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In vitro propagation of *Bambusa vulgaris* cv. Wamin by axillary shoot proliferation

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Abstract

Development of rapid *in vitro* cloning through axillary shoot proliferation from mature clump for the production of quality planting material has been described. Explants from 12-year old field grown clump of *Bambusa vulgaris* cv. Wamin (BVW) from Institute of Wood Science and Technology, Bangalore were used for initiation of cultures. Axillary bud break was achieved 95% of cultures in BVW on MS (Murashige and Skoog) liquid medium supplemented with additives and various combination of auxins IAA, NAA(0.25mgl^{-1}) and cytokinins viz., Kinetin, BAP (0.5-2.5mgl^{-1}), TDZ (0.05-0.25mgl^{-1}) . Additives ascorbic acid (50mgl^{-1}), citric acid (25mgl^{-1}) and cysteine (25mgl^{-1}) were added during shoot initiation and shoot multiplication. The proliferated shoots (2-3 weeks old) showed rapid multiplication with 27-28 shoots/clump at fifth passage on MS liquid medium supplemented with additives and BAP (5.0mgl^{-1}). A clump of atleast 3 shoots were used for *in vitro* root induction in half- strength MS medium with NAA (1.0mgl^{-1}). Field transfer of *in vitro* rooted plantlets were carried out by transferring the plantlets to pots containing sand: soil: FYM (farmyard manure) in 1:1:1 ratio, maintained in nursery at 30±2°C and relative humidity of 80±5%. Micropropagated plants achieved a height of 30-35 cm with 3-4 tillers (shoots) with miniature rhizome within four months.

Keywords: Bamboo, *B. vulgaris* cv. Wamin, Micropropagation, axillary shoot proliferation

Abbreviations used

BAP: N6-Benzyl amino purine	IAA: Indole-3-acetic acid	IBA: Indole-3-butyric acid	Kn: Kinetin
MS: Murashige and Skoog medium	NAA: Naphthalene acetic acid	TDZ: Thidiazuron	

1. Introduction

Bambusa vulgaris Schrad. ex J.C.Wendl. f. waminii T.H.Wen (BVW) belonging to the family Poaceae is commonly known as "Pitcher's bamboo" and "Buddha's belly bamboo"[1]. It is medium-sized clump forming bamboo found in the Northern Shan States of China. The plant is cultivated in North-eastern parts of India such as Orissa, Arunachal Pradesh, Manipur[2] and considered as unique ornamental plant because of the presence of dramatic swollen internodes. It is one of the important commercial species of bamboo used for making miniature landscapes and decorative materials, reported to be associated with erosion control. Propagation of BVW by seeds is limited as there is no report on its flowering[3]. Vegetative propagation by making use of offsets and culm cuttings has proved to be unsuccessful. Hence propagation and mass-multiplication of BVW require special attention and tissue culture method seems to be most effective approach. Research on tissue culture of bamboo was reported on embryo culture of *Dendrocalamus strictus*[4]. In bamboo, majority of the successful achievements are made through micropropagation studies[5-10]. Recent report on *in vitro* regeneration and *in vitro* flowering of BVW[11] is also available.

The present investigation describe the procedure for micropropagation of BVW by enhancing the proliferation of axillary bud from mature nodal explants collected from field grown clumps.

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2. Materials and Methods

2.1 Establishment of cultures

Nodal segments having single axillary bud along with part of internodal segments on either side were obtained during March-April from newly emerging lateral branches from 12 year-old clumps of BVW maintained at Bambusetum, Institute of Wood Science and Technology (IWST), Bengaluru, Karnataka, India. The plant material was authenticated by the authorized taxonomist, FRLHT, Bangalore. The nodal segments were thoroughly wiped all along their surface using cotton swab dipped in 70% (v/v) ethanol. The nodal segments were cut into pieces of 2.5-3.0cm length and 2-3mm diameter and subjected to 0.01% (v/v) liquid detergent Tween 80 followed by 0.01% (w/v) Bavistin (systemic fungicide), 70% ethanol (v/v) (for 20 s) and mercuric chloride (0.1% w/v) individually treated for 5 min respectively with intermittent washing with sterile distilled water. The nodal segments were then aseptically cultured onto 10-15 ml of liquid Murashige & Skoog's medium[12] containing 3% sucrose with different combinations of auxins and cytokinins. The auxins used were NAA and IAA at 0.25mgl^{-1} and cytokinins like BAP and Kn were used at 0.5-2.5mgl^{-1} and TDZ used at 0.05-0.25mgl^{-1}. Ascorbic acid (50mgl^{-1}), citric acid (25mgl^{-1}) and cysteine (25mgl^{-1}) were added as additives in the medium. The pH of the medium was adjusted to 6.3 prior to autoclaving at 15 psi for 20 min. The cultures were incubated at 25±2°C under 16h photoperiod with light intensity of 3000 lux. The chemicals used for the study were of analytical grade (Himedia).

The cultures were observed after 2 weeks for the number of shoots proliferated after the axillary bud break and the average length of shoots were recorded. The shoots proliferated from the nodal explant were excised and cultured on the media supplemented with different combinations of cytokinins (BAP, TDZ, Kn) for further multiplication of shoots.

2.2 In vitro *shoot multiplication*

The shoots obtained from nodal explant were excised and cultured on MS liquid medium containing ascorbic acid (50mgl^{-1}), citric acid (25mgl^{-1}) and cysteine (25mgl^{-1}) additives, auxin NAA 0.25mgl^{-1} and different concentrations of cytokinin BAP 2.5-5.0 mg^{-1} and TDZ 0.25-0.5mgl^{-1} for 5 passages. Sub culturing was carried out at an interval of 2 weeks on fresh liquid medium. The average number of propagules derived at the end of each subculture cycle out of a single propagule (5-6 shoot clusters) was regarded as rate of multiplication or the multiplication fold.

2.3 In vitro *rooting and Hardening*

The shoots were separated into clusters bearing 2-4 shoots and transferred to half strength MS agar gel medium incorporated with different auxins (NAA, IAA and IBA 0.5- 1.0 mgl^{-1}). After 8 weeks of culture, the number of roots per propagule, root length and rooting percentage were recorded.

The *in vitro* rooted plantlets with each plantlet having 2-4 tillers with shoot length of 5.0-6.0cm were carefully removed from the culture bottle. The roots were thoroughly washed under tap water to remove the adhering medium. The plants were kept in 0.1% (w/v) Bavistin for 5-10 min before transferring to polybags. Hardening of *in vitro* rooted plantlets were carried out by transferring the plantlets to pots containing sand: soil: FYM (farmyard manure) in 1:1:1 ratio and maintained in the green-house at 30±2°C with relative humidity of 80±5%.

2.4 Statistical treatment

Each treatment consisted of ten explants cultured separately in culture tubes for *in vitro* axillary bud proliferation. For *in vitro* shoot multiplication, each treatment consists of five culture bottles, each containing shoot clump of 5-6 shoots and for *in vitro* rooting, each treatment consists of four culture bottles, each containing 2-3 shoots / clump . The experiments were performed in triplicates.

Analysis of variance (one way or single factor) with standard error were determined followed by the least significant difference (LSD) test at p = 0.05 level to compare means.

3. Results and Discussion

3.1 Plant material

Surface sterilization of nodal segments of BVW with 0.1% mercuric chloride for 5 min and 70% alcohol for 20 s reduced bacterial contamination and yielded 95% aseptic cultures. Sterilization of the explants with 0.1% Bavistin for 5 min prevented fungal contamination. Mercuric chloride and 70% ethanol have been used to disinfect the explants of *B. wamin*, *B. balcooa* and *Dendrocalamus farinosus* respectively [9,10,13].

In *B. wamin* March - April was found to be the best season for collection of explants[9]. Even in *B. nutans* the bud break was highest in summer season followed by winter season[14]. Pre-summer appeared to be the best in terms of least phenol production together with frequency of response in *D. asper*[15]. However, maximum bud break in *B. balcooa* was observed in the month of October[16].

3.2 Shoot initiation

Among the various concentrations and combinations of auxins NAA ,IAA- 0.25mgl^{-1} and cytokinins BAP, Kn 0.5-2.5mgl^{-1}, TDZ 0.05-0.25mgl^{-1} supplemented with additives like ascorbic acid (50mgl^{-1}), citric acid (25mgl^{-1}

$^{-1}$) and cysteine (25mgl^{-1}) tried on MS liquid medium, NAA (0.25mg l^{-1}) with BAP (2.5 mg l^{-1}) induced bud break in 94- 95% of the cultures within 2 weeks (**Table I**). Although bud break appeared in the first week of culture, sufficient number of shoots were obtained only after 2 weeks (**Fig. I**). The average number of shoots induced per nodal explant were 6.63 with shoot length 6.45 (**Table I**). The combined use of NAA and BAP proved better than Kn and TDZ for high frequency of shoot initiation and subsequent shoot growth. Incorporation of additives like ascorbic acid (50mgl^{-1}), citric acid (25mgl^{-1}) and cysteine (25mgl^{-1}) in the medium improved rate of shoot induction, number of shoots/explant and shoot growth. Additives are known to have antioxidant properties and minimize leaching and browning problem associated with field-grown explants. The effect of antioxidants in *B. pallida* is in agreement with the findings[17].

3.3 Shoot multiplication

Among the various concentrations and combinations of cytokinins BAP 2.5-5.0mgl^{-1}, TDZ 0.25-0.50mgl^{-1} supplemented with additives like ascorbic acid (50mgl^{-1}), citric acid (25mgl^{-1}) and cysteine (25mgl^{-1}) tried for shoot multiplication, NAA (0.25mg l^{-1}) and BAP (5.0 mg l^{-1}) was most suitable and showed an average multiplication rate of 4.72-fold . Incorporation of 5.0 mgl^{-1} BAP to the medium promoted highest shoot number of 27.77 with shoot length of 7.36 cm (**Table II**). Incorporation of additives ascorbic acid (50 mgl^{-1}), citric acid (25mgl^{-1}), cysteine (25 mgl^{-1}) proved effective for shoot multiplication of shoots. Sub-culturing was found crucial and essential within 2 week period for 5 passages for maintaining growth and vigor of the shoots. During subculturing the concentration of BAP was increased gradually from lower (2.5mgl^{-1}) to higher concentration (5.0mgl^{-1}) for sustained growth. Although TDZ concentration influenced in increased number of shoots, the shoots were found to be dwarf and vitrified. Subculturing of shoots on Kn containing medium (5.0mgl^{-1}) resulted in vitrified shoots with low survivability of shoots.

In accordance to the results obtained, in *Pseudoxytenanthera stocksii* Munro, *B. balcooa*, *A. callosa* Munro, *B. glaucescens*, *D. hamiltonii* and *Guadua augustifolia*, BAP was found to be effective for

Table I. Effect of plant growth regulators on multiple shoot induction

Sl. No.	Plant growth regulators (mgl^{-1})	Response (%)	Shoot no.	Shoot length (cm)
1	Control (HF)	51.32^{o}	1.75^{x}	2.06^{s}
2	NAA 0.25 + BAP 0.5	90.33^{d}	3.60^{u}	5.31^{e}
3	IAA 0.25 + BAP 0.5	89.26^{e}	3.09^{v}	4.91^{e}
4	NAA 0.25 + BAP 1.5	92.40^{b}	4.58^{n}	5.78^{e,f}
5	IAA 0.25 + BAP 1.5	91.20^{c}	3.74^{t}	5.56^{e}
6	**NAA 0.25 + BAP 2.5**	**94.31^{a}**	**6.63^{a}**	**6.45^{a}**
7	IAA 0.25 + BAP 2.5	91.60^{c}	5.62^{g}	5.74^{e}
8	NAA 0.25 + TDZ 0.05	84.46^{h}	3.22^{u}	2.64^{g}
9	IAA 0.25 + TDZ 0.05	83.31^{i}	2.92^{w}	2.41^{h}
10	NAA 0.25 + TDZ 0.15	86.72^{g}	4.02^{r}	2.93^{h}
11	IAA 0.25 + TDZ 0.15	85.42	3.83^{s}	2.82^{g,h}
12	NAA 0.25 + TDZ 0.25	88.64^{e,f}	5.41^{i}	3.60^{m}
13	IAA 0.25 + TDZ 0.25	87.75^{f}	4.53^{o}	3.42^{n}
14	NAA 0.25 + Kn 0.5	87.63^{f}	4.95^{l}	4.71^{j}
15	IAA 0.25 + Kn 0.5	87.23^{f}	4.75^{m}	4.66^{i}
16	NAA 0.25 + Kn 1.5	89.62^{d}	5.42^{i}	5.43^{f}
17	IAA 0.25 + Kn 1.5	88.22^{e,f}	4.36^{p}	5.39^{g}
18	NAA 0.25 + Kn 2.5	90.87^{c}	5.75^{f}	5.69^{e}
19	IAA 0.25 + Kn 2.5	90.44^{c}	5.55^{g}	5.61^{e}
		SE = 0.08 CD = 0.16 (0.05)	SE=0.07 CD=0.14	SE=0.07 CD=0.14

Additives: Ascorbic acid 50 mgl^{-1}+ Citric acid 25mgl^{-1}+ Cysteine 25 mgl^{-1}; Treatments followed by the similar alphabets do not differ significantly from each other.

Table II. Effect of plant growth regulators on shoot multiplication

Sl. No.	Treatments (PGR's mgl^{-1})	Number of shoots/ explant	Shoot length (cm)	Shoot Multiplication (fold)
1	HF	3.97^{f}	2.67^{e}	0.75
2	NAA 0.25+BAP 2.5	18.53^{e}	2.97^{e}	3.16
3	NAA 0.25+BAP 5.0	22.50^{b}	6.30^{b}	3.72
4	NAA 0.25+TDZ 0.25	20.03^{d}	3.53^{d}	3.55
5	NAA 0.25+TDZ 0.5	21.37^{c}	5.40^{c}	3.16
6	**NAA 0.25+BAP 5.0 +Additives**	**27.77^{a}**	**7.36^{a}**	**4.72**
		SE = 0.22 CD = 0.40 (0.05)	SE = 0.13 CD = 0.25	SE = 0.08 CD = 0.12

Additives: Ascorbic acid 50 mgl^{-1} +Citric acid 25mgl^{-1} + Cysteine 25 mgl^{-1}; Treatments followed by the similar alphabets do not differ significantly from each other; Observations taken after four weeks of culture.

Table III. Effect of auxins on *in vitro* root induction

Sl. No.	Treatments (auxins mgl^{-1})	No. of roots per clump	Root length (cm)	Rooting percentage
1	MS/2+HF	1.60^{g}	2.70^{f}	43.80
2	MS/2+NAA 0.5	5.89^{b}	5.63^{b}	86.50
3	**MS/2+NAA 1.0**	**7.52^{a}**	**6.20^{a}**	**95.67**
4	MS/2+IAA 0.5	2.60^{e}	4.17^{e}	63.72
5	MS/2+IAA 1.0	3.72^{d}	4.60^{d}	65.80
6	MS/2+IBA 0.5	2.33^{f}	5.30^{c}	74.64
7	MS/2+IBA 1.0	3.80^{c}	5.57^{b}	82.20
		SE = 0.03 CD = 0.05 (0.05)	SE = 0.18 CD = 0.30	

Additives: Ascorbic acid 50 mgl^{-1} +Citric acid 25mgl^{-1} + Cysteine 25 mgl^{-1}; Treatments followed by the similar alphabets do not differ significantly from each other

Figure I. Micropropagation using mature nodal explants of *B. vulgaris* cv. Wamin. A: Source material; B: Multiple buds proliferated from a single nodal explant cultured on MS basal medium supplemented with 2.5 mgl^{-1} BAP and additives under liquid conditions after 2 weeks of culture; C: Shoot multiplication on MS medium supplemented with 5.0 mgl^{-1} BAP and additives under liquid conditions after 4 weeks of culture; D: *In vitro* rooting on half-strength MS medium with NAA 1.0mgl^{-1} ; E: Hardening of *in vitro* plants; F: *In vitro* raised field-grown clump (1-yr

shoot multiplication[6, 16, 18-21]. In *B. bambos*, MS medium supplemented with BAP (3.0mgl^{-1}) proved best for multiplication of *in vitro* differentiated shoots[22]. However, in *B. wamin* lower concentrations of BAP induced multiplication of shoots in MS medium[11]. Liquid MS medium with 5 mgl^{-1} BAP and 40 mgl^{-1} Adenine sulphate has proved best for axillary bud proliferation in *D. asper*[15]. In *D. strictus*, shoot proliferation was achieved in liquid MS medium with high BAP and Kn without addition of coconut water[23]. In liquid cultures, a greater uptake of BAP was possible due to the larger surface of absorption provided by the partially submerged shoots[24] .

3.4 In vitro *rooting and Hardening*

Shoot clump (2-4 shoots/clump) showed better response for *in vitro* rooting when compared with single shoots cultured for rooting. Various auxins IAA, IBA, NAA in varying concentrations were tested for *in vitro* rooting of shoot clump in the half-strength MS agar medium. NAA (1.0 mgl^{-1}) induced rooting with maximum number of roots (**Table III**) and best in terms of rooting percentage (95.67%), root number (7.52) and root length (6.20 cm). NAA favored higher rooting due to photostability, less disrupted by autoclaving and exhibit long tissue half-life[25.26].

Varied rooting responses have also been reported by several workers. In *D. strictus* the highest number of root (1.36) was regenerated on full-strength MS medium supplemented with 3 mgl^{-1} NAA [23]. In *Thamnocalamus spathiflorus* Munro a two-step procedure was reported by IBA treatment and subsequent transfer to auxin-free half-strength MS medium[27]. The best rooting percentage (84.7%) in *B. nutans* was reported in MS medium with IBA[28].

In BVW high rate (> 95%) of plant survivability was observed when hardened in greenhouse conditions. The plants were kept for an initial three weeks in a polytunnel followed by one week in a greenhouse and two weeks in shade proved the most suitable method. The micropropagated plants achieved a height of 30-35 cm with 3-4 tillers (shoots) with miniature rhizome within four months. The leaves showed lush green with healthy appearance showing expanded leaf lamina. In *B. pallida* similar method was followed with a high rate (>95%) of plant survival[17]. The survival rate of *in vitro* plants was 90% observed in *B. nutans* and 70% in *A. callosa* [14, 18].

Conclusion

The *in vitro* cloning protocol developed by axillary shoot proliferation using nodal explants in *Bambusa vulgaris* cv. Wamin, an economically important bamboo species has been taken up with the objective of enhanced shoot multiplication, good rooting and improved survival rate of *in vitro* propagated plants. A further study on genetic fidelity of these *in vitro* regenerated plants is under progress. Studies on *in vitro* production of BVW plants provide sufficient material for further utilization of the same on commercial scale.

Conflict of interest

The author's declares none.

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