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Rapid and mass propagation of economically important Bamboo *Dendrocalamus hamiltonii*

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Abstract

An efficient and reproducible protocol for the large scale propagation of *Dendrocalamus hamiltonii* is described. To establish aseptic cultures, the seeds were disinfected with sodium hypochlorite (4%) for 20 min. For shoot induction, the seeds were further inoculated on MS medium supplemented with cytokinins. Multiple shoots were formed within 3-5 weeks of seed culture. 7-8 shoots were obtained when seeds were inoculated on MS medium supplemented with 35 µM BAP. The initiated shoots were excised from mother explants and further multiplied on MS medium, supplemented with defined plant growth regulators. Best shoot multiplication was observed on MS medium supplemented with BAP (10 µM). A regular subculture in every 3-4 weeks increased the rate of multiplication. To initiate *in- vitro* rooting, pulse treatment was given in a 2- step procedure. Excised propagules of 3-5 shoots were inoculated on MS medium supplemented with high concentration of auxin (IBA) for 7 days, later on these *in vitro* shoots were transferred to half strength MS medium without auxin for 10-15 days to obtain well rooted plants. Plantlets were hardened, acclimatized and established in soil, where they exhibited normal growth. As Bamboo rapidly fixes atmospheric carbon into biomass, it can reverse the effect of fossil fuel emission by vehicular usage.

Keywords: *Dendrocalamus hamiltonii,* Bamboo, Micropropagation, Bamboo chips.

Introduction

The bamboos occupy a special place in the lives of rural poor and rural industries, especially in Asia. The most important use of bamboo is as a raw material in pulp, paper and rayon industries and recently for bamboo chips as energy source (Anonymous, 1978; Rao *et al*., 1990).The multifarious uses of bamboos have increased their demand much beyond the availability. Bamboo is threatened because of its monocarpic habit and increased market demand. It has traditionally been propagated through seed or through vegetative means, but these methods besets with many problems. The conventional vegetative propagation through cuttings and rhizome is undependable due to the bulky size of the propagules in the required number (Rao *et al.,* 1990). The potential of micropropagation has raised high hopes and a lot of research has been focused on the development of protocols for a rapid and a large scale propagation (Rao *et al.,* 1985; Nadgauda *et al.,* 1990; Godbole *et al.,* 2002; Sood *et al.,* 2002). Micropropagation of bamboo species using seed has the advantage of having greater number of genotypes in culture, from where propagation may proceed ensuring greater diversity of the species. The population obtained is clonal as long as the plants derived from a single embryo are kept separately. The *Dendrocalamus hamiltonii* is one such economically important species which is distributed in the North-West Himalaya, Sikkim, Arunachal Pradesh, Assam, Manipur, Meghalaya, Mizoram, Nagaland, Tripura of India, Bhutan and Bangladesh. Flowering cycle is reported to be 30-40 years. It is popular for its strong culms that are used for construction purposes, the tender shoots are used for the preparation of 'hiyup' a sour pickle by the tribals of Arunachal Pradesh in India. Its leaves also serve as a fodder for animals. The present study was undertaken to establish a protocol for efficient *in-vitro* propagation of *D. hamiltonii* by high frequency shoot proliferation from the seeds since seed propagated plants are expected to last a full life span.

Materials and methods

Seed germination

Mature seeds of *Dendrocalamus hamiltonii were* obtained from The Sheel Biotech Company, New Delhi, India. After dehusking carefully, the healthy seeds were selected and washed with cetrimide for five minutes and then surface sterilized with sodium hypochlorite (4%) for 20 minutes followed by three to four rinses with sterile distilled water to remove the traces of sterilant. Sterilized seeds were then cultured aseptically on semi-solid MS medium containing 3% sucrose supplemented with varying concentrations of cytokinin alone (5 to 45µM BAP) and in combination. The medium was gelled with agar (0.7%), adjusted to pH 5.8 prior to autoclaving at 121^{o}C for 15 min. Cultures were maintained at 25^{0}C ± 2^{0}C under a 16h photoperiod with a photon flux density of 2500 lux from white fluorescent tubes.

One seed was inoculated in each culture tube. For each experiment a minimum of 24 replicates were taken. Observations were recorded after an interval of 4 weeks.

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Multiple shoot formation

The germinating seeds were transferred to the culture medium for further growth and development of the multiple shoots. The regenerated shoots were excised and sub-cultured on fresh medium with suitable cytokinin (BAP) for further *in vitro* shoot multiplication. In 3-4 weeks these shoots were further multiplied and were cut into shoot clusters of 3-4 shoots and were again subcultured on semi-solid MS medium supplemented with BAP (5-30µM).These subcultured *in vitro* shoots were multiplied every three weeks. Experiments were conducted to obtain maximum *in vitro* shoot multiplication rate that largely consisted of experiments pertaining to cytokinins *i.e.* effect of BAP and Kn alone and in combination. For this, multiplied shoots were subcultured in propagules consisting of 3-4 shoots. For each experiment a minimum of 12 replicates were taken. Observations were recorded after an interval of three weeks. Cultures were maintained at $25^0C \pm 2^0C$ under a 16h photoperiod with a photon flux density of 2500 lux from white fluorescent tubes. The number of propagule cultured and number of propagule derived at the end of subculture was regarded as the rate of multiplication.

In vitro rooting

Propagules consisting of 3-4 shoots were excised from multiple shoots and transferred on MS medium containing various concentrations of auxins like NAA and IBA for root induction. Rooting response was recorded in terms of rooting percentage, average number of roots produced and average root length. Variance was calculated as standard error and CRD design was applied to each observation.

Hardening and acclimatization

In vitro raised plantlets need to be hardened and acclimatized before field transplantation as they are heterotrophic in their mode of nutrition and cannot withstand the environmental conditions without proper hardening and acclimatization because they grew under controlled condition. For hardening these, plantlets were transferred to autoclaved 250 ml screw cap glass bottle containing 1/3 volume of autoclaved vermiculite. These plantlets were nurtured half strength MS medium (without organics) twice a week for two weeks and were kept in tissue culture room. After two weeks these were shifted to mist chamber having relative humidity of 80-90% with a temperature of 30 ± 2^0C. The caps of bottles were removed and plantlets were allowed to remain in the bottle for 1 week before they were transferred to polybags containing a mixture of sand, farmyard manure and soil in a ratio of 1:1:1.Here plantlets were kept for three weeks and were irrigated with half strength MS medium. Later on, these polybags were shifted to open shade house for acclimatization.

Statistical analysis

Data were recorded after 3-4 weeks. Experiments were repeated thrice and the value represents the mean of three experiments. 24 replicates were taken for each experiment. The statistical methodology was the same as described earlier in general material and methods.

Results

Initiation of shoot cultures

It was found that the ability of the seeds to form multiple shoots was dependent on the concentration of BAP in the medium. Seeds were cultured on semi-solid MS medium supplemented with different concentrations of cytokinin BAP (5-45µM).All viable seeds germinated and formed shoots, without root formation on MS medium containing BAP. At lower concentrations of BAP (5-15µM) in the MS medium 10-15% seed germination rate was observed with 3-4 shoot developing from a seed. On hormone free medium root was also developed along with single shoots. A maximum germination of 37.50% was recorded at a concentration of 35 µM BAP supplemented in the MS medium where 9-10 shoots were produced within 3 weeks of culture (Table-1). BAP when used along with Kn in the MS medium did not improve the seed germination response as compared to BAP when used alone in the MS medium. Multiple shoots developed from the seeds were excised and subcultured for further multiplication on MS medium containing 5-30 µM BAP (Plate 1, Fig. A-D).

Table 1. Effect of BAP concentration in MS medium on *in vitro* seed germination proliferation of *D.hamiltonii*

BAP (µM)	Response %	Mean shoot number	Mean Shoot Length (cm)
0µM	11.11 ±1.39	2.22 ±0.16	1.72± 0.03
5µM	13.89 ±1.39	2.57 ±0.12	2.13± 0.04
15µM	15.27±1.39	3.57 ±0.26	2.25± 0.22
25µM	18.05±1.39	6.62 ±0.16	2.51± 0.05
35µM	37.50±2.40	9.66± 0.10	3.39± 0.01
45 µM	34.77±1.44	8.29± 0.13	3.21± 0.05
One Way Anova	**F= 49.94****	**F= 362.34****	**F=197.47****

** = P<0.01, *= P<0.05

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Plate 1. Micropropagation of *Dendrocalamus hamiltonii* through seed culture
Fig.A Seeds of *Dendrocalamus hamiltonii.*
Fig.B Germination of seed.
Fig.C shoot formation from seed cultured on MS + 7.0mg/l BAP.
Fig.D Shoot and root formation from seed cultured on Hormone free MS medium.

In vitro shoot multiplication

Multiple shoots developed from the seeds were excised and subcultured in propagules of 3-5 shoots on agarified MS medium supplemented with 05-30 µM BAP for shoot multiplication.The *in vitro* shoots were successfully multiplied and subcultured on MS medium supplemented with 10µM BAP (Plate 2, Fig.A).In this medium, the maximum shoot multiplication rate of 8-9 folds was obtained in three weeks along with sizeable shoot elongation (Table- 2).These shoots were carefully separated into propagules (cluster of 3-5 shoots) and were again subcultured on shoot multiplication medium. On decreased levels of BAP the shoot multiplication rate decreased with increase in shoot length. Effect of the size of propagule on shoot multiplication rate was also studied. It was found that the shoot multiplication rate declined sharply, if propagules of less than or more than 3-4 shoots were cultured. Maximum multiplication rate was obtained when the shoot cultures were regularly subcultured on fresh medium in 3 weeks of time. The cultures incubated for longer duration showed necrosis.

In vitro rooting

In vitro shoots when transferred onto the MS medium supplemented with auxin produced *in vitro* roots. A varied effect on *in vitro* rooting was observed by auxin (IBA, NAA and IAA) when incorporated in the MS medium at different concentrations. For *in vitro,* rooting shoot clusters of 3-4 shoots (2-3 cm in length) were cultured on *in vitro* rooting medium. Initially, less percentage of rooting was obtained; therefore *in vitro* roots were obtained by pulse treatment of high concentrations (25.0-150µM) of auxins and then subsequent transfer of treated shoots on hormone free MS medium. Initially, the *in vitro* shoots were kept in this medium for seven days. As a result root primordia developed at the base of propagules. It was observed that the treatment with IAA did not show any development of primordia. The IBA or NAA supplemented medium gave positive results *i e.,* showed the primordial initiation. These shoots with developing root primordia required transfer on basal medium (MS medium without hormones) for elongation of primordium into roots. Therefore, these *in vitro* shoots with root primordia were transferred to the liquid MS medium without any auxin. The high percentage of rooting response was observed in those *in vitro* shoots which were given pulse treatment in medium supplemented with IBA. MS medium supplemented with 100µM IBA gave the maximum rooting response (93.93%) with 8-9 roots on an average per shoot propagule (Table 3, Plate 2 Fig. B). *In vitro* rooting was also induced when NAA was supplemented in the MS medium but the percentage response was very less. So it can be concluded that IBA proved to be superior auxin as compared to NAA

Plate 2. *In vitro* multiplication and rooting of *Dendrocalamus hamiltonii.* **Fig.A** *In vitro* shoots multiplication on MS + 15µM BAP.
Fig. B *In vitro* rooting.

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and IAA for obtaining *in vitro* rooting in *D. hamiltonii*.

Hardening and acclimatization

Four-five week old tissue culture raised plantlets with well developed root system were hardened and acclimatized prior to field transfer. For hardening the *in vitro* rooted plantlets, they were first washed with water so as to remove adhered agar/medium and then transferred to autoclaved culture bottles containing vermiculite. These plantlets were supplied with half strength MS medium without organics twice a week (Plate 3, Fig. E) and later they were transferred to mist chamber at 80-90% relative humidity and temperature of 30±2^{0}C.Plants were then transferred into polybags containing sand: soil: FYM in 1:1:1 ratio. These plantlets were kept in mist chamber for four to five weeks. After mist chamber stage the plants became hardened and were shifted to open shade house conditions for acclimatization to outer environmental conditions. During hardening, the shoots elongated, leaves turned greener and expanded. In shade house the plants were further transferred to bigger polybags or earthen pots and were irrigated with water (Plate 3, Fig. F). Plants' were hardened, acclimatized and established in soil, where they exhibited normal growth.

Table 3. Effect of IBA (pulse treatment) on *in vitro* rooting of *D.hamiltonii.*

IBA (µM)	Response %	Mean root number	Mean root length (cm)
10 µM	09.09±0.00	2.11 ±0.04	05.03± 0.09
20 µM	12.12±1.51	2.55 ±0.08	05.39± 0.06
40 µM	22.74±2.62	2.74 ±0.13	05.63± 0.08
60 µM	30.30±1.51	3.27 ±0.16	06.10± 0.07
80 µM	54.54±2.62	4.65 ±0.21	05.89± 0.08
100 µM	93.93±1.52	9.77 ±0.08	07.15± 0.10
150 µM	37.87±1.51	4.14 ±0.05	05.64±0.07
200 µM	06.06±1.52	1.55 ±0.12	04.11±0.12
One Way Anova	F= 273.74**	F= 460.92**	F=104.25**

** = P<0.01, *= P<0.05

Plate 3. Hardening and Acclimatization. **Fig. E** *In vitro* hardening of plantlets **Fig. F** *In vitro* raised plants in polybags

Discussion

In the present study for *in vitro* propagation of *Dendrocalamus hamiltonii* through aseptic seed culture, seeds of *D. hamiltonii* procured from Sheel Biotech Ltd., New Delhi were used. Micropropagation of bamboo species using seed or seedlings derived materials have earlier been reported by a number of workers (Nadgir *et al*., 1984; Jerath, 1986; Rao and Rao, 1988; Saxena, 1990; Mrudul *et al.,* 1996; Maity and Ghosh, 1997; Arya and Arya, 1997; Arya *et al.,* 2002; Ravikumar *et al*., 1998; Arya *et al.,* 1999; Kapoor and Rao, 2006).

Maximum germination of 37.50% was recorded at a concentration of 35 µM BAP supplemented in the MS medium. These results are in agreement with the findings of other workers who have also noted the effectiveness of BAP for the initiation of shoot cultures from seeds in different bamboo species (Nadgir *et al*., 1984; Saxena, 1990; Mrudul *et al.,* 1996; Arya and Arya, 1997; Maity and Ghosh, 1997; Ravikumar *et al*., 1998; Arya *et al.,* 2002; Kapoor and Rao, 2006).

In the present investigation, effect of different cytokinins was tested either alone or in combination for *in vitro* shoot multiplication rate. BAP was selected as the most suitable phytohormone for *in vitro* shoot multiplication in *Dendrocalamus hamiltonii.* These results are supported by the earlier reports on *in vitro* multiplication of bamboos, where BAP had been used extensively for shoot multiplication (Ramanayake *et al.* 2006; Yasodha *et al.,* 2008; Ramanayake *et al.* 2008; Arya *et al.,* 2008; Mudoi and Borthakur, 2009). The higher dose of BAP produced the highly

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reduced shoots of *D. hamiltonii.* Subculturing at higher concentration of BAP fortified medium caused stagnant and unhealthy growth of the shoots and leaf lamina and were almost condensed.

In vitro shoot multiplication was carried out in liquid as well as on semisolid MS medium routinely in *Dendrocalamus hamiltonii.* Multiplication rate in liquid medium was found better than on semisolid medium. According to Debergh (1983), poor shoot multiplication on semisolid medium may be due to binding of water and adsorption of minerals and PGRs, by gelling agents resulting in the depleted supply to growing shoots. Earlier Das and Pal (2005), in *B. balcooa*; Sanjaya *et al.* (2005), in *Pseudoxytenanthera stocksii*; Arya *et al.* (2006), in *D. giganteus*; Ramnayake *et al.* (2006), in *B. vulgaris;* Diab and Mohammed (2008) in *Oxytenanthera abyssinica* have reported higher rate of multiplication in liquid medium as compared to semisolid medium. However, later on due to several problems like falling of shoot propagule in liquid medium, unfolding of paper bridges, evaporation of medium during autoclaving and vitrification, they were transferred to semisolid medium after few rapid subculturing in liquid medium, where shoots showed normal growth and were not vitrified.

Maximum shoot multiplication rate of *D. hamiltonii* was obtained when the pH of the medium was adjusted to 5.8 as at this pH maximum ionic exchange is possible, thus nutrient uptake is maximum and deviation from this pH either towards acidic or basic leads to precipitation or accumulation of toxins in medium. These results are supported by other workers who reported the similar results on different species of bamboos (Sanjaya *et al.*, 2005; Ramanayake *et al.,* 2006; Arya *et al.,*2006; Ramanayake *et al.,*2008; Arya *et al.,* 2008; Yasodha *et al.*2008). pH of the medium also declines rapidly on subculture in the first few weeks. Moreover, Yasodha *et al.* (1997) in *Bambusa nutans* and *D. membranaceous* reported shoot multiplication on medium with pH 5. Arya *et al.,* (2006) in *D. giganteus* also obtained multiplication on 4.5 pH medium.

During the present investigation for the yield of maximum number of shoots and better overall growth of cultures a subculture frequency of three weeks in *D. hamiltonii was* found to be the most suitable. This is in accordance with Yasodha *et al*. (1997), who performed subculturing at 15-20 days interval for *Bambusa nutans* and *Bambusa arundinacea* cultures. Delaying of the subculturing period resulted in gradual browning of the shoots followed by blackening of the basal portion. Hence, sub culture duration was recorded as the most crucial factor for obtaining optimal and desired level of regeneration of shoots.

In the present investigation, experiments were conducted to get the minimum number of shoots in a propagule which when subcultured on fresh medium produced the multiple shoots in a large number and eventually resulted in maximum multiplication rate. Single nodal segments produced very few lateral shoots, giving a less multiplication rate and after that, all shoots died or become chlorotic. On the other hand, dividing the shoot clumps in groups of 3-5 shoots yielded a more or less constant multiplication rate. In the present investigation also a propagule of 4 shoots was used. These results were supported by Jimenez *et al.,* 2006, and Agnihotri & Nandi (2009), who in *Guadua angustifolia* and *D. hamiltonii,* respectively reported 3-5 shoots in a propagule for a better multiplication rate.

In the present study, maximum rooting percentage obtained after a two-step treatment of 7 days on liquid MS medium supplemented with 100µM IBA and then transferred *in vitro* shoots to basal MS medium. Later on, these shoots also showed simultaneous shoot elongation, this is due to "cytokinin-carry over effect" in the shoots as there is sufficient residual cytokinin in shoots. IBA was found to be the most favourable root inducer compared to NAA and IAA. These results are in line with earlier reports on several bamboos such as *Drepanostachym falcatum* (Arya *et al.,* 2008), *Oxytenanthera abyssinica* (Diab and Mohammed, 2008), *Dendrocalamus hookeri* (Ramanayake *et al.* 2008), *Dendrocalamus hamiltonii* (Agnihotri and Nandi, 2009), *Melocanna baccifera* (Kant *et al*., 2009).

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