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IMPROVED CLONAL PROPAGATION OF SUPERIOR *DENDROCALAMUS HAMILTONII* NEES GERMPLASM THROUGH *IN VITRO* TECHNIQUES

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ABSTRACT
Dendrocalamus hamiltonii is an economically important bamboo species of North East India. Being an important species, demand for quality propagules is escalating day by day which is not possible to meet using conventional propagation techniques alone. Therefore, optimization of an efficient micropropagation technique has become need of the hour. A very efficient technique is developed where nodal explants are washed with $HgCl_2$ with a pre treatment of fungicides and post treatment of antibiotic. For bud breaking and initiation of shoot multiplication, liquid Murashige and Skoog's medium fortified with 1.0 mg/L BAP + 0.25mg/L TDZ was found best among different hormonal combinations assessed. For further multiplication of shoots after detachment of the mother node, liquid MS medium fortified with 2.0 mg/L BAP proved to be the best in terms of both shoot number and shoot length. Agar gelled (6%) half strength MS medium fortified with 5.0 mg/L IBA, 10.0 mg/L Coumarin and 75.0 mg/L Putrescine was found to produce maximum number of roots. Primary hardening was done in in vermiculite for three weeks. 100% survivability was recorded at this stage. Secondary hardening was done in nursery beds or in polybags under agro shade net where more than 95% survivability was recorded. The protocol is so efficient that it can be used for large scale propagation of the species.

KEY WORDS: *Dendrocalamus hamiltonii*, micropropagation, sterilization, $HgCl_2$, Cytokinins, Coumarin, Putrescine, Hardening

INTRODUCTION

Dendrocalamus hamiltonii is an economically important multipurpose bamboo which is mainly used for construction, pulping, handicrafts and fuel purposes. Culms are 12–25 m high and 10–18.5 cm in diameter, naked below and heavily branched above. Being an important species, demand for quality propagules is escalating day by day which cannot be met using conventional propagation techniques alone. In this paper, an efficient and cost effective micropropagation technique in *D. hamiltonii* is reported. Though, few reports on micropropagation of *D. hamiltonii* from mature clump exist (Agnihotri *et al*, 2009; Singh *et al*, 2009, Jha *et al*, 2013) all these protocols rely on semi solid medium where either agar or phytagel is used for solidification. Use of solidifying agent increases the cost of production. For successful large scale commercial propagation, protocol must result high rooting and survival percentage too. This report describes an efficient micropropagation protocol which does not rely on the use of solidifying agents in induction and multiplication stage and results more than 90 percent rooting.

MATERIALS AND METHODS

Collection, preparation and surface sterilization of explants

Explants from a single Candidate Plus Clump (CPC), from Bamboo Germplasm Bank of Rain Forest Research Institute, Jorhat was utilized for the study. The culm cuttings of this selected CPC were maintained in the nursery bed by regular pruning for growth of new branches. Actively growing 30-60 days old lateral branches having 2.5-3.0 mm diameter were collected for initiating cultures. For explants, single nodal cuttings of about 3-4 cm long were cut and the leaf sheaths were removed using a sterilized scalpel without causing any damage to the dormant bud. The explants were washed with 1% (V/V) solution of Tween 20 by shaking for five minutes in a sterile bottle and rinsed with sterile distilled water for 3-4 times. Pretreatment of explants was done by dipping in solution of Bavistin (0.5%) and Indofil M - 45 (0.5%) for one hour. Sterilization of the explants was done using $HgCl_2$ (0.125%) for 10 minutes and rinsed 4-5 times followed by Cefotaxime (50 mg/L) treatment for another one hour. These sterilized explants were then placed in culture tubes containing 10 ml of MS medium for different experiments. The inoculated culture materials were kept in growth room and maintained within 25±2°C temperature under 16-hour light period was maintained by photoperiodic timer with light intensity of 2000 Lux.

Effect of cytokinins for shoot multiplication from nodal buds

Surface sterilized explants were inoculated in MS liquid medium with 16 different treatments. In these treatments liquid MS medium was fortified with 5 different doses of BAP (1, 2, 3, 4 & 5 mg/L) alone and in combination with 0.25 mg/L kinetin or 0.25 mg/L TDZ. Hormone free liquid MS medium was kept as control.

Effect of cytokinins for shoot multiplication from clumps:

Detachment of the newly initiated clump of multiple shoots from the mother node was done with a sharp scalpel after 6 weeks *i.e.* at the time of third subculture. These bunches of multiple shoots or shoot clumps were inoculated in liquid MS medium supplemented with 2mg/L BAP and 0.25 mg/L kinetin in culture bottles. They

were further subcultured at two weeks interval for three times to generate enough multiple shoots required for various experiments. In order to enhance the production of shoots after removal of mother node, different experiments were carried out.

To study the effect of cytokinins on shoot multiplication, shoot clumps were inoculated in MS liquid medium containing 8 different concentrations of BAP (0.5 mg/L - 4.0 mg/L) along with a control where only MS liquid medium was used without BAP.

Effect of auxins on root induction:

Three auxins NAA, IBA & IAA were used singly in MS semi-solid medium (6 gm/L Agar) to assess their effect on rooting. Each of the auxins was used in 5 different concentrations (1, 2, 3, 4 & 5 mg/L).

Effect of media strength on rooting:

Effect of different strength (Full, 1/2, 1/3 and 1/4) of MS semi-solid media (6 gm/L Agar) fortified with 5.0 mg/L IBA on rooting were experimented to ascertain the optimum strength of the media for rooting.

Effect of additives on enhancement of rooting:

Some additives are known to have synergistic effect on rooting. Therefore, two additives coumarin (5, 10, 15 & 20 mg/L) and putrescine (25, 50 & 75 mg/L) were used in 16 different concentrations and combinations to assess their effect. MS media fortified with 5.0 mg/L IBA was used.

Hardening

The primary hardening was done in vermiculite after washing thoroughly in water to remove the traces of agar from the rooted propagules and kept under agroshade net. The plants were kept covered with white polythene sheets to maintain the humidity (around 80%) for initial 7 days. Watering was done at 3 days interval and spraying of 1/4 strength liquid MS (macro and micro salts without organics) done at 7 days interval. After three weeks, the plants were taken out of the trays and transplanted in pots containing soil or in nursery beds under agro shade net for another three months for secondary hardening. Regular watering was done at this stage.

Experimental Design and data analysis:

Experiments with nodal explants were carried out in culture tubes with one explant each (one replicate). Total 18 replicates were used in the studies of surface sterilization and shoot multiplication in nodal explants. Experiment for shoot multiplication studies after removal of the mother node were carried out in 400 ml culture jam bottles (one replicate) each carrying 4 shoot clumps (4-5 shoots/clump). 6 replications were kept against each treatment. For *in vitro* rooting studies, four culture bottles (replications) each with four shoot clumps (3-4 shoots/clump) were used.

The data were analyzed with 2-way ANOVA and the best treatments medium were determined using Student-Newman-Keuls test at 5% probability level.

RESULTS AND DISCUSSION

All the treatments including the control induced multiple shoots (Table 1). However, among the different treatments 1.0mg/L BAP in combination with 0.25 mg/L TDZ (treatment no.12) produced highest number of shoots per node (5.06) with maximum shoot length (3.48 cm). Synergistic effect of two cytokinins has also been demonstrated earlier by Saxena and Bhojwani, (1991); Ramanayake and Yakandawala, (1997) and Das and Pal, (2005) in different species of bamboos. However, Mishra *et al.* (2008) carried out bud breaking and shoot multiplication of *B. tulda* in MS liquid medium fortified with both cytokinin (10 µM BA) and auxin (0.1 µM IAA).

TABLE 1: Effect of cytokinins on shoot multiplication from nodal explants of *D. hamiltonii* after 4 weeks of culture

Treatment No.	Cytokinins (mg/L)	Shoot number per node	Shoot length (cm)
1	Control (Hormone free)	0.56^{e}	0.56^{d}
2	1.0 BAP	1.28^{de}	1.07^{c}
3	2.0 BAP	1.94^{cd}	1.49^{c}
4	3.0 BAP	3.94^{b}	2.55^{b}
5	4.0 BAP	3.83^{b}	2.40^{b}
6	5.0 BAP	2.94^{bc}	2.32^{b}
7	1.0 BAP + 0.25 Kinetin	2.78^{bc}	2.29^{b}
8	2.0 BAP + 0.25 Kinetin	2.72^{bc}	2.24^{b}
9	3.0 BAP + 0.25 Kinetin	2.72^{bc}	2.38^{b}
10	4.0 BAP + 0.25 Kinetin	3.17^{bc}	2.60^{b}
11	5.0 BAP + 0.25 Kinetin	3.39^{b}	2.67^{b}
12	1.0 BAP + 0.25 TDZ	5.06^{a}	3.48^{a}
13	2.0 BAP + 0.25 TDZ	3.56^{b}	2.92^{b}
14	3.0 BAP + 0.25 TDZ	3.11^{bc}	2.87^{b}
15	4.0 BAP + 0.25 TDZ	2.83^{bc}	2.80^{b}
16	5.0 BAP + 0.25 TDZ	3.11^{bc}	2.67^{b}
SEd		0.40	0.22
CD		0.66	0.36
P- value		2.24E-25	2.43E-38

Treatments followed by different letters are significantly different from each other.
Numbers of replicates for each treatment were 18 and each replicate consisted of single nodal explant.
SEd: Standard Error of Difference & CD: Critical Difference at 5% probability level

Maximum average number of shoots per clump (13.21) with highest average shoot length (5.86 cm) was produced in MS liquid medium supplemented with 2.0 mg/L of BAP. Lowest average shoot number and length was observed in the control (Table 2). However, both Saxena and Bhojwani (1991) and Mishra *et al.* (2008) found highest shoot multiplication of *Bambusa tulda* in MS liquid medium supplemented with both cytokinins and auxins. This variation may be due to use of different clones or ecotypes in the studies.

TABLE 2: Effect of BAP on shoots numbers and shoots length of the clump after 2 weeks of culture

Treatment No.	Treatments (PGRs) mg/L	Shoot nos.	Shoot length (cm)
1	Control (Hormone free)	7.33[d]	2.87
2	0.5 BAP	7.96[d]	3.17
3	1.0 BAP	8.33[d]	4.53
4	1.5 BAP	8.50[d]	4.43
5	2.0 BAP	13.21[a]	5.86
6	2.5 BAP	10.67[b]	4.82
7	3.0 BAP	10.63[b]	4.96
8	3.5 BAP	9.67[c]	4.62
9	4.0 BAP	9.67[c]	4.59
SEd		0.362	0.064
CD		0.611	0.107
P-value		2.82E-18	9.27E-36

Treatments followed by different letters are significantly different from each other.
Numbers of replicates for each treatment were 6 and each replicate consisted of 4 shoot clumps.
SEd: Standard Error of difference & CD: Critical Difference at 5% probability level

All the treatments induced roots except the hormone free MS medium (Table 3). However, 5.00 mg/L IBA was found to be the most effective in producing highest average number of roots per clump (6.31) with maximum average root length (2.66 cm). In accordance to our result, Saxena (1990) achieved rooting in IBA with coumarin. But Sharma and Sarma, (2013) reported that no root was initiated in IBA and IAA in *B. tulda* but obtained in NAA.

TABLE 3: Effect of auxins on root induction from *in vitro* multiplied shoots

Treatment No.	Auxins (mg/L)	Response (%)	Root Nos./clump	Root Length(cm)
1	Control	0	0.00[h]	0.00[f]
2	1.0 NAA	56.25	2.00[f]	1.10[de]
3	2.0 NAA	62.5	2.50[e]	1.43[cde]
4	3.0 NAA	75	2.87[de]	1.70[bcd]
5	4.0 NAA	75	3.56[c]	2.04[abc]
6	5.0 NAA	81.25	3.87[c]	2.13[abc]
7	1.0 IBA	68.75	2.62[e]	1.18[de]
8	2.0 IBA	68.75	3.12[d]	1.85[bcd]
9	3.0 IBA	75	3.75[c]	2.13[abc]
10	4.0 IBA	81.25	5.25[b]	2.37[ab]
11	5.0 IBA	87.5	6.31[a]	2.66[a]
12	1.0 IAA	50	1.44[g]	0.75[e]
13	2.0 IAA	62.5	2.06[f]	1.59[bcd]
14	3.0 IAA	68.75	2.44[e]	1.73[bcd]
15	4.0 IAA	75	2.50[e]	1.72[bcd]
16	5.0 IAA	75	2.69[e]	2.05[abc]
SEd			0.1576	0.118
CD			0.268	0.2
P value			3E-20	4E-12

Treatments followed by different letters are significantly different from each other.
Numbers of replicates for each treatment were 4 and each replicate consisted of 4 shoot clumps.
SEd: Standard Error of difference & CD: Critical Difference at 5% probability level

To study if there is any synergistic effect of additives, coumarin (5.0, 10.0, 15.0 & 20.0 mg/L) and putrescine (25.0, 50.0 &75.0 mg/L) were added to MS medium supplemented with 5 mg/L IBA (Table 4/Figure 1). It was observed that addition of coumarin and putrescine had a positive effect on rooting. All the treatments containing additives produced higher number of roots as well as root length. Maximum average root number (10.62) and length (3.32 cm) was obtained in medium supplemented with 5.0 mg/L IBA and 10.0 mg/L coumarin and 75.0 mg/L putrescine.

Coumarin is a natural phenol that has been implicated in both promotion and inhibition of plant growth (Pollock *et al.*, 1954). It affects IAA biosynthesis and increases the endogenous-free IAA level during the induction phase of rooting to initiate more roots in *Vigna radiata* (Tartoura *et al.*, 2004). Synergistic effect of coumarin in rooting of bamboo species under *in vitro* condition is well established. In *B. tulda*, Mishra *et al.* (2008) reported 98% rooting in liquid MS supplemented with 40 µ M coumarin alone and Saxena (1990) reported rooting in MS media with IAA ($1X10^{-5}M$) and coumarin ($6.8X10^{-5}M$). Ramanayake and Yakandawala, (1997) reported 71.5% rooting on $^1/_2$ MS with 3 mg/L IBA and 10 mg/L coumarin in 27 days in *D. giganteus.* Saxena and Bhojwani, (1993) reported rooting (73%) in *D. longispathus* on $^1/_2$ MS + IAA (10 µM) + coumarin (68 µM) within 14 – 21 days.

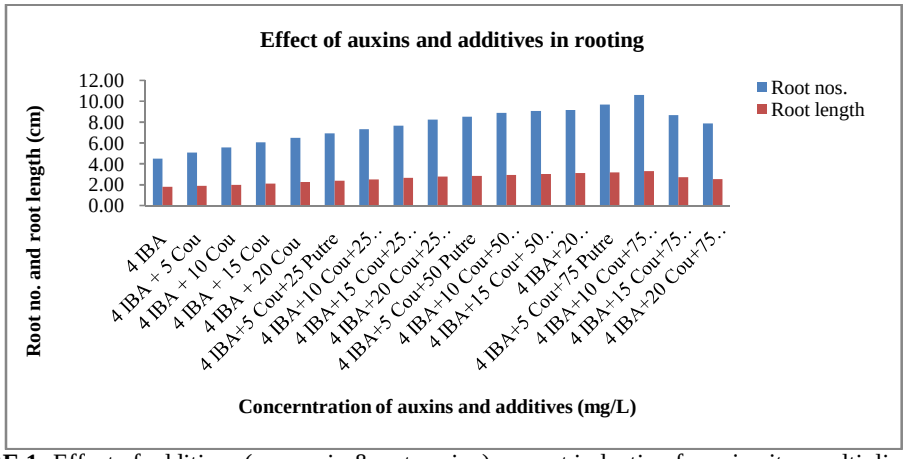

FIGURE 1: Effect of additives (coumarin & putrescine) on root induction from *in vitro* multiplied shoots

TABLE 4: Effect of additives (coumarin & putrescine) on root induction from *in vitro* multiplied shoots

Treatment No.	Additives (mg/L)	Response (%)	Root No./clump	Root Length (cm)
1	5.0 IBA	81.25	4.50^{m}	1.77^{k}
2	5.0 IBA + 05.0 Coumarin	81.25	5.06^{l}	1.91^{j}
3	5.0 IBA + 10.0 Coumarin	87.5	5.56^{kl}	1.98^{j}
4	5.0 IBA + 15.0 Coumarin	81.25	6.06^{jk}	2.12^{i}
5	5.0 IBA + 20.0 Coumarin	87.5	6.50^{ij}	2.24^{i}
6	5.0 IBA + 05.0 Coumarin + 25.0 Putrescine	81.25	6.94^{hi}	2.38^{h}
7	5.0 IBA + 10.0 Coumarin + 25.0 Putrescine	75	7.31^{gh}	2.50^{gh}
8	5.0 IBA + 15.0 Coumarin + 25.0 Putrescine	81.25	7.69^{fg}	2.64^{fg}
9	5.0 IBA + 20.0 Coumarin + 25.0 Putrescine	87.5	8.25^{def}	2.77^{ef}
10	5.0 IBA + 05.0 Coumarin + 50.0 Putrescine	68.75	8.50^{cd}	2.85^{de}
11	5.0 IBA + 10.0 Coumarin + 50.0 Putrescine	87.5	8.87^{cd}	2.95^{cd}
12	5.0 IBA + 15.0 Coumarin + 50.0 Putrescine	81.25	9.06^{bc}	3.03^{bc}
13	5.0 IBA + 20.0 Coumarin + 50.0 Putrescine	87.5	9.19^{bc}	3.11^{bc}
14	5.0 IBA + 05.0 Coumarin + 75.0 Putrescine	81.25	9.69^{b}	3.17^{b}
15	5.0 IBA + 10.0 Coumarin + 75.0 Putrescine	93.75	10.62^{a}	3.32^{a}
16	5.0 IBA + 15.0 Coumarin + 75.0 Putrescine	81.25	8.69^{cd}	2.72^{ef}
17	5.0 IBA + 20.0 Coumarin + 75.0 Putrescine	87.5	7.87^{ef}	2.55^{g}
SEd			0.318	0.068
CD			0.5405	0.115
P-value			9E-15	9.37E-19

Treatments followed by different letters are significantly different from each other.
Numbers of replicates for each treatment were 4 and each replicate consisted of 4 explants.
SEd: Standard Error of difference & CD: Critical Difference at 5% probability level

With the above mentioned factors (5.0 mg/L IBA + 10 mg/L coumarin + 75 mg/L putrescine) as constant, MS salt strength (both macro and micro) were varied to study the effect on root induction (Table 5/Figure 2). 100% root induction was observed in half strength MS. Significantly, highest average number of roots (6.81) was produced in $^{1}/_{2}$ strength MS as compared to other salt strengths.

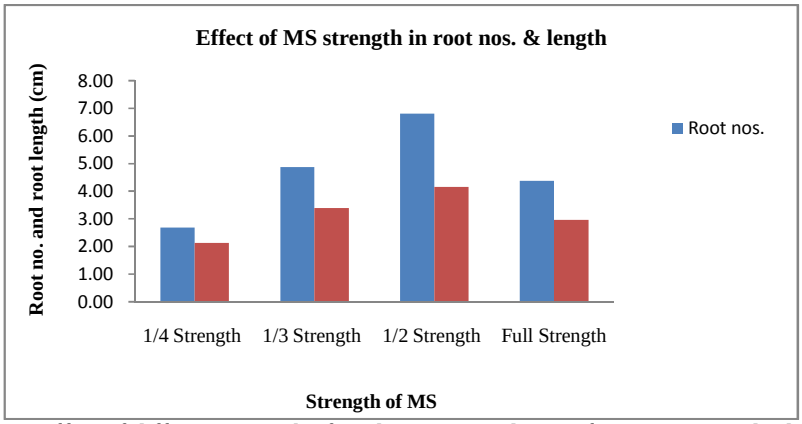

FIGURE 2: Effect of different strength of media on root induction from *in vitro* multiplied shoots

The same strength also produced maximum average root length (4.16 cm). In bamboo, very few reports exist where study has been carried out to evaluate the effect of salt concentration on rooting. However, there exist several report where different species of bamboo has been rooted in ½ strength MS successfully. In contrast several species have been rooted in full MS strength successfully. In our comparative study, rooting was achieved in all the concentration. However, highest percentage was achieved in $^{1}/_{2}$ (half) strength. Under field conditions, reduced nutrient strength signals the cuttings to induce roots so that the plant is equipped to survive. Reduced ionic concentration in $^{1}/_{2}$ MS might have simulated similar condition and attributed towards better rooting performance. However, further reduction of ionic strength of salts in $^{1}/_{3}$ and $^{1}/_{4}$ MS did not yield good result probably because of reduction of nutrient level beyond optimum level.

TABLE 5: Effect of different strength of media on root induction from *in vitro* multiplied shoots

Treatment No.	MS Salt Strength	Response (%)	Root Nos./clump	Root Length (cm)
1	$^{1}/_{4}$ Strength	75.00	2.69^{d}	2.13^{d}
2	$^{1}/_{3}$ Strength	68.75	4.87^{b}	3.40^{b}
3	$^{1}/_{2}$ Strength	93.75	6.81^{a}	4.16^{a}
4	Full Strength	50.00	4.37^{c}	2.96^{c}
SEd			0.27	0.08
CD			0.57	0.18
P-Value			0.003319	0.000474

Treatments followed by different letters are significantly different from each other.
Numbers of replicates for each treatment were 4 and each replicate consisted of 4 explants.
SEd: Standard Error of & CD: Critical Difference at 5% probability level

Hardening

The plantlets growing under *in vitro* condition are physiologically different from the plant growing outside. Under *in vitro* condition plantlets rooted but photosynthesis system are almost non-functional and they remain in a very high humid condition. Stomata remain always open and cuticular wax is also not developed. Therefore, the plants from tissue culture are not transplanted directly in the field; a hardening step is provided. In our study, primary hardening was carried out in vermiculate with 100% survivability. For secondary hardening the plantlets were transferred to soil beds or to polybags where more than 95% survivability was recorded. Vermiculite promotes maximum root growth since it is well aerated and retains moisture and nutrients.

FIGURE 3: (a) Sprouting of buds from nodal explants of *D. hamiltonii*. (b) 3 weeks old multiple shoots of *D. hamiltonii*. (c) 4-5 weeks old explants initiating multiple shoots development from a single node in liquid MS media. (d) Profuse multiplication of shoots. (e) *In vitro* rooting of *D. hamiltonii* initiation. (f) Rooted propagules ready for primary hardening. g. Primary hardening of rooted propagules in vermiculite. (h-i) Secondary hardening of propagules in polypots. (j) Field plantation.

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