Somatic embryogenesis in 'maggar' bamboo (*Dendrocalamus hamiltonii*) and field performance of regenerated plants

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***In vitro* regeneration through somatic embryogenesis using explants taken from field-grown 10- and 45-year-old elite maggar bamboo (*Dendrocalamus hamiltonii*) plants has been demonstrated. The concentration of plant growth substances used in the culture medium, and age of the mother plant (MP) were found to significantly influence callus formation, and subsequent embryogenesis. Eighteen months after transfer to soil, *in vitro* propagated plants of the two age groups, and the corresponding MPs were compared in respect of gas and water vapour exchange rates, related parameters, morphological features and leaf anatomy. The rate of photosynthesis was significantly influenced by the age of the MPs and was found to be higher in the tissue culture (TC)-raised plants; plants (both TC-raised and MPs) of the younger age group performed better than the corresponding plants of the older age group. The same trend was found when the water-use efficiency was taken into consideration. Thus, based on various parameters studied in the present investigation, *in vitro* propagated and hardened plants were found to be generally comparable with the corresponding mother plants. The *in vitro* propagation methodology reported here has already been used for mass-scale production of maggar bamboo.**

Keywords: Bamboo, *in vitro* regeneration, mother plant, somatic embryogenesis, tissue culture.

DENDROCALAMUS HAMILTONII Nees et Arn. Ex Munro, family Poaceae, commonly called 'maggar', is a multipurpose bamboo with many well-known uses, and a source of nutritive green fodder for the cattle, especially during winter – a lean period for the greens in the hills. Like other bamboos, it is also propagated through seed, stem or rhizome cuttings. A method of mass propagation has also been developed through the use of single node culm cuttings in maggar bamboo[1,2]. However, the traditional methods of propagation limit the number of propagules that can be produced, and is both labour-intensive and time-consuming[3]. Further, there is also risk involved in bamboo propagation through vegetative means using material from plants of unknown age because of the peculiar behaviour of mass flowering and death of the flowered clumps. Thus the use of tissue culture (TC) techniques for rapid multiplication of bamboos has been recommended; it offers many advantages, particularly when the explants are taken from physiologically young and field-tested elite clones[4]. *In vitro* shoot multiplication of bamboos has been carried out using varied explants like zygotic embryo, seed, seedling explants[5–7], as well as from nodal segments taken from mature culms[4,8,9]. Plant regeneration through callus cultures obtained from nodal segments[10,11] and young leaves[12] has also been demonstrated. There are also reports of somatic embryogenesis and plant regeneration using inflorescence segments[13], anthers[14] and zygotic embryos[15,16]. For *in vitro* studies of maggar bamboo, earlier workers have reported multiple shoot formation and flowering using single-node cuttings taken from 2-year-old seedlings[6] and shoot-bud regeneration through callus derived from nodal segments of a 7-year-old plant[17]. Somatic embryogenesis using explants taken from field-tested seedlings has also been reported[10,18]. On transfer of TC-raised plants from laboratory to field conditions, even after undergoing a period of hardening, such plants take time to adapt to the changed environmental conditions; sometimes failure to acclimatize resulting in heavy mortality of TC-raised plants has also been reported[19]. It is, therefore, essential that TC-raised plants are gradually hardened and properly acclimatized prior to field transfer[5,20].

The present article reports regeneration of maggar bamboo through somatic embryogenesis using explants taken from both physiologically young (10 year old), as well as mature, elite (45 year old) plants. The influence of plant growth substances (PGSs) and plant age on micropropagation of maggar bamboo has been studied. Eighteen-month-old TC-raised plants growing under *ex vitro* environment were also compared with the corresponding mother plants (MPs), of both age groups, on the basis of some physiological and anatomical characters.

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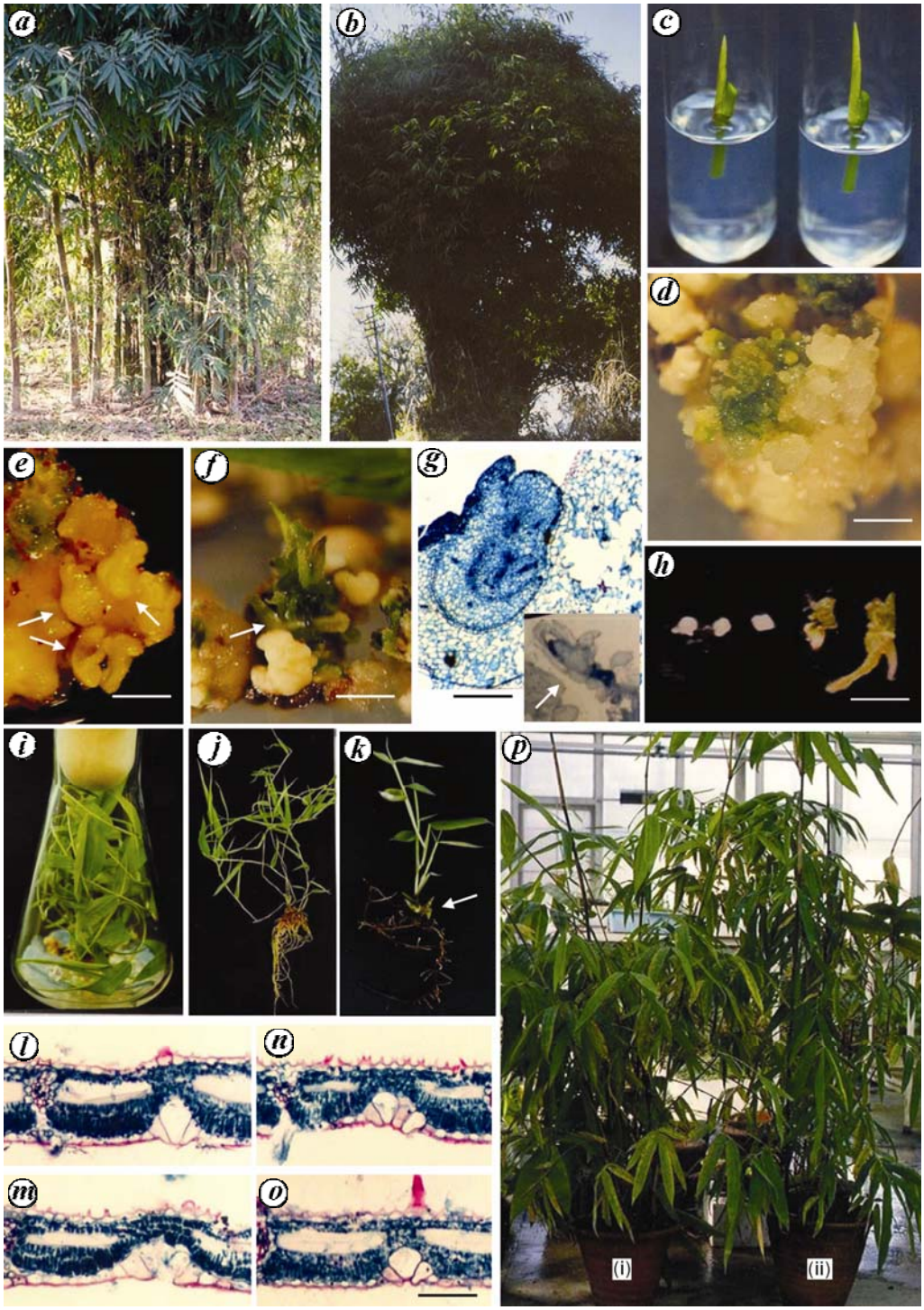

Figure 1. Plant regeneration through somatic embryogenesis of field-grown maggar bamboo (*Dendrocalamus hamiltonii*). ***a***, A field-grown, 10-year-old mother plant (MP) growing in the G.B. Pant Institute nursery at Kosi, Almora. ***b***, A field-grown, 45-year-old MP growing in the District Court premises in Almora. ***c***, Sprouted buds from nodal explants cultured on half-strength MS medium without plant growth substances. ***d***, Embryogenic callus derived from the culture of basal segments of a sprouted bud on MS medium supplemented with 6-benzylaminopurine (BAP) and 2,4-dichlorophenoxyacetic acid (2,4-D; 5.0 μM each, bar = 4 mm). ***e***, Somatic embryogenesis through callus phase on MS medium supplemented with 5.0 μM BAP and 7.5 μM 2,4-D (bar = 4 mm). ***f***, Somatic embryos showing germination under *in vitro* conditions on the same medium as in ***e*** (bar = 4 mm). (***g*** and inset), Longitudinal section of a somatic embryo showing bipolar organization and vasculature (bar = 400 μm). ***h***, Individual somatic embryos showing sequential stages of germination (bar = 4 mm). ***i***, Plantlets derived from somatic embryos. ***j***, *In vitro* propagated bamboo showing profuse roots after culture on half strength MS medium containing 5 μM indole-3-butyric acid (IBA). ***k***, Rhizome formation in a somatic embryo-derived plant after culture in half strength MS liquid medium supplemented with 20.0 μM IBA. ***l*** and ***m***, Transverse section of leaves of 10-year-old MP and corresponding tissue culture (TC)-raised plant respectively (bar = 80 μm). ***n***, ***o***, Transverse section of leaves of 45-year-old MP and corresponding TC-raised plant, respectively (bar = 80 μm). ***p***, Hardened TC-raised plants, 18 months after transfer to pots and propagated using explants from (i) a 10-year-old and (ii) a 45-year-old MP.

Materials and methods

A vegetatively propagated plant from a 7-year-old elite bush of maggar bamboo (Figure 1 *a*) was procured from Himachal Pradesh Agricultural University, Palampur[17] (32°6′9″N/76°32′49″E, 1309 m amsl) and planted in the G.B. Pant Institute nursery at Kosi, Almora (29°37′30″N/79°37′15″E, 1130 m amsl). The nodal cuttings from a 42-year-old 'mature', elite plant (Figure 1 *b*; approximate age found from several elderly local residents), growing in the District Court premises in Almora (29°35′24″N/79°40′24″E, 1760 m amsl), were collected and planted in the Institute nursery. After 3 years of plant establishment, explants were taken from a single and identified plant of each age group; now 10- and 45-year-old. Single-node stem segments (approx. 25–30 mm long) with healthy axillary buds, well before sprouting in April, having internodal portions on either side were excised from minor branches and used for culture establishment.

Initiation of culture and culture conditions were as described previously[7]. After 3 weeks of incubation, sprouted buds (about 20–25 mm long) were excised from the nodal explants and segmented transversely, using a sharp blade, into small pieces (about 5 mm) under aseptic conditions. These were then placed in petri dishes (100 mm diameter, 7 mm height; 10 pieces per petri dish) on MS medium containing various concentrations of 6-benzylaminopurine (BAP; 2.5–5.0 μM) and 2,4-dichlorophenoxyacetic acid (2,4-D; 2.5–10.0 μM; both from Sigma Chemical Co, USA). The petri dishes were sealed with parafilm and the number of explants forming callus was recorded after 8 weeks. The culture conditions in the incubation room were as described earlier[7]. After 5–6 subcultures, creamish-white callus was obtained; unless otherwise mentioned, subculturing was routinely performed at 4-weeks interval. Data on the formation of somatic embryos (SEs), number of embryos formed per callus lump (approximately 12–15 mm across and 120 mg in weight), number of shoots per callus lump and shoot length, etc. were recorded after 12 weeks of subculture.

Individual germinating SEs (approx. 3 cm shoot length) were carefully removed from the callus mass and cultured in 250 ml conical flasks (10 SEs per flask), containing half strength MS medium supplemented with various concentrations of indole-3-butyric acid (IBA; 0–100 μM; Sigma, USA). After 8 weeks of culture, percentage of root development, number of roots formed per shoot derived from individual SEs and root length were recorded. For rhizome formation, these were placed in 250 ml conical flasks closed with cotton plugs, containing half strength MS liquid medium supplemented with IBA (0–100 μM), and kept over a gyratory shaker (100 rpm) placed inside the incubation room. Transfer of *in vitro*-raised plantlets to soil was carried out as previously described[7].

Evaluation of MP and TC plants

Four TC-raised and four plants obtained through vegetative propagation of the original single MPs of each category (10- and 45-year-old; total $4 \times 4 = 16$ plants) were used for studies of carbon assimilation and water-vapour exchange. Measurements were conducted on five uppermost undamaged, fully expanded and healthy leaves with the help of a closed portable photosynthetic system (model LI-6400; LI-COR, Lincoln, Nebraska, USA). In preliminary experiments, 25°C was found to be the optimum temperature for photosynthetic measurements. All subsequent gas and water vapour exchange measurements were, therefore, carried out at 25°C. To determine the effect of light on gas and water vapour exchange, leaves were exposed to different photosynthetic photon flux densities (PPFDs), viz. 100, 500, 1000, 1500 and 2000 μmol m^{-2} s^{-1} from an artificial light source (model LI-6400-02 light emitting silicon diode; LI-COR), fixed on top of the leaf chamber (3 cm × 2 cm). Photosynthetically active radiation (PAR) was recorded using a quantum sensor kept in the range 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 experiments. Water-use efficiency (WUE) was determined by calculating the ratio of the rates of photosynthesis (*Pn*) and of transpiration (*E*).

Anatomical details and stomatal density were compared in well-established, TC-raised (18-month-old) plants and the respective MPs. Leaf segments of fully expanded fourth leaf from the top of each branch, were taken for this study. Hand-cut sections were serially dehydrated in different concentrations of ethanol and stained with Safranin (1%, w/v; S.D. Fine-Chem Ltd, Mumbai, India) followed by light green (0.5%, w/v, Sigma) solutions as described earlier[21] and mounted in DPX.

For the determination of stomatal density (SD), the method described earlier[7] was used, and for all sets of plants SD was calculated using the formula:

$$\mathrm{SD} = \frac{X}{\pi Y^2} \times 10^6 \text{ per mm}^2,$$

where X is the number of stomata per microscopic field of view and Y the radius of the microscopic field of view (μm).

For the determination of chlorophyll content, leaf tissue (200 mg) taken from the fourth leaf from the tip of a branch was homogenized in 20 ml (80%, w/v) aqueous

Table 1. Effect of 6-benzylaminopurine (BAP) and 2,4-dichlorophenoxyacetic acid (2,4-D) concentration in the medium, and age of the mother plants on callus induction from the segments of *in vitro* sprouted buds in maggar bamboo

Plant growth substance (PGS) concentration		% Explants forming callus[#]	
BAP (μM)	2,4-D (μM)	10-year-old	45-year-old
2.5	2.5	20.0 ± 4.7^{b}	10.0 ± 0.0^{b}
2.5	5.0	53.3 ± 2.7^{c}	43.3 ± 2.7^{d}
2.5	10.0	33.3 ± 2.7^{b}	16.7 ± 2.7^{c}
5.0	2.5	0.0 ± 0.0^{a}	0.0 ± 0.0^{a}
5.0	5.0	80.0 ± 4.7^{d}	73.3 ± 2.7^{e}
5.0	10.0	33.3 ± 2.7^{b}	10.0 ± 0.1^{b}
LSD ($P = 0.05$)		14.09	3.85

[#]Means within a column followed by the same letters are not significantly different ($P = 0.05$) as determined by *F*-LSD; those with different letters are significantly different.

ANOVA

Source of variation	df	MS	*F*	*P*-value
Treatment	4	1281.462	58.17289***	0.000847
Age	1	443.556	20.13555***	0.010929
Error	4	22.0285		
Total	9			

***$P < 0.001$. Each treatment consisted of 10 explants per petri dish (100 × 7 mm) in triplicate and all values are an average of 30 explants. Basic constituents of the medium were MS salts with sucrose (2%, w/v) and phytagel (0.2%, w/v). Results were recorded after 8 weeks of culture in light (40 μmol m^{-2} s^{-1}; 16 h/day).

acetone, kept for 10 min at room temperature in the dark, and the tissue residue removed by centrifugation. The absorbance of the supernatant was measured at 663 and 645 nm using a spectrophotometer (Uvikon 931, Kontron Instruments, Italy) and the chlorophyll (Chl) *a* and *b* levels were calculated using the formula described previously[22].

$$\text{Chl } a = [(9.78 \times A_{663}) - (0.99 \times A_{645})] \times (V/1000 \times \text{Fw})/0.1 \text{ g},$$

$$\text{Chl } b = [(21.4 \times A_{645}) - (4.65 \times A_{663})] \times (V/1000 \times \text{Fw})/0.1 \text{ g}.$$

where A_{663} is the absorbance at 663 nm; A_{645} the absorbance at 645 nm; V the total volume and Fw the fresh weight. The Chl *a* and Chl *b* concentrations have been expressed in mg/g Fw.

Water status and specific leaf area were determined using the procedure described earlier[7], and the statistical analyses were carried out using standard methods[23].

Results

Axillary buds of the nodal explants, taken from plants of two age groups (10- and 45-year-old; Figure 1 *a* and *b* respectively), sprouted within 7–10 days. Normally, a single bud sprouted per explant (Figure 1 *c*); however, occasionally 2–4 buds were also seen sprouting from the same explant. Small segments, cut transversely from sprouted buds (about 20–25 mm long; 3 weeks after the start of incubation), responded differently following culture on MS medium supplemented with various PGSs. It was observed that the bud segments derived from 'physiologically young' 10-year-old MPs performed significantly better ($P < 0.001$; Table 1) than those taken from the 'mature', 45-year-old MPs. In both cases callus initiation occurred 15–20 days following subculture. Amongst various PGS concentrations used, MS medium supplemented with 5.0 μM BAP and 5.0 μM 2,4-D resulted in the best callus induction with 80.0 and 73.3% response in segments derived from 10- and 45-year-old-plants respectively (Table 1). During the initial 16–20 weeks of culture, growth of callus was found to be slow, but it picked up afterwards. After attaining a stable growth rate (22–25 weeks from the time of callus initiation), the callus volume was found to double after each subculture (Figure 1 *d*). The callus cultures were, therefore, maintained on MS medium supplemented with BAP and 2,4-D, 5.0 μM each, to obtain sufficient material for the next step.

Friable, yellowish-white callus gradually turned creamish-white and slightly compact when subcultured on MS medium supplemented with 5.0 μM BAP and 5.0–7.5 μM 2,4-D. After 8–10 weeks on this medium, numerous small

Table 2. Effect of BAP and 2,4-D concentrations in the medium, and age of the mother plants on somatic embryogenesis and plantlet development in maggar bamboo

PGS concentration (μM)		% Calluse lumps showing embryogenesis[#]		No. of embryos/ callus lump[#]		No. of shoots/ callus lump[#]		Length of the tallest shoot[#] (mm)	
BAP	2,4-D	10-year-old	45-year-old	10-year-old	45-year-old	10-year-old	45-year-old	10-year-old	45-year-old
1.0	1.0	16.7 ± 2.7^{a}	13.3 ± 2.7^{ab}	1.0 ± 0.1^{ab}	0.4 ± 0.1^{a}	0.1 ± 0.7^{a}	0.1 ± 0.1^{a}	0.7 ± 0.3^{ab}	0.4 ± 0.2^{a}
1.0	2.5	16.7 ± 2.7^{a}	6.7 ± 2.7^{a}	0.8 ± 0.1^{a}	0.4 ± 0.2^{a}	0.2 ± 0.1^{a}	0.0 ± 0.0^{a}	0.6 ± 0.1^{a}	0.0 ± 0.0^{a}
1.0	5.0	23.3 ± 2.7^{ab}	26.7 ± 2.7^{c}	3.6 ± 0.5^{b}	3.1 ± 0.2^{ab}	0.3 ± 0.1^{ab}	0.3 ± 0.1^{a}	2.3 ± 0.3^{c}	1.8 ± 0.2^{b}
1.0	7.5	16.7 ± 2.7^{a}	20.0 ± 4.7^{bc}	9.0 ± 1.4^{d}	5.2 ± 0.8^{b}	0.2 ± 0.1^{a}	0.5 ± 0.1^{ab}	0.3 ± 0.1^{a}	0.4 ± 0.1^{a}
1.0	10.0	30.0 ± 0.0^{b}	26.7 ± 2.7^{c}	4.0 ± 0.2^{c}	3.5 ± 0.2^{b}	0.0 ± 0.0^{a}	0.0 ± 0.0^{a}	0.0 ± 0.0^{a}	0.0 ± 0.0^{a}
2.5	2.5	33.3 ± 2.7^{bc}	33.3 ± 2.7^{cd}	7.7 ± 1.1^{d}	8.3 ± 0.5^{c}	0.9 ± 0.1^{b}	0.8 ± 0.1^{bc}	5.0 ± 0.2^{d}	5.2 ± 0.2^{d}
2.5	5.0	53.3 ± 2.7^{e}	40.0 ± 4.7^{d}	15.6 ± 0.5^{e}	14.0 ± 1.7^{d}	2.5 ± 0.4^{d}	2.2 ± 0.2^{d}	5.1 ± 0.3^{d}	4.4 ± 0.4^{c}
2.5	7.5	63.3 ± 2.7^{fg}	63.3 ± 2.7^{g}	25.4 ± 1.4^{g}	25.2 ± 0.2^{f}	4.1 ± 0.2^{f}	4.4 ± 0.2^{e}	6.1 ± 0.3^{d}	7.1 ± 0.8^{e}
2.5	10.0	53.3 ± 2.7^{e}	46.7 ± 2.7^{ef}	20.4 ± 0.9^{f}	16.8 ± 1.1^{d}	3.2 ± 0.1^{e}	2.2 ± 0.1^{d}	6.2 ± 0.3^{d}	5.2 ± 0.5^{d}
5.0	2.5	46.7 ± 2.7^{de}	43.3 ± 2.7^{de}	9.3 ± 1.0^{d}	9.0 ± 0.3^{c}	1.7 ± 0.3^{c}	1.3 ± 0.2^{c}	5.5 ± 0.4^{d}	4.5 ± 0.7^{c}
5.0	5.0	70.0 ± 4.7^{g}	66.7 ± 2.7^{g}	20.9 ± 0.9^{f}	21.2 ± 1.0^{e}	6.4 ± 0.2^{g}	6.5 ± 0.3^{f}	11.2 ± 1.3^{e}	10.6 ± 0.4^{g}
5.0	7.5	93.3 ± 2.7^{h}	90.0 ± 4.7^{h}	38.7 ± 0.6^{h}	37.3 ± 0.2^{g}	11.9 ± 0.3^{h}	11.3 ± 0.4^{g}	15.7 ± 0.2^{f}	17.1 ± 0.5^{h}
5.0	10.0	56.7 ± 2.7^{ef}	53.3 ± 2.7^{fg}	19.5 ± 0.6^{f}	19.2 ± 0.3^{e}	4.8 ± 0.2^{g}	4.5 ± 0.3^{e}	10.3 ± 0.8^{e}	9.6 ± 0.6^{f}
LSD ($P = 0.05$)		10.06	11.72	2.97	2.98	0.68	0.71	1.95	1.25

[#]Means within a column followed by the same letters are not significantly different ($P = 0.05$) as determined by F-LSD; those with different letters are significantly different.

ANOVA

Source of variation	% calluses showing embryogenesis				No. of embryos per callus			No. of shoots/callus			Length of the tallest shoot (mm)		
	df	MS	*F*	*P*-value	MS	*F*	*P*-value	MS	*F*	*P*-value	MS	*F*	*P*-value
Treatment	12	1105.29	99.71***	4.24E-10	245.72	268.40***	1.19E-12	22.76	348.42***	2.51E-13	48.18	178.19***	1.36E-11
Age	1	72.11	6.51**	0.03	5.82	6.36**	0.03	0.19	2.85^{NS}	0.12	0.28	1.04^{NS}	0.33
Error	12	11.08			0.92			0.07			0.27		
Total	25												

***$P < 0.001$; **$P < 0.05$; [NS]Not significant. Each treatment consisted of 10 callus lumps per 250 ml conical flask in triplicate, and all values are an average of 30 explants (150 ± 20 mg fresh weight per callus lump). Basic constituents of the medium were MS salts with sucrose (2%, w/v) and phytagel (0.2%, w/v). Results were recorded after 12 weeks of culture in light (40 μmol m^{-2} s^{-1}; 16 h/day).

SEs with smooth surface could be seen developing on the surface of embryogenic callus (Figure 1 *e*; see arrows). The SEs matured after 8–12 weeks of culture on the same medium and started to germinate while still attached with the parent tissue (Figure 1 *f* and *h*; see arrow). Histological examination of the embryos revealed a bipolar organization with an integrated coleoptile–coleorhiza axis (Figure 1 *g*; see arrow). Amongst various combinations of PGSs used, maximum embryogenesis (93.3% and 90.0%) with highest number of SEs (38.7 and 37.3 per callus lump) and regenerated plantlets (11.9 and 11.3) per callus lump, in the case of 10- and 45-year-old plants respectively, was recorded on MS medium supplemented with 5.0 μM BAP and 7.5 μM 2,4-D (Table 2). Slightly lower concentration of 2,4-D (5.0 μM) with 5.0 μM BAP also gave good response. It was observed that medium composition as well as the age of the MPs significantly influenced the embryogenic process. From the ANOVA analysis (Table 2), it can be seen that calculated *F* value is greater than the critical *F* [F_{05} (1, 12) = 4.75] for embryogenesis and the number of embryos formed. Thus, embryogenesis and the number of embryos formed were significantly influenced by the treatment (BAP and 2,4-D concentrations in the medium) and age of the MPs (Table 2); explants taken from the 10-year-old MPs exhibited significantly higher response than those taken from the 45-year-old MPs. On the other hand, treatments and the age of the MPs had only slight or no effect on the number of shoots formed per callus lump and the length of regenerated shoots.

The plantlets developed from germinated SEs (Figure 1 *i*) were sometimes (approx. 10% cases) found to have reasonable root system while still in the regeneration medium containing high cytokinin (5.0 μM BAP) along with auxin (7.5 μM 2,4-D). In most cases germinated SEs had only rudimentary root system, and sometimes roots were small, thick and finger-like without branching. Medium containing half strength MS with IBA (5.0 μM) accelerated the development of roots with the formation of long and branched, normal looking roots (Figure 1 *j*). Different concentrations of IBA resulted in significant differences in root development, number of roots formed

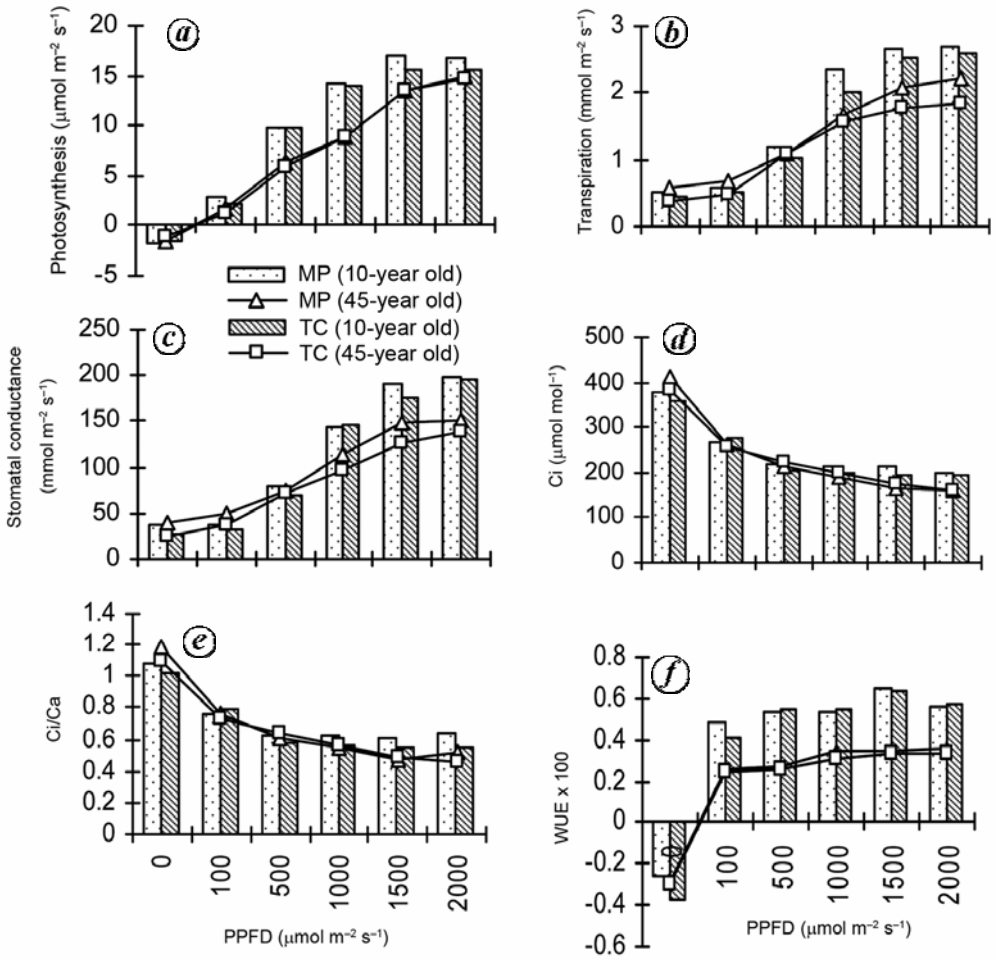

Figure 2. Gas and water vapour exchange studies in *in vitro* propagated and corresponding mother plants of *Dendrocalamus hamiltonii*. MP, mother plant; TC, Tissue culture-raised plant (18 months after transfer to *ex vitro* conditions. Data were recorded on young, fully expanded fourth leaf from the shoot tip).

per rooted plant and in terms of the length of the longest root ($P < 0.05$); however, the age of MPs did not appear to affect the process. Medium containing half strength MS salts with 5.0–25.0 μM IBA resulted in 100% root development. IBA at 5.0 μM in the medium was found to be the lowest concentration to obtain 100% and good root formation following this procedure. This concentration was, therefore, used routinely. Rhizome formation was low (8%; Figure 1 *k*, see arrow) and could only be observed when half strength liquid MS medium containing 20.0 μM IBA was used. Survival of the acclimatized plants was found to be over 70% after 6 months of *ex vitro* transfer (Figure 1 *p*).

Evaluation of MP and TC plants

The effect of different light intensities on photosynthesis, and the dark respiration of MP and TC-raised maggar bamboo of both age groups is shown in Figure 2. The rate of net photosynthesis (*Pn*) was found to increase with increasing PAR, and the maximum (A_{max}) *Pn* was found at 2000 μmol m^{-2} s^{-1} PPFD for both types of plants (TC-raised as well as MPs) of both age groups (Figure 2 *a*). A_{max} was 7.53% higher in the 10-year-old MP than in its corresponding TC-raised plants; however, ANOVA analysis revealed that the effect was not statistically significant. Similarly, there was no significant difference in A_{max} of 45-year-old MP and its TC-raised counterparts. The A_{max} was 10.62% higher in 10-year-old MP in comparison of 45-year-old MP, whereas this value was 5.84% higher in TC-raised plants derived from the MPs of corresponding age groups. The ANOVA analyses (tables not shown), revealed that *Pn* was significantly influenced ($P = 0.05$) by the age in respect of the MPs as well as their TC-raised counterparts; the 10-year-old plants (and the corresponding TC-raised plants) performed better than their 45-year-old counterparts. The rate of transpiration (*E*) under different light intensities in the MPs as well as TC-raised plants, from both the age groups (Figure 2 *b*), clearly indicated its effect on transpiration. *E* increased with increasing PPFD from 100 to 2000 μmol m^{-2} s^{-1}.

Transpiration was generally higher in the MPs compared to the corresponding TC-raised plants. At the highest light intensity, *E* was 23% and 29% higher in 10-year-old MP in comparison to 45-year-old MP and between their TC-raised counterparts respectively.

The stomatal conductance was found to increase at higher light intensities, in both types of plants of the two age groups (Figure 2 *c*). Like *E*, stomatal conductance was also higher in the MPs over their TC-raised counterparts of both age groups, and it was higher in 10-year-old MP when compared to 45-year-old MP. The same was true for TC-raised plants of both age groups. Both intercellular CO_2 concentration (*Ci*) and the ratio of intercellular CO_2 concentration to ambient CO_2 (*Ci*/*Ca*) were found to decrease with increase in PPFD, in both types of plants of the two age groups. Although there was no significant difference in *Ci* (Figure 2 *d*) and *Ci*/*Ca* (Figure 2 *e*) between the MPs and TC-raised plants, the values were about 15% higher in the MPs compared to TC-raised plants of the two age groups.

Based on *Pn* and *E*, recorded at different light intensities, WUE (Figure 2 *f*) was calculated. A sharp increase in WUE was recorded in MPs as well as TC-raised plants of the two age groups in response to light (100 μmol m^{-2} s^{-1}). A more or less steady state was recorded at 500 μmol m^{-2} s^{-1} and higher light intensities for the two types of plants of both age groups. WUE was maximum (0.65 ± 0.04) at 1500 μmol m^{-2} s^{-1} in the 10-year-old MP, followed by TC-raised plants of the same age group (0.632 ± 0.05).

Specific leaf mass and relative water content (RWC) values of MPs of both age groups were significantly higher compared to TC-raised plants of the corresponding age groups (Table 3). In contrast, these values were not significantly different between MPs and their TC-raised counterparts of the two age groups. Transverse section of leaves of TC-raised and corresponding MPs of both age groups showed similar tissue arrangement. However, leaves of MPs were thicker than their TC-raised counterparts, for both age groups (Table 3); the thickness was about 19% and 15% higher in 10-year-old and 45-year-old plants respectively. Stomatal density of TC-raised plants was 496 and 482 mm^{-2} for TC-raised plants derived from 10- and 45-year-old MPs respectively, and 498 and 563 mm^{-2} in the corresponding MPs (Table 3). The stomatal length was similar in all types of plants of both the age groups (data not shown). Epidermal cells were found to be elliptical/oval in shape with well-developed cuticle. No major qualitative difference was observed in the structure of spongy and palisade mesophyll cells (Figure 1 *l*–*o*). Approximately 8% and 10% higher Chl *a* and Chl *b* levels were recorded in TC-raised plants in comparison to the corresponding MPs. When Chl *a* and Chl *b* levels were compared between the TC-raised plants derived from 10- and 45-year-old MPs, the values were about 20% more in TC-raised plants derived from the 10-year-old MP (Table 3).

Discussion

Micropropagation work on bamboos in the past has focused mainly on developing protocols for multiple shoot formation or embryogenesis using juvenile explants (seed, excised zygotic embryos, or explants taken from aseptically or field-grown seedlings of unknown potential in terms of subsequent field performance). However, it would be desirable to take explants from field-tested elite plants. A few reports are now available on bamboo propagation using nodal explants taken from field-grown culms[4,10,11,20,24]. Plant regeneration, as described in this study, through somatic embryogenesis has the added advantage of producing a large number of plants within a short period. This represents an improvement over an earlier work on maggar bamboo[11], which also used physiologically young plants as a source of explants. Several concentrations of BAP and 2,4-D were evaluated in the present study to optimize SE formation, and unlike the earlier report[11], addition of gibberellic acid (GA_3) in the medium was not required for induction of SEs. Amongst various concentrations and combinations of PGSs tried, the medium with relatively higher concentration (5.0–10.0 μM) of 2,4-D along with (5.0 μM) BAP favoured callusing and embryogenesis – these observations are consistent with previous reports[13,16]. Also, 100% root formation was observed in the present study with lower concentration of IBA (5.0 μM), a considerable improvement over an earlier work which reported inconsistent and much lower (25–30%) rooting of shoots under *in vitro* conditions in bamboo[10].

Among the MPs of two age groups, the explants derived from 10-year-old MP performed better in comparison to those from 45-year-old MP, reflecting clearly the effect of age (and possibly that of genotype). *In vitro* response of explants taken from mature and young plants of *Prosopis* species also showed that the explants taken from physiologically young plants performed better[25]. The role of plant age on morphogenesis is generally believed to be mediated by the differences in endogenous levels of PGSs; clear experimental evidence in favour of such an explanation is, however, lacking[26,27]. Plants regenerated from SEs, like the zygotic embryos, can be expected to last the full lifecycle of a given bamboo species[28]. This is, however, still a speculation, as there are no reports regarding the long-term fate of plants regenerated through somatic embryogenesis using explants taken from an adult bamboo. Long-term observations are, therefore, needed on such plants. It would be important to know if the TC-raised plants of bamboo, including those of SE origin flower simultaneously when the corresponding MP flowers. It remains to be seen whether the SE-derived plants 'remember' the age of the MP like the vegetatively propagated plants, or that these are truly juvenile like the plants of seed origin.

The comparison of field performance of TC-raised plants with the corresponding MPs in respect of photo-

Table 3. Comparison of some morphological features, chlorophyll content, relative water content (RWC) and specific leaf mass of *in vitro* propagated plants and mother plants of maggar bamboo

Plants		Leaf thickness (μm)	Stomatal frequency (no./mm^2)	Chlorophyll *a* (mg/g fr. wt)	Chlorophyll *b* (mg/g fr. wt)	RWC (%)	Specific leaf mass (mg/cm^2)
10-year-old	MP	127.20 ± 1.13	498.20 ± 3.43	2.26 ± 0.02	1.03 ± 0.26	97.12 ± 0.46	3.80 ± 0.10
	TC	102.40 ± 0.82	496.40 ± 8.10	2.45 ± 0.08	1.16 ± 0.02	93.08 ± 0.46	3.39 ± 0.05
45-year-old	MP	138.61 ± 1.22	563.34 ± 3.52	1.84 ± 0.02	0.84 ± 0.02	96.75 ± 0.51	3.95 ± 0.09
	TC	116.90 ± 0.75	482.73 ± 8.67	1.99 ± 0.03	0.93 ± 0.01	94.37 ± 0.36	3.07 ± 0.04
LSD (P = 0.05)		3.31	21.50	0.14	0.11	1.51	0.25

Each treatment consisted of three replicates. Results were recorded 18 months after transfer of TC-raised plants to *ex vitro* conditions. MP, Mother plant; TC, Tissue culture-raised plant.

synthetic rate and related gas exchange parameters reported in this study is of value and novel; these physiologically important parameters have been suggested as selection criteria for plants[29]. In the only other field performance study reported on TC-raised bamboos, comparative growth performance was monitored over a period of 6 years, and the study revealed that *in vitro*-raised plants showed better growth performance in terms of physical parameters (e.g. number and height of the culms) compared to that of their cutting-raised counterparts[10]. The present study while confirming this trend substantially furthered evidence, in terms of physiological parameter, and TC-raised plants performed better than the corresponding of MPs, irrespective of age. During this study it was also observed that the MP and the corresponding TC-raised plants from 10-year-old, physiologically young and elite stock performed better than their counterparts from the 45-year-old stock. This is indicative of some kind of ageing effect in bamboos. Like MPs, TC-raised plants also maintained a high rate of photosynthesis at higher light intensities, confirming tolerance to high irradiance. It has been suggested[30] that plants possessing improved WUE are likely to be more productive and better competitors than those having lower WUE. Plant survival, growth and productivity are intimately coupled with the aerial environment through processes such as energy exchange, loss of water in transpiration and uptake of carbon dioxide in photosynthesis. Water vapour exchange rate affects the physiology of the whole plant. Therefore, data on physiological parameters such as reported in the present study are likely to provide valuable information regarding the suitability of TC-raised plants for developing plantations. The present study suggests that TC-raised plants perform at par or slightly better than the corresponding MPs, irrespective of the age and/or genotypic differences between the MPs, in terms of important physiological and morphological parameters.

In summary, maggar bamboo has tremendous scope for use in eco-friendly agro-forestry projects in the hills (up to 1800 m amsl), to bring marginal lands into use. The present study substantially supplements existing data on field performance of TC-raised plants, and substantiates the use of TC technology for mass propagation of physiologically young, elite material for the economic benefit of the rural population. This study describes a useful protocol for mass propagation of maggar bamboo via somatic embryogenesis, through the proliferation of embryogenic callus. The protocol has also been applied successfully to raise a large number of plantlets of maggar bamboo for field plantation in Uttarakhand under the National Bamboo Mission (S. K. Nandi, unpublished work). Like other bamboos, vegetatively propagated plants of maggar bamboo are known to 'remember' the age of the MP. It remains to be seen if this 'age memory' is retained when the plants are propagated through somatic embryogenesis or multiple shoot proliferation using TC technology.

1. Sharma, O. P., Mass multiplication of *Dendrocalamus hamiltonii* Munro – a critical evaluation. *Ind. For*., 1986, **112**, 517–523.
2. Sharma, O. P. and Kaushal, S. K., Exploratory propagation of *Dendrocalamus hamiltonii* Munro by one-node culm cuttings. *Ind. For.*, 1985, **111**, 135–139.
3. Kumar, A., Pal, M. and Kumar, S., Mass production of field planting stock of *Dendrocalamus hamiltonii* vegetatively through macro-proliferation. *Ind. For*., 1992, **118**, 638–642.
4. Saxena, S. and Bhojwani, S. S., *In vitro* clonal multiplication of 4-year-old plants of bamboo, *Dendrocalamus longispathus* Kurz. *In Vitro Cell. Dev. Biol*., 1993, **29**, 135–142.
5. Nadgauda, R. S., Parasharami, U. A. and Mascarenhas, A. F., Precocious flowering and seeding behavior in tissue-cultured bamboos. *Nature*, 1990, **344**, 335–336.
6. Chambers, S. M., Heuch, J. H. R. and Pirrie, A., Micropropagation and *in vitro* flowering of the bamboo *Dendrocalamus hamiltonii* Munro. *Plant Cell Tiss. Org. Cult*., 1991, **27**, 45–48.
7. Bag, N., Chandra, S., Palni, L. M. S. and Nandi, S. K., Micropropagation of Dev-ringal [(*Thamnocalamus spathiflorus* (Trin.) Munro] – a temperate bamboo, and comparison of *in vitro* propagated plants and seedlings. *Plant Sci*., 2000, **156**, 125–135.
8. Chaturvedi, H. C., Sharma, M. and Sharma, A. K., *In vitro* regeneration of *Dendrocalamus strictus* Nees through nodal segments taken from field-grown clumps. *Plant Sci*., 1993, **91**, 97–101.
9. Lin, C. S. and Chang, W. C., Micropropagation of *Bambusa edulis* through nodal explants of field-grown culms and flowering of regenerated plants. *Plant Cell Rep*., 1998, **17**, 617–620.
10. Sood, A., Ahuja, P. S., Sharma, M., Sharma, O. P. and Godbole, S., *In vitro* protocols and field performance of elites of an important bamboo *Dendrocalamus hamiltonii* Nees et Arn. Ex Munro. *Plant Cell Tiss. Org. Cult*., 2002, **71**, 55–63.

11. Rout, G. R. and Das, P., Somatic embryogenesis and *in vitro* flowering of 3 species of bamboo. *Plant Cell Rep*., 1994, **13**, 683–686.
12. Jullien, F. and Van, K. T. T., Micropropagation and embryoid formation from young leaves of *Bambusa glaucescene* 'Golden goddess'. *Plant Sci*., 1994, **98**, 199–207.
13. Yeh, M. L. and Chang, W. C., Somatic embryogenesis and subsequent plant regeneration from inflorescence callus of *Bambusa beecheyana* Munro var. beecheyana. *Plant Cell Rep*., 1986, **5**, 409–411.
14. Tasy, H. S., Yeh, C. C. and Hsu, J. Y., Embryogenesis and plant regeneration from anther culture of bamboo [*Sinocalamus latiflora* (Munro) McClure]. *Plant Cell Rep*., 1990, **9**, 349–351.
15. Rao, I. U., Rao, I. V. R. and Narang, V., Somatic embryogenesis and regeneration of plants in the bamboo *Dendrocalamus strictus*. *Plant Cell Rep*., 1985, **4**, 191–194.
16. Woods, S. H., Phillips, G. C., Woods, J. E. and Collins, G. B., Somatic embryogenesis and plant regeneration from zygotic embryo explants in mexican weeping bamboo, *Otatea acuminata* aztecorum. *Plant Cell Rep*., 1992, **11**, 257–261.
17. Sood, A., Sharma, O. P. and Palni, L. M. S., Improved methods of propagation of maggar bamboo (*Dendrocalamus hamiltonii* Nees et Arn. ex Munro) using single node cuttings taking from juvenile culms of elite seedlings. *J. Am. Bamboo Soc*., 1992, **9**, 17–24.
18. Godbole, S., Sood, A., Thakur, R., Sharma, M. and Ahuja, P. S., Somatic embryogenesis and its conversion into plantlets in a multipurpose bamboo, *Dendrocalamus hamiltonii* Nees et Arn. ex Munro. *Curr. Sci*., 2002, **83**, 885–889.
19. Pandey, A., Palni, L. M. S. and Bag, N., Biological hardening of tissue culture raised tea plants through rhizosphere bacteria. *Biotechnol. Lett.*, 2000, **22**, 1087–1091.
20. Purohit, V. K., Tamta, S., Chandra, S., Vyas, P., Palni, L. M. S. and Nandi, S. K., *In vitro* multiplication of *Quercus leucotrichophora* and *Q. glauca*: Important Himalayan oaks. *Plant Cell Tiss. Org. Cult.*, 2002, 121–133.
21. Jensen, W. A., *Botanical Histochemistry*, W.H. Freeman Co., San Francisco, USA, 1962, p. 408.
22. Arnon, D. I., Copper enzymes in isolated chloroplasts. Polyphenoloxidase in *Beta vulgaris*. *Plant Physiol.*, 1949, **24**, 1–15.
23. Snedecor, G. W. and Cochran, W. G., *Statistical Methods*, Oxford and IBH Publishing Co Pvt Ltd, New Delhi, 1968, p. 593.
24. Prutpongse, P. and Gavinlertvatana, P., *In vitro* micropropagation of 54 species from 15 genera of bamboo. *Hortic. Sci*., 1992, **27**, 453–454.
25. Shekhawat, N. S., Rathore, T. S., Singh, R. P., Deora, N. S. and Rao, S. R., Factors affecting *in vitro* clonal propagation of *Prosopis cineraria*. *Plant Growth Regul*., 1993, **12**, 273–280.
26. Nehra, N. S. and Kartha, K. K., Meristem and shoot tip culture: requirements and applications. In *Plant Cell and Tissue Culture* (eds Vasil, I. K. and Thorpe, T. A.), Kluwer Academic Publishers, The Netherlands, 1994, pp. 37–70.
27. Diro, M. and vanStaden, J., The type of explant plays a determining role in the micropropagation of *Ensete ventricosum*. *S. Afr. J. Bot*., 2005, **71**, 154–159.
28. Rao, I. V. R., Yusoff, A. M., Rao, A. N. and Sastry, C. B., *Propagation of Bamboo and Rattan through Tissue Culture*, The IDRC Bamboo and Rattan Research Network, Canada, 1989, p. 60.
29. Lapido, D. O., Grace, J., Sandford, A. P. and Leakey, R. R. B., Clonal variation in photosynthetic and respiration rates and diffusion resistance in the tropical hardwood *Triplochiton scleroxylom* K. Schum. *Photosynthetica*, 1984, **18**, 20–27.
30. Neals, T. F., Patterson, A. A. and Hastney, V. J., Physiological adaptation to draught in the carbon assimilation and water loss of xerophytes. *Nature*, 1968, **219**, 469.

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