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Micropropagation of Dev-ringal [*Thamnocalamus spathiflorus* (Trin.) Munro] — a temperate bamboo, and comparison between in vitro propagated plants and seedlings

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Abstract

An efficient protocol for in vitro propagation of *Thamnocalamus spathiflorus* (Trin.) Munro, an evergreen temperate bamboo, through multiple shoot formation from zygotic embryos excised from germinating seeds, as well as from nodal explants taken from a 2-year-old plant has been developed. Multiple shoot formation was standardized in both the cases on Murashige and Skoog (MS) medium supplemented with 5.0 μM 6-benzylaminopurine (BAP) and 1.0 μM indole-3-butyric acid (IBA). Clumps of three to four microshoots when transferred to plant growth regulator-free medium following 14 days' initial culture on medium supplemented with 150.0 μM IBA, resulted in 100% rooting with five to 12 roots per rooted plant. Then, 18 months following the transfer of in vitro propagated plants into earthen pots containing soil and growth under outdoor conditions, gas and water vapour exchange rates were measured at different light levels (0, 100, 500, 1000, 1500, 2000 $\mu mol^{-2}\ s^{-1}$), and compared with seedlings of the same age. Light optima for photosynthesis were recorded $\sim 1000\ \mu mol\ m^{-2}\ s^{-1}$ in in vitro propagated plants as well as seedlings. Water use efficiency increased with increase in the light intensity up to 1000 $\mu mol\ m^{-2}\ s^{-1}$ and then decreased subsequently at higher light intensities. Stomatal conductance and transpiration also increased with the increase in light intensity up to 2000 $\mu mol^{-2}\ s^{-1}$. Intercellular CO_2 concentration (C_i) and the ratio of intercellular CO_2 concentration to ambient CO_2 (C_i/C_a) decreased with the increase in light intensity up to 500 $\mu mol^{-2}\ s^{-1}$ and then reached nearly constant levels at higher light intensities. The results show that in vitro propagated and hardened plants of *T. spathiflorus* are morphologically as well as functionally comparable to seed raised plants of the same age, within the limits of this study. © 2000 Elsevier Science Ireland Ltd. All rights reserved.

Keywords: Micropropagation; *Thamnocalamus spathiflorus*; Temperate bamboo; Gas and water vapour exchange; Water use efficiency

1. Introduction

Thamnocalamus spathiflorus (Trin.) Munro, an evergreen, temperate bamboo grows as a thick under canopy shrub in the Himalayan region extending from the river Sutlej eastward to Bhutan and probably in Assam at 800–3300 m altitude [1]. The culms are strong, 3.5–6.0 m in height and 1.5–3.5 cm in diameter, smooth glaucous green when young, yellow or reddish brown when mature, and the plants can survive winter temperatures as low as −13°C [2]. Split culms are locally used for making household and agricultural implements; its quality fibre has found use in the paper industry [3]. Being considered a hill-specific bamboo with substantial soil binding properties, it requires little post plantation care. In view of its importance, it is considered a valuable plant for afforestation and soil conservation programmes in the hills, particularly at higher altitudes. It has potential for use in slope stabilization, through mountain risk engineering techniques (unpublished results) and in social forestry programmes, and the plant is entwined with the culture of this region.

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The conventional propagation of bamboos is through seeds and by culms or rhizome/offset cuttings. The habit of unpredictable and gregarious flowering and seeding, followed by death, makes bamboo propagation uncertain through seeds which have short viability, and improvement by inter/intra specific hybridization very difficult [4]. The seedling populations are also highly heterogeneous. *T. spathiflorus* flowers only once in its lifetime after 16–17 years [5]. Also, the genetic constitution of seeds from open-pollinated wild plants is unreliable due to heterozygosity. For conventional vegetative propagation, rhizomes/offsets are not available in sufficient numbers required for large scale plantations; propagation through nodal segments has not been developed for this species. Tissue culture has tremendous potential in this context and could be useful in overcoming the above difficulties. So far micropropagation of this species has not been reported; however, considerable progress has been made in developing micropropagation protocols for several other bamboos. In vitro shoot multiplication of bamboos using various explants [6–13], somatic embryogenesis and plant regeneration from zygotic embryos of *Dendrocalamus strictus* [14] and *Otatea acuminata* subsp. *aztecorum* [15], and from floral explants of *Bambusa beecheyana* [16] have been reported.

One of the most important and critical steps in field transfer of micropropagated plants is their transition during hardening from in vitro to an ex vitro environment, and subsequent field performance. Poor survival of in vitro propagated plants under ex vitro conditions is mainly due to poor development of cuticular waxes, non-functional stomata, water loss due to excessive transpiration [17], poor root system and susceptibility to pathogens. In the course of hardening, the micropropagated plants gradually overcome these inadequacies and adapt to ex vitro conditions.

Plant survival, growth and productivity are reported to be intimately coupled with the aerial environment through processes such as energy exchange, loss of water vapour in transpiration and uptake of carbon dioxide in photosynthesis [18,19]. Water vapour exchange rate affects the energy budget and transpiration of leaf, and consequently the physiology of the whole plant [20–22]. Therefore, data on physiological parameters such as gas and water vapour exchange are likely to provide valuable information regarding the suitability of tissue culture raised plants for field plantations.

In the present study an attempt has been made, for the first time, to develop an efficient in vitro micropropagation method through multiple shoot formation (and subsequent rooting) from excised zygotic embryos as well as from nodal explants taken from a 2-year-old plant of *T. spathiflorus*. Subsequently, 18 months after the transfer to soil and hardening of in vitro propagated (IVP, tissue culture raised) plants of *T. spathiflorus* in the institute nursery, their performance was evaluated on the basis of selected morphological and physiological parameters, and compared with seedlings (SED) of the same age. The effects of different light intensities on photosynthesis and water vapour exchange characteristics of these plants have also been examined.

2. Materials and methods

2.1. Seed germination

Mature seeds of *T. spathiflorus* (Trin.) Munro (syn. *Arundinaria spathiflora* Trin.; common names: Dev-ringal, Ringal; local names: Deoningal, Tham, Parikh, Myoosay) were collected from Khaljhuni village (30°6′20″ N, 79°58′18″ E; 2300 m altitude) near Pindari glacier, Central Himalaya, India, during the 1st week of July 1996. Seeds were carefully dehusked and wetted with soft detergent solution (Extran 0.05%, v/v; Merck, India) for 10 min and then washed with tap water ($\times 5$). Thereafter the seeds were rinsed in distilled water and imbibed for 4 h. Finally, they were surface-disinfested with mercuric chloride (0.1%, w/v; 7 min), rinsed with sterilized distilled water ($\times 4$) and cultured in Petri dishes (17-mm depth, 100-mm diameter) containing 30 ml of plant growth regulator (PGR)-free Murashige and Skoog's (MS) [23] basal medium supplemented with sucrose (2%, w/v), myo-inositol (100 mg l^{-1}), thiamine-HCl (0.1 mg l^{-1}), nicotinic acid (0.5 mg l^{-1}), pyridoxine-HCl (0.5 mg l^{-1}) and glycine (2.0 mg l^{-1}). The pH was adjusted to 5.8 with 0.1 M KOH, and the medium was gelled with 0.2% phytagel (w/v, Sigma), and autoclaved (1.05 kg cm^{-2}; 20 min) before use. The Petri dishes containing ten seeds per dish were incubated at 25 ± 1°C in the dark.

2.2. Multiple shoot formation from excised embryos

After 8 days of incubation in the dark, Petri dishes containing germinated seeds were placed under cool white fluorescent light (16-h photoperiod, 40 μmol m^{-2} s^{-1}; Philips, India) for 4 days and the emerging shoots were allowed to grow to a height of 5–6 mm. At this stage the germinating embryos were carefully excised under aseptic conditions and further cultured in 250-ml conical flasks containing 60 ml MS medium supplemented with 2% sucrose with or without 6-benzylaminopurine (BAP; 0–20.0 μM) and indole-3-butyric acid (IBA; 0–2.5 μM) in various combinations. After 8 weeks of culture, the number of explants (excised embryos) responding to various treatments, rate of shoot multiplication, and the length of the tallest shoot, were recorded. At least 20 excised embryos were cultured per treatment for multiple shoot induction. Number of shoots per embryo and the average length of the tallest shoots, were recorded for statistical analysis. The least significant difference ($P = 0.05$) among means was calculated according to Snedecor and Cochran [24].

2.3. Bud sprouting from nodal segments and shoot multiplication

Single-node stem segments (1.5–2.5 mm in diameter, 20–25 mm long) taken from a single 2-year-old seed raised plant were used for this experiment. Nodal segments with healthy axillary buds, with sufficient internodal portion on either side, were cultured on 1/2 strength MS medium after removal of the leaf sheath and surface disinfestation as mentioned above. After 1 week, 'infection-free' explants were transferred to the multiplication medium (MS containing 5.0 μM BAP and 0.1 μM IBA) and further incubated for 12–15 days. The sprouted buds were then excised from the nodal explants and cultured on the same medium for multiplication.

2.4. Rooting

For induction of adventitious roots, clumps of three to four microshoots (3.5–4.0 cm in height) were separated from the mother clump and placed on 1/2 strength MS medium supplemented with 0–300 μM IBA. These shoot clumps were cultured for 14 days on medium supplemented with IBA before transfer to PGR-free 1/2 strength MS medium. After 8 weeks of culture on this medium, per cent rooting, number of roots per clump and the length of the longest root were recorded. Each treatment consisted of at least 20 shoot clumps. Data were subjected to statistical analysis.

2.5. Transfer of in vitro propagated plants to soil

Well-rooted plants were taken out from the culture flasks and washed thoroughly in running tap water to remove all traces of medium attached to the roots. These microplants were then transplanted in plastic pots (7 cm in diameter, 8 cm in height) containing a mixture of soil and sand (3:1, v/v; pH 6.5); a transparent plastic bag was placed on the top of each pot to maintain adequate moisture during hardening in a net-house (50% shade). Plants were watered at 15-day intervals with tap water. After a month the plastic covering was gradually removed and the plantlets were allowed to grow under natural conditions. The fully acclimatized plants were then transferred and maintained in earthen pots (20 cm in diameter; 18 cm in height) containing normal garden soil.

2.6. Gas and water vapour exchange studies

Gas and water vapour exchange studies (as well as other comparisons) of in vitro propagated plants and seedlings of same age were carried out on plants maintained for 18 months in the institute nursery at Kosi, Almora (1150 m altitude, 79°38′10″ E and 29°38′15″ N) under outdoor conditions. For such comparisons micropropagated plants obtained through the culture of excised zygotic embryos were used. In each category four plants were used for studies of carbon assimilation and water vapour exchange. Measurements were conducted on five upper most undamaged, fully expanded and healthy leaves with the help of a closed portable photosynthesis system (model LI-6400; LI-COR, Lincoln, USA). Following preliminary experiments on seedlings, 25°C was found optimum for growth of *T. spathiflorus*, and all subsequent gas and water vapour exchange measurements were carried out at 25 ± 0.5°C. To determine the effect of light on gas and water vapour exchange, leaves were exposed to different photosynthetic photon flux densities (PPFD), i.e. 100, 500, 1000, 1500 and 2000 μmol m^{-2} s^{-1} from an artificial light source (model 6400-02 light emitting

silicon diode; LI-COR), fixed on the top of leaf chamber. PPFD was recorded using a quantum sensor kept in the range of 660–675-nm short wave radiation, fixed at the leaf level. The rate of dark respiration was measured by maintaining the leaf in the cuvette at zero irradiance. The leaf chamber was covered with a black cloth throughout the course of respiration measurements to avoid any external radiation. Flow rate (500 mmol s^{-1}), CO_2 concentration inside the leaf chamber (350 ± 5 ppm), temperature (25 ± 0.5°C) and relative humidity (50 ± 5%) were maintained at a constant level throughout the experiment. Since the steady state of photosynthesis is reached within 30–45 min, the plants were kept for ~ 1 h under each set of light conditions before the observations were recorded. Water use efficiency (WUE) was determined by calculating the ratio of the rates of photosynthesis and that of transpiration.

2.7. Water status and specific leaf area

Fresh, turgid and dry weight of a total of 75 leaves (in triplicate of 25 leaves) for each plant and five plants for each set were measured. Samples were collected at 09:30 h and their fresh weight (Fw) was recorded immediately. Following 12-h soaking of leaves in deionized water (Milli-Q water) in the dark and after the removal of excess water with tissue paper, the turgid weight (Tw) of the leaves was measured. Dry weight (Dw) of leaves (80°C, 48 h) and the leaf area (leaf area meter; model LI-3000, LI-COR) were also recorded. The specific mass of the leaves was calculated by dividing the leaf dry weight by leaf area. Relative water content (RWC) of the leaves was calculated according to the equation: $\text{RWC}\ (\%) = (\text{Fw} - \text{Dw})/(\text{Tw} - \text{Dw}) \times 100$.

2.8. Leaf anatomy

Anatomical details and stomatal frequency were compared in well-established plants. Leaf segments of fully expanded fourth leaf from the top of each branch were taken for anatomical work. Hand-cut sections were serially dehydrated in different concentrations of ethanol and stained with Safranin (1%, w/v, Sigma) followed by Light Green (0.3%, w/v, Sigma) solution and mounted in DPX (Qualigens Fine Chemicals, India).

For the determination of stomatal frequency, impressions of both the adaxial and abaxial surfaces of the leaf were taken on 'White Glue' (Home Bond, Hardware Stores, St. Jacobes, Ont., Canada). Stomatal frequency was counted following random observations under a light microscope (Labophot-2, Nikon, Japan) and the final frequency was calculated for both sets of plants.

2.9. Chlorophyll estimation

Leaf tissue (200 mg) from the fourth leaf of a branch was homogenized in 80% aqueous acetone, kept for 10 min at room temperature (dark), and the cells were removed by centrifugation. The absorbance was measured at 663 and 645 nm using spectrophotometer (Uvikon 931, Kontron Instruments, Italy) and the chlorophyll a and b levels were calculated as described by Kirk [25].

3. Results

3.1. Seed germination

Within 12 days of inoculation 50% of seeds germinated on PGR-free MS medium; gradually a single (rarely two or three) unbranched shoot (without any root) emerged from each seed (Fig. 1A). Incorporation of BAP (0–2.5 μM), 2,4-D (0–1.0 μM) and GA_3 (0–1.0 μM) in the medium increased the germination rate but quite often resulted in callus formation from the embryonic axis.

Fig. 1. In vitro multiplication of *T. spathiflorus* (Trin.) Munro using zygotic embryos as initial explants. (A) A germinating seed on MS medium (12 days after inoculation; bar, 1 mm). (B) Proliferation of shoots from excised and germinated embryo after 3 weeks of culture. (C) Radial emergence of shoots without callusing at the base. (D) Profuse multiple shoot formation from a single excised embryo after 8 weeks of culture. (E) A well-rooted plantlet (cultured on PGR free 1/2 MS medium for 8 weeks following an initial exposure for 14 days on medium containing 150 μM IBA; bar, 1 cm). (F) Acclimatized plants 2 months after transfer to pots.

Fig. 2. In vitro multiplication of *T. spathiflorus* (Trin.) Munro using nodal segments taken from a 2-year-old plant as explants. (A) Sprouted buds from nodal explants taken from a 2-year-old plant. (B) Shoot multiplication using sprouted buds removed from the nodal explants. (C) Well-rooted plantlets prior to the transfer to soil. (D) 18-month-old seedlings (SED) and in vitro propagated plants (IVP). (E, F) Transverse section of a leaf of seedling (E; bar, 18 μM) and in vitro propagated plant (F; bar, 15 μM).

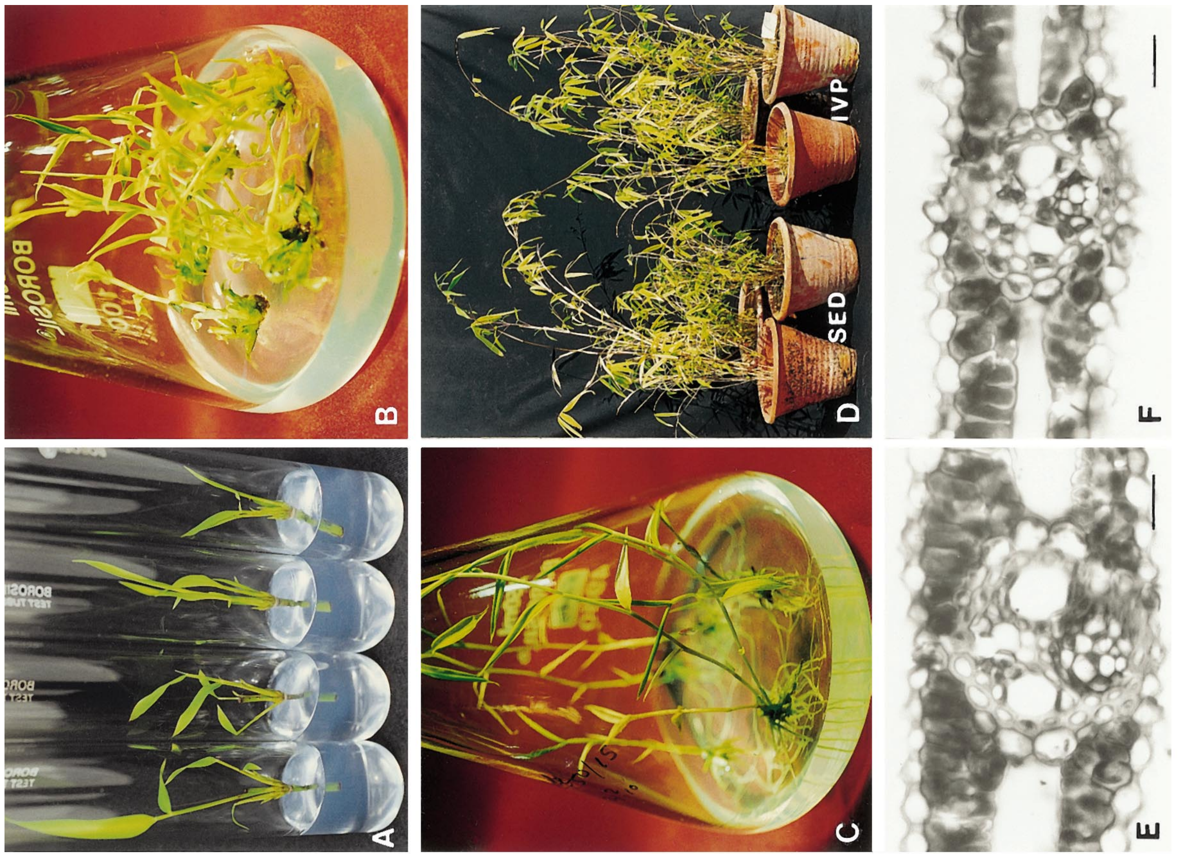

Fig. 2

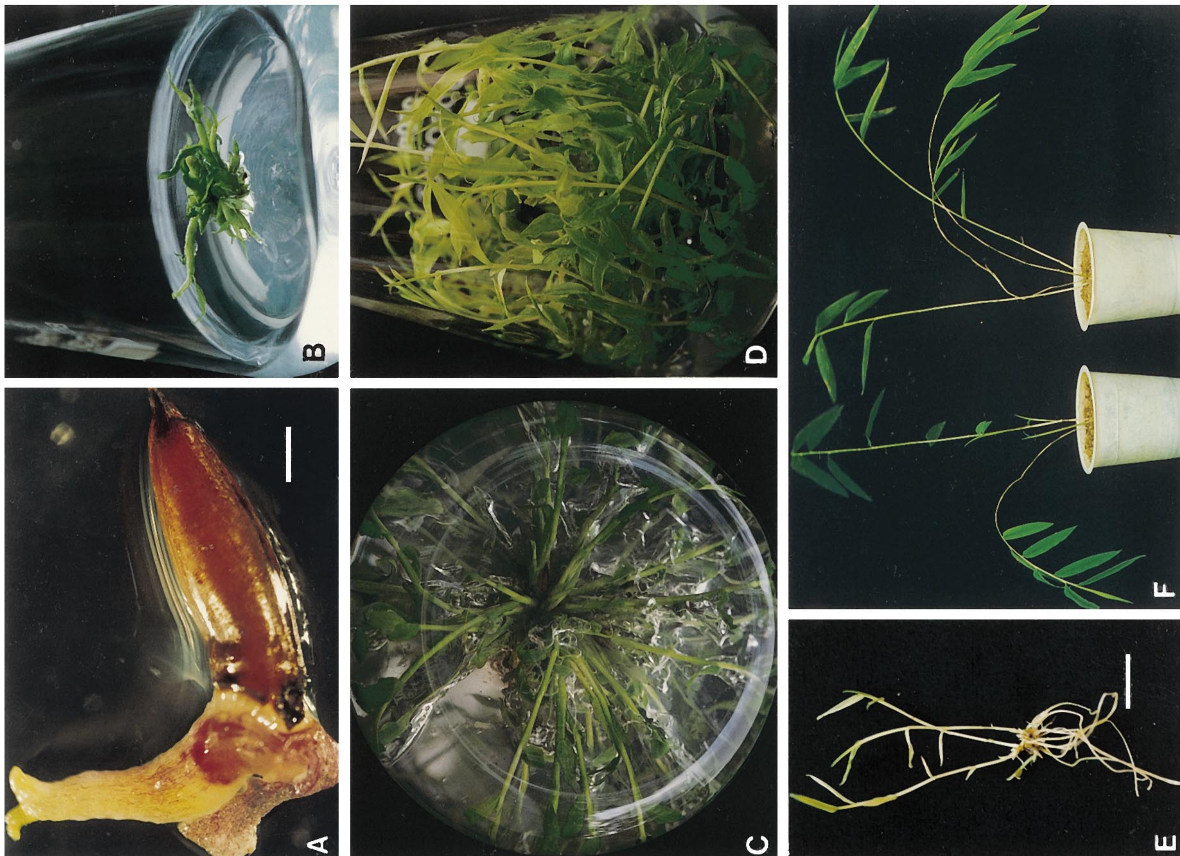

Fig. 1

3.2. Multiple shoot formation from excised zygotic embryos

The excised germinating embryos did not survive for more than 3–4 weeks on PGR-free medium. During the initial 10–20 days, the seedlings remained green with one or two shoots but subsequently died without showing any visible signs of growth. The presence of PGRs (BAP and IBA) in the medium improved the survival as well as shoot multiplication. On addition of BAP (2.0–20.0 μM) and IBA (1.0–2.5 μM) to the medium, multiple shoot formation was induced from excised embryos (Fig. 1B); no callusing was observed. Shoots were found to develop radially from a single point (Fig. 1C) from excised embryos cultured on the multiplication medium. Among various PGR combinations tested, best results were recorded on medium containing 5.0 μM BAP and 1.0 μM IBA. On this medium ~90% of explants proliferated with 16–38 (average 28.6) shoots per explant (Table 1) in 8 weeks. In an exceptional case, a maximum of 117 shoots were recorded from one clump (Fig. 1D). Apart from this, medium supplemented with 10.0 μM BAP and 1.0 μM IBA gave the second best response exhibiting 70% explants proliferation with an average of 23.3 shoots per clump. It should be mentioned that higher concentrations of BAP (15.0–20.0 μM) and IBA (2.5 μM) adversely affected the shoot multiplication rate.

3.3. Bud sprouting from nodal segment and shoot multiplication

Nodal segments without a leaf sheath cover sprouted within 4–5 days of culture on 1/2 strength MS medium. Generally four to 16 buds were present on a nodal explant and almost all the buds sprouted (Fig. 2A) but occasionally one or two remained suppressed. After 2–3 weeks, sprouted buds of 30–35-mm length were excised and cultured on medium; best shoot multiplication was obtained on MS medium supplemented with 5.0 μM BAP and 1.0 μM IBA. Little response and practically no multiplication was observed during the first 25–30 days incubation of these excised buds on the multiplication medium. However, following the first subculture, shoot multiplication started and the frequency gradually increased with time. Maximum multiplication rate was found to be six to 11 shoots per explant (Fig. 2B) in 8 weeks. The degree of proliferation also depends on the inoculum size. Propagules containing a minimum of three to four shoots proliferated at a maximum rate where as single shoots proliferated at a much slower rate (one to three shoots per explant). Microshoots (clump of three to four, 3.5–4.0 cm in height) were separated and subjected to rooting or cultured for further shoot multiplication.

Table 1
Effect of BAP and IBA on multiple shoot formation in excised embryos of *T. spathiflorus* following culture on MS medium for 8 weeks

Treatment		% Response of germinating embryos[a]	No. of shoots per excised embryo[b] ± S.E.	Average length of tallest shoot[b] (mm) ± S.E.
BAP (μM)	IBA (μM)			
0.0	0.0	0.0	0.0 ± 0.0	0.0 ± 0.00
2.0	1.0	30.0	4.8 ± 0.44	25.8 ± 2.22
5.0	1.0	90.0	28.6 ± 1.41	33.7 ± 1.06
10.0	1.0	70.0	23.3 ± 0.97	28.4 ± 1.24
15.0	1.0	50.0	9.6 ± 0.97	26.2 ± 1.50
20.0	1.0	50.0	11.5 ± 0.91	24.6 ± 1.76
2.0	2.5	30.0	3.2 ± 0.44	9.7 ± 0.96
5.0	2.5	35.0	8.0 ± 0.76	20.5 ± 1.26
10.0	2.5	40.0	6.5 ± 0.68	21.5 ± 1.38
15.0	2.5	30.0	8.6 ± 1.12	22.3 ± 2.13
20.0	2.5	55.0	10.2 ± 0.58	23.6 ± 1.20
LSD value ($P = 0.05$)			3.8	4.7

[a] Response in respect of multiple shoot formation was recorded from 20 germinating embryos per treatment.

[b] Data on the number of shoots and the average length of tallest shoot were recorded per excised embryo, 8 weeks after transfer to the medium; initially the embryos were carefully dissected from germinating seeds, 12 days after incubation of imbibed seeds on PGR-free medium.

Table 2
Effect of IBA on rooting of in vitro raised shoots of *T. spathiflorus*, 8 weeks after transfer to PGR-free medium

Initial conc. of IBA (μM) (2 weeks)	% Survival[a]	% Rooting[a]	Average no. of roots/rooted plant[b] ± S.E.	Average length of longest root[b] (mm) ± S.E.
0.0	100	0.0	0.0 ± 0.00	0.0 ± 0.00
25.0	100	0.0	0.0 ± 0.00	0.0 ± 0.00
50.0	100	30.0	3.0 ± 0.33	10.7 ± 1.58
75.0	100	55.0	3.2 ± 0.35	10.5 ± 1.41
100.0	100	85.0	5.7 ± 0.49	14.4 ± 1.22
125.0	100	85.0	8.1 ± 0.77	19.8 ± 1.68
150.0	100	100.0	10.6 ± 0.76	31.4 ± 1.92
175.0	90	100.0	12.0 ± 0.62	21.4 ± 1.77
200.0	90	100.0	11.1 ± 0.92	18.4 ± 1.20
300.0	60	100.0	11.6 ± 0.90	18.4 ± 3.03
LSD value ($P = 0.05$)			2.12	5.2

[a] Response was recorded on the basis of 20 explants per treatment.
[b] Data on the average number of roots per rooted plant and the average length of tallest root were recorded after a total of 10 (2+8) weeks.

3.4. Rooting

No root formation occurred on the shoot multiplication or PGR-free medium. Microshoots were rooted on a PGR-free 1/2 strength MS medium following initial incubation for 14 days on medium containing IBA. Initial exposure to IBA and subsequent withdrawal of auxin from the medium was found very effective for overall root development. This two-step rooting procedure (first the root induction on IBA containing medium followed by root elongation on PGR-free medium) resulted in good root growth within 7–10 days after transfer to auxin-free medium.

Generally five to 12 roots emerged from the base of the shoot clump (Fig. 1E and Fig. 2C). It is important to note that no callusing was observed during this process. Among the various concentrations of IBA tested (Table 2), rooting (30–100%) was observed at concentrations ranging from 50 to 300 μM IBA; maximum rooting (100%) was obtained on 150–300 μM IBA.

3.5. Transfer to soil

In vitro rooted plants were carefully hardened under normal climatic conditions in a net-house (50% shade) in the institute nursery. The plants generally took 8–10 weeks to establish; this could be seen with the emergence of new sprouts (Fig. 1F) and the rhizome formation. The micropropagated plants appeared morphologically uniform with normal leaf shape and growth pattern compared to seedlings (Fig. 2D).

3.6. Gas and water vapour exchange

The effect of different light intensities on photosynthesis (P_n), dark respiration and transpiration (E) of the two types of plants is shown in Fig. 3A. P_n increased with increasing light intensity up to 1000 μmol m^{-2} s^{-1} in both cases. Maximum rate of photosynthesis (A_{max}) was recorded to be 9.09 ± 0.76 μmol m^{-2} s^{-1} for seedlings, whereas it was found to be slightly lower (8.29 ± 0.18 μmol m^{-2} s^{-1}) for in vitro propagated plants at 1000 μmol m^{-2} s^{-1}. Light saturation of photosynthesis was recorded in the range of 1000–1500 μmol m^{-2} s^{-1} PPFD. CO_2 exchange was found to be adversely affected by light intensities beyond 1500 μmol m^{-2} s^{-1} PPFD. Dark respiration (at 0 μmol m^{-2} s^{-1} PPFD) was ~15% higher in seedlings as compared to in vitro propagated plants. The rate of transpiration (Fig. 3A) increased considerably with an increase in light intensity (up to 2000 μmol m^{-2} s^{-1}) in both sets of plants.

Similar to transpiration, stomatal conductance (g_s) also increased with light intensity in all cases up to 2000 μmol m^{-2} s^{-1} in both sets of plants (Fig. 3B). However, increment of stomatal conductance (g_s) was pronounced at lower light intensities below 1000 μmol m^{-2} s^{-1}. Contrary to stomatal conductance (g_s), intercellular CO_2 concentration (C_i) was maximum, i.e. 361 ± 9.39 and 392 ± 9.87 μmol mol^{-1} at zero light for in vitro propagated plants and seedlings, respectively (Fig. 3B). The values gradually decreased up to 500 μmol m^{-2} s^{-1} and subsequently remained constant at higher light intensities. It was interesting to note that C_i was

similar in both sets of plants at light intensities higher than 500 μmol m^{-2} s^{-1}.

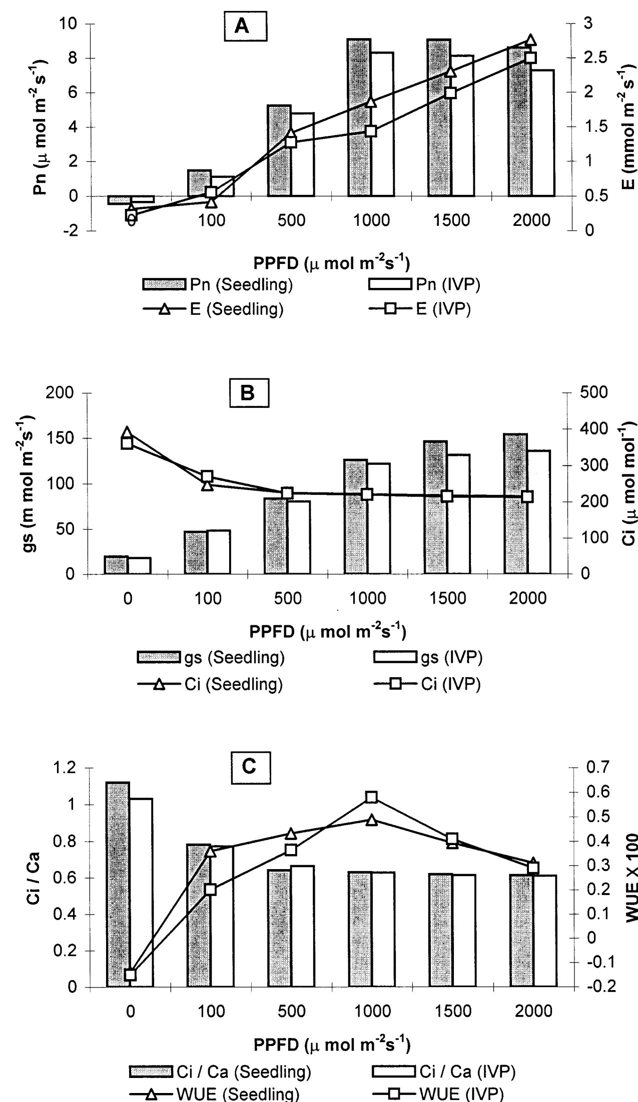

Fig. 3. Gas and water vapour exchange studies in 18-month-old seedlings and in vitro propagated plants of *T. spathiflorus* (Trin.) Munro. (A) Light dependent photosynthesis (P_n) and transpiration (E) in seedlings and in vitro propagated plants of *T. spathiflorus* at 25°C and 50% RH. IVP, in vitro propagated plant; standard deviation (S.D.) bars not shown for clarity; pooled S.D. values are given below: S.D. (P_n seedling) = 0.33, S.D. (P_n IVP) = 0.19; S.D. (E seedling) = 0.14, S.D. (E IVP) = 0.10. (B) Effect of different light intensities on stomatal conductance (g_s) and on intercellular CO_2 concentration in conventionally propagated and in vitro propagated plants of *T. spathiflorus* at 25°C and 50% RH. Average S.D. values are given below: S.D. (g_s seedling) = 3.11, S.D. (g_s IVP) = 6.43; S.D. (C_i seedling) = 12.02, S.D. (C_i IVP) = 8.12. (C) Variation in intercellular CO_2 concentration to ambient CO_2 (C_i/C_a) and water use efficiency (WUE) in conventionally propagated and in vitro propagated plants of *T. spathiflorus* at 25°C and 50% RH. Average S.D. values are given below: S.D. (C_i/C_a seedling) = 0.23, S.D. (C_i/C_a IVP) = 0.20; S.D. (WUE seedling) = 0.18, S.D. (WUE IVP) = 0.12.

Table 3
Comparison of some morphological features, chlorophyll content, RWC and specific leaf mass of in vitro propagated plants and seedlings of *T. spathiflorus*[a]

Parameters	In vitro propagated plants[b]	Seedlings[c]
Leaf thickness (μm)	57.20 ± 1.84	82.4 ± 1.19
Stomatal frequency (stomata/mm²)	431.00 ± 4.83	496.40 ± 18.23
Chlorophyll a (mg/g fresh weight)	1.71 ± 0.1	1.51 ± 0.01
Chlorophyll b (mg/g fresh weight)	0.61 ± 0.01	0.55 ± 0.01
RWC (%)	87.77 ± 0.52	93.55 ± 0.12
Specific leaf mass (mg/cm²)	2.23 ± 0.02	2.16 ± 0.07

[a] Data were recorded in triplicate for each plant and five plants were used for both the cases.
[b] Measurements were carried out on 18-month-old plants after successful hardening of in vitro propagated plants.
[c] Measurements were carried out on 18-month-old plants after hardening of seedlings.

Variations in the ratio of intercellular CO_2 concentration to ambient CO_2 concentration (C_i/C_a) and water use efficiency (WUE) of both plants are shown in Fig. 3C. Similar to C_i, C_i/C_a ratio at 0 μmol m^{-2} s^{-1} light intensity was maximum (1.12 ± 0.34) for seedlings, where as it was 1.03 ± 0.67 for in vitro propagated plants. The C_i/C_a ratio gradually decreased with light intensity up to 500 μmol m^{-2} s^{-1} and then became nearly constant (~ 0.61) for both sets of plants at higher light intensities (2000 μmol m^{-2} s^{-1}).

In the two sets of plants, a gradual increase in WUE was recorded with increase in light intensity up to 1000 μmol m^{-2} s^{-1} and maximum values were recorded at this light level. A sharp decline in WUE was observed at light intensities higher than 1000 μmol m^{-2} s^{-1} in both sets of plants.

3.7. Leaf anatomy, chlorophyll content, RWC and specific mass

Comparison of some morphological features, chlorophyll content, RWC and specific mass of in vitro propagated plants and seedlings is summarised in Table 3. Cross-section of leaves showed similar tissue arrangements but the stomatal frequency was higher in seedlings, and in the case of in vitro propagated plants leaf lamina was thinner (57 μm, Fig. 2F) than that of seedlings (82 μm,

Fig. 2E) even after a considerable period of transfer to the field (18 months). In both sets of plants epidermal cells were found to be elliptical/oval in shape with well-developed cuticle; major qualitative differences were not observed in respect of spongy and palisade mesophyll cells.

4. Discussion

Traditionally bamboos are propagated through seeds, offsets and culm cuttings. Propagation by seeds is unreliable due to the long and unpredictable flowering habit, and also undesirable on account of a large variation found in the seedling population [9]. While an improved method of vegetative propagation is available for some species [26], only a limited number of plants can be raised using this method, inadequate to meet the growing demand due to short supply of propagules. Therefore, in order to supplement the conventional methods, an efficient in vitro propagation method using explants taken from established and selected mature plants would offer a desirable alternative for large-scale multiplication of 'plus' or 'elite' bamboo. Being a clonal method, it highly reduces or eliminates the variation inherent in seed-raised population.

Another reported approach for in vitro propagation of bamboos is through the formation of multiple shoots using excised embryos from germinating seeds as explants. The multiple shoots can be subcultured and further multiplied. This method is quite common and also popular. It has been observed that multiple shoot induction from zygotic embryos is easy and the potentiality of multiplication rate is also higher than reported for other methods. Thus the population obtained is clonal as long as the plants derived from a single embryo are kept separately. This is important because the genetic constitution of bamboo seeds from open pollinated plants in the wild is unpredictable, and consequently the seedling populations are highly heterozygous and unsuitable for large scale afforestation programmes [9,26].

Since bamboo plants are required in large numbers for mass scale plantation, an efficient micropropagation protocol would be useful to generate large number of plants within a short time. A schematic representation of various steps taken up for in vitro propagation of *T. spathiflorus* is depicted in Fig. 4. Most reported studies of bamboo micropropagation have used embryonal tissues [6,7,14,15]. The procedure reported here is based on direct shoot multiplication from excised zygotic embryos as well as from nodal explants taken from a 2-year-old plant (physiologically young and field tested). The shoot multiplication was optimized on MS medium; using the two approaches ∼ 28 shoots (Table 1) per embryo and four to 16 shoots per nodal explant were obtained after 2–3 weeks of culture. The observed direct shoot multiplication through axillary branching, without the callus for-

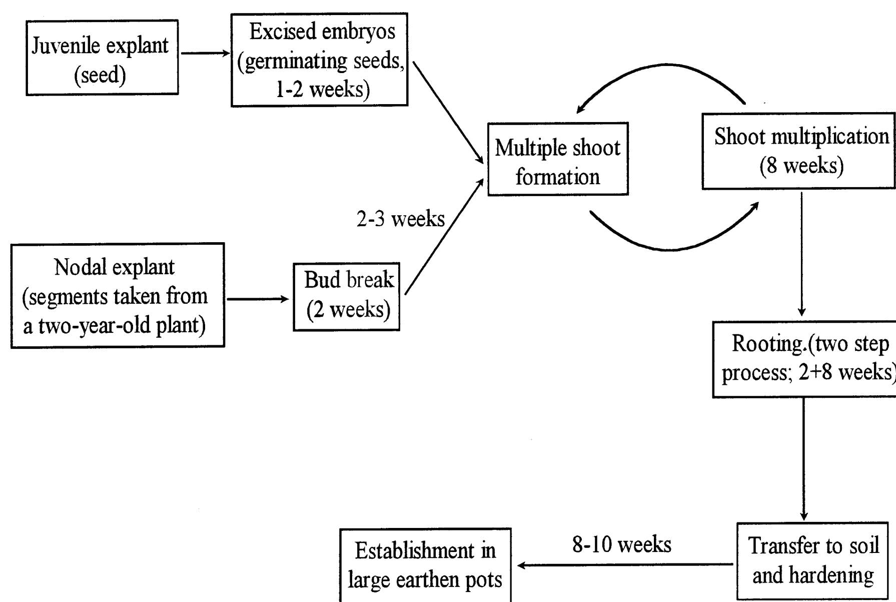

Fig. 4. Schematic representation of in vitro propagation of *T. spathiflorus*.

mation, would appear to be an interesting feature of this study as also reported in *Bambusa tulda* [7], *Dendrocalamus hamiltonii* [9], and *Dendrocalamus giganteus* [11]. The results of this investigation suggest that nutrients in the culture medium, particularly the growth regulators are important for survival and multiplication of *T. spathiflorus* embryos under in vitro conditions. Several workers have reported [4,7,9] higher rates of shoot multiplication and improved growth in liquid medium in comparison to solid or semi-solid medium. The slower growth or poor shoot multiplication on solid or semi-solid medium vis-a-vis liquid medium may be because solubilized agar binds water, absorbs nutrients and PGRs resulting in reduced uptake of nutrients, PGRs and other essential constituents [27]. However, a continuous culture in liquid medium may cause vitrification associated with reduced rate of multiplication [28]. It is known that high relative humidity promotes vitrification even when the shoots are not submerged [29]. It has been observed that culture in agar gelled medium resulted in the secretion of phenolics by the explants and was associated with the blackening of medium and reduced multiplication. This could be overcome by culture on medium gelled with phytagel which resulted in comparable multiplication efficiency. Such plants showed normal growth and were free from vitrification.

For the development of any successful in vitro micropropagation protocol, root induction of excised shoots and subsequent survival of plantlets in the soil are crucial. A two-step rooting procedure followed in this study resulted in excellent rooting. It was observed that continuous culture on medium with higher doses of IBA for relatively longer periods adversely affected growth and subsequent survival of shoots (Table 2). Although the rooting efficiency has been reported to greatly improve (up to 92%) with a combination of auxins and phenols [7,9], in the present study, 100% rooting efficiency was achieved following 150.0 μM IBA treatment and subsequent culture of shoots on auxin-free medium.

Generally a variation in the photosynthetic rate reflects the adjustment of plants to the environment. The data on gas and water vapour exchange indicate that *T. spathiflorus* can survive light intensities of $\sim 1000-1500$ μmol m^{-2} s^{-1} PPFD, commonly encountered under outdoor conditions. Although seedlings exhibited slightly higher rates of transpiration (E), net photosynthesis (P_n), stomatal conductance (g_s), intercellular CO_2 concentration, C_i/C_a ratio and water use efficiency were comparable to those of in vitro propagated plants. Thus in vitro propagated plants would appear to be 'normal' in respect of the functional characteristics examined.

It has been reported that the leaves of in vitro propagated plants are smaller, thinner, and often translucent with poorly developed epidermal and mesophyll tissue and reduced cuticle [29–31]. The stomatal frequency and stomatal apparatus of epidermal tissues of micropropagated plants were reported to differ markedly from nursery and field grown plants [32]. In in vitro propagated plants the mesophyll cells had poorly developed chloroplasts, with low chlorophyll, proteins and disorganized grana [30,33]. In spite of some structural differences which may be present initially, during the course of hardening in vitro propagated plants of *T. spathiflorus* would seem to have adapted well in respect of physiological, anatomical and functional features which were comparable to seedlings of same age (Table 3). In this investigation it was observed that slightly lower leaf thickness and stomatal frequency were found in in vitro propagated plants in comparison to seedlings; however, higher chlorophyll level was recorded in tissue culture raised plants. Although the RWC of seedlings was 6.6% more than that of in vitro propagated plants, there was no significant difference in the specific mass of leaves.

In conclusion, the present study describes, for the first time, an effective regeneration and multiplication protocol for in vitro propagation of *T. spathiflorus*, a temperate bamboo. The comparison of several physiological and morphological parameters in micropropagated plants and seedlings showed considerable similarities. High multiplication efficiency, good rooting, easy establishment in the soil and normal growth performance of micropropagated plants, as reported in this study, are features necessary for the adoption of in vitro propagation technology for large scale multiplication of a species.

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