In vitro propagation of *Gigantochloa atroviolaceae* Widjaja through nodal explants

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Abstract: A procedure for the regeneration of complete plantlets of *Gigantochloa atroviolaceae* through axillary shoot proliferation is described. Axillary bud break was accomplished in full strength liquid MS medium fortified with 25.0 µM BAP. Axillary shoots produced were multiplied on semi-solid MS medium supplemented with BAP (20µM) + NAA (3.0µM) giving a multiplication rate of 2.39. *In vitro* shoots were rooted on full strength MS medium supplemented with varying concentrations of auxins. Optimal rooting was achieved on medium supplemented with 35.0µM IBA. Regenerated plantlets were successfully hardened and acclimatized under net house conditions with over 80% survival. [Journal of American Science 2010;6(10):1019-1025]. (ISSN: 1545-1003).

Keywords: *Gigantochloa atroviolaceae*, *in vitro* propagation, nodal explants, axillary shoot multiplication

Abbreviations: MS: Murashige and Skoog (1962); **PGR:** Plant Growth Regulator; **BAP:** 6, Benzylaminopurine; **Kn**: 6- Furfurylaminopurine; **NAA:** α-Naphthalene Acetic Acid; **IBA:** Indole-3 Butyric Acid.

1. Introduction

Bamboo, the world's fastest growing and environment-friendly giant grass, is now internationally recognized as an important, cost effective and safe alternative to wood. In India, the consumption of bamboo has increased considerably from 2.2 million tones in 1980 (Varmah and Bahadur, 1980) to 13.48 million tones in 2003 (Planning Commission, 2003) due to rapid growth of bamboo based industries. In the current scenario, the largest consumer of bamboo in the country is construction sector (3.4 million tones) followed by an escalating demand for a variety of products viz. paper production, laminates, flooring, boards, ply etc. However, the sectors consuming bamboos are facing a shortage of bamboo supply. Due to limitations in conventional methods of propagation the potential of micropropagation has raised high hopes and a lot of research has been focused on the development of protocols for rapid and large scale propagation. Stimulation of axillary meristems into shoots using nodes with pre-formed buds in axils is the most commonly used method for mass propagation of bamboos. Axillary shoots proliferating from these pre-formed meristems have the advantage of being the true compliment of explant source and hence selected superior stocks can be multiplied on mass scale.

Gigantochloa atroviolaceae Widjaja (*G.atter sensu* Kurz.), commonly known as Black Bamboo, is a highly attractive ornamental bamboo from Java and Sumatra. It is an impressive, loosely tufted, clumped, sympodial branched bamboo having thin but strong and durable black culms with narrow white bands at the nodes and large leaves. The timber is used for building and furniture construction, musical instruments, craft items and ornamental purposes.

The rapidly increasing bamboo-based industries have resulted in severe loss of forest stocks. Consequently, there is always a shortage of planting stock material of this bamboo due to its tremendous economical importance. The species is mainly propagated vegetatively by rhizome or culm cuttings. However cuttings are bulky, difficult to handle and transport. Moreover, propagules obtained via vegetative methods are successful only if they root. Root promoting substances (auxins) have little effect on bamboo rooting (mature cuttings) and planted material (vegetatively propagated plants) have been found to develop very slowly (Hassan, 1980). Further year round, vegetative propagation is difficult due to seasonal specificity of material (Saxena and Bhojwani, 1993). Therefore, it becomes imperative to adopt alternative methods for its rapid multiplication and tissue culture techniques offer a potent tool for the purpose. The present work reports a suitable

method for rapid *in vitro* propagation of *G.atroviolaceae* through axillary shoot proliferation.

2. Material and Methods

2.1 Initiation of cultures

Young juvenile shoots were collected from the mature clump of *Gigantochloa atroviolaceae* growing at Tissue Culture Discipline, FRI, Dehradun (Figure A). Nodal segments containing single axillary bud were used as the source material for micropropagation. Explants were swabbed with alcohol soaked cotton and then pretreated with 1% Bavistin (50% carbendazim WP) for 15-20 minutes. Surface sterilization was done with 0.1% $HgCl_2$ solution for 12-14 minutes followed by 3-4 times washing with sterilized distilled water to remove the traces of steriliant. Surface disinfected explants were inoculated on liquid MS medium supplemented with BAP or Kn (5- 35µM) in different seasons of the year. The effect of cytokinins on bud-induction was assessed after a period of 4 weeks.

2.2 *In vitro* Shoot Multiplication

After 4 weeks of inoculation, the axillary shoots sprouted from the nodal explant were excised and transferred on to semisolid MS medium supplemented with BAP or Kn (5- 30µM) alone and BAP (15, 20 25 µM) in combination with NAA (1.0, 3.0, 5.0 µM) to test the effect of various PGRs on *in vitro* multiplication of shoots. Propagules of 3 shoots were used for the purpose. Observations were recorded after an interval of 4 weeks. Regular subculturing was carried out at periodic interval of 4 weeks using MS medium supplemented with 20µM BAP + 3µM NAA.

2.3 *In vitro* Rooting

For *in vitro* rooting, clusters of 2-3 shoots (2-3cm) were kept on full- strength MS medium augmented with different concentrations of IBA (5-40 µM) and NAA (5- 30 µM). Rooting response was recorded in terms of rooting percentage, average number of roots produced and average root length after a period of 4 weeks.

All culture media contained 3% sucrose (w/v) and agarified with 0.7% agar in case of semi-solid media. The pH of the medium was adjusted to 5.8 prior to autoclaving the medium at 121^0C and 1.0×10^5 Pa for 15 minutes.

2.4 Culture conditions

All the cultures were grown under a photoperiod of 16 hours in light (illuminated by cool white fluorescent tubes (Philips, India) and for 8 hours in dark in a culture room maintained at 25 ± 2^0C.

2.5 Hardening and acclimatization

In vitro rooted shoots were transferred to autoclaved 250 ml screw cap glass bottles containing 1/3 volume of vermiculite and kept in culture room for three weeks. The plantlets were nurtured with half strength MS medium (without organics) twice a week. Later these bottles were shifted to mist chamber under relative humidity of 80-90% maintained at 30 ± 2^0C temperature. The caps of bottles were removed and plantlets were allowed to remain in the bottle for 3-4 days before being transferred to polybags containing a mixture of sand, farmyard manure and soil in a ratio of 1:1:1.The polybags were subsequently shifted to shade house conditions and acclimatized plantlets were irrigated with tap-water

2.6 Statistical analysis

Data collected in the Completely Randomized Design (CRD) of experiments (Compton, 1994) was analyzed using Microsoft Excel ver. 2007 © Microsoft Technologies, USA. Experiments were repeated thrice and data represents the mean of three experiments. Each treatment consisted of minimum twelve replicates. Data was subjected to one way Analysis of Variance (ANOVA). Degree of variation was shown by Standard Error (SE), Critical Difference (CD) at 5%. The significance level was tested at 5%, 1% and 0.1%. The significance of the data as ascertained by F-test and the CD values computed, were used for comparing differences in means of various treatments

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3. Results

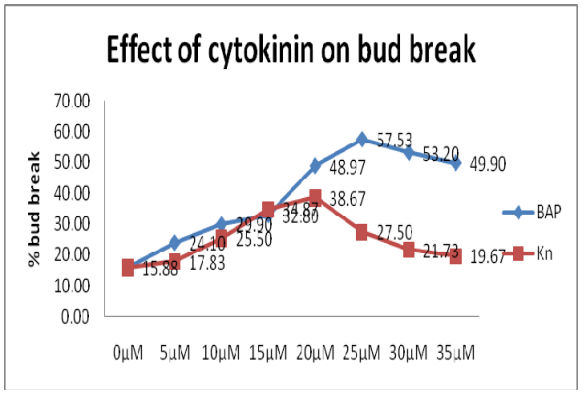
3.1 Culture Initiation

Treatment with 0.1% $HgCl_2$ solution resulted in 85-90% aseptic and viable cultures. Nodal segments when cleaned with ethyl alcohol (70%) dipped cotton, prior to $HgCl_2$ treatment, improved surface sterilization of explants. Pretreatment with fungicide (Bavistin) led to a significant reduction in fungal contamination.

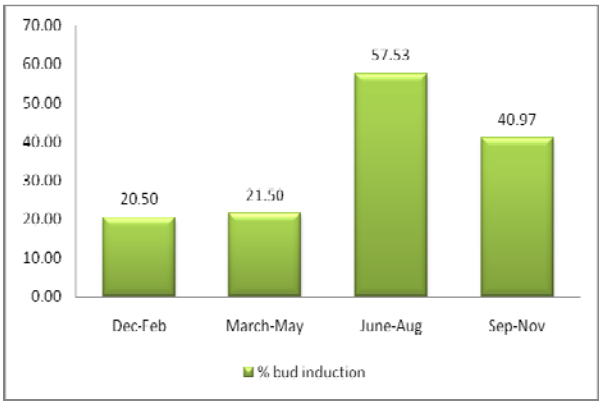
Bud break was obtained in both the cytokinins (BAP and Kn) fortified liquid medium within 2-3 weeks and number of shoots ranged from 1-3. Axillary buds cultured on basal MS medium (without any plant growth regulator) yielded 15.88% bud break response, producing only 1.12 ± 0.08 axillary shoots which did not survive. BAP treatment significantly increased the percent bud break

response. The optimal medium for axillary bud culture was MS medium (liquid) supplemented with 25µM BAP, yielding maximum (57.53%) percentage bud break with maximum average number of shoots 2.64 ± 0.07 and maximum average shoot length (cm) 2.60 ± 0.18 after a period of 4 weeks (Graph 1, Figure B). The bud break response declined with increased concentration of BAP (30 - 35µM) resulting in small condensed shoots, most of them showing no further development. On Kn supplemented MS medium a maximum bud break response of only 38.67% was obtained with 20µM Kn with average shoot number 2.27 ± 0.15 and average shoot length of 1.39 ± 0.03 cm. However, the shoots were small, thin and weak and most of them did not show any further development.

Significant effect of season was noted on percent bud- induction. Best results (57.53% bud-induction) were obtained in the month of June-August followed by 40.97% bud-break in the months of September-November. The months of December-February and March –May proved to be unsuitable for obtaining optimal bud-induction, giving only 20.50% and 21.50 % bud break, respectively (Graph 2).



Graph 1: Effect of PGRs on percent bud induction in nodal explants of *G. atroviolaceae*. Data recorded after 4 weeks.



Graph 2: Effect of Season on percent bud induction in nodal explants of *G. atroviolaceae* on MS + 25 µM BAP. Data recorded after 4 weeks.

3.2 *In vitro* shoot multiplication

The excised axillary shoots when cultured on basal MS medium (without PGR) exhibited a drastic declination in multiplication rate (0.70 ± 0.11) and shoots gradually died. This necessitated the incorporation of cytokinins in the medium. For *in vitro* shoot multiplication. MS medium supplemented with BAP at 20 µM concentration gave a maximum multiplication rate of 1.94 ± 0.15 folds with average shoot number 5.82 ± 0.04 and shoot length of 2.23 ± 0.15 cm. An increase in BAP concentration beyond this level resulted in decreased multiplication potential (Table 1). In Kn supplemented medium, shoot multiplication rate significantly declined as compared to BAP fortified medium. A best response of only 1.32 ± 0.05 fold multiplication with mean shoot number 3.95 ± 0.16 and mean shoot length 1.88 ± 0.11 cm was obtained on MS medium supplemented with 20µM Kn.

A significant improvement in shoot multiplication response was observed when BAP in combination with lower concentrations of NAA was tried (Table 1). This is due to the synergistic effect of interaction of cytokinin with auxin in the medium. An average number of 7.17 ± 0.21 shoots with mean shoot length 2.17 ± 0.18 cm and multiplication rate of 2.39 ± 0.07 folds was obtained on MS medium supplemented with 20µM BAP + 3µM NAA (Figure C). Since this interaction proved to be most effective, for maintenance of cultures regular subculturing of shoots was done in this media combination at periodic interval of 4 weeks.

3.3 *In vitro* Rooting

Full strength MS medium supplemented with 35 µM IBA gave the maximum rooting response (47.67%) with an average of 4.33± 0.22 roots per propagule and mean root length of 2.90 ± 0.22 cm (Table 2, Figure D). At lower concentrations of IBA (5-15µM) no rooting was obtained whereas higher concentration of IBA (40 µM) exhibited a decrease in *in vitro* root production.

On medium fortified with NAA 16.98% - 31.93% rooting was obtained. The pattern of root induction and rooting percentage was found to be similar to that of IBA supplemented medium. Best response (31.93%) was on 15µM NAA supplemented MS medium with 3.29 ± 0.10 roots on an average per shoot propagules and average root length of 2.86 ± 0.06 cm.

3.4 Hardening and Acclimatization

The tissue culture raised plants are heterotrophic in their mode of nutrition as they grow on medium rich in minerals and sugar and thus, cannot withstand the environmental conditions without proper hardening and acclimatization. In 4-5 weeks of transfer to rooting medium, healthy plantlets with good roots and shoot system developed. For hardening the *in vitro* rooted plantlets were transferred to autoclaved culture bottles containing vermiculite. These plantlets were irrigated with half - strength MS medium without organics and maintained in culture room for three weeks. This was followed by transfer to mist chamber for three weeks at relative humidity of 80-90% and 30 ± 20C temperature. Plants were then shifted to polybags containing Sand: Soil: FYM in 1:1:1 proportion and maintained in the mist chamber where a survival rate of over 80% was recorded (Figure E).

Table 1: Effect of PGRs in MS medium on shoot multiplication of *G. atroviolaceae*. Data recorded after 4 weeks (*: significant at 95%, * : significant at 99.9%).**

PGR Concentration(µM)	Mean shoot number	Mean shoot length (cm)	Multiplication rate
Control	2.10 ± 0.32	1.42 ± 0.09	0.70 ± 0.11
BAP			
5.0	2.97 ± 0.29	1.92 ± 0.10	0.99 ± 0.10
10.0	4.12 ± 0.11	1.95 ± 0.15	1.37 ± 0.04
15.0	5.50 ± 0.10	2.10 ± 0.25	1.83 ± 0.03
20.0	5.82 ± 0.04	2.23 ± 0.15	1.94 ± 0.07
25.0	5.62 ± 0.04	2.22 ± 0.09	1.87 ± 0.05
30.0	4.35 ± 0.09	1.98 ± 0.14	1.45 ± 0.03
Kn			
5.0	2.52 ± 0.15	1.72 ± 0.10	0.84 ± 0.05
10.0	3.41 ± 0.13	1.75 ± 0.09	1.14 ± 0.04
15.0	3.89 ± 0.18	1.98 ± 0.14	1.30 ± 0.06
20.0	3.95 ± 0.16	1.88 ± 0.11	1.32 ± 0.05
25.0	3.63 ± 0.15	1.91 ± 0.10	1.21 ± 0.05
30.0	3.38 ± 0.09	1.66 ± 0.12	1.13 ± 0.03
BAP + NAA			
15.0 + 1.0	5.97 ± 0.27	2.13 ± 0.14	1.99 ± 0.09
15.0 + 3.0	6.77 ± 0.20	1.97 ± 0.15	2.26 ± 0.07
15.0 + 5.0	6.02 ± 0.16	1.90 ± 0.13	2.01 ± 0.05
20.0 + 1.0	6.44 ± 0.13	2.25 ± 0.18	2.15 ± 0.04
20.0 + 3.0	7.17 ± 0.21	2.17 ± 0.18	2.39 ± 0.07
20.0 + 5.0	6.90 ± 0.17	2.05 ± 0.13	2.30 ± 0.06
25.0 + 1.0	6.48 ± 0.16	2.20 ± 0.23	2.16 ± 0.05
25.0 + 3.0	6.17 ± 0.24	2.07 ± 0.19	2.06 ± 0.08
25.0 + 5.0	5.53 ± 0.20	1.93 ± 0.17	1.84 ± 0.07
Significance	***	*	***
CD at 5%	**0.15**	**0.12**	**0.05**

Table 2: Effect of auxins in MS medium on *in vitro* rooting in *G. atroviolaceae.* Data recorded after 4 weeks. (* :significant at 99.9%)**

Auxin concentration(µM)	Response %	Mean root number	Mean root length (cm)
Control	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
IBA			
5.0	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
10.0	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
15.0	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
20.0	12.27 ± 4.36	1.50 ± 0.10	1.52 ± 0.14
25.0	20.78 ± 4.79	2.58 ± 0.13	2.40 ± 0.26
30.0	31.64 ± 3.45	3.94 ± 0.65	2.69 ± 0.14
35.0	47.67 ± 3.98	4.33 ± 0.22	2.90 ± 0.22
40.0	39.10 ± 3.18	3.89 ± 0.18	2.42 ± 0.29
NAA			
5.0	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
10.0	16.98 ± 1.66	2.17 ± 0.22	1.58 ± 0.15
15.0	31.93 ± 3.67	3.29 ± 0.10	2.86 ± 0.06
20.0	28.41 ± 3.21	2.41 ± 0.13	2.59 ± 0.17
25.0	26.05 ± 2.09	2.27 ± 0.12	2.46 ± 0.07
30.0	21.29 ± 2.45	2.37 ± 0.06	2.28 ± 0.26
Significance	***	***	***
CD at 5%	**4.12**	**0.30**	**0.26**

4. Discussion

Rapid growth, short rotation cycles and the development of newer bamboo products has led to bamboo gaining importance in social forestry programmes. However, cultivation of bamboo in India is still in its prime. About 99% of annual bamboo production in the country comes from natural stands in forests. Propagation of bamboos by a reliable alternative method of tissue culture, thus, becomes imperative. In the present study, axillary nodes from lateral branches were taken as the starting material for micropropagation. The suitability of nodal segments having axillary buds has been reported in micropropagation of several bamboos (Saxena, 1990; Bag *et al*., 2000; Arya *et al*., 2006).

BAP is the most common cytokinin that is used for promotion of bud differentiation and further development. In our study also, incorporation of BAP into the medium improved the incidence of bud-break. Maximum bud break (57.53%) was achieved with 25 µM BAP. Response of bud-induction on Kn supplemented medium was significantly lower as compared to the results in medium augmented with BAP. These results are in line with those of other workers indicating the efficiency of BAP for shoot culture initiation in several bamboos (Huang and Huang, 1995; Arya and Arya, 1997; Devi *et al*., 2009). Besides, percentage of bud-induction was found to vary with seasonal change and best response was recorded in the months of June-August. The observations are compatible with previous investigations which have established that seasonal variations influence bud-break response in explants (Saxena and Bhojwani, 1993; Ramanayke and Yakandawa, 1997; Das and Pal, 2005; Yadav *et al.,* 2008).

In the present study, browning was observed at the cut ends of explants because of which majority of explants turned black and eventually died. Such browning of explants was earlier reported by Nadgir *et al.,* 1984; Chaturvedi *et al*., 1993; Bisht *et al*., 2000. To overcome this problem, media were changed at every 2-3 days interval from the beginning of the cultures.

In our study, cytokinins when tried alone for shoot multiplication, BAP gave superior results as compared to Kn. However, best results of *in vitro* shoot multiplication, in terms of multiplication rate, mean shoot number and mean shoot length,were obtained on full strength MS medium containing BAP (20 µM) and NAA (3.0µM). A reason of the

positive effect of auxin at low concentration in the culture media is that it nullifies the effect of higher cytokinin (Hu and Wang, 1983). A cyokinin /auxin combination has earlier proved to be efficient for *in vitro* shoot proliferation in *Pseudoxytenanthera stocksii* (Sanjaya *et al*., 2005), *Thamnocalamus spathiflorus* (Bag *et al.,* 2000), *Bambusa nutans* (Yadav *et al*., 2008). Contrastingly, in some studies on micropropagation of bamboos, either BAP alone or a combination of BAP and Kn was found to give best results of shoot multiplication *in vitro* (Nadgir *et al.,*1984; Ramanayke and Yakandwala , 1997; Sanjay *et al*., 2005; Das and Pal, 2005; Kapoor and Rao, 2006).

In the present study, apart from optimal supplement of the medium towards production of healthy shoots, regeneration capacity of the *in vitro* shoots was also found to be dependent upon the time of subculturing. 4 weeks period was found to be the best gestation period for recycling of shoots. Delaying of subculturing period resulted in gradual browning of the shoots followed by total blackening, mostly from the basal portion. Henceforth, subculturing period was recorded as the most crucial factor for obtaining optimal and described level of shoot regeneration.

In vitro rooting was successfully achieved in regenerated shoots of *G. atroviolaceae.* The role of auxins in root development is well established (Torrey, 1976; Scott, 1972). A varied effect of auxins was observed in our study by incorporating different concentrations of IBA and NAA in full strength MS medium. Effective role of IBA in bamboos for rooting has previously been reported in *D. giganteus* and *D. strictus* (Das and Rout,1991), *Bambusa nutans* and *D. memranaceous* (Yashodha *et al*.,1997), *D. asper* (Arya *et al.*, 2002), *Pesudoxytenanthera stocksii* (Sanjay *et al.*, 2005). Similar are the results obtained in the present study on *G. atroviolaceae* where IBA at 35.0 µM concentration gave response with regards to all rooting parameters. Rooted plantlets were subsequently hardened under green house conditions followed by acclimatization in shade house with over 80% survival rate.

To our knowledge, this is the first report on *in vitro* propagation of *Gigantochola atroviolaceae* via axillary shoot proliferation from nodal segments derived from field-grown material. The method can be used for rapid and mass propagation of this important bamboo species.

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Micropropagation of *Gigantochloa atroviolaceae.* Fig. A: Mature Mother Clump; Fig. B: Axillary Bud Break:Fig. C: *In vitro* Multiplication; Fig. D: *In vitro* Rooting; Fig. E: Hardened Plants.