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In vitro micropropagation of *Bambusa balcooa* Roxb. through nodal explants from field-grown culms and scope for upscaling

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An efficient and reproducible *in vitro* procedure for large-scale multiplication of *Bambusa balcooa* Roxb. has been described. Multiple shoot formation (8–10) was observed from excised tender node (12–18 mm in length) containing axillary bud isolated from secondary branches of 1½-yr-old culms, when implanted on Murashige and Skoog (MS) medium containing 6-benzylaminopurine (BAP, 1.0 mg l^{-1}). Continuous shoot proliferation, tenfold, every 4 weeks was achieved by sub-culturing shoot clumps (2–3 shoots/cluster) in BAP (1.0 mg l^{-1}) fortified medium. Seventy-five per cent of shoots could be rooted efficiently on excised propagules when transferred to MS medium supplemented with BAP (1.0 mg l^{-1}) and naphthaleneacetic acid (3.0 mg/l). Using this protocol, the first batch of plantlets can be obtained after six months from the initial establishment and then every month new batches of plantlets can be regenerated, depending upon the availability of efficient shoots. After hardening, rooted plantlets were successfully transferred to the soil in polythene sleeves with over 90% survivability and recorded normal growth. Macroproliferation can be done after a period of 5 months from the soil transfer, by which propagules can be increased by more than three times. The tech-economic viability was studied with consideration that the production will be executed in a well-established plant tissue culture laboratory. Factors for characterization at various stages for tissue culture work of *B. balcooa* have also been studied and evaluated.

Keywords: *Bambusa balcooa*, culms, micropropagation, nodal explants.

BAMBUSA BALCOOA Roxb., an indigenous widespread bamboo of North East India, is considered as the best, tallest, strongest, and highly durable, and is utilized mostly for structural use and pulping. It has maximum girth of culms and rind thickness among all species of the genus *Bambusa.* The species is also valued for its edible tender shoots, mainly for food and pickle industry. But, the rapidly increasing bamboo-based industries have resulted in severe loss of forest stocks, which demands adequate re-plantation of elite bamboo species to strengthen the raw material stock.

Seed production in *B. balcooa* is generally not recorded after gregarious flowering[1]. Moreover, its flowering cycle is reported as 40–100 years and only once during its life time[2]. In this regard, large-scale elite bamboo replantation necessitates sufficient ready stock of planting material, for which application of plant tissue culture technique is the only justified methodology. Alternative methods of vegetative propagation using offsets or culms are slow, expensive and also cumbersome to handle and liable to desiccation.

Research on tissue culture of bamboo was reported by Alexander and Rao[3] on embryo culture of *Dendrocalamus strictus*. Since then, starting from Huang and Murashige[4], a good start has been made on bamboo tissue culture and a number of laboratories have begun to make progress. But a majority of the successful achievements are made through somatic embryogenesis and/or regeneration from juvenile seedlings. Consequently, reports on regeneration from mature/field source materials followed by upscaling work for commercial use are limited. To the best of our knowledge, reports on clonal multiplication from six species of *Dendrocalamus* and three species of *Bambusa* are available, among which large-scale field transfer (quantity-wise, total 30,000 nos) was reported for *Dendrocalamus asper*, *D. membranaceus*, *D. strictus*, *D. giganteus*, *Bambusa arundinacea* and *B. vulgaris*[5].

However, reports on *in vitro* regeneration of *B. balcooa* are limited, except those of Das and Pal[6], and Gillis *et al.*[7], where protocols about field transfer and survivability are not available. The present study reports an upscaling procedure for mass multiplication of this commercially important bamboo species.

Tender nodes (12–18 mm in length) obtained from minor branches of 1½-year-old culms of *B. balcooa* regenerated from approximately 80-year-old elite plants were used as explants.

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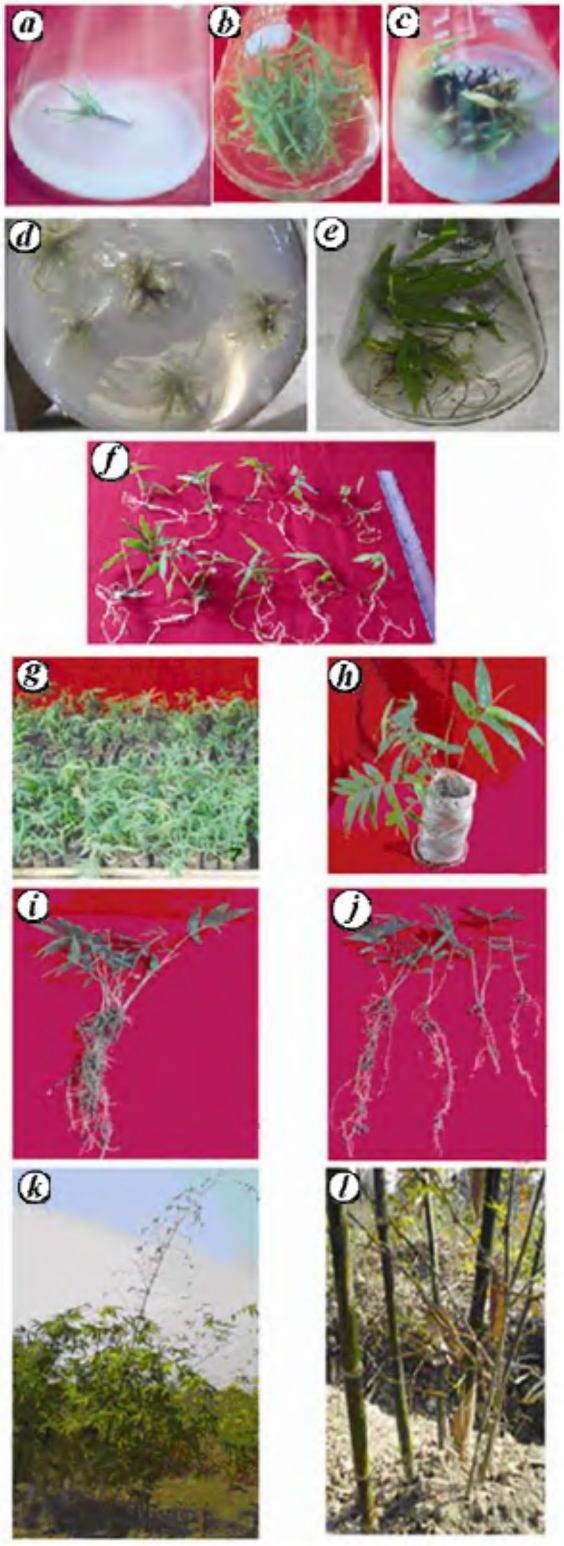

Figure 1. Stages in the micropropagation of *Bambusa balcooa*. ***a***, Shoot initiation from axillary bud breaking. ***b***, Shoot multiplication. ***c***, Browning of *in vitro* shoots. ***d***, Root initiation in solid medium. ***e***, Hardening of rooted shoot on liquid medium. ***f***, Rooted plantlets after hardening. ***g***, *In vitro*-raised plantlets in poly sleeves. ***h***, Four-month-old plant in poly sleeve. ***i***, Plant with tillers after removing the poly sleeve. ***j***, Splitting of shoots (macro proliferation). ***k***, Three-year-old *in vitro*-raised bamboo plants in field. ***l***, Culms girth of 3-year-old bamboo with tillers.

After removal of leaf sheath, the node containing the axillary bud was dipped in 5% (v/v) Tween 20 solution for 3 h followed by thorough washing under running tap water for 20 min. Explants were then disinfected with 0.1% mercuric chloride solution for 5–7 min and rinsed thoroughly with sterile distilled water prior to culture *in vitro*. Pretreatment of the explants with a mixture of an antifungal and an antibiotic (0.1% solution each of mancozeb and gentamycine) was done, depending upon the time of collection of the explants.

For initial axenic establishment, nodal explants were placed on 25 ml of culture media in culture tubes (25 × 150 mm OD × length) and plugged with cotton wool. Murashige and Skoog (MS) medium[8] containing 30 g l^{-1} sucrose and 8.0 g l^{-1} agar was used as the basal medium. Additional supplements, viz. 6-benzylaminopurine (BAP), kinetin (Kn), 2-isopentenyl adenine (2ip, Hi Media Chemicals, India) were tested separately.

All cultures were maintained under 16 h photoperiod with a light intensity of 10 µmol m^{-2} s^{-1} (cool white fluorescent light) and a temperature of 25 ± 2°C.

For axillary bud break, preliminary experiments were conducted by culturing node explants in MS medium containing two different concentrations of BA. Thereafter, proliferated shoot clumps (8–10 nos) from primary cultures were divided into clusters of shoots (2–3 shoots/cluster) and these propagules were sub-cultured again in MS medium containing various cytokinins, viz. BA, Kn and 2ip individually, at different concentrations to select the best and optimal hormonal supplement for shoot multiplication. After selecting the best treatment, shoot clumps were cut into groups of 2–3 shoot clusters and were sub-cultured into the same optimal medium regularly at an interval of 3–4 weeks. The process was repeated to get the desired number of shoots.

Shoot clusters (two shoots, 2–3 cm long) were transferred for rooting into a root-inducing, hormone-fortified MS medium such as naphthaleneacetic acid (NAA), indole-3-butyric acid (IBA), indole-3-acetic acid (IAA), and indole-3-propionic acid (IPA) for screening the best and optimal one. The number of individual shoot clusters (propagules) which responded to rooting was counted and expressed as per cent rooting.

The hardening procedure followed involved few steps process, i.e. transfer of rooted shoot to MS basal liquid medium for 15–20 days, followed by exposing the rooted shoots in half-strength MS basal liquid medium for another 15 days. Then plantlets were kept in unsterilized filtered water for a total of 30 days; 15 days in the culture room followed by another 15 days in ambient room temperature (28 ± 2°C) conditions. During that stage rooted shoots were washed thoroughly in running tapwater to remove the adhered agar from the roots and the caps of the flasks were removed.

Hardened plants were planted in polythene sleeves containing soil : sand : cow-dung mixture (1 : 1 : 2) and

kept in a netted greenhouse for acclimatization before transferring to the field.

Macro shoots that regenerated after 3–5 months of transfer of micro shoots in polybags were split individually, each containing intact roots having root hairs and a well-developed shoot system. These macro shoots were replanted in the soil in polythene sheets and were kept in shade and well-watered conditions.

Different factors associated with various phases of successful establishment of plant tissue culture of *B. balcooa* were characterized taking data at various steps. Results were inferred from the data of 20×2 observations of five replicates.

The sterilization procedure followed in this study yielded 65–70% axenic cultures. However, in certain lots the percentage of microbial contamination was recorded to be more than 50. Re-sterilization of the explants by treating with 0.1% solution containing an antifungal (mancozeb) and an antibacterial agent (gentamycine) for 5 min helped in the recovery of more cultures. However, due to availability of sufficient source material, rejection of the infected material was preferred. August–September was found to be the best season for collection of explants.

Axillary bud break was noticed after 10 days of incubation in all the healthy cultures. A cluster of shoots at varied number and shoot length was recorded within 20 days from the cultures grown on MS medium supplemented with two different concentrations of BA (1.0 and 5.0 mg l^{-1}). Percentage of culture response, time taken for bud break, number of shoots initiated and shoot length were also found to vary in the two different concentrations of BA. Once the axillary bud break was achieved, shoot proliferation could be increased and maintained by regular sub-culturing at 4 weeks interval on MS medium supplemented with lower concentration of BA (1.0 mg l^{-1}). Sub-culturing at higher concentrations of BA-fortified medium caused stagnant and unhealthy growth of the shoots, leading to ultimate death of the cultures. Among the three cytokinins tested, BA was selected as the most suitable hormone to induce shoot multiplication. Incorporation of 1.0 mg l^{-1} BA to the medium promoted the highest shoot multiplication rate of 20.0 ± 3.8 with shoot length of 3.3 ± 0.2 cm (Figure 1 *a*; Table 1). Increase in the level of BA (2.0, 3.0 and 5.0 mg l^{-1}) in the medium had the tendency for regeneration of more shoots, but regenerated shoots had shorter shoot length and leaf lamina and were also almost inconspicuous and condensed. This may be due to the supra-optimal concentration of the hormones which are toxic, and hence shoots became smaller with unexpanded leaves. Effectiveness of the medium containing BA alone or in combination with an auxin was reported earlier in a number of Bamboo species[9–12], but unlike the present study, they had reported multiplication efficiency at higher doses of BA (5.0–10 mg l^{-1}). However, considering all aspects, 1.0 mg l^{-1} BA proved to be ideal for healthy shoot multiplication and growth of *B. balcooa* (Figure 1 *b*).

Shoot regeneration was also achieved from the medium containing Kn (1.0, 2.0 and 4.0 mg l^{-1}), but the rate of multiplication was low. Moreover, within 15–20 days of culture, the leaves turned brown and led to the ultimate death of the shoots. Effectiveness of 2ip was almost similar to that with Kn. Shoots when cultured on the basal medium initially showed increase in length, but after few days it stopped.

Apart from optimal supplement of the medium towards production of healthy shoots, regeneration capacity of the *in vitro* shoot was found to be dependent upon the size and number of shoots/clump and also the time of sub-culturing.

Clusters having more or less 2–3 shoots were observed to affect the multiplication potentiality (Table 2). Moreover, 25–30 days was found to be the best gestation period for recycling of shoots. Delaying of sub-culturing period resulted in gradual browning of the shoots followed by total blackening, mostly from the basal portion (Figure 1 *c*). Hence sub-culturing period was recorded as the most crucial factor for obtaining optimal and desired level of regeneration of shoots. Rate of shoot multiplication remained constant up to 3 years of the study period.

The results of root induction achieved by culturing shoot clumps (two shoots/clump) on different auxins with

Table 1. Morphogenic response of nodal explants of *Bambusa balcooa* at different concentrations of cytokinins supplemented to MS medium

Growth regulator (mg l^{-1})	Shoot numbers/culture	Shoot length (cm)	Total leaf number
MS basal	1.5 ± 0.5	1.1 ± 0.1	2.3 ± 1.2
Kin 1.0	2.3 ± 1.2	1.4 ± 0.4	3.8 ± 0.9
Kin 2.0	2.3 ± 2.7	1.5 ± 0.4	5.0 ± 2.0
Kin 4.0	5.0 ± 2.9	1.5 ± 0.3	6.0 ± 1.9
2ip 1.0	1.5 ± 0.5	1.1 ± 0.1	3.8 ± 0.9
2ip 2.0	2.5 ± 1.6	1.1 ± 0.7	5.3 ± 1.4
2ip 4.0	1.8 ± 0.9	1.6 ± 0.6	5.0 ± 2.0
BA 0.5	7.3 ± 1.6	1.1 ± 0.1	8.3 ± 0.9
BA 1.0	20.0 ± 3.8	3.3 ± 0.2	26.0 ± 4.0
BA 2.0	7.3 ± 1.6	1.4 ± 0.2	7.8 ± 1.6
BA 3.0	8.8 ± 0.9	1.3 ± 0.4	8.8 ± 0.9
BA 5.0	29.5 ± 3.9	1.5 ± 0.4	15.3 ± 5.2

Table 2. Effect of multiplication and growth on culturing varied number of shoot clumps

No. of shoots/ clump	Shoot number	Shoot length (cm)	Leaf number
1	19.3 ± 1.5	3.0 ± 1.2	39.3 ± 10.4
2	20.0 ± 3.8	3.3 ± 0.2	45.6 ± 13.1
3	18.3 ± 1.3	2.6 ± 0.8	26.3 ± 13.5
4	17.0 ± 4.0	2.9 ± 0.9	33.8 ± 15.5
5	26.3 ± 2.5	3.0 ± 0.9	33.3 ± 12.6

Table 3. Effect of BA and auxins on rooting of *in vitro* shoots of *B. balcooa*

BA (1.0 mg l^{-1}) + auxins (mg l^{-1})						
NAA	IAA	IBA	IPA	No. of roots	Root length (cm)	Percentage of rooting
0.5				–	–	–
1.0				0.4 ± 0.7	0.4 ± 0.8	25
2.0				0.5 ± 0.9	0.4 ± 1.3	25
3.0				2.1 ± 2.0	1.5 ± 1.1	75
4.0				0.5 ± 0.5	0.7 ± 0.9	50
5.0				0.5 ± 0.8	0.3 ± 0.4	37.50
	0.5			–	–	–
	1.0			–	–	–
	2.0			–	–	–
	3.0			–	–	–
	4.0			–	–	–
	5.0			–	–	–
		0.5		–	–	–
		1.0		–	–	–
		2.0		–	–	–
		3.0		–	–	–
		4.0		–	–	–
		5.0		–	–	–
			0.5	–	–	–
			1.0	–	–	–
			2.0	–	–	–
			3.0	–	–	–
			4.0	–	–	–
			5.0	–	–	–

Table 4. Estimated cost and profit for production of *B. balcooa* plantlets

Parameter	Cost involvement
Manpower	25% of total cost
Utility	36.7% of total cost
Equipment use	3.3% of total cost
Consumable	25% of total cost
Office expenditure	6.7% of total cost
Miscellaneous	3.3% of total cost
Total production cost	Rs 6.0 lakhs
Total sales realization @ Rs 8.00/plantlet	Rs 8.0 lakhs
Profit	Rs 2.0 lakhs/annum

varied levels in combination of BA (1.0 mg l^{-1}) are given in Table 3. Among the four auxins tested, NAA was found to be the most effective for normal and efficient root formation, which was observed at the base of the shoots after 15–25 days of culture period. Within 40 days, a well-developed root system was seen in almost 75% of the cultures. No root formation was observed when shoots were cultured in MS media containing IAA, IBA and IPA. Combination of 1.0 mg l^{-1} BA and 3.0 mg l^{-1} NAA induced the highest frequency of rhizogenesis (Figure 1 *d*) with maximum root number (2.1 ± 2.0 roots per shoot) and average root length (1.5 ± 1.1 cm) along with simultaneous shoot growth (Table 3). Higher concentration of auxins (more than 3.0 mg l^{-1}) showed reduction in rooting number. Singular supplementation of NAA (3.0 mg l^{-1}) resulted in good response towards root formation, but shoot growth was retarded, resulting in ultimate browning to blackening of the shoots and the leaves. Similar to our findings, Arya *et al.*[5] also reported favourable response of NAA for *in vitro* rooting of *B. arundinacea.* However, Das and Pal[6] reported effectiveness of IBA for root initiation in *B. balcooa*, which we have not recorded in our study.

After root initiation, when the roots attained a length of 0.5–1.0 cm, the shoots were transferred to MS basal liquid medium for 15–20 days for root elongation, which was followed by exposing the rooted shoots in half-strength MS basal liquid medium for another 15 days (Figure 1 *e*). After this, the plantlets were kept in unsterilized filtered water for a total 30 days, the first 15 days in culture room followed by another 15 days in ambient room temperature (28 ± 2°C). During this phase, white-coloured, new secondary roots developed, which resulted in higher survival frequency. During this stage, rooted shoots were washed thoroughly in running tapwater to remove adhered agar from the roots and were transferred to the poly sleeves (Figure 1 *f*). More than 90% survivability was recorded from well-established rooted *B. balcooa* shoots (Figure 1 *g*). Hardened plants were planted in polythene sleeves containing 1 : 1 : 2 soil : sand : cow-dung mixture and kept in netted poly-house for acclimatization before transferring to the field (Figure 1 *h*).

In general, rooting and transplantation of plantlets to the field is the most important and difficult task in micro-

Table 5. General description of mother plant/source material for axenic establishment

Factors	Standard
Suitable material for axenic establishment	Node containing axillary bud from secondary and tertiary branches (1½-year-old)
Nature of initial material	Healthy, slightly tender branches, free from inborn microbial infestation
Quality of mother stock	Average yield and growth, continuously for 10 years

Table 6. General description in hardening stage

Factors	Standard
General appearance	Healthy, greenish
Height of plantlets	2.5–6.0 cm
Tillers/plantlet	1–4
Leaves/plantlet	3–7
Visible nodes/plantlet	3–5
Nature of roots	Fibrous type
Number of roots	1–4 with initiation of white-coloured secondary roots
Length of roots	3–10 cm

Table 7. General description of tissue culture-raised plants

Factors	Standard	
	4–6-month-old	10–12-month-old
General appearance	Healthy, greenish	Healthy, greenish
Height of plantlets (cm)	15–22	20–35
Tillers/plantlet	1–6	1–8
Leaves/plantlet	12–18	20–25
Visible nodes/plantlet	5–6	10–12
Insect/disease pest infestation	Less than 5%	Less than 5%

propagation[13]. Except for a few reports of bamboo, viz. *D. strictus*[14,15], all the others involved a callus phase which could lead to loss of genetic fidelity.

Macroproliferation can be suitably adapted for the well-established *B. balcooa* plantlets after 3–5 months of transfer of the micro plants. By splitting the rooted tillers it was possible to increase the production up to three times (Figure 1 *i* and *j*). Recycling the macroproliferation procedure resulted in continuous plantlet production in *B. balcooa*.

Economic/commercial viability of this protocol was estimated for production in a well-established plant tissue culture laboratory, where adequate facilities are available. The selling cost of the plant was considered as Rs 8.00 (Indian rupee)/plant (Table 4). Total production capacity was considered as one lakh of plantlets/year in five batches @ 20,000 plantlets per batch with 300 working days in a year.

In conclusion, we have described a continuous mass multiplication protocol of *B. balcooa*, which is cost-effective, easy to raise, economic to adopt and convenient to transport, thus serving commercial interest. Moreover, after 3 years of study in both laboratory and field conditions, it was observed that after successful field transfer, *in vitro*-raised plantlets attained a better plant growth and culms girth at the experimental farm of our institute (Figure 1 *k* and *l*). We have characterized a few factors for certification of tissue culture work on *B. balcooa* (Tables 5–7).

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