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Micropropagation of the giant bamboo (*Dendrocalamus giganteus* Munro) from nodal explants of field grown culms

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Abstract

Propagules of *Dendrocalamus giganteus* were produced by a process of in vitro axillary shoot proliferation followed by rooting. Axillary bud break and the presence of culture contaminants in single-node segments of secondary branches were strongly influenced by the seasonal rainfall pattern. Cultures of axillary shoots were initiated during peak bud-break periods in a semi-solid MS medium with 2 mg l^{-1} benzylaminopurine (BAP), 0.1 mg l^{-1} kinetin and 1 g l^{-1} Benlate (benomyl). Continuous shoot proliferation for a period of 1 year was achieved in a liquid MS medium with 6 mg l^{-1} BAP, 0.1 mg l^{-1} kinetin and 8% (v/v) coconut water. Shoot proliferation at a 1.8-fold increase in shoot number every 13 days took place after a lag phase of 65 days. Shoots (77.5%) rooted when transferred from the shoot proliferation medium containing 3 mg l^{-1} indole butyric acid in the last two passages, to a rooting medium with MS modified to half strength major salts, 3 mg l^{-1} IBA and 10 mg l^{-1} coumarin. After a period of in vitro hardening the propagules were transferred to soil. © 1997 Elsevier Science Ireland Ltd.

Keywords: Bamboo; *Dendrocalamus giganteus*; Micropropagation; In vitro bud-break; Root induction; Seasonal effects

1. Introduction

Dendrocalamus giganteus is a woody perennial that belongs to the subfamily Bambusoideae of the family Poacea, the grasses. It is the largest of the bamboos and is economically desirable in that it could provide a high biomass of wood that can be processed to develop many new products to replace timber. Propagules are limited. Seeds are rare due to the unpredictable and long flowering cycle of 76 years [1]. Unlike other bamboos, vegetative propagation is unprofitable and difficult [2]. Bamboos with their segmented structure of intervening nodes and internodes provide a large number of axillary buds that are potential plants [3]. In vitro methods are the most suitable in realizing this potential. There are a few reports on forced

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axillary branching and complete plantlet development in species of bamboo such as *D. strictus* [4,5], *Bambusa tulda* [6] and *D. brandisii* [7] where the seedling material whose performance is unknown, was used as the initial explant. Prutpongse and Gavinlertvana [8] reported that 54 species of bamboo, including *D. giganteus*, were micropropagated, but this report lacks details on the source of explant, rate of propagation and rooting. There is only one report where axillary buds from a mature culm of *D. longispathus* was used to produce plantlets [9]. The paucity of reports on plantlet production in bamboo through tissue culture lies mainly in the difficulty of initiating axenic cultures and in attaining a high rooting percentage [10]. This investigation determined the factors that contributed to the successful initiation of cultures, continuous and rapid proliferation of axillary shoots and finally to a high rooting percentage from the axillary buds in a 5-year-old field grown clump of *D. giganteus*.

2. Materials and methods

2.1. Culture initiation

Single node segments 2.5–3.0 cm in length, with unsprouted buds from the third to fifth nodes of secondary branches, from a 5-year-old field grown clump of giant bamboo bearing 20 primary culms of a mean height of 6 m, were used to initiate cultures. The outer prophylls covering the axillary buds were removed and the nodes washed under running water. They were surface-sterilised with the supernatant from a saturated solution of bleaching powder for 10 min, rinsed in sterile water, and kept in a 1 g l^{-1} suspension of Benlate (benomyl, Baur and Co. Ltd.) for 1 h. They were surface-sterilised again in a 0.3% solution of mercuric chloride for 10 mins and rinsed in sterile water. Nodes were cultured singly in culture tubes containing 10 ml of a basal Murashige and Skoog medium (MS) [11] incorporated with 3% sucrose, 0.35% agargel (Sigma) and supplemented with different levels and combinations of 6-benzylaminopurine (BAP), kinetin (K), gibberellic acid (GA3), indole acetic acid (IAA), thidiazuron (TDZ) and coconut water (CW). Some treatments were incorporated with 1 g l^{-1} of Benlate alone, or in combination with different levels of streptomycin (50–100 mg l^{-1}), chloramphenicol (5–10 mg l^{-1}) and rifampicin (20 mg l^{-1}). A hot water treatment at 45, 50, and 55°C for 15 or 30 min prior to surface sterilization was also carried out to reduce contaminants.

To investigate the seasonal effects on in vitro bud break, a sample of 40 nodes was cultured fortnightly, from April 1994 to October 1996. The medium with the supplements that induced the highest bud break and minimised culture contaminants were used. The nodes were transferred to fresh medium whenever the medium turned brown. In vitro bud break and the presence of microbial contaminants were recorded weekly. The number of axillary shoots that sprouted and the length of the longest shoot per node were recorded.

2.2. Shoot proliferation

Nodal segments with in vitro formed axillary shoots were transferred to a basal MS medium with 3% sucrose and supplemented as follows to induce axillary shoot proliferation.

8% CW + 0.1 mg l^{-1} K	M_1
8% CW + 6 mg l^{-1} BAP + 0.1 mg l^{-1} K	M_2
8% CW + 6 mg l^{-1} BAP + 0.1 mg l^{-1} K + 0.38% agar	M_3
8% CW + 12 mg l^{-1} BAP + 0.1 mg l^{-1} K	M_4
6 mg l^{-1} BAP + 0.1 mg l^{-1} K	M_5
8% CW + 6 mg l^{-1} BAP + 0.1 mg l^{-1} K + 1000 mg l^{-1} casein hydrolysate (CH)	M_6
8% CW + 6 mg l^{-1} BAP + 1500 mg l^{-1} CH + 0.1 mg l^{-1} K	M_7

The shoots were cultured in magenta GA7 vessels (Sigma) containing 50 ml of medium and incubated at 24 ± 2°C and a 16 h photoperiod. The medium was incorporated with 0.5 and 0.25 g l^{-1} Benlate during the first and second transfers, respectively. The liquid cultures were kept on a shaker at 55 strokes per min. The shoots in liquid cultures were supported on 3 × 3 cm pieces of

luffa (the reticulate mass of fibre within dry pods of *Luffa acutangular* (L) Roxb.) as the submerged shoots showed signs of vitrification. The entire mass of luffa fibre removed from dry pods was boiled, bleached, rinsed in distilled water, autoclaved, dried and stored. These were cut into pieces and placed in the medium before autoclaving. Once the shoots recovered, the luffa pieces were discarded.

The shoots were transferred to fresh medium whenever the medium turned brown every 9–15 days. The mother node was removed when the shoot number increased. Large clusters of shoots were separated at their internodes into smaller clusters having at least five shoots. Single shoots and smaller clusters that were accidentally separated were also included in the medium and kept under observation.

The total number of shoots per vessel at the end of each subculture cycle was recorded separately for each mother node that initiated them. The growth curve was plotted with the mean shoot number from all mother nodes per culture vessel at the end of each successive subculture cycle against the subculture cycles in days. The growth rate was calculated from the regression line obtained by plotting Ln (mean shoot number per vessel) vs. number of days in shoot proliferation medium.

2.3. Rooting

The shoots were separated into clusters bearing 2–7 shoots (propagules) and transferred to root induction medium. This was a modified MS medium with the macro nutrients reduced to half strength and incorporated with 2 mg l^{-1} biotin, 2 mg l^{-1} calcium pantothenate, 2% sucrose (basal medium), and other supplements such as indolebutyric acid (IBA), coumarin (1,2-Benzopyrone) and IAA as follows to form the eight treatments R_1–R_8.

3 mg l^{-1} IBA	R_1
10 mg l^{-1} IBA for 2 days in the dark and then transferred to basal medium with 2mg l^{-1} IBA	R_2
3 mg l^{-1} IBA + 10 mg l^{-1} coumarin	R_3
3 mg l^{-1} IBA + 10 mg l^{-1} coumarin + 2 mg l^{-1} IAA	R_4
3 mg l^{-1} + 10 mg l^{-1} coumarin + 2 mg l^{-1} IAA + 0.02% activated charcoal	R_5
5 mg l^{-1} IBA + 10 mg l^{-1} coumarin + 2 mg l^{-1} IAA	R_6
3 mg l^{-1} IBA was incorporated in the shoot proliferation medium for the last two sub cultures and then transferred to R_3	R_7
Same as R_7 but transferred to R_4	R_8

The propagules were supported on filter paper bridges in culture tubes containing 15 ml of liquid medium. A total of 20 tubes each were cultured for treatment R_1 and R_2 while 40 tubes were cultured in each of the other treatments. The shoots were transferred to fresh medium at 2–3 week intervals. The number of roots and new shoots that developed, and the number of days taken to initiate these were recorded.

2.4. Hardening and acclimatization

The rooted shoots were transferred to growth regulator free MS medium with macro nutrients at half strength and with 2% sucrose, under aseptic conditions. After 1 week they were transferred to sucrose free medium and the caps of the tubes were removed periodically until signs of wilting stopped, and then transferred to soil in polythene bags containing coir dust and loam (1:1). These were kept in the shade and gradually exposed to sunlight.

3. Results

3.1. Culture initiation

The MS medium containing 2.0 mg l^{-1} BAP and 0.1 mg l^{-1} K induced the highest percentage of axillary bud-break whereas GA3, IAA, TDZ and CW reduced bud-break. Incorporation of Benlate at 1 g l^{-1} in the medium reduced culture contaminants significantly. The antibiotics and

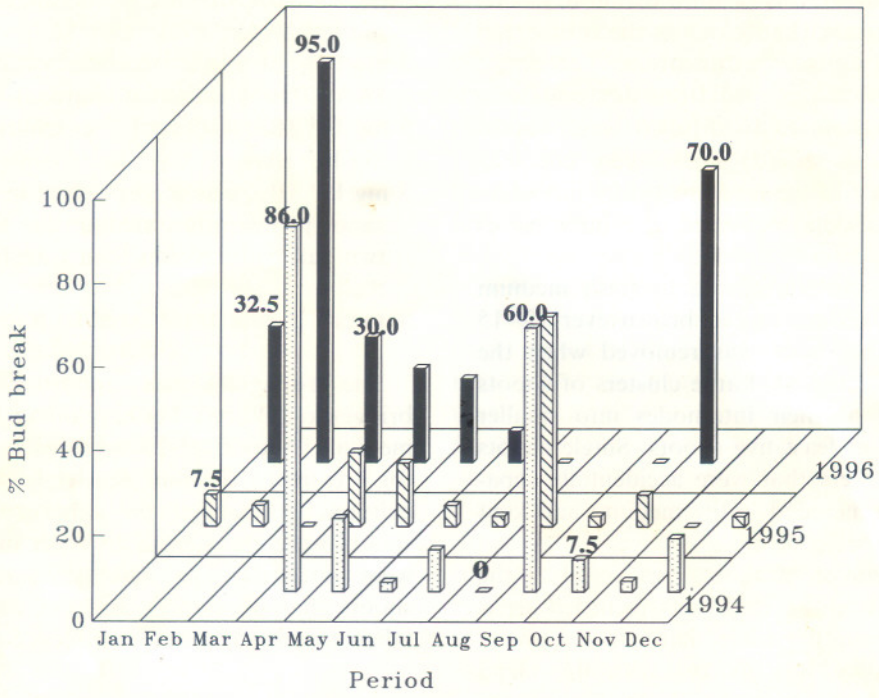

Fig. 1. Variations in in vitro bud-break in nodal segments from April 1994 to October 1996.

the hot water treatments used were not as effective. Therefore, MS medium with 2 mg l^{-1} BAP, 0.1 mg l^{-1} K and 1 g l^{-1} Benlate was used to study the seasonal effects on in vitro bud-break.

In vitro bud-break was not uniform and showed a mean of $19.8 \pm 4.8\%$ ranging from 0 to 86.7% during the period under investigation (Fig. 1). Two periods of peak bud-break were observed each year. In 1994 these were in April (86.7%) and September (62.5%). In 1995 bud-break was lower and the peak periods were in April (17.5%) and August (50.0%). In 1996, they were in February (95.0%) and October (70.0%). The rainfall pattern during these years (Fig. 2) showed that peak bud-break took place in the month just before the onset of the two monsoon rains (south-west and north-east monsoons) each year, except in April 1995 when there was only 17.5% bud-break during a rainy month. At other times bud-break could not be induced or it was less than 10%.

Culture contaminants of a systemic nature appeared after the third week of culture initiation. They were bacteria or fungi that appeared at the cut-ends of the node or near the axillary buds. Although a significantly strong correlation was not found between bud-break and contamination throughout the period, an inverse relationship was observed during periods of high or low bud-break each year (Fig. 2). During the period of highest contamination of 90% in Aug 1994, in vitro bud-break did not take place in any of the cultured nodes. Whenever contamination exceeded 50%, bud-break was less than 10%. Contaminants were generally less than 30% during the periods of peak bud break. Axenic culture initiation was possible only during these periods of peak bud-break. A mean of 1.4 buds ranging from one to five in number sprouted at each node (Fig. 4A). Shoots that sprouted had a mean length of 62.7 mm ranging from 43 to 170 mm. They turned brown and died even with frequent transfer to fresh medium in the culture tubes. But on transfer to Magenta vessels containing a higher volume (50 ml) of medium more axillary buds sprouted from the mother node and also from the nodes of elongating shoots. Liquid cultures in the shaker

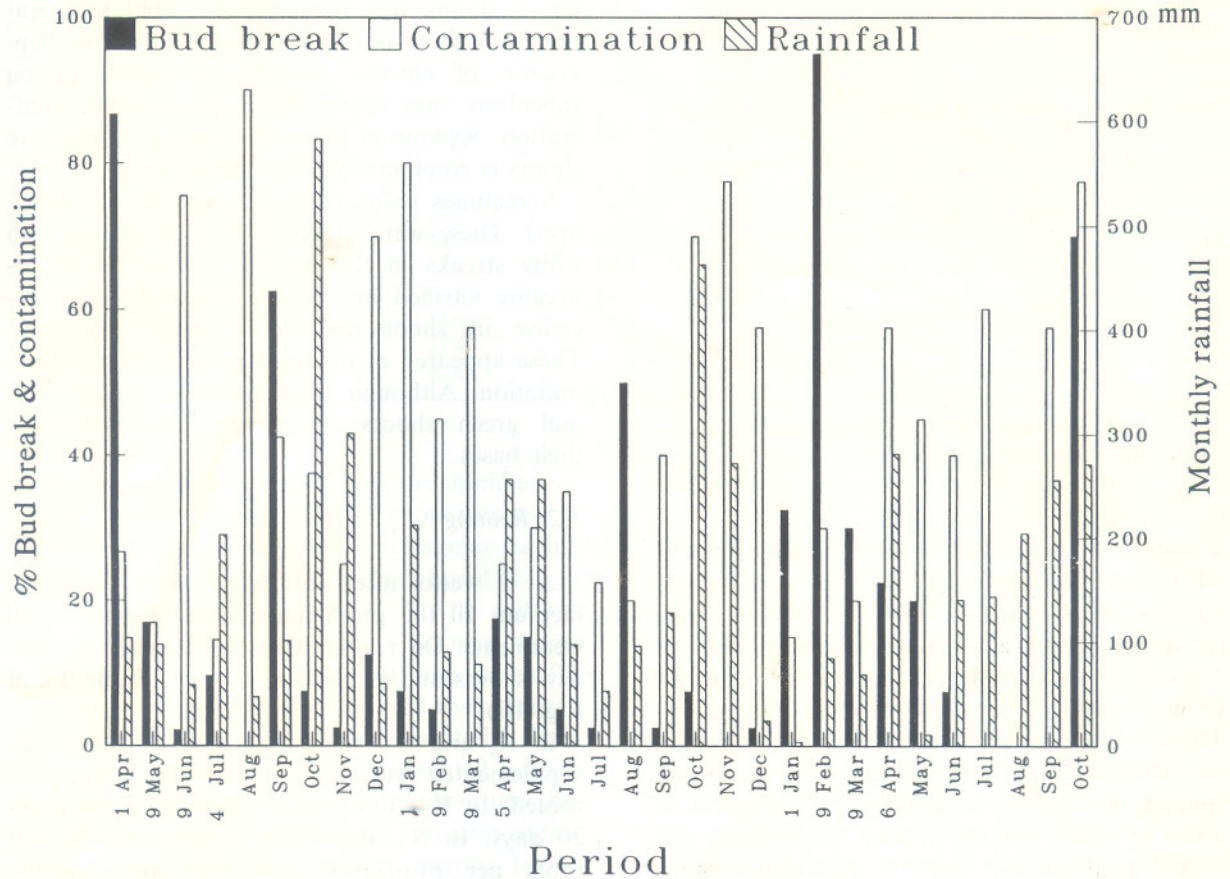

Fig. 2. Monthly variations in in vitro bud-break and contamination in nodal segments and the rainfall pattern from April 1994 to October 1996.

responded faster to axillary shoot proliferation than stationary cultures. During the initial stages there was browning of the medium and vitrification of shoots which were overcome by frequent transfer to fresh medium and the use of luffa pieces to support the shoots. Once the shoots recovered they continued to proliferate rapidly over a period of 1 year under investigation (Fig. 4B).

Table 1 shows the increase in the number of axillary shoots during the first four subculture cycles, C_1–C_4, in media, M_1–M_7, formulated to induce shoot proliferation. When the sprouted nodes were transferred to larger vessels, none of these media significantly induced a high shoot number to develop in the first cycle. But from C_2 there were significant differences in the media in their ability to induce shoots. M_1 and M_3 (the medium with no BAP and the semi-solid medium, respectively) consistently gave the lowest shoot numbers while M_2, M_4, M_6 and M_7 scored the highest shoot numbers in the different cycles and these were often not significantly different from each other. In the absence of coconut water (M_5) a significantly lower number of shoots developed. The effect of casein hydrolysate was manifested late when M_6 or M_8 scored the highest number of shoots in C_4. At this stage the responses in these two media were not significantly different from M_4 which had a higher level of BAP (12 mg l^{-1}).

Fig. 3 shows the growth curve of shoots in medium M_2. The lag phase of 65 days corre-

Table 1
Mean shoot number during four sub culture cycles in different media

Treatments	Mean shoot number			
	C_1	C_2	C_3	C_4
M_1	5.86[a]	9.57[c]	11.29[d]	12.33[c]
M_2	5.33[a]	15.83[a]	42.67[b]	65.17[ab]
M_3	6.00[a]	9.25[c]	11.63[d]	13.71[c]
M_4	5.50[a]	14.38[ab]	54.00[a]	70.00[ab]
M_5	5.57[a]	12.38[b]	23.50[c]	54.14[b]
M_6	5.50[a]	13.88[ab]	29.38[c]	71.00[ab]
M_7	5.86[a]	13.38[b]	29.88[c]	75.71[a]

The same letters within a column means treatments are not significantly different at $P = 0.05$.

sponded to the axillary shoot induction period when there was browning of the medium and vitrification of shoots. This was followed by a rapid growth phase when the shoot number increased. Subsequently a period of constant growth was achieved with periodic fluctuations. The growth rate calculated from the regression line showed a rate of 0.77 shoots per 13 day subculture period corresponding to a 1.8-fold increase in shoot number at each subculture.

A similar growth pattern was seen when a single shoot separated from a proliferating cluster of shoots was subcultured. Rapid proliferation took place only after a long lag phase. Separation of clusters into single shoots during subculture thus slowed the rate of shoot proliferation. Separation of nodes bearing at least five shoots is recommended for subculturing.

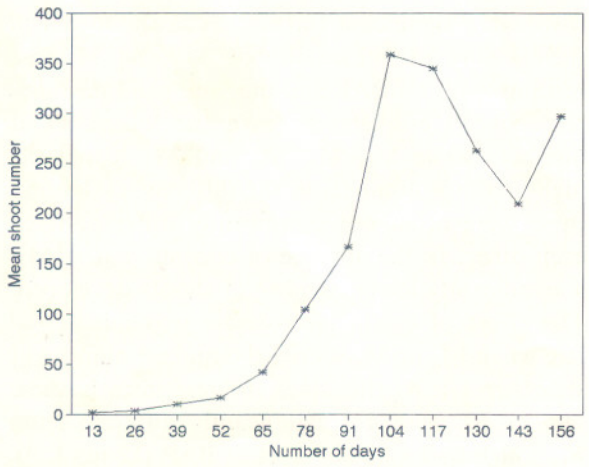

Fig. 3. Mean shoot proliferation per nodal segment at each subculture in medium M_2.

Sometimes chlorophyll deficient shoots developed. These were albino shoots or shoots with white streaks in the leaves. The albino shoots became vitrified and showed over a 4-fold increase in shoot number at each subculture. These appeared at different periods after culture initiation. Although these never recovered, normal green shoots sometimes developed from their bases.

3.2. Rooting

At 2 weeks after transfer to root induction medium all the propagules turned brown in all treatments. On regular transfer to fresh medium new shoots and/or roots developed (Table 2 and Fig. 4C).

In R_1 and R_2, where the basal medium was supplemented with IBA only, a few propagules rooted. In R_1, 30% of the propagules rooted in 20 days. In R_2, there was a larger number of roots per propagule, but only 10% of the propagules rooted. There was no further development of roots or shoots and the propagules did not survive.

Incorporation of 10 mg l^{-1} coumarin with 3 mg l^{-1} IBA (R_3) induced new shoots and roots. The rooting was higher (45%) than in R_1 or R_2 but root induction took a longer period of 76.5 mean days. All the rooted propagules developed new shoots in a mean number of 42.5 days. The mean root number was also higher with 4.3 roots per propagule. Although 65.5% of the propagules developed new shoots, all of them did not survive as some did not develop roots. When 2 mg l^{-1} IAA was incorporated (R_4) in addition to other media supplements in R_3, percentage rooting (37.5%) and the mean root number (2.6 per propagule) was less than in R_3. Activated charcoal (R_5) did not induce roots but a few propagules (17.5%) developed new shoots. At a higher level of IBA at 5 mg l^{-1} (R_6),

Table 2
Effect of treatments on rooting and development of new shoots

Medium	Roots			Shoots			Plantlets
	%	Mean No.	Mean days	%	Mean no.	Mean days	
R_1	30.0	1.3	20.0	0	0	—	—
R_2	10.0	3.0	20.0	0	0	—	0
R_3	45.0	4.3	76.5	65.5	2.2	42.5	45.0
R_4	37.5	2.6	57.1	60.0	1.7	48.6	35.0
R_5	0	0	—	17.5	1.6	48.9	0
R_6	50.0	2.5	53.9	37.5	1.7	46.2	30.0
R_7	77.5	6.0	27.4	72.5	2.1	47.3	70.0
R_8	32.0	2.8	42.8	28.0	2.6	44.6	24.6

rooting increased to 50% but the number of propagules that developed new shoots was less at 37.5% resulting in only 30% propagules having both new shoots and roots. The treatment R_7 had the highest percentage rooting (77.5%) and the highest number of propagules with new shoots and roots (70%). The number of days taken for induction of roots was also low and took a mean number of 27.4 days. In this treatment the shoot proliferation medium was supplemented with 3 mg l^{-1} IBA during the last two subcultures before the shoots were transferred to a root induction medium with IBA and coumarin. In R_8 where a similar treatment was given before transfer of propagules to the root induction medium with IBA, coumarin and IAA, only 24.6% plantlets with new shoots and roots developed and the mean number of days for rooting was 42.8.

3.3. Hardening and acclimatization

The shoots turned greener and elongated during in vitro hardening. In some propagules a large number of roots developed that elongated and matted. In these the new shoots showed very little or no further growth. Only 30% of the propagules survived in vitro hardening. All these well developed propagules that were transferred to the potting mix survived. These plants appeared normal and tillered after some time, indicating the development of rhizomes (Fig. 4D).

4. Discussion

4.1. Bud-break and proliferation

The major problem encountered in this attempt to develop a protocol for micropropagating *D. giganteus* was culture initiation. McClure [3], observed that in nature dormancy and breaking dormancy in buds of bamboo varied with their position in the plant, the season of the year and the species. The midculm nodes of secondary branches that were used have been reported to be the best explants for axillary shoot initiation [9,12]. In the present study these showed a seasonal response to in vitro axillary bud-break in the month just before the onset of the two major rainy seasons, the south-west and the north-east monsoons in Kandy. In *Bambusa vulgaris*, bud-break was induced more frequently throughout the year, and was strong and positively correlated with rainfall [13]. Saxena and Bhojwani [9] found that in vitro bud-break in *D. longispathus* took place during the monsoon period in the Haryana State of India. Civinova and Sladsky [14] suggested that the differential responses of cultured winter and spring buds of temperate species such as oak, aspen and black locust could be linked to the dynamics of endogenous substances in the tree. In tropical species including bamboo, changes in the environment such as those caused by rainfall or its onset may trigger the synthesis or breakdown of endogenous substances that control growth responses. The inverse relationship be-

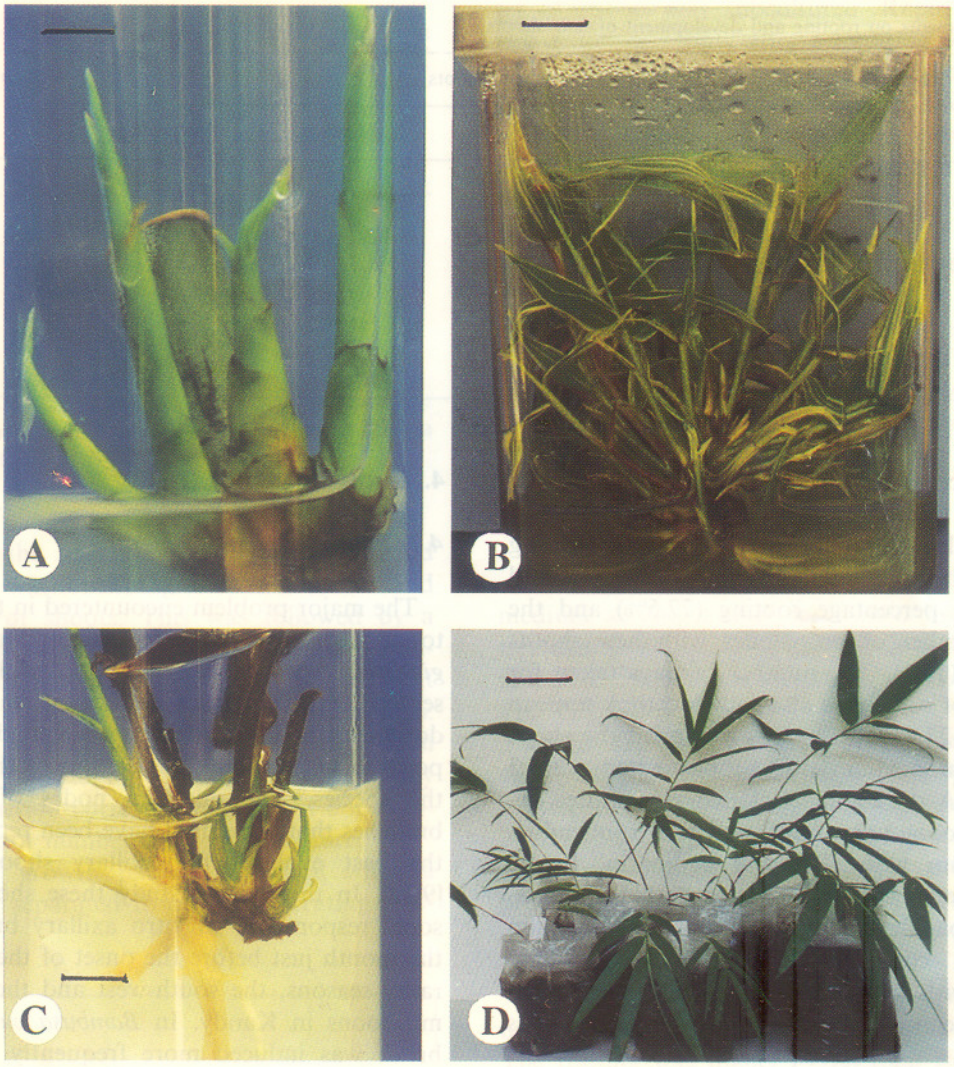

Fig. 4. (A) Axillary shoots sprouting from a single-node segment after 1 month in culture. Scale bar = 25 mm; (B) Profliferating axillary shoots in liquid medium ready for subculturing (after 13 days of culture). Scale bar = 1 cm; (C) Roots and new shoots developing in a cluster of axillary shoots after 2 weeks in rooting medium. Scale bar = 25 cm; (D) Established plants of *D. giganteus*, 2 months following transplanting in soil. Scale bar = 10 cm.

tween in vitro bud-break and systemic culture contaminants in *D. giganteus* in the present study and in *B. vulgaris* [13] indicates that these substances could also control the growth of microflora harboured within the tissues. In *D. strictus* too culture initiation of mature field grown culms was difficult due to contaminants [4,15]. There are reports that the season of explant collection influenced the rooting responses. Chaturvedi et al [16] found that in *D. strictus* the rooting response was better in cultures initiated from explants collected in July–August than in those collected in September–October.

Induction of axillary shoots took place during the lag phase that lasted 65 days. Nadgir et al [15] and Chaturvedi et al [16] reported that the few shoots that regenerated from the mature nodes of *D. strictus* senesced and turned brown even after

transfer to a fresh medium. Kumar [17] who initiated axillary shoot cultures of *D. strictus*, *B. bambos* and *Thyrsostachis siamensis* suggested that browning and death was due to the lack of meristematic tissues at the base of the cultures. We observed that if the sprouted nodes were allowed to remain in the culture tubes, even with frequent transfer to fresh medium browning of shoots occurred, but the removal of the browned leaves and leaf sheaths and transfer to larger vessels containing liquid medium stimulated axillary shoot proliferation. The removal of leachates, good aeration and replenishment of nutrients during the lag phase stimulated growth. Saxena and Bhojwani [9] controlled vitrification of shoots by supporting them on pieces of foam. We used the natural plant material from luffa and it provided better aeration and flow of medium through the larger pores of its matrix. According to Chang-Ho and Martinez [18] luffa did not have any harmful effects even on isolated cells.

A relatively high level of BAP (6–12 mg l^{-1}) in a liquid medium brought about a high rate of shoot proliferation. High levels of BAP have been used by Saxena and Bhojwani [9] to induce axillary shoot proliferation in *D. longispathus* and by Prutpongse and Gavinlertvana [8] who micropropagated over 50 species of bamboo. BAP is known to be a difficult substance for absorption by plant cells. In liquid cultures a greater uptake of BAP was possible due to the larger surface of absorption provided by the partially submerged shoots. In the present study, the addition of CH to the medium with 6 mg l^{-1} BAP had the same effect as the higher level of BAP (12 mg l^{-1}) suggesting the possibility of CH substituting BAP at high levels. The promotory effect of coconut water on bamboo shoot cultures have been reported by others. Saxena and Bhojwani [9] found that 10% CW enhanced shoot multiplication in *D. longispathus*. Nadgauda et al [19] and Rajapakse [12] induced shoot proliferation in seedling derived cultures of *B. arundinacea* (*B. bambos*) and *D. brandisii* and in *D. giganteus*, respectively, in media supplemented with CW, from which in vitro flowers were also induced. We did not observe in vitro flowering in *D. giganteus* in the present study but proliferating *D. asper* shoots initiated from seeds, cultured in the same medium with CW flowered (unpublished data). CW and casein hydrolysate are undefined substances containing nutrients. Coconut water is reported to have cytokinins [20]. Their effect on shoot proliferation may have been due to the availability of nutrients and growth regulators.

4.2. Rooting and acclimatization

Saxena and Bhojwani [9] achieved a high percentage (73%) of rooting in *D. longispathus* in a medium incorporated with IBA, IAA and coumarin. In our study coumarin was essential for the development of roots and shoots when supplemented with IBA in the rooting medium. The ratio of IBA to coumarin was also important to get a high percentage of rooting. Other phenolic compounds such as caffeic acid and phloroglucinol were reported to regulate rooting and shoot proliferation [20,21]. These have been used to induce rooting in species that are difficult to root [16,22,23]. Most of these have involved a synergism with added auxins and it is reported that they act as substrates for IAA-oxidase, enhancing endogenous levels of IAA. Incorporation of IAA to the rooting medium with IBA and coumarin however reduced rooting in the present study.

Chaturvedi et al [16] reported that prolonged incubation in the rooting medium brought about abnormal knotting of roots caused by the inhibitory effects of auxins on their further development. Our observations of elongation and matting of roots with suppressed shoot growth that resulted in low survival during in vitro hardening may have been due the long period of incubation in the rooting medium (Table 2). Further studies on improvement of rooting and survival of rooted propagules are under investigation.

The appearance of albino shoots in bamboo is common. Saxena [6], attributed this to the conversion of chloroplasts to amiloplasts at higher sucrose levels. The recovery of normal shoots from these is worth investigation as they showed a 4-fold rate of shoot proliferation which was more than double that of normal shoots.

We report here for the first time a complete protocol for the in vitro micropropagation of *D.*

giganteus by continuous and rapid proliferation of axillary shoots followed by a high percentage (77.5%) of rooting and establishment of plants in soil. The use of pre-existing axillary buds for propagation reduces the possibility of any variation among the progeny in a clone and therefore safely applied for rapidly cloning selected field grown clumps. It is worth noting that axillary bud break is strongly influenced by climatic factors and the season for culture initiation could vary with localities. The influence of these seasonal effects on axillary shoot proliferation on in vitro maintained cultures is also worth investigation.

Acknowledgements

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