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Effective and Large Scale *In vitro* Propagation of *Dendrocalamus strictus* (Roxb.) Nees using Nodal Segments as Explants

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ABSTRACT

Dendrocalamus strictus (Roxb.) Nees belongs to the family Poaceae is the most important and fastest growing bamboo species. An effort has been made to propagate *D. strictus* using nodal explants selected from mature mother plants. The disinfected nodes were inoculated on agar gelled Murashige and Skoog's (MS) basal medium with various concentrations of 6-benzylaminopurine (BAP), while maximum 4-5 shoots were induced on 4.0 mg/l BAP. The newly formed buds were transferred to solidified MS basal medium containing 3.0 mg/l BAP and 0.5 α-Naphthalene acetic acid (NAA) to achieve about 26 multiple shoots. These experiments were conducted with a cluster of minimum 3-5 shoots. The induction of roots from *in vitro* regenerated shoots was found difficult in this plant system. Three types of auxins i.e. indole-3 butyric acid (IBA), indole-3 acetic acid (IAA) and NAA were used in various combinations for rooting of shoots. About 93 % rooting was archived on half strength MS medium augmented with 3.0 mg/l NAA. The rooted shoots were hardened in the greenhouse for two months and the secondary hardened plantlets were successfully shifted to the farmer's field. More than 90 % survival rate was observed under the natural conditions in this plant species.

***Keywords*:** *Dendrocalamus strictus*, axillary shoots, *in vitro* propagation, rooting, hardening

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1. INTRODUCTION

Bamboo is a woody grass and belongs to the family Poaceae. Bamboos are versatile, arborescent, perennial and non-wood forest trees, with tremendous eco-sociological and commercial importance. The worldwide interest in bamboo as a source of biomass in sustainable agriculture and agro forestry system has increased rapidly in recent years (Pandey and Singh, 2012).

In India, bamboo cultivation is widely spread from Northeast to the Southwest. About half of the bamboo in India is used by pulp, paper, rayon, mat boards, furniture, musical instruments, agricultural implements, baskets, bridges, coffins, beds, toys and weapons industries (Reddy, 2006). The leaves of bamboo is used as a quality forage as an alternative to the traditional sources of food to the cattle due to high amount of digestive nutrients and digestive crude protein (Chobey and Sharma, 2011).

Dendrocalamus strictus (Roxb.) Nees (family Poaceae) also known as solid bamboo or calcata bamboo is a native to Southeast Asia. It is a medium-sized bamboo species with culms of about 8-20 meters tall and 2.5-7 cm in diameter. The internodes are generally 32-47 cm long and thick-walled in nature. The plant is having many clustered branches with one large and dominant branch. New branches arise from the lower nodes of the larger branch. There are large variations in the size (5-20 cm long and 1.5-3.5 cm broad) of the leaves, as they are smaller in dry habitats and bigger in moist conditions. *Dendrocalamus strictus* is growing in deciduous forests up to 3,500 ft. It is a tightly tufted bamboo, with strong, flexible, thick walled or hard culms. The young shoots are greenish-brown in color and covered with white sheaths, turn brown when mature (Mahapatra *et al.*, 2008).

The demand of *D. strictus* is increasing day by day as it contains 85% cellulose and used in the paper and pulp industry. It is extremely drought tolerant bamboo therefore; it is also suitable for law rainfall areas and poor soil conditions. The leaves decoction is used as traditional medicine for wound healing (Mahapatra *et al.*, 2008), abortifacient (Sharma and Borthakur, 2008) and to treat cold, cough and fever (Kamble *et al.*, 2010).

The natural propagation of bamboo is hampered due to short seed dormancy period, high seed sterility, low seed viability, high seed-borne infections and large-scale consumption by wild animals especially rodents. *Dendrocalamus strictus* has long and unpredictable flowering cycle of 35–40 years and the propagation through seeds is relatively difficult. As per Saxena and Bhojwani (1993) there are several other factors that check effective multiplication of *D. strictus* via vegetative/stem cuttings and sexual methods.

In vitro techniques provide extensive applications of *in vitro* regeneration methods for shoot multiplication, secondary metabolite production, conservation and propagation of medicinal plants (Li *et al.*, 2012; Shekhawat and Manokari, 2016a). Micropropagation technology is a technique through which groups of genetically identical plants all derived from a selected individual multiplies vegetatively and rapidly by aseptic culture of meristematic regions under controlled nutritional and environmental conditions *in vitro*. Nowadays, unlike the conventional propagation method, it is the only realistic means of achieving rapid and large scale production of disease free quality planting materials (Lodha *et al.*, 2015) and an alternative approach for fast multiplication of a variety in its original form (Patel *et al.*, 2016).

It is very effective in entire disease cleansing, rejuvenation and subsequent mass propagation of well adapted and promising varieties facing gradual deterioration in yield, quality and vigor due to accumulation of pathogens during prolonged vegetative cultivation and

hence sustains the productive potential of crops for a longer period (Wang *et al.*, 2009; Shekhawat and Manokari, 2016b).

Bamboo micropropagation has certain limitations, especially when explants from adult plant are used, the major problem being the induction of roots (Paranjothy *et al.*, 1990). Biotechnological interventions are needed to fulfill the increasing demand of *D. strictus* by the various industries and to overcome problems related to the regeneration. Therefore, the present report describes an effective regeneration methodology for large scale propagation of *D. strictus,* an industrially important bamboo species.

2. MATERIALS AND METHODS

2. 1. Collection of explants

About 3 years old and disease free mother plants of *Dendrocalamus strictus* were selected from the campus of Bamboo research Center, Vkai division Rajpipla, District Narmada, Gujarat State of the India. The fresh spouts of the healthy plants were collected and transported to the plant tissue culture laboratory. The nodal segments of about 2-3 cm long were used as explants. The explants were collected year round to find out the seasonal effect on initiation of cultures under *in vitro* conditions. The explants were cut with help of sharp cutter which was wiped with 70% ethyl alcohol. The explants were hard enough therefore extra care was taken to make the explants of suitable sizes.

2. 2. Surface sterilization of explants

The nodal explants were thoroughly washed under running tap water. These were then washed in fungicide (Copper Chloride 50% WP) and Bactericide (Streptocycline) with few drops of Tween - 20 for 15 minutes and rinsed with distilled water for three times. Above exercise was performed in the semi cleaned area of the laboratory. Further surface sterilization of the explants was done under Laminar airflow bench.

The explants were treated with 0.1 % $HgCl_2$ (mercuric chloride) solution for 8 minutes and thoroughly washed with sterile distilled water under the LAF. The explants were then given 60 seconds dip in 70 % ethyl alcohol and again rinsed in sterile distilled water for 4-5 times.

2.3. Initiation of cultures

Murashige and Skoog basal medium (Murashige and Skoog, 1962) gelled with agar powder (8 gm/l) at 5.9 pH with 3% sucrose was used in this study. The medium was autoclaved for 20 minutes at 15 psi pressure. The surface sterilized explants were inoculated aseptically to the autoclaved medium (10 ml in boiling tubes / 60 ml in culture bottles) under Laminar airflow bench. Both cytokinins (6-benzylaminopurine, BAP and kinetin) ranging from 1.0 to 6.0 mg/l was used to induce the shoots from the axillary buds of the explants. The cultures were kept in the culture room at 25±2 °C temperature with illumination of 20-50 µmol $m^{-2}s^{-1}$ Spectral Photon Flux Density (SPFD) and 60-70% relative humidity.

2. 4. Multiplication of shoots

Proliferated auxiliary clusters of shoots were excised and transferred to fresh MS medium supplemented 1.0 to 5.0 mg/l BAP and/or kinetin along with 0.5 to 2.5 mg/l NAA. The shoots

multiplication was performed using 3-5 auxiliary clusters of shoots per culture bottle. The multiple shoots were transferred to the fresh medium at regular interval of 14 to 16 days under sterile conditions. The physical conditions of the culture room remained same as mentioned for establishment of cultures.

2. 5. *In vitro* rooting of shoots

Experiments for *in vitro* rooting of the shoots were conducted on clusters only. No individual shoots were rooted in the medium but the rooted shoots were separated before transferring them for the hardening in the greenhouse. Full strength MS basal medium with 3% sucrose with different concentrations of various auxins [indole-3 butyric acid (IBA), indole-3 acetic acid (IAA) and α-Naphthalene acetic acid (NAA)] were used for rooting. All cultures were transferred in growth chamber for 3 to 4 weeks to induce roots.

2. 6. Hardening and acclimatization of *in vitro* rooted shoots

Rooted shoots from 3-4 week old cultures were taken from the solidified medium very carefully so that the roots should not damage. The roots were washed very carefully under running tap water to remove any adhered agar. These plants were transplanted to a low EC and pH (low) cocopeat (kelteck India Ltd.) contained containers for 25 to 30 day for primary hardening. The primary hardened plantlets were then transfer in the Net houses for secondary hardening process for another 1-2 months.

3. RESULTS AND DISCUSSION

In the present investigation, experiments were conducted to establish cultures from selected plants of *D. strictus*. The stem segments were hard enough; therefore extra care was taken while selecting the explants, cutting and surface sterilizing them. The explant age (physiological status), the season of explant collection, explants size and plant quality are some of the factors which play important role in the initiation and multiplication of cultures (Kurtz *et al.*, 1991; Piqueras and Debergh, 1999; Smith, 2000). The explants harvested during the months of July, 2018 – October, 2018 were found most suitable for the initiation of *in vitro* cultures of *D. strictus*.

3. 1. Sterilization of explants and establishment of cultures *in vitro*

Explants sterilized with 0.1% mercuric chloride for 8 minutes were found to eliminate bacterial contamination. The explants were treated with systemic fungicide before mercuric chloride to kill the fungi. Nodal shoot explants observed to be most suitable for culture initiation than the apical shoot tips in present investigation. Shoot buds appeared within 7-10 days from the axillary meristem of the explants. Maximum numbers of shoots were appeared from the nodes (Figs. 1A), which were in direct contact with the medium as compared to the node which is not in contact to the nutrient medium.

Explants cultured on growth regulator free MS medium exhibited one shoot only. However, when MS medium was supplemented with different cytokinins alone, multiple shoot formation occurred within two weeks of cultures. Of the two cytokinins tested (BAP and Kin), BAP at the concentration of 4.0 mg/l was found the most effective growth regulator in inducing

multiple shoot buds from the nodes as well as from shoot tip explants. The maximum number of shoots and the highest shoot regeneration frequency was achieved at 4.0 mg/l BAP, on this medium maximum 4.6 ± 0.18 shoots (average) with 5.4 ±0.30 cm length was induced per explant (Table 1). Reduction in the number of shoots and shoot length was observed at higher levels of BAP. Percentage of response varied on MS medium supplemented with BAP. Comparatively less percentage of response was observed on kinetin enriched media (Table 2).

Superiority of BAP over kinetin in culture induction response was also reported in number of economically important species such as plantain (Buah *et al.,* 2010), hybrid of almond × peach (Arab *et al.*, 2014) and grapes (Khan *et al.,* 2015).

Table 1. Effect of BAP and kinetin concentrations on shoots induction response of *D. strictus* explants on MS medium.

Concentration of BAP (mg/l)	Response (%)	Number of Shoots / Explant ± SE	Length of Shoots / Explant ± SE
Control (0.0)	0	0.0 ± 0.00	0.0 ± 0.00
1.0	69	1.6 ± 0.10	2.5 ± 0.21
2.0	81	2.3 ± 0.21	3.3 ± 0.13
3.0	87	3.7 ± 0.26	4.0 ± 0.19
4.0	92	4.6 ± 0.18	5.4 ± 0.30
5.0	82	3.0 ± 0.25	4.2 ± 0.10
6.0	70	2.9 ± 0.11	4.0 ± 0.27
7.0	63	2.4 ± 0.30	3.7 ± 0.29

Table 2. Effect of kinetin concentrations on induction of shoots from *D. strictus* explants on MS medium.

Concentration of kinetin (mg/l)	Response (%)	Number of Shoots / Explant ± SE	Length of Shoots / Explant ± SE
Control (0.0)	0	0.0 ± 0.00	0.0 ± 0.00
1.0	20	1.0 ± 0.19	1.2 ± 0.28
2.0	44	1.1 ± 0.00	1.7 ± 0.11
3.0	59	2.0 ± 0.11	2.2 ± 0.15

4.0	65	3.1 ± 0.17	3.9 ± 0.23
5.0	60	2.9 ± 0.19	3.4 ± 0.15
6.0	51	2.7 ± 0.14	2.9 ± 0.11
7.0	49	2.0 ± 0.16	2.3 ± 0.14

Pandey and Singh (2012) developed propagation protocol for *D. strictus* using nodal explants from mature plants. The explants, after surface sterilization, were inoculated on solidified MS basal medium with 2 mg/l BAP. Kapruwan et al (2014) developed an accelerated protocol for large-scale propagation of *D. strictus.* For initiation of aseptic cultures, various concentrations of mercuric chloride for different time period was studied and it was found that 0.2% $HgCl_2$ was found to be effective. The axillary shoots of *D. strictus* containing single axillary bud were inoculated in semisolid MS medium fortified with different concentration of growth regulator, 6-benzylaminopurine (BAP).

3. 2. *In vitro* proliferation of shoots

Microshoots of 3.0-4.0 cm length obtained from the node explants were used in shoot proliferation on MS medium supplemented with various concentrations of plant growth regulators. The effect of different concentrations of plant growth regulators (auxin and cytokinin) on shoot elongation and further proliferation was evaluated by measuring the shoot length and numbers after four weeks of subculture.

According to the number of shoots and shoot length, the significant variations were observed between treatments, which were tested with analysis of variance by One-Way ANOVA. Three mg/l BAP combined with 0.5 mg/l NAA produced 26.0 ±0.13 shoots under the multiplication stage. Increase of BAP concentration to 4 mg/l fastened the rate of shoots multiplication. But further increase of BAP reduced number of shoots in this study (Figs. 1B, 1C and Table 3).

Table 3. Combined effects of Cytokinin (BAP) concentrations on multiplication of shoots from *in vitro* regenerated shoots on MS Medium containing 0.5 mg/L NAA.

Concentration of BAP + 0.5 mg/L NAA	Number of Shoots ± SE	Shoot Length (cm) ± SE
1.0	11.4 ± 0.31	5.0 ± 0.26
1.5	19.7 ± 0.49	5.5 ± 0.19
2.0	16.0 ± 0.25	7.4 ± 0.33
2.5	21.6 ± 0.20	5.0 ± 0.38

3.0	26.0 ± 0.13	4.2 ± 0.11
3.5	23.3 ± 0.27	3.8 ± 0.15
4.0	16.3 ± 0.27	3.0 ± 0.14
5.0	9.3 ± 0.27	2.6 ± 0.11

Pandey and Singh (2012) transferred the shoots to the solidified MS basal medium with 4 mg/l BAP and 15 mg/l Ads to achieve about three fold multiplications. Single shoots or 2 shoot clusters did not survive and therefore all the experiments were conducted with a cluster of minimum 3-shoots. The combined use of various cytokinins with auxins in shoot multiplication was also adopted in *Daphne mezereum* (Nowakowska et al. 2019), *O. corymbosa* (Revathi et al. 2019), *Conocarpus erectus* (Dewir et al. 2018) and *Elephantopus scaber* (Abraham and Thomas 2015)

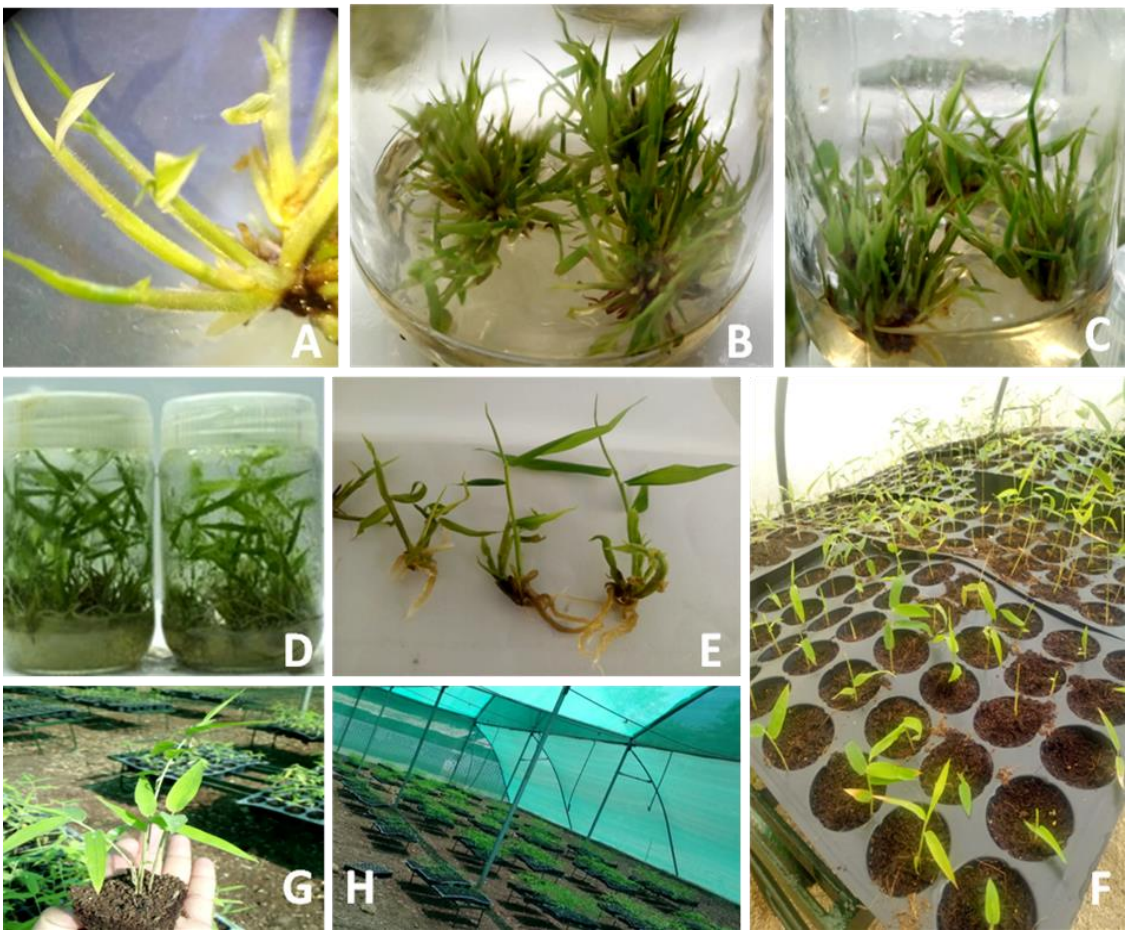

Fig. 1. (A) Bud breaking from the nodal explants, (B and C) Multiple shoots induction, (D) Elongated shoots *in vitro*, (E) *In vitro* rooting of the shoots, (F to H) Different stages of hardening of plantlets.

The shoots were further multiplied when the newly formed shoots were harvested, randomly selected and their nodal segments were subcultured on the fresh MS medium containing 3.0 mg L^{-1} BAP in the first set of experiments. Significant improvements in shoot numbers per explant were observed in this set than the initial stage of culture establishment (Fig. 1D).

3. 3. *In vitro* rooting of the shoots

Rooting was found difficult in this plant species with IBA and IAA. The regenerated shoots acquired sufficient length (5-6 cm) after the end of the four weeks of incubation under multiplication stage and were ready for transfer to the rooting phase. Three types of auxins were tested (IAA, IBA and NAA), among which NAA was found most suitable for *in vitro* root induction. Emergence of roots primordia were observed from the shoot base within 15 days in the medium augmented with auxins.

The complicated process of rooting was induced by auxins by maintaining the polarity of plants and promoting lateral root formation due to repetitive cell division as envisaged by George et al. (2008). The regenerated shoots were rooted maximally on half strength MS medium incorporated with various concentrations of NAA (Table 4). Only 3 mg/l NAA produces highest percentage of rooting.

The maximum percentage of rooting response (93%) and the total number of roots (10.2 ±0.17 roots with 4.7 ±0.30 cm length) was recorded on half strength MS medium with 3.0 mg/L NAA (Fig. 3E). But superiority of IBA on *in vitro* rooting was recorded in many reports such as *Olive europea* (Haq *et al.*, 2009), *Camellia sinensis* (Bidarigh *et al.*, 2012), strawberry (Anuradha *et al.*, 2016), *Oldenlandia corymbosa* (Revathi *et al.*, 2018) etc. The rooting efficacy of the shoots decreased as the auxins concentrations increased in the medium.

Table 4. Effect of NAA concentrations on root induction from *in vitro* produced shoots on half strength MS medium.

Concentration of NAA (mg L^{-1})	Response (%)	Number of roots / shoot ± SE	Length of roots/ shoot ± SE
Control (0.0)	0	0.0 ± 0.00	0.0 ± 0.00
1.0	65	5.4 ± 0.10	1.7 ± 0.16
2.0	70	8.1 ± 0.23	2.3 ± 0.18
2.5	89	8.8 ± 0.11	3.0 ± 0.36
3.0	93	10.2 ± 0.17	4.7 ± 0.30
3.5	80	5.0 ± 0.39	3.0 ± 0.22
4.0	72	4.2 ± 0.21	2.5 ± 0.10
5.0	60	4.0 ± 0.38	1.4 ± 0.24

Pandey and Singh (2012) also found difficulty in rooting in this plant. They used three hormones viz. IAA, IBA and NAA with a concentration of 1, 3 and 5 mg/l were used in various combinations for rooting. Out of the total 9 treatments, only 20% rooting was found in one treatment, namely in the medium with 5 mg/l indole butyric acid (IBA) by them. Kapruwan *et al.* (2014) observed maximum rooting in 5 mg/l BAP. A maximum of 100 % shoots were effectively rooted when transferred to liquid MS medium supplemented with 2.5 mg/l 6-benzylaminopurine and 5 mg/l IAA. Efficacy of NAA in rooting was reported in *Inula japonica* (Yong-Mei *et al.,* 2008) and *Saussurea lappa* (Johnson *et al.,* 1997).

3. 4. Hardening and acclimatization of plantlets in greenhouse

The rooted plants were transferred to eco-friendly paper cups containing soilrite® irrigated with 1/4th solution of MS salts and incubated in greenhouse for 4 weeks to acclimatize the plants under *ex vitro* conditions for four weeks (Fig. 1F). While the plants are hardened, these should get physical support and they also require habitat soil for survival of the plants. When the plants are transferred to pots/soil/polybags there should be minimum disturbance to the root system. After one month, the micropropagated plants were planted in poly bags containing garden soil and vermicompost (1:1) and maintained in a greenhouse (Figs. 1G and 1H). Water logging during hardening showed a negative effect on the growth of the plantlets.

Successful micropropagation depends on the efficient establishment of plantlets outside the culture vessels. Acclimatization of *in vitro* plantlets is often difficult because of poorly developed structural parameters (Wardle and Short, 1983; Hazarika, 2003). Greenhouse acclimatization under slightly higher irradiance, fluctuating light and temperature less than field environment have been reported to reduce excessive evapo-transpiration by the gradual development of physiological adaptation.

4. CONCLUSIONS

An efficient micropropagation protocol has been developed for commercial production of *Dendrocalamus strictus* in this study. The nodal shoot segments were used as explants which were sterilized by use of 0.1% mercuric chloride. The shoots were induced on MS medium augmented with BAP and these were further multiplied on MS medium fortified by BAP with NAA. The in vitro induced shoots were rooted in vitro on MS medium contained NAA. The protocol is effective enough for large scale propagation of this industrially important bamboo species. The conditions should be further improved to enhance number of shoots by use of liquid culture systems.

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