	50.00 $\pm$ 0.14 ^{bc}	2.60 $\pm$ 0.26 ^b	4.00 $\pm$ 0.39 ^b	5.28 $\pm$ 0.37 ^b	50.00 $\pm$ 0.14 ^{bc}	2.60 $\pm$ 0.26 ^b	4.00 $\pm$ 0.39 ^b	5.28 $\pm$ 0.37 ^b
T3	0	1.07	90.00 $\pm$ 0.14 ^a	3.70 $\pm$ 0.26 ^{bed}	4.20 $\pm$ 0.61 ^{bc}	5.04 $\pm$ 0.20 ^{bed}	90.00 $\pm$ 0.14 ^a	3.40 $\pm$ 0.26 ^b	4.30 $\pm$ 0.47 ^{bed}	4.98 $\pm$ 0.27 ^{bc}	90.00 $\pm$ 0.14 ^a	3.40 $\pm$ 0.26 ^b	4.30 $\pm$ 0.47 ^{bed}	4.98 $\pm$ 0.27 ^{bc}
T4	0	2.69	80.00 $\pm$ 0.00 ^{ab}	3.70 $\pm$ 0.26 ^{bc}	4.70 $\pm$ 0.47 ^{bc}	4.99 $\pm$ 0.29 ^{bed}	80.00 $\pm$ 0.28 ^a	3.00 $\pm$ 0.20 ^b	5.30 $\pm$ 0.57 ^{bed}	4.26 $\pm$ 0.35 ^{bc}	80.00 $\pm$ 0.28 ^a	3.00 $\pm$ 0.20 ^b	5.30 $\pm$ 0.57 ^{bed}	4.26 $\pm$ 0.35 ^{bc}
T5	4.44	0	50.00 $\pm$ 0.14 ^c	3.20 $\pm$ 0.20 ^{bed}	5.00 $\pm$ 0.36 ^{bc}	4.65 $\pm$ 0.27 ^{bed}	50.00 $\pm$ 0.14 ^{bc}	3.20 $\pm$ 0.20 ^{bc}	5.00 $\pm$ 0.36 ^{bed}	4.65 $\pm$ 0.27 ^{bed}	50.00 $\pm$ 0.14 ^{bc}	3.20 $\pm$ 0.20 ^{bc}	5.00 $\pm$ 0.36 ^{bed}	4.65 $\pm$ 0.27 ^{bed}
T6	4.44	0.54	50.00 $\pm$ 0.14 ^c	4.00 $\pm$ 0.25 ^b	4.70 $\pm$ 0.59 ^{bc}	4.08 $\pm$ 0.23 ^{def}	60.00 $\pm$ 0.00 ^b	4.00 $\pm$ 0.20 ^b	4.70 $\pm$ 0.59 ^{bed}	4.08 $\pm$ 0.23 ^{def}	60.00 $\pm$ 0.00 ^b	4.00 $\pm$ 0.20 ^b	4.70 $\pm$ 0.59 ^{bed}	4.08 $\pm$ 0.23 ^{def}
T7	4.44	1.07	60.00 $\pm$ 0.00 ^{bc}	3.60 $\pm$ 0.49 ^{bc}	4.50 $\pm$ 0.67 ^{bc}	4.05 $\pm$ 0.21 ^{def}	40.00 $\pm$ 0.28 ^{bc}	3.60 $\pm$ 0.49 ^b	4.50 $\pm$ 0.67 ^{bed}	4.05 $\pm$ 0.21 ^{def}	40.00 $\pm$ 0.28 ^{bc}	3.60 $\pm$ 0.49 ^b	4.50 $\pm$ 0.67 ^{bed}	4.05 $\pm$ 0.21 ^{def}
T8	4.44	2.69	80.00 $\pm$ 0.28 ^{ab}	3.20 $\pm$ 0.20 ^{bed}	3.70 $\pm$ 0.39 ^b	4.36 $\pm$ 0.20 ^{bed}	50.00 $\pm$ 0.14 ^{bc}	4.20 $\pm$ 0.32 ^{bc}	2.50 $\pm$ 0.61 ^{bc}	5.70 $\pm$ 0.18 ^{defg}	50.00 $\pm$ 0.14 ^{bc}	4.20 $\pm$ 0.32 ^{bc}	2.50 $\pm$ 0.61 ^{bc}	5.70 $\pm$ 0.18 ^{defg}
T9	8.87	0	80.00 $\pm$ 0.28 ^{ab}	3.00 $\pm$ 0.25 ^{bed}	5.30 $\pm$ 0.57 ^{bed}	4.26 $\pm$ 0.35 ^{bed}	90.00 $\pm$ 0.14 ^a	7.10 $\pm$ 0.26 ^a	7.20 $\pm$ 0.47 ^a	6.99 $\pm$ 0.29 ^a	90.00 $\pm$ 0.14 ^a	7.10 $\pm$ 0.26 ^a	7.20 $\pm$ 0.47 ^a	6.99 $\pm$ 0.29 ^a
T10	8.87	0.54	90.00 $\pm$ 0.14 ^a	6.20 $\pm$ 0.32 ^a	7.50 $\pm$ 0.61 ^a	6.02 $\pm$ 0.18 ^a	30.00 $\pm$ 0.14 ^c	3.20 $\pm$ 0.20 ^{bc}	3.70 $\pm$ 0.39 ^{def}	4.36 $\pm$ 0.20 ^{bed}	30.00 $\pm$ 0.14 ^c	3.20 $\pm$ 0.20 ^{bc}	3.70 $\pm$ 0.39 ^{def}	4.36 $\pm$ 0.20 ^{bed}
T11	8.87	1.07	60.00 $\pm$ 0.00 ^{bc}	3.60 $\pm$ 0.63 ^{bc}	4.00 $\pm$ 0.49 ^{bed}	3.90 $\pm$ 0.18 ^{ef}	30.00 $\pm$ 0.14 ^c	3.60 $\pm$ 0.63 ^b	4.00 $\pm$ 0.49 ^{bed}	3.90 $\pm$ 0.18 ^{ef}	30.00 $\pm$ 0.14 ^c	3.60 $\pm$ 0.63 ^b	4.00 $\pm$ 0.49 ^{bed}	3.90 $\pm$ 0.18 ^{ef}
T12	8.87	2.69	90.00 $\pm$ 0.14 ^a	2.20 $\pm$ 0.24 ^d	1.90 $\pm$ 0.17 ^f	3.65 $\pm$ 0.18 ^f	50.00 $\pm$ 0.14 ^{bc}	2.20 $\pm$ 0.24 ^c	1.90 $\pm$ 0.17 ^g	3.65 $\pm$ 0.18 ^{fg}	50.00 $\pm$ 0.14 ^{bc}	2.20 $\pm$ 0.24 ^c	1.90 $\pm$ 0.17 ^g	3.65 $\pm$ 0.18 ^{fg}
T13	21.48	0	80.00 $\pm$ 0.00 ^{ab}	2.90 $\pm$ 0.31 ^{bed}	4.20 $\pm$ 0.46 ^{bc}	3.33 $\pm$ 0.35 ^f	90.00 $\pm$ 0.14 ^a	2.90 $\pm$ 0.31 ^{bc}	4.20 $\pm$ 0.46 ^{bed}	3.33 $\pm$ 0.35 ^{fg}	90.00 $\pm$ 0.14 ^a	2.90 $\pm$ 0.31 ^{bc}	4.20 $\pm$ 0.46 ^{bed}	3.33 $\pm$ 0.35 ^{fg}
T14	21.48	0.54	70.00 $\pm$ 0.14 ^{abc}	2.80 $\pm$ 0.41 ^{bed}	3.40 $\pm$ 0.54 ^{def}	3.61 $\pm$ 0.28 ^f	30.00 $\pm$ 0.14 ^c	2.80 $\pm$ 0.41 ^{bc}	3.40 $\pm$ 0.54 ^{defg}	3.61 $\pm$ 0.28 ^{fg}	30.00 $\pm$ 0.14 ^c	2.80 $\pm$ 0.41 ^{bc}	3.40 $\pm$ 0.54 ^{defg}	3.61 $\pm$ 0.28 ^{fg}
T15	21.48	1.07	50.00 $\pm$ 0.14 ^c	3.00 $\pm$ 0.25 ^{bed}	2.40 $\pm$ 0.33 ^{df}	3.66 $\pm$ 0.25 ^f	30.00 $\pm$ 0.14 ^c	3.00 $\pm$ 0.20 ^{bc}	2.40 $\pm$ 0.33 ^{fg}	3.66 $\pm$ 0.25 ^{fg}	30.00 $\pm$ 0.14 ^c	3.00 $\pm$ 0.20 ^{bc}	2.40 $\pm$ 0.33 ^{fg}	3.66 $\pm$ 0.25 ^{fg}
T16	21.48	2.69	50.00 $\pm$ 0.14 ^c	3.60 $\pm$ 0.74 ^{bc}	2.60 $\pm$ 0.40 ^{def}	3.45 $\pm$ 0.30 ^f	30.00 $\pm$ 0.14 ^c	3.60 $\pm$ 0.74 ^b	2.60 $\pm$ 0.40 ^{defg}	3.62 $\pm$ 0.32 ^{fg}	30.00 $\pm$ 0.14 ^c	3.60 $\pm$ 0.74 ^b	2.60 $\pm$ 0.40 ^{defg}	3.62 $\pm$ 0.32 ^{fg}

BAP = 6-benzylaminopurine; NAA = naphthaleneacetic acid

Parameters recorded after 12 wk of transfer in multiplication media

Data are shown as mean $\pm$ SDMeans with the same lowercase superscript in a column are not significantly different ($p \geq 0.05$).**Table 2** Effect of various concentrations of Murashige and Skoog (1962) medium (MS) and indole-3-butyric acid (IBA) on *in vitro* root induction in *Bambusa vulgaris* 'wamin' and *Bambusa bambos*

Treatment	MS	Plant growth regulator (μM)		<i>B. vulgaris</i> 'wamin'				<i>B. bambos</i>			
		Choline chloride	IBA	Rooting (%)	Number of roots	Length of roots (cm)	Rooting (%)	Number of roots	Length of roots (cm)	Rooting (%)	Number of roots
TR1	$\frac{1}{2}$	53.71	0	60.00 $\pm$ 0.00 ^b	1.5 $\pm$ 0.15 ^{bc}	1.28 $\pm$ 0.32 ^{ef}	60.00 $\pm$ 0.00 ^e	1.4 $\pm$ 0.20 ^d	1.13 $\pm$ 0.15 ^{cd}	60.00 $\pm$ 0.00 ^e	1.4 $\pm$ 0.20 ^d
TR2	$\frac{1}{2}$	53.71	5.37	70.00 $\pm$ 0.14 ^a	1.1 $\pm$ 0.20 ^{de}	1.48 $\pm$ 0.12 ^{cd}	50.00 $\pm$ 0.42 ^f	1.4 $\pm$ 0.23 ^d	1.40 $\pm$ 0.26 ^b	50.00 $\pm$ 0.42 ^f	1.4 $\pm$ 0.23 ^d
TR3	$\frac{1}{2}$	53.71	10.74	70.00 $\pm$ 0.14 ^a	1.4 $\pm$ 0.27 ^{bed}	1.43 $\pm$ 0.25 ^{de}	100.00 $\pm$ 0.00 ^a	2.5 $\pm$ 0.12 ^a	2.66 $\pm$ 0.23 ^a	100.00 $\pm$ 0.00 ^a	2.5 $\pm$ 0.12 ^a
TR4	$\frac{1}{2}$	53.71	21.48	70.00 $\pm$ 0.14 ^a	4.4 $\pm$ 0.18 ^a	2.60 $\pm$ 0.30 ^b	90.00 $\pm$ 0.14 ^b	2.0 $\pm$ 0.25 ^b	1.32 $\pm$ 0.44 ^{bc}	90.00 $\pm$ 0.14 ^b	2.0 $\pm$ 0.25 ^b
TR5	Full	53.71	0	30.00 $\pm$ 0.14 ^c	0.5 $\pm$ 0.21 ^f	1.15 $\pm$ 0.25 ^f	20.00 $\pm$ 0.00 ^g	1.7 $\pm$ 0.55 ^c	0.45 $\pm$ 0.26 ^c	20.00 $\pm$ 0.00 ^g	1.7 $\pm$ 0.55 ^c
TR6	Full	53.71	5.37	70.00 $\pm$ 0.14 ^a	1.0 $\pm$ 0.29 ^{de}	1.62 $\pm$ 0.41 ^{bc}	60.00 $\pm$ 0.28 ^e	2.0 $\pm$ 0.52 ^b	1.00 $\pm$ 0.42 ^d	60.00 $\pm$ 0.28 ^e	2.0 $\pm$ 0.52 ^b
TR7	Full	53.71	10.74	60.00 $\pm$ 0.28 ^b	0.7 $\pm$ 0.23 ^{ef}	1.64 $\pm$ 0.16 ^b	70.00 $\pm$ 0.14 ^d	1.2 $\pm$ 0.02 ^e	0.96 $\pm$ 0.24 ^d	70.00 $\pm$ 0.14 ^d	1.2 $\pm$ 0.02 ^e
TR8	Full	53.71	21.48	60.00 $\pm$ 0.00 ^b	1.7 $\pm$ 0.20 ^b	1.40 $\pm$ 0.25 ^{de}	80.00 $\pm$ 0.00 ^f	1.8 $\pm$ 0.22 ^c	0.99 $\pm$ 0.23 ^d	80.00 $\pm$ 0.00 ^f	1.8 $\pm$ 0.22 ^c

IBA- Indole-3 butyric acid

Parameters recorded after 4 wk of transfer in rooting media

Data are shown as mean $\pm$ SDMeans with the same lowercase superscripts in a column are not significantly different ($p \geq 0.05$)

Table 3 Polymerase chain reaction amplicons obtained from various random amplified polymorphic DNA markers in *in vitro*-raised *B. vulgaris* ‘wamin’ and *B. bambos* plantlets

Primer	Primer sequence	<i>B. vulgaris</i> ‘wamin’			<i>B. bambos</i>		
		Band size (bp)	Number of bands amplified	Number of monomorphic bands	Band size (bp)	Number of bands amplified	Number of monomorphic bands
OPA-3	5' AGT CAG CCAC 3'	375–2134	7	7	376–1650	8	8
OPA-4	5' AAT CGG GCT G 3'	310–1200	8	8	422–1298	11	11
OPA-20	5' GTT GCG ATC C 3'	760–1802	8	8	250–2024	16	16
OPC-2	5' GTG AGG CGT C 3'	255–2000	13	13	240–1992	13	13
OPC-4	5' CCG CAT CTA C 3'	245–1100	12	12	310–1730	16	16
OPC-5	5' GAT GAC CGC C 3'	264–1255	9	9	750–1510	12	12
OPC-8	5' TGG ACC GGT G 3'	420–2035	10	10	270–1400	9	9
OPM-2	5' ACA ACG CCT C 3'	265–1320	11	11	362–1559	9	9
OPM-4	5' GGC GGT TGT C 3'	320–1328	11	11	250–2505	11	11
OPN-1	5' CTC ACG TTG G 3'	249–1670	8	8	375–1980	8	8

Statistical analysis

The data were subjected to one-way analysis of variance using the Microsoft Excel software package (Microsoft Corp.; USA) followed by Duncan's multiple range test using the DSAASTAT software (Onofri and Pannacci, 2014). Data were reported as the mean $\pm$ SD unless otherwise noted.

DNA extraction and polymerase chain reaction amplification

Total DNA was extracted from the mother plant (aged 36 mth) and the tissue culture-raised plantlets (aged 6 mth) of both bamboo species using the method described by Doyle and Doyle (1990). The quantity and purity (absorbance at 260 nm: absorbance at 280 nm) of DNA was measured using the Nanodrop N.D.1000 (Thermo Scientific; U.S.A.). The DNA samples were diluted to 20 ng/ μ L with TE buffer and stored at 4°C. RAPD primer series A, C, M and N (Eurofins MWG Operon Inc.; India) were screened for the selection of suitable RAPD markers for clonal fidelity testing (Table 3).

RAPD amplification was performed in a 25 μ L reaction system containing 100 ng DNA, 1X DreamTaq Green polymerase chain reaction (PCR) buffer with 2 mM MgCl₂ (Thermo Fisher Scientific; U.S.A.), 0.2 mM each of dNTPs (Thermo Fisher Scientific; U.S.A.), 0.5U Taq DNA polymerase (Kapa Biosystems; U.S.A.), 4 pmol MWG Operon primers (OPA-3, OPA-4, OPC-2, OPC-4, OPC-5, OPC-8, OPM-2, OPM-4, OPN-1, OPN-9) and sterile distilled water. Amplification was automated using a PCR machine (Applied Biosystems; USA). The PCR cycles involved an initial denaturation step at 94 °C for 5 min followed by 42 cycles of denaturation at 94 °C for 1 min, primer annealing at 38 °C for 1 min, extension at 72 °C for 1 min and final extension at 72 °C for 5 min. Agarose gel (1.5 %) was prepared in 1X TBE buffer to separate the amplified products. A gel documentation system (Alpha Innotech; USA) was used for visualization of amplified DNA fragments (Gajera et al., 2010).

Results

Surface sterilization

Axenic cultures were established by the sequential application of Carbendazim-50% (25 min), Cefotaxime (18 min), Kanamycin (18 min), Streptocycline (15 min) and 0.1 % HgCl₂ (18 min). Although the initiations were performed during July to September, when the climate was very humid (monsoon) in Gujarat, more than 75% of axenic cultures were obtained for both bamboo species. The axenic cultures obtained for *B. vulgaris* and *B. bambos* were 78.13% and 94.93%, respectively.

Culture initiation and mass multiplication

The incidence of bud break was observed in almost all explants within 7 d of initiation. The sprouting percentages for the different treatments were in the ranges 20–90% and 30–90% for *B. vulgaris* and *B. bambos*, respectively. The problem of exudation in the cultures was resolved by regular subculture in fresh medium. The maximum shoot proliferation was observed in the treatment supplemented with 8.87 μ M BAP + 0.54 μ M NAA for *B. vulgaris* and 8.87 μ M BAP for *B. bambos* (Figs. 1A–1C and Figs. 2A–2C, respectively). The numbers of shoots recorded were 6.20 ± 0.32 and 7.10 ± 0.26 for *B. vulgaris* and *B. bambos*, respectively. The lengths of shoots measured were 6.02 ± 0.18 cm and 6.99 ± 0.29 cm and the numbers of leaves formed were 7.50 ± 0.61 and 7.20 ± 0.47 for *B. vulgaris* and *B. bambos*, respectively (Table 1).

Rooting

In vitro rooting was induced on clumps containing three to four shoots. Among the different treatments used, treatment TR₄ ($\frac{1}{2}$ MS + 53.71 μ M choline chloride + 21.48 μ M IBA) for *B. vulgaris* and TR₃ ($\frac{1}{2}$ MS + 53.71 μ M choline chloride + 10.74 μ M IBA) for *B. bambos* produced maximum numbers of roots and highest root lengths (Fig. 1D and Fig. 2D, respectively). The rooting percentages for the different treatments were in the ranges 20–90 and 30–70 for *B. vulgaris* and *B. bambos*, respectively. The number of roots and length of roots recorded for *B. vulgaris* were 4.4 ± 0.18 and 2.60 ± 0.30 cm, respectively and those recorded for *B. bambos* were 2.5 ± 0.12 and 2.66 ± 0.23 cm, respectively (Table 2).



Fig. 1 (A) Culture initiation of *Bambusa vulgaris*; (B) propagule of *B. vulgaris*; (C) shoot proliferation of *B. vulgaris*; (D) rooting of *B. vulgaris* on solid media; (E) plantlet of *B. vulgaris*; (F) acclimatized plants of *B. vulgaris* in polyhouse, where scale bar = 1 cm

Hardening and transplantation

Cocopeat is a multipurpose soil conditioner and growing medium. The shoots turned greener and were elongated during *in vitro* hardening. A large number of roots were elongated and matted. Fungal and bacterial contaminants were removed using aerial spray of antifungal and antibacterial agents. The survival percentages recorded for *B. vulgaris* and *B. bambos* during *ex vitro* hardening were 85% and 80%, respectively. Plantlets were then transferred to the polyethylene bags containing soil mixtures. These plants appeared normal and tillered in a very short time, with the gradual development of multiple stems (Figs. 1E–1F and Figs. 2E–2F, for *B. vulgaris* and *B. bambos*, respectively).

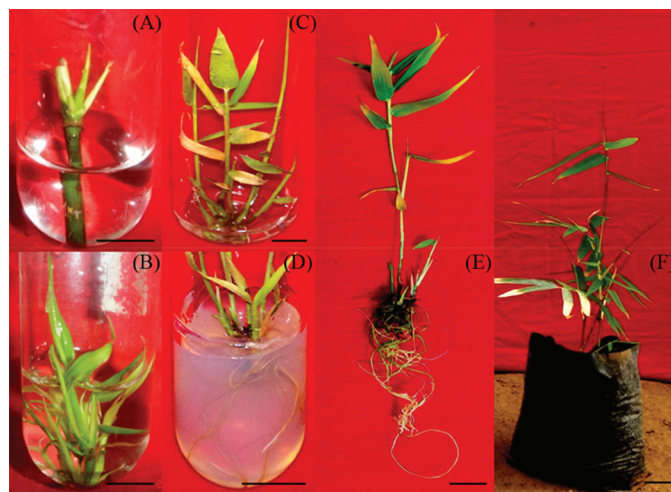


Fig. 2 (A) Culture initiation of *Bambusa bambos*; (B) propagule of *B. bambos*; (C) shoot proliferation of *B. bambos*; (D) rooting of *B. bambos* on solid media; (E) plantlet of *B. bambos*; (F) acclimatized plants of *B. bambos* in polyhouse, where scale bar = 1 cm

Genetic fidelity

Out of the various RAPD primers screened, 10 primers which resulted with six or more scorable bands were selected for the present study. The 10 RAPD primers generated 245–2134 bp sized amplicons for *B. vulgaris* ‘wamin’ and 240–2505 bp sized amplicons for *B. bambos*. (Table 3). All the bands generated using 10 decamers were monomorphic and confirmed the genetic purity of clonally propagated bamboo plants (Figs. 3A and 3B).

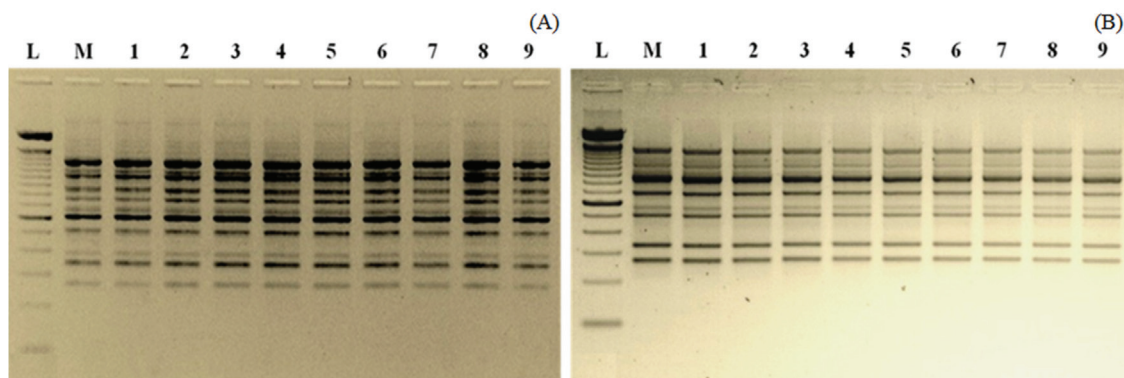


Fig. 3 Polymerase chain reaction amplification products obtained with a random amplified polymorphic DNA primer OPM-2 of: (A) *Bambusa vulgaris* ‘wamin’; (B) *Bambusa bambos*, where lane L represents 100 bp ladder, lane M represents mother plant DNA and lanes 1–9 represent *in vitro* raised clones of *B. vulgaris* ‘wamin’ and *B. bambos*

Discussion

An efficient micropropagation protocol was developed for *B. vulgaris* and *B. bambos* as there has been an ever-increasing demand for bamboo by the pulp and paper, wood chip, construction and horticultural industries. The present study yielded significantly higher frequencies of both aseptic culture establishment (78.13% for *B. vulgaris* and 94.93% for *B. bambos*) and bud break initiation (90.00 ± 0.14 for *B. vulgaris* and 90.00 ± 0.14 for *B. bambos*). Mudoi and Borthakur, (2009) obtained only 65–70% axenic cultures of *Bambusa balcooa* from the explants initiated during August to September. Das and Pal (2005a) also reported very low frequencies of bud break (50%) and axenic culture establishment (50%) from *B. balcooa* explants initiated during June to October. Similar correlation between rainfall and axenic culture development (79%) was reported for *Bambusa tulda* by Mishra et al. (2008). Propagule size was found to be crucial for shoot proliferation as well as *in vitro* rooting. As in earlier *in vitro* studies related to *B. balcooa* (Das and Pal, 2005b; Mudoi and Borthakur, 2009) and other bamboo species (Saxena and Bhojwani, 1993; Jimenez et al., 2006), the present study also realized the requirement of a propagule size of three or more shoots for proliferation and rooting. Further, the combinational effect of BAP and NAA assisted shoot multiplication in both bamboo species. Similar investigation was carried out by Kant et al. (2009) and Baskaran et al. (2014) in their independent studies and both yielded similar results. This indicated an important role of the auxin/cytokinin ratio during the initiation and multiplication of bamboo. Moreover, it was observed that *in vitro* cultures performed better in liquid medium compared to semi-solid medium. (Saxena, 1990) also obtained highest shoot multiplication and growth in liquid medium for *B. tulda*. The problem of phenolic exudation in the cultures was alleviated by frequently transferring the shoots to fresh liquid medium. Devi and Sharma (2009) practiced quick transfer of *Arundinaria callosa* ‘Munro’ cultures to fresh media to overcome the problem of browning. Both root induction and elongation were achieved in a single step within 21 d. The rooting response was improved significantly by the inclusion of IBA and choline chloride to the medium. Only half strength MS was sufficient to obtain rooting frequencies of 70% and 100% for *B. vulgaris* and *B. bambos*, respectively. Use of choline chloride in conjunction with IBA for vigorous rooting in the present investigation was well supported by the work of Sood et al. (2002) who used 64.46 μM choline chloride and 4.90 μM IBA which produced the highest number of primary, secondary and tertiary roots. Ramanayake et al. (2006) obtained a rooting frequency of 77.5% on half-strength MS supplemented with 14.76 μM IBA during propagation of *Dendrocalamus giganteus* Munro. Cocopeat proved an excellent autoclavable substrate for the hardening of the *in vitro* propagated plants of both the bamboo types. The many porous particles and fibers in cocopeat can assist in providing channels for aeration and drainage (Hume, 1949). The plants were gradually adapted to atmospheric temperature after their transfer to the polyhouse and this was maintained until their transplantation in the field. The appearance of the monomorphic bands in the gel lanes against the RAPD primers in the present study confirmed that all the plants produced through tissue culture were true-to-type. Similarly, Das and Pal (2005a) detected clonal fidelity in micropropagated plantlets of *B. tulda* using five RAPD decamers. Anand et al. (2013) assessed genetic fidelity in the edible bamboo ‘*B. bambos*’ using 15 RAPD markers and obtained monomorphic amplicons.

Conflicts of Interest

The authors declare that there are no conflicts of interest

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