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Micropropagation of *Dendrocalamus membranaceus* Munro. through axillary shoot proliferation and confirmation of clonal fidelity of *in vitro* raised plants

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Abstract
The aim of the present study was to standardize an efficient protocol for mass multiplication of an important bamboo *Dendrocalamus membranaceus* categorized under "Important" category by United Nations Environment Programme – World Conservation Monitoring Centre, (UNEP – WCMC) and ascertaining the clonal fidelity of *in vitro* raised plants by molecular markers. Axillary buds from the field grown plants were taken as explants to induce multiple shoots on Murashige and Skoog (MS) medium containing 4.4 µM N_6-Benzylaminopurine (BAP) alongwith 1.16 µM Kinetin (KN). A clump comprising of a minimum of 3 shoots was inoculated on to half strength MS medium containing 5.4 µM á-Naphthaleneacetic Acid (NAA) in addition to low concentrations of 4.4 µM BAP for root induction. About sixty five percent success was achieved in plant establishment after acclimatization in green house. Total chlorophyll and carotenoids ($17.92^{a} \pm 1.33$) mg/g FW (fresh weight) and electrolyte leakage percentage ($76.01^{a} \pm 3.9$) was found to be higher in *ex vitro* raised plants whereas relative water content percentage ($68.8^{a} \pm 4.0$) was higher in *in vitro* grown plants depicting role of physiological factors in overall growth and development of plants. An assessment of clonal fidelity using 35 Random Amplified Polymorphic DNA (RAPD) and 20 Inter Simple Sequence Repeats (ISSR) as molecular markers revealed that the bands were shared by both the parent clump and *in vitro* raised plants confirming the genetic stability of the Tissue Culture (TC) raised plantlets. Micropropagation protocol reported here along with all the molecular and physiological factors studied is the first report in this very important bamboo species.

Keywords: Acclimatization, bamboo, clonal fidelity, *Dendrocalamus membranaceus*, micropropagation, molecular markers

INTRODUCTION

In vitro conservation and sustainable management of bamboo should be a high priority as they play an important role in local economies besides having more than 1500 documented uses (Paranjothy and Mariam, 2000). In 2011, an amendment to Indian

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Forest Act, 1927 was approved and Union Ministry of Environment and Forests declared bamboos as a minor forest produce to address the livelihood issues of the forest dwellers. *Dendrocalamus membranaceus* Munro, a sympodial edible bamboo native to Myanmar is listed among 18 additional taxa under 'Important' category (Bystriakova *et al*., 2003) Kennard and Freyre (1957), after studying 27 bamboos for edibility of shoots, considered this bamboo to be excellent from processing point of view. According to Yuming *et al*. (2004) besides numerous uses of bamboos, their shoots possess high nutritional value having high protein content of upto 15.23%, low fat content of less than 2.4% and high edible cellulose content (6-8%). Probably more than 2 million tonnes of bamboo shoots are consumed annually (Kleinhenz *et al*., 2000). Figures for bamboo timber will be multiple times greater, e.g. 3 million t/ year in India (Subramanian,1995) and most likely about 30 million t/year worldwide. Although initial reports on regeneration of bamboo plantlets through embryo culture appeared in the late sixties (Alexander and Rao, 1968), advances in tissue culture studies on bamboos is fairly recent. The complete protocol on bamboo micropropagation by Mehta *et al*. (1982) using seeds of *Bambusa arundinacea* laid the foundation for bamboo micropropagation and *in vitro* conservation, as it is a quick method with high multiplication rate. Since then a lot of advances in clonal propagation of bamboos using both juvenile and adult tissue have been made (Thiruvengadam *et al*., 2011; Banerjee *et al*., 2011). In the present communication, we report a protocol for large scale propagation of *D. membranaceus* with high rates of shoot multiplication, rooting of microshoots in a difficult to root species, comparative account of physiological characters of *in vitro* and *ex vitro* grown plants viz. relative water content (RWC), electrolyte leakage (EL) and chlorophyll content and finally, checking the clonal fidelity of *in vitro* raised and acclimatized plantlets by use of molecular markers. Micropropagation from nodal explants and the study of physiological parameters were conducted for the first time in *D. membranaceus*.

MATERIALS AND METHODS

Selection and preparation of explants

The explants for *in vitro* cultures were obtained as single node segments (3-4 cm long) from healthy field growing culms of *D. membranaceus* (1 year old) assembled at the experimental farm of the CSIR - Institute of Himalayan Bioresource Technology, Palampur, India, at an elevation of (1300 masl) and 32º7'11' N latitude and 76º31'48'' E longitude, during the year 2011.

Initiation of aseptic cultures

After removing the leaf sheaths carefully, the nodal segments were rinsed with 0.01% Tween 20 (Polyxyethylene sorbitan monolaurate, Qualigens, Mumbai) for 10 minutes. Explants were given treatment in Carbendazim (0.25%) first, followed by Streptomycin

sulphate (0.25%) and Tetracycline (0.05%) for 15 minutes each. Final disinfection was done with 10% Sodium hypochlorite (NaOCl) for 10 min followed by 0.1% Mercuric chloride ($HgCl_2$) for 8 min. Each treatment was followed by repeated washings in sterile distilled water. Explants were inoculated on Murashige and Skoog medium (MS, 1962) having pH 5.75 before autoclaving containing 3% sucrose, 0.8% agar after removing about 0.5 cm of cut ends from both sides of node. Cultures were incubated under cool, white 40 W fluorescent lamps with irradiation at 70 µmol m^{-2} s^{-1} photosynthetic photon flux density (PPFD) at a temperature of 25 ± 2 ºC with 16/8 h light:dark cycle. In order to study the influence of season on culture establishment explants were collected in winter, spring, summer and rainy season during the year.

Investigation of cytokinin effects on shoot multiplication

Different cytokinins were tested for their efficiency for shoot multiplication. Basal MS medium (BMS) consisting of macronutrients, micronutrients, vitamins and a carbon source ie sucrose as devised by Murashige and Skoog (1962) was supplemented with BAP ranging from 0.0 to 13.2 µM and KN from 0.0 to 4.65 µM. Shoots were sub-cultured to fresh medium at a regular interval of 20 days in 250 ml capacity Erlenmeyer flasks (Borosil, Mumbai, India) containing 100 ml of media.

Effect of NAA and BAP on rooting

Shoots were excised in clumps of 4-5 from multiple shoot bunches and transferred to MS media of full, half and quarter strength containing low concentration of BAP (0.0 to 4.4 µM) alongwith NAA (0.0 to 10.74 µM) for induction of roots. The rate of root proliferation was dependent upon type and concentration of auxin and the strength of media.

Acclimatization and *ex vitro* transfer

After 30 days, the rooted plantlets were removed from culture tubes, washed in lukewarm water and the plantlets were placed inside a polytunnel in riverbed sand under shade for acclimatization initially. After 14 days, the plantlets were transferred to polybags containing suitable potting mixture (Soil:Sand:FYM ::1:1:1, v/v/v). Stomatal studies were conducted after 3 months for comparing the *in vitro* and *ex vitro* acclimatized plantlets.

Relative water content

Relative water content (RWC) was determined according to Perl-Treves and Galum, (1991). From each leaf, 9 discs were cut and their fresh weights (FW) were measured. Leaf discs were then immersed in distilled water for 20-24 hours and their hydrated weights were also measured. The samples were then dried overnight in an oven maintained at 70ºC and their dry weights measured. The RWC was calculated by the

formula: 100 x (actual weight-dry weight) / (hydrated weight–dry weight).

Electrolyte leakage

For estimating cell membrane stability, electrolyte leakage was measured using an electrical conductivity meter with slight modifications in the method described by Wright and Simon, (1973). Eight leaf discs were cut, rinsed and immersed in distilled water for 22 hours. Initial readings in $\mu S.cm^{-1}$were taken before boiling. Instead of autoclaving we boiled the discs for 5-10 min and the reading were taken using conductivity meter (Model 510 PC, Cyberscan, China). Results were expressed as percentage of electrolye leakage.

Electrolyte leakage % = 100 x (EL before boiling) / (EL after boiling).

Chlorophyll content

The green epidermis from culms of *in vitro* and *ex vitro* grown plants was peeled and ground to a fine powder in a pestle and mortar. Forty mg fresh weight (FW) of the powder was added to sample vial containing 25 ml of the solvent acetone (MacKinney, 1941); DMF (Moran and Porath,1980) and DMSO (Hiscox and Israelstam, 1979) and the chlorophyll extracted using an ultrasonicator for 3 minutes. The chlorophyll solutions were analysed with an ultraviolet (UV)-Vis Spectrophotometer (Biotek Synergy, HT).

Genomic DNA isolation and PCR amplification

DNA was extracted from fresh expanded leaves (100 mg) using the CTAB method following Doyle and Doyle (1990). Thirty five RAPD primers and twenty ISSR primers at a concentration of 1 µM/ reaction were scanned in the present study to ascertain the clonal fidelity of *in vitro* raised plants. PCR was performed in a volume of 25 µl PCR reaction containing 10X Taq buffer A (2.5 µl), 2 mM $MgCl_2$ (1 µl), 0.15 µl of dNTPs in a concentration of 10 mM each, One unit of Taq DNA polymerase (Bangalore GeNei Pvt. Ltd), 17.68 µl autoclaved distilled water and 2 µl (20-25 ng) of template DNA in a DNA thermal cycler (S 1000 BioRad). For RAPD analysis an initial denaturation step at 94ºC for 5 minutes and 45 cycles were carried out as per details : 94ºC for 1 min (Denaturation), 37 ºC for 1 min (Annealing), 72ºC for 2 min (Extension) and one final extension of 1 min at 72ºC. ISSR analysis was performed with denaturation at 94ºC for 4 min, followed by 44 cycles of denaturation at 94ºC for 1 min, annealing temperature (Tm) specific to primer for 1 min and extension at 72ºC for 1 min and finally at 72ºC for 7 minutes. Aliquots of 5 µl sample plus gel loading buffer (6X) were loaded on 1.8% Agarose gel for electrophoresis in 1X TBE buffer for RAPD and ISSR analyses. Gel photographs were analysed through Gel Doc system (Molecular Imager, Gel Doc XR Imaging System).

Statistical analysis

All the investigations were based on Complete Randomized Block Design with a minimum of 3 replicates. The data shown represent the mean ± SD. Significance of treatment was determined by one way ANOVA, and the means were compared with Duncan's Test at a significance of $p \leq 0.05$ using Statistica system version 7.

RESULTS

Intiation of aseptic cultures

Axillary bud break was influenced by climatic factors and monthly variation in the bud break efficiency was recorded throughout the year 2011. It was found to be equally good during spring and rainy seasons (Fig. 1a). The preferred season for raising aseptic cultures was during the months of late February to April with a bud break of 90%. Rainy season (80% bud break) was not preferred as there was a corresponding increase in the rate of contamination. Explant size also affected the bud break, being maximum when explants of the size of 25 mm were used. The larger explants probably contained more nutrient reserves and PGR to sustain themselves during the initiation phase of cultures. Very small sized (5mm) explants did not respond well in initiation of cultures (Fig. 1 b) which was achieved on both solid and liquid MS media. Agarified media

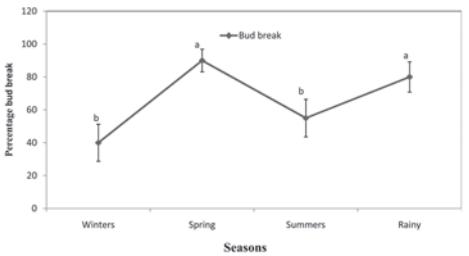

Figure1. a Effect of season on bud break. Spring season gave maximum 90% bud break.

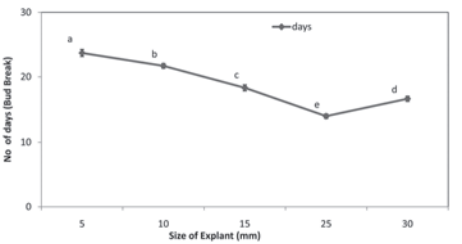

Figure1. b Effect of size of explants on bud break showing best response when size of explants used is 25 mm.

(0.8%) was preferred which resulted in better growth of sprouts, low rates of contamination, less browning of the medium and needed lesser number of sub-culturing.

Investigation on cytokinin effect on shoot multiplication

Best results were obtained on solid MS medium supplemented with 4.4 µM BAP and 1.15 µM KN where a mean number of shoots per explant obtained was $13.40^{a} \pm 1.5$ in about 30 days with a shoot length of $2.92^{a} \pm 0.31$ cm and the number of leaves was $15.50^{a} \pm 0.97$ with no signs of albinism (Table 1). Higher doses of BAP (13.2 µM) induced thin leaf like shoots many of which them did not develop further. Repeated

subculture of shoots in liquid medium resulted in vitrification of shoots, more contamination rate and release of phenolic compounds in the media and therefore, solid medium was preferred for repeated sub-culturing.

Table 1. Effect of different concentration of BAP and KN on rate of proliferation of shoots

BAP/KN (μM)	No of shoots	Shoot Length (cm)	Total no of leaves
0/0	$2.80^{f} \pm 1.2$	$1.20^{g} \pm 0.25$	$4.20^{h} \pm 0.78$
0/4.6	$2.60^{fg} \pm 0.6$	$1.10^{g} \pm 0.17$	$2.40^{1i} \pm 0.5$
0/1.16	$2.50^{fg} \pm 0.5$	$1.17^{g} \pm 0.23$	$3.50^{h} \pm 0.52$
0/2.2	$2.50^{fg} \pm 0.7$	$1.10^{g} \pm 0.12$	$2.50^{i} \pm 0.52$
4.4/0	$7.50^{c} \pm 0.8$	$1.60^{ef} \pm 0.61$	$9.90^{c} \pm 0.73$
4.4/0.46	$9.30^{b} \pm 1.1$	$2.20^{bc} \pm 0.71$	$12.10^{b} \pm 0.73$
4.4/1.16	$13.40^{a} \pm 1.5$	$2.92^{a} \pm 0.31$	$15.50^{a} \pm 0.97$
4.4/2.2	$8.80^{b} \pm 1.3$	$2.45^{b} \pm 0.43$	$9.10^{cd} \pm 1.52$
8.8/0	$5.60^{d} \pm 0.9$	$2.00^{cd} \pm 0.40$	$7.30^{f} \pm 1.15$
8.8/0.46	$6.20^{d} \pm 0.7$	$2.45^{b} \pm 0.43$	$7.70^{ef} \pm 1.15$
8.8/1.16	$7.20^{c} \pm 0.9$	$2.35^{bc} \pm 0.33$	$8.85^{de} \pm 1.08$
8.8/2.2	$3.80^{e} \pm 0.9$	$2.35^{bc} \pm 0.24$	$7.30^{f} \pm 0.67$
13.2/0	$2.20^{fg} \pm 0.7$	$1.65^{def} \pm 0.41$	$5.80^{g} \pm 0.91$
13.2/0.46	$2.50^{fg} \pm 0.7$	$1.70^{def} \pm 0.34$	$7.30^{f} \pm 0.67$
13.2/1.16	$2.70^{f} \pm 0.9$	$1.80^{de} \pm 0.25$	$7.60^{f} \pm 1.26$
13.2/2.2	$1.70^{g} \pm 0.8$	$1.40^{fg} \pm 0.39$	$5.20^{g} \pm 0.78$

Cell values differing by a letter in superscript within each column are significantly different at $p \leq 0.05$

Effect of auxin on root induction

In vitro raised shoots in a cluster of 3-4 shoots were used as propagules for root induction. Root development was noticed within 30-45 days of inoculation. NAA was found to be more effective than IBA for rooting of microshoots. Half-strength MS medium fortified with 4.4 μM BAP and 5.4 μM NAA gave the best response for induction of roots with mean number $6.00^{a} \pm 1.00$ of roots and root length of $6.68^{ab} \pm 0.72$ as depicted in Table 2. Occasionally short clumps having 12-13 roots were also formed when explants were taken during rainy season for culture establishment. Combining NAA and BAP favoured rooting response as compared to NAA alone. NAA (0.53 to 10.74 μM) when used singly resulted in necrotic shoots. Various modifications in medium are underway for improvement in rooting percentage. Further, the transfer of *in vitro* rooted shoots to BMS after 20-25 days produced good quality roots and plantlets.

Hardening and acclimatization

The plantlets (10-12 weeks old) were acclimatized in green house under controlled atmospheric conditions of low irradiance, high RH (60-80%) and temperature not exceeding 28 ºC to promote the plant growth. Survival rate after acclimatization was

Table 2. Effect of NAA and BAP combinations on rooting in *D. membranaceus*

NAA/BAP (µM)	Mean no of roots	Root length (cm)	Root growth response
0.0/0.0	$0.00^{d} \pm 0.0$	$0.00^{i} \pm 0.00$	-
0.0/2.2	$0.00^{d} \pm 0.0$	$0.00^{i} \pm 0.00$	-
0.0/4.4	$0.00^{d} \pm 0.0$	$0.00^{i} \pm 0.00$	-
0.0/8.8	$0.00^{d} \pm 0.0$	$0.00^{i} \pm 0.00$	-
0.53/0.0	$0.00^{d} \pm 0.0$	$0.00^{i} \pm 0.00$	-
0.53/2.2	$0.00^{d} \pm 0.0$	$0.00^{i} \pm 0.00$	-
0.53/4.4	$0.5^{c} \pm 0.5$	$5.30^{de} \pm 0.67$	+
0.53/8.8	$1.3^{b} \pm 0.04$	$5.00^{efl} \pm 0.8$	++
5.37/0.0	$1.4^{b} \pm 0.5$	$7.10^{a} \pm 0.73$	+++
5.37/2.2	$1.4^{b} \pm 0.5$	$7.22^{a} \pm 0.82$	++++
5.37/4.4	$6.0^{a} \pm 1.0$	$6.68^{ab} \pm 0.72$	++
5.37/8.8	$1.7^{b} \pm 0.4$	$6.32^{bc} \pm 0.70$	++
10.74/0.0	$1.6^{b} \pm 0.5$	$5.82^{cd} \pm 0.86$	++
10.74/2.2	$1.4^{b} \pm 0.5$	$4.70^{fg} \pm 0.63$	+
10.74/4.4	$1.4^{b} \pm 0.5$	$4.35^{g} \pm 0.94$	+
10.74/8.8	$1.3^{b} \pm 0.4$	$3.65^{1h} \pm 0.4$	+

– depicts absence of roots, + depicts presence of root/s, ++ depicts fair response, +++ depicts fair response with more healthy roots, ++++ depicts good rooting response. Cell values differing by a letter in superscript within each column are significantly different at $p \leq 0.05$

67 per cent. Hence, a complete protocol for micropropagation was established (Fig. 2 a-h). There was no significant difference in stomata number and density in *in vitro* grown and *ex vitro* grown plants. It was shown from our results that stomata were more on abaxial side and more number of stomata were opened as compared to adaxial side. Stomatal shape was typical of monocots represented by the dumbbell shape. Silicified cells were also present throughout on both the surfaces. In addition, abaxial surface has conspicuous papillae present in costal and intercostal region of the leaves (Figs. 3 a-f).

Relative water content

RWC percentage was found to be higher in *in vitro* grown plantlets with values of $68.8^{a} \pm 4.09$ (Table 3) that could be explained as being the result of the controlled environmental conditions. There was an inverse relationship of water saturation deficit

Table 3. Effect of *in vitro* and *ex vitro* environment on physiological parameters in micropropagated plants

Physiological parameters	*In vitro*	*Ex vitro*	Remarks
Relative water content %	68.8±4.09	56.6±3.2	Plant water status in terms of cellular hydration
Water saturation deficit	31.2±4.09	43.4±3.2	Plant water balance
Electrolyte leakage %	68±2.9	76±3.9	Cell membrane stability

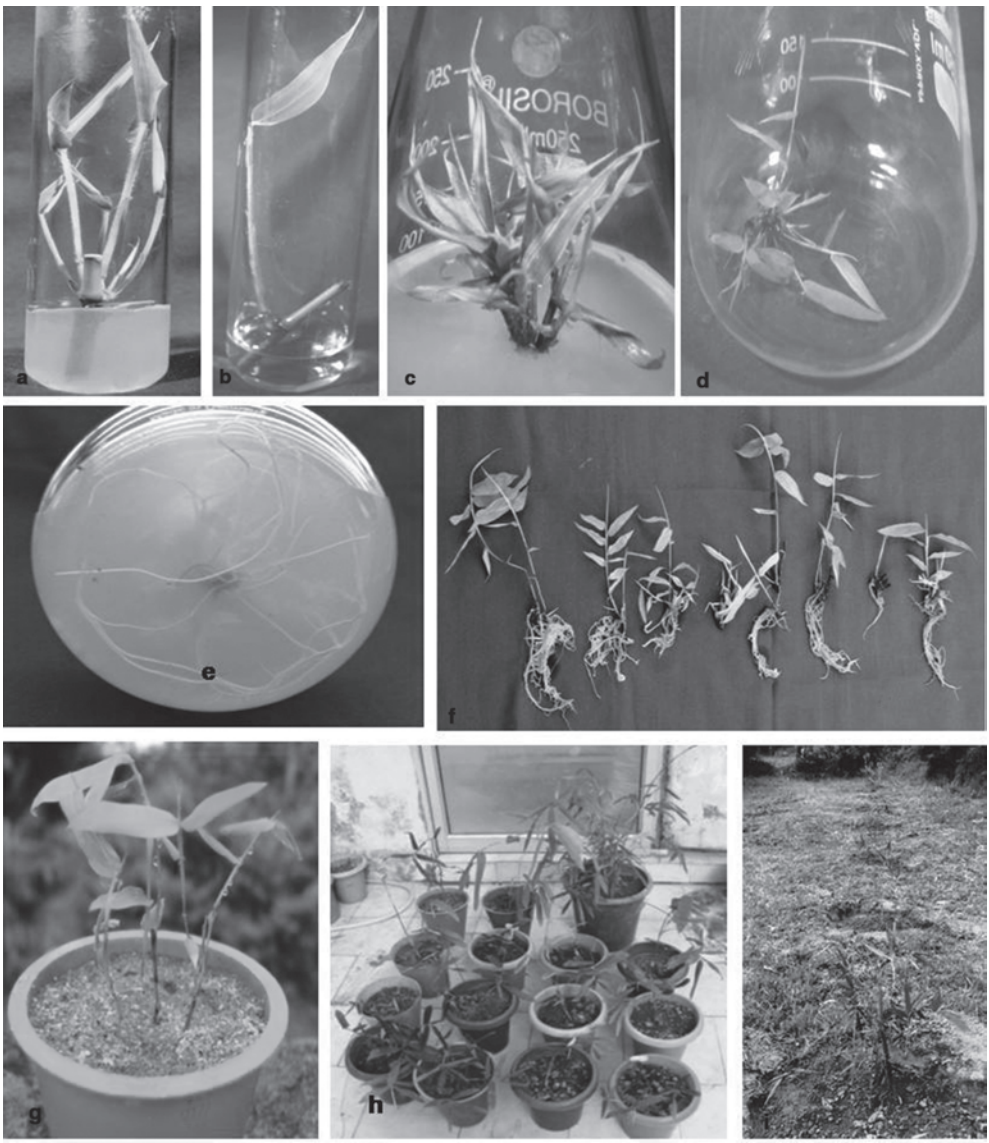

Figure 2. a. Bud break on BMS. **b**. Bud break on liquid Basal MS Medium **c.** Multiple shoot proliferation on 4.4 µM N_6-Benzylaminopurine (BAP) and 1.15 µM Kinetin (Kn). **d**. Shoot proliferation on liquid medium containing same composition of hormones as solid medium. **e-f.** Rooting on 5.4 µM á Naphthaleneacetic Acid (NAA) and 4.4µM BAP. **g.** Hardening of plantlets on Soil: Sand: FYM ::: (1:1:1) **h.** Acclimatization in green house. **i**. Hardening of plants in field.

(WSD) with RWC. However, water status of the plants may stabilize after some days during acclimatization. Low RWC in *in vivo* conditions ($56.6^b \pm 3.2$) indicated a lag phase in the growth of plants when shifted from a high Relative humidity (RH) of 70% to low RH (~60%) in green house. This directly affects the leaf conductance, gas

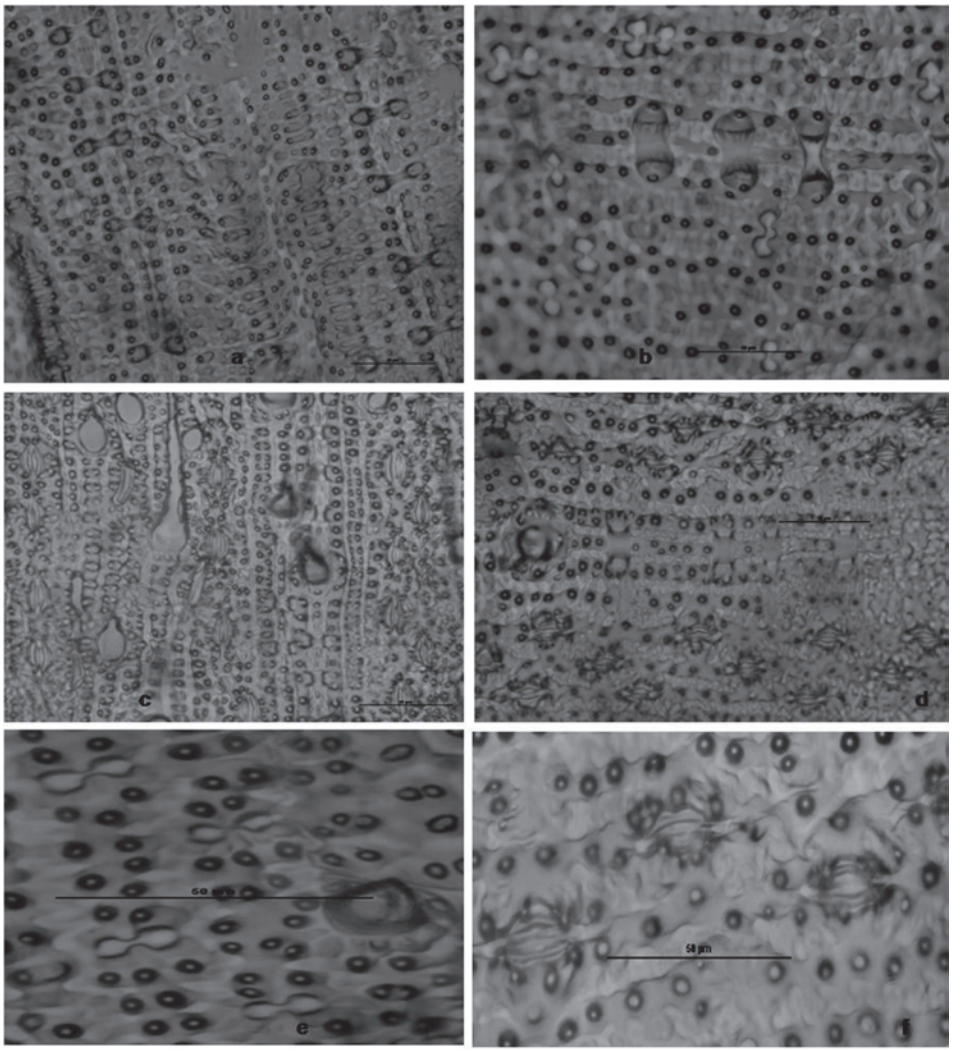

Figure 3. a,b. Stomata on adaxial surface of *in vitro* and *ex vitro* grown leaves. **c,d.** Stomata on abaxial surface of leaves of *in vitro* and e*x vitro* grown plants. Abaxial surface have more stomatal density, stomatal number and more number of stomata are open. Silica cells were observed on both the surfaces but more developed in *ex vitro* grown plants. **e.** Elliptical atomata on adaxial surface **f.** Ring shaped stomata on abaxial surface.

exchange, net assimilation rates and photosynthesis during first week of hardening.

Electrolyte leakage

Electrolyte leakage is a measure of poor cell integrity. The exposure of plants to *ex vitro* conditions (stressed environment) resulted in a significant increase ($76.01^{a} \pm 3.9$) in the percentage of electrolyte leakage (Table 3) as compared to *in vitro* grown plants.

Table 4. Chlorophyll Content in culms of *in vitro* and *ex vitro* grown plants using different solvents by ultrasonics

Solvents	*In vitro* (mg/g FW)	*Ex vitro* (mg/g FW)	Remarks
Acetone	$9.58^{a} \pm 0.83$	$17.92^{a} \pm 1.33$	More stable and less abrasive solvent
DMSO	$7.15^{b} \pm 0.28$	$12.98^{b} \pm 2.99$	Less toxic than DMF
DMF	$6.00^{c} \pm 0.54$	$11.25^{b} \pm 3.46$	More viscous and less stable

Cell values differing by a letter in superscript within each column are significantly different at $p \leq 0.05$

Chlorophyll content and carotenoids

Maximum value for total chlorophyll and carotenoid content mg/g FW using ultrasonics was found to be ($17.92^{a} \pm 2.1$) in *ex vitro* conditions (Table 4). Increased levels of CO_2 in green house promote synthesis of chlorophyll and carotenoids. The results revealed that the extraction efficiency of chlorophyll is related to solvent type, extraction method and properties of bamboo culm meal. Among the solvents, extraction efficiencies were found to be in order Acetone >DMSO>DMF due to different stabilities of these solvents.

Clonal fidelity

For ascertaining the clonal fidelity, 15 randomly selected plants, after 5 months of hardening in green house were subjected to RAPD and ISSR analyses. Out of 35 scanned RAPD markers, only 30 primers produced 73 amplified products (Table 5). For ISSR analysis of 20 markers, only 15 ISSR primers gave 38 scorable bands (Table 6). For a total of 45 scanned markers, 111 amplified products were obtained. The size

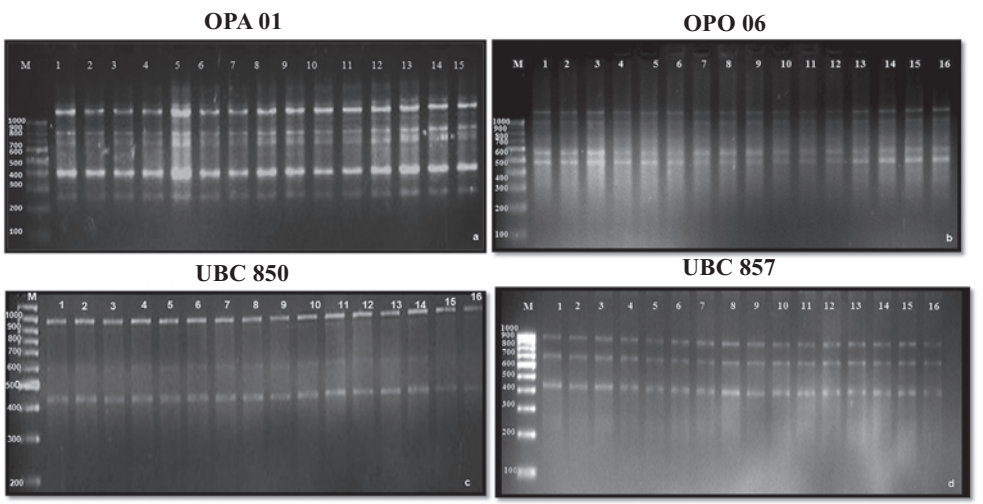

Figure 4a. Agarose gel electrophoresis of RAPD amplification profiles with primer OPA 01 **b.** OPO 06. **c.** ISSR amplification profile with UBC 850 **d**.UBC 857. M is molecular weight marker , 1 is mother or control plant. 2-16 are Tissue Culture (TC) raised plants. All bands were observed at the same positions confirming the genetic fidelity of TC raised plants.

Table 5. Random Amplified Polymorphic DNA (RAPD) primers used to verify *D. membranaceus* clones

Primers	5'-3' motif	No of scorable bands	No of monomorphic bands	No of polymorphic bands	Range of amplification (bp)
OPA 01	CAG GCC CTT C	7	7	0	250, 400, 700, 750, 800,1100, 1150
OPA 02	TGC CGA GCT G	3	3	0	400,600,800
OPA 03	AGT CAG CCA C	2	2	0	350, 700
OPA 04	AAT CGG GCT G	5	5	0	350, 600, 700, 800, 1100
OPA 05	AGG GGT CTT G	5	5	0	250, 350, 500, 600, 700
OPA 08	GTG ACG TAG G	5	5	0	250, 500, 600, 700, 800
OPA 09	GGG TAA CGC C	6	6	0	300,400, 450, 500, 550, 900
OPA 11	CAA TCG CCG T	1	1	0	500
OPA 12	TCG GCG ATA G	1	1	0	1000
OPA 15	TTC CGA ACC C	5	5	0	350, 400, 450, 500, 600
OPA 17	GAC CGC TTG T	2	2	0	500, 1000
OPA 18	AGG TGA CCG T	4	4	0	500, 600, 1000, 1100
OPO 02	ACG TAG CGT C	3	3	0	600, 800, 1300
OPO 04	AAG TCC GCT C	1	1	0	1000
OPO 06	CCA CGG GAA G	4	4	0	500, 600,1000, 1100
OPO 10	TCA GAG CGC C	1	1	0	350
OPO 12	CAG TGC TGT G	1	1	0	1100
OPO 13	GTC AGA GTC C	1	1	0	1150
OPO 16	TCG GCG GTT C	1	1	0	500
OPO 18	CTC GCT ATC C	1	1	0	1100
OPT 02	GGA GAG ACT C	3	3	0	550, 700, 900
OPT 04	CAC AGA GGG A	1	1	0	400
OPT 05	GGG TTT GGC A	1	1	0	500
OPT 06	CAA GGG CAG A	1	1	0	500
OPT 07	GGC AGG CTG T	1	1	0	350
OPT 10	CCT TCG GAA G	1	1	0	1200
OPT 11	TTC CCC GCG A	1	1	0	750
OPT 15	GGA TGC CAC T	1	1	0	1000
OPT 17	CCA ACG TCG T	1	1	0	400
OPT 19	GTC CGT ATG G	3	3	0	100, 1100,1500
Total	**30**	**73**	**73**	**0**	**250-1500**

OP Series sequences of Operon Technologies-Alameda, USA

of fragments varied in the range of 200 bp to more than 1 kb (Fig 4). Optimum Tm for RAPD markers falls near 37 ºC and that for ISSR markers, the range falls between 45.5 to 54 ºC. For RAPD analyses, OPA series gave the best amplification followed by OPO and OPT series. For ISSR markers UBC 834 gave maximum amplified products in the range of 400-800 bp. All the bands scored were monomorphic across the mother plant and its tissue raised progenies thereby, indicating the genomic conservation during *in vitro* multiplication.

Table 6. The Inter Simple Sequence Repeats (ISSR) markers utilized to verify *D membranaceus* clones

Primer	5'-3' motif	T_m °C	T_a °C	No of scorable bands	No of monomorphic bands	No of Polymorphic bands	Range of Amplification (bp)
UBC 807	$(AG)_8T$	47.0	44.5	1	1	0	400
UBC 808	$(AG)_8C$	48.8	45.5	1	1	0	700
UBC 810	$(GA)_9T$	45.4	43.5	3	3	0	600, 700, 800
UBC 811	$(GA)_8C$	46.8	44.5	3	3	0	300, 500, 700
UBC 812	$(GA)_8A$	45.7	43.0	1	1	0	600
UBC 818	$(CAC\ ACA)_2CAC\ AG$	51.0	48.0	2	2	0	450, 800
UBC 830	$(TG)_8G$	52.0	50.0	3	3	0	400, 700, 850
UBC 834	$(AG)_8YT$	49.2	47.0	5	5	0	400, 450, 600, 700, 800
UBC 835	$(AG)_8C$	48.8	44.0	2	2	0	800, 900
UBC 836	$(AG)_8YA$	48.9	45.5	1	1	0	850
UBC 840	$(GA)_8YT$	47.4	42.0	4	4	0	500, 600, 1000, 1100
UBC 844	$(CT)_8RC$	48.6	44.5	3	3	0	300, 500, 600
UBC 850	$(GT)_8YC$	52.7	49.5	3	3	0	450, 600, 1000
UBC 857	$(AC)_8YG$	54.3	50.0	3	3	0	450, 700, 900
UBC 888	$BDBC(AC)_5A$	47.3	44.4	3	3	0	450, 500, 700
Total	**15**	**-**	**-**	**38**	**38**	**0**	**300-1100**

B = (C,G,T ie not A); D = (A, G, T ie not C); R = (A, G); Y = (C, T)
UBC series sequences of University of British Columbia, Canada

DISCUSSION

Bamboo is a critical natural resource which has not easily lent itself to modern methods of mass propagation and genetic improvement owing to its long vegetative phase and monocarpic flowering behaviour (Rao and Rao, 1988). Micropropagation was earlier thought to be difficult to achieve in material beyond the seedling stage (Aderkas and Bonga, 2000). It is well known that the plant growth regulators effective for one species may not be equally effective for other cultivar or species (Singh *et al*., 2004) for obtaining the same response. Therefore, standardizing a protocol for micropropagation is the best available method for cultivation and propagation of selected genotypes characterized by superior agronomic traits. No protocol for micropropagation from nodal explants has earlier been reported for this species. Our results show that many factors limit the final output of the protocol and includes the organ that serves as the tissue source, the physiological and ontogenic age of the organ, the season in which the explants are obtained, size of explants, the overall quality of the plant from which the explants are taken and stomatal structure and efficiency of their functioning at the plantlet and the hardening stage. Time of explant collection is known to influence *in vitro* propagation (Lin *et al*., 1991; Kumari and Ramanayake, 1996; Mehta *et al*., 2010). The best season for bud break was found to be spring and the least preferred season being winter. The number of shoots per explant varied between 15-25, low contamination rates and more healthy cultures resulted during spring thus showing better proliferation of *in vitro* cultures. The best time for acclimatizing micropropagated plants in field was found to be the rainy season when

adequate humidity prevails. A good number of bamboo species have been reported to regenerate *in vitro* through axillary branching such as *Dendrocalamus hamiltonii* (Godbole *et al*., 2002; Sood *et al*., 1994, 2004), *Dendrocalamus asper* (Arya *et al*., 2002), *Bambusa bambos* (Arya and Sharma, 1998), *Dendrocalamus giganteus* (Ramanayake and Yakandawala, 1997). Besides these, there is only a single report on micropropagation of *D. membranaceus* using seeds as explants (Yasodha *et al*., 1997). In general, rooting and transplantation of plantlets to field is the most important and difficult task in micropropagation (Murashige,1974) and specifically in woody species like bamboos. Earlier Dekkers and Rao, (1989) had obtained multiple shoots in *D. strictus* but with poor root induction. Similarly, multiple shoots were obtained by using seedling cultures of *D. strictus* by Kumar, (1994), which could not be continued for long due to lack of meristematic tissue at the base of the explants. Hence, establishment of an efficient micropropagation protocols could help the regeneration of plants in large numbers in a short time for afforestration programmes in the country.

Based on an extended literature review (Wright, 1974; Lugojan and Ciulca, 2011) and in several previous experiments, comparative analysis of physiological parameters such as RWC and electrolyte efflux, as a screening test of membrane tolerance to water stress was done to compare *in vitro* grown plantlets with the ones growing *ex vitro*. Plants grown *in vitro* have significantly less water stress than plants grown in green house, less physiological stress since an exogenous carbon source (sucrose) is provided thereby; reducing the need of photosynthesis. The aseptic environment *in vitro* also reduces the stress due to any pathogenic organism. All these factors contribute to low values of electrolyte leakage, high RWC in *in vitro* grown plants. Chlorophyll content is directly related to its gross primary productivity (GPP) (Gitelson *et al*., 2006). According to Chang *et al*. (1998) ultrasonics is preferred for extraction of chlorophyll as compared with traditional methods of grinding and centrifugation. Comparison of chlorophyll content using three different solvents viz Acetone, DMSO and DMF by ultrasonics depicted that acetone was a preferred choice for extraction of pigments in bamboos also. The observed difference in the chlorophyll content gave a valuable information regarding the adaptation of plants to *ex vitro* conditions of increased light and CO_2 levels etc (Emerson, 1929). One of the adaptation being the increased production of chlorophyll in their leaves. It is well observed that the primary productivity of the plants is directly related to chlorophyll content and their use as a forest species for wood and pulp. The physiological parameters including water status and electrolyte leakage will stabilize when the plantlets get fully acclimatized in the field and can be tested by noting their field performance again after 3-6 months.

During micropropagation of any plant species, the most crucial step is to retain the genetic integrity with respect to mother plant. In the present study, RAPD and ISSR markers were selected. The use of Inter-simple sequence repeats (ISSR) as markers is more recent than RAPD and involve the use of microsatellite sequences directly in polymerase chain reaction (PCR) for DNA amplification (Gupta *et al*., 1994). RAPD

markers have been used earlier in bamboo for confirming the fidelity (Agnihotri *et al*., 2009; Das and Pal, 2005). Similarly, Joshi and Dhawan (2007); Bhattacharya *et al*. (2010); Nayak *et al*. (2011); Negi and Saxena (2010) reported fidelity of micropropagated plants using ISSR markers. Although, all tissue cultured plants especially those arising from axillary bud proliferation, are expected to be genetically identical, the possibility of genetic variations emerging during the *in vitro* process cannot be ruled out (Negi and Saxena, 2010). Micro-mutations occurring in the somatic cells can also be the probable reason for variability in the clones. Biotechnological techniques like use of molecular markers (Gielis *et al*., 2002) are crucial for the future programmes on improvement of perennials like bamboos where any undesirable variant would last for several years. Our results confirmed tissue culture raised plantlets to be similar with the mother plant supporting the fact that axillary proliferation is the safest mode of multiplication of true to type plants.

Henceforth, the *in vitro* propagation method devised here using mature preformed tissue will be helpful in producing plants in large numbers all the year round and ensuring conservation of this important species. Finally, ensuring the genetic integrity of micropropagated plants will be useful for its commercialization for quality paper and pulp and for various academic needs.

CONCLUSION

The results revealed the significance of nodal explants in maintaining the prolonged *in vitro* cultures. The effectiveness of the micropropagation protocol was dependent upon the season, size of explants, plant growth regulators and on the successful acclimatization of the plantlets. Physiological studies conducted in the course of this study helped in understanding the transition of plantlets form the *in vitro* conditions to the soil. RAPD and ISSR analyses revealed that no additional variation originates during process of *in vitro* propagation and hence, qualifies the quality check for multiplication of true to type plants.

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