In vitro shoot Proliferation and Somatic Embryogenesis : Means of Rapid Bamboo multiplication

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Abstract

The conventional methods of propagation of bamboos sexual as well as vegetative have lots of problems that restrict their multiplication in large numbers. Bamboo is now a commercial crop and hence there is shortage of availability of planting stock material. To overcome this tissue culture offers rapid and reliable method to produce plants in large number. Tissue culture protocol has been developed in three economically important bamboo viz. *Dendrocalamus asper* (edible bamboo), *Drepenostachyum falcatum* (hill bamboo) and *D. hamiltonii*. Multiple shoots were induced from aseptic *in vitro* culture of nodal shoot segments through axillary branching. In vitro shoots were proliferated from axillary buds in two weeks on MS medium supplemented 1-10mg/lBAP. In case of *D. asper* a consistent 14-16 fold shoot multiplication was obtained after 4 weeks subcultured cycle. In *D. hamiltonii* and *D. falcatum* shoot multiplication was 6-10 folds. Rooting was obtained on MS supplemented with 1.0-5.0 mg/l NAA or 5-10mg/l IBA. Embryogenic callus was developed from explants taken from nodal tissues and basal part of leaves that were taken from *in vitro* multiplied shoots. Somatic embryos were induced on MS medium supplemented with 20-30 µM 2,4-D and 2% sucrose in dark conditions. In *D. asper* callus proliferated to 3-5 folds every 4 week on MS + 9µM 2,4-D + 2.85µM NAA + 0.88µM BAP medium. In *D. hamiltonii* embryogenic callus was multiplied on MS + 10 µM 2,4-D + 5µM BAP medium. In *D. falcatum* callus proliferated to 2-3 folds on MS + 10µM 2,4-D + 0.88µM BAP. Somatic embryos matured to scutellar and coleoptillar embryos on 5µM ABA medium. In *D. asper* somatic embryos developed into plantlets within 30 days on MS medium with 4.4µM BAP and 2.8µM GA3 with a conversion rate of 70%. In *D. hamiltonii* and *D. falcatum* somatic embryos germinated on MS with 5µM BAP. The plantlets developed from germination of somatic embryos and through in vitro shoot proliferation were hardened and acclimatized and field transferred.

Key words: *In vitro*, Tissue Culture, Bamboo's, Micropropagation, Somatic Embryogenesis

Introduction

In view of constant increase in the demand of the planting material and the limitations of the conventional methods of propagation, research into newer and more rapid methods of propagation for different bamboo species is urgently called for. In such condition, *in vitro* methods remained an alternative solution. Tissue culture offers rapid and reliable methods such as micropropagation to rapidly produce bamboo plants in large numbers. Micropropagation via *in vitro* shoot proliferation and somatic embryogenesis offers method for mass propagation. Tissue culture protocol has been developed in three economically important bamboo viz. *Dendrocalamus asper* (edible bamboo), *Drepenostachyum falcatum* (hill bamboo) and *D. hamiltonii*.

Materials and Methods

Nodal segments containing single axillary bud were used as source material for tissue culture. Prior to sterilization procedures, the basal sheath covering the bud was removed with the help of a scalpel. The nodal segments with axillary buds were first swabbed with alcohol soaked cotton and then surface sterilized with 0.1% $HgCl_2$ solution for 8-10 minutes, followed by 3-4 times washing with autoclaved distilled water to remove the traces of sterilant. The surface sterilized axillary buds were inoculated on MS (Murashige and Skoog, 1962) medium supplemented with cytokinin (BAP/Kn). The pH of the medium was adjusted to 5.8 prior to autoclaving the medium at 121^0C for 15 minutes. Cultures were maintained at 25 ± 2^0C temperature with 16 hours illumination with a photon flux density of 2500 lux from white fluorescent tubes.

The proliferated *in vitro* shoots were excised and sub-cultured on MS medium for further *in vitro* shoot proliferation. In 3-4 weeks these shoots multiplied and were cut into shoot clusters of 3-4 shoots and were again subcultured. Different sets of experiments were conducted to obtain maximum *in vitro* shoot proliferation rate. The proliferated *in vitro* shoots were cultured on MS medium containing various concentrations of auxins like NAA and IBA for *in vitro* root induction. A propagule of three shoots were inoculated on MS medium supplemented with different concentrations of auxins. Three propagules (shoot clusters of 3 shoots) were cultured per conical flask to induce *in vitro* rooting.

Embryogenic callus was developed from explants taken from nodal tissues and basal part of leaves taken from *in vitro* multiplied shoots. 8-10 weeks old compact callus was cultured for differentiation and multiplication of somatic embryos from callus. For this, callus was transferred to other hormonal combinations. 2, 4-D was used (5-50 µM) alone and in combination with BAP. IAA and NAA was also used with 2, 4-D and BAP in different concentrations. Cultures were scored for embryogenic frequency and multiplication rate of callus after 4 weeks of incubation in dark. The effect of subculture duration was assessed for multiplication of embryos from 2-8 weeks. Embryogenic callus were scored for mean number of embryos per embryogenic culture. Approximately 50 mg of embryogenic callus was inoculated in all the related experiments and after 4 weeks duration the final weight of the callus was recorded. Globular embryos in callus need maturation for further development of embryos to scutellar and colleoptillar stages.Callus with somatic embryos were transferred on MS medium fortified with different concentration of sucrose (2-10%) for maturation of embryos. Within 4 weeks on sucrose enriched medium, the mature somatic embryos were then transferred on germination medium containing different concentrations of BAP (5-30 µM) for germination of somatic embryos. Germination was carried out at 25 ± 2^0C under 16/8 hrs (light/dark) photoperiod for 4 weeks. Germinated embryos and rooted plantlets were transferred to vermiculite for hardening.
In vitro raised plantlets need to be hardened and acclimatized before field transplantation. 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 with 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. All experiments were repeated thrice and the value represents the mean of three experiments. 24 replicates were taken for each experiment.

Results and Discussion

Establishment of cultures

Out of the various sterilizing agents tried mercuric chloride proved to be the best sterilant as it produced the maximum number of aseptic explants as well as the maximum bud break response. Nodal segments containing axillary buds were surface sterilized with 0.1 % $HgCl_2$ for 8-10 min. which resulted in 60-70% aseptic cultures, the remaining treated buds dried out or produced very delayed bud break response. Nodal segments when cleaned with ethyl alcohol (70%) swabbed cotton prior to Mercuric chloride solution treatment improved the surface sterilization of the axillary buds. The treated nodal segments required 3-5 washing with autoclaved distilled water, without these washing bud break response was either delayed or axillary buds died after sometime. The nodal segments sterilized with NaOCl or H_2O_2 yielded significantly less percentage of aseptic buds (30-40%).

Axillary bud break was achieved in 2-3 weeks in all the aseptic cultures on MS medium supplemented with 10-50 µM BAP. The number of proliferating shoots ranged from 1 to 6 per axillary bud. BAP treatments significantly increased the percentage of bud break and the number of shoots that proliferated. Maximum bud break response of 70% was obtained in MS medium supplemented with 30µM BAP where 2-3 shoots proliferated during bud break in case of *D. asper* and *D.hamiltonii*. The bud break response decreased with increased concentration of BAP (40-50µM) resulting in small condensed shoots. It was observed that at lower concentration of BAP (10-20 µM) in MS medium, 1-2 axillary shoots proliferated in case of *D falcatum*.

Nodal segments collected during February-March and September-October showed maximum bud break response up to 60-70% on MS medium supplemented with cytokinin. Nodal segments collected during November to January (winter months) produced reduced bud break response (35-45%).

In vitro shoot multiplication

The proliferated axillary shoots were excised from mother explant and subcultured on MS medium supplemented with 10-50 µM BAP for further *in vitro* shoot multiplication. These multiplied *in vitro* shoots were later dissected out into propagule (a group of 3 shoots) and were subcultured on MS medium supplemented with 10-50 µM BAP for further *in vitro* shoot multiplication. A number of experiments were performed with respect to *in vitro* shoot multiplication. BAP at 20-30 µM when incorporated in the MS medium gave good shoot multiplication (Fig.1A). On hormone free medium the *in vitro* shoot multiplication rate drastically decreased and the shoot cultures died. At increased level of BAP (40-50 µM) in MS medium the shoot multiplication decreased along with reduction in the shoot length of the multiplied shoots. In case of *D. asper* a consistent 14-16 fold shoot multiplication was obtained after 4 week subcultured cycle (Table 1). In case of *D. hamiltonii* and *D. falcatum* the shoot multiplication was 6-10 folds. 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 *D.asper*, *D. hamiltonii* and *D. falcatum*. 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.,* 2008a; Mudoi and Borthakur, 2009;). The higher dose of BAP produced the reduced 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.

For the *in vitro* multiplication of shoots, carbon source in the form of sugars has to be added to the nutrient medium for growth and proliferation of shoots as the photosynthetic capability of in vitro shoots are limited. In the present case, different concentrations of sucrose (0-5%) in the MS medium was tested for the growth and development of *in vitro* shoots. Sucrose at 3% in the MS medium gave the best results for in *in vitro* shoot multiplication. On sucrose free medium the *in vitro* shoots did not multiply and with the passage of time, leaves and shoots became pale green.

In vitro rooting

In vitro rooting was attempted by culturing the *in vitro* multiplied shoots on MS medium supplemented with different auxins like IBA or NAA. During *in vitro* rooting experiments a propagule of 3 shoots were cultured on rooting medium. In vitro rooting was obtained in 80-90% of *in vitro* shoots on 2-3 mg/l NAA or 8-10mg/l IBA supplemented MS medium in 5 weeks in case of *D. asper* and *D. falcatum* (Fig.1B). Whereas for *D. hamiltonii* 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 was followed for *in vitro* rooting. In the present study 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). On rooting medium shoots also elongated and good root and shoot system developed in 5-7 weeks (Fig. 1D).

Somatic embryogenesis

In the present study, induction of somatic embryogenesis was influenced by the addition of BAP along with 2, 4-D. Subculture interval of more than 4 weeks resulted in the formation of non-embryogenic callus with a gradual browning of the cultures. Embryogenic callus proliferated faster in dark conditions than the light conditions as reflected from increased multiplication folds (2-3 folds) in dark conditions. In *D. asper* callus proliferated to 3-5 folds every 4 week on MS + 9µM 2, 4-D+ 2.85 µM NAA + 0.88 µM BAP. In *D. hamiltonii* embryogenic callus was multiplied on MS + 10µM 2, 4-D + 5µM BAP medium. In *D. falcatum* callus proliferated to 2-3 folds on MS + 10µM 2, 4-D + 0.88µM BAP. Somatic embryos matured to scutellar and coleoptillar embryos on 5µM ABA supplemented medium. In *D. asper* somatic embryos developed into plantlets within 30 days on MS medium supplemented with 4.4 µM BAP and 2.8 µM GA_3 with a conversion rate of 70%. In *D. hamiltonii* and *D. falcatum* somatic embryos germinated on MS medium supplemented with 5µM BAP (Table 2).

Furthermore, for optimal utilization of all globular embryos to attain maturity to scutellar embryos and to coleoptillar staged embryos, a maturation step was needed (Fig.1E). For this the somatic embryos obtained on multiplication medium were transferred on MS medium supplemented with varying concentrations of sucrose. Sucrose at lower concentration (1-3%) in MS medium promotes multiplication of globular somatic embryos, whereas higher sucrose concentration (4-6%) promoted the maturation of embryos present in callus. Sucrose at 6% level with complete elimination of 2, 4-D from the medium showed highest efficiency (9.00 embryos/culture) of scutellar and coleoptillar embryos. Concentrations higher than 6% of sucrose in MS medium resulted in decreased maturation rate and browning of callus.

Coleoptillar stage somatic embryos developed shoots and roots when these were transferred to MS medium supplemented with BAP (5 µM -15 µM). Maximum germination frequency observed on MS medium supplemented with 5 µM BAP under 16/8 hrs (light/dark) photoperiod and 25 ± 2^0C temperature. Within 4-6 weeks complete plantlets with well developed shoot and roots developed. 15% of coleoptillar stage embryos regenerated to plantlets on germination medium, other 85% embryos formed only shoots. Germinated embryos (somatic embryo plants) were placed in polybags containing soil, sand and FYM in 1:1:1 proportion and placed in the mist chamber for 7-15 days with a RH of 80-90% and temperature 30 ± 2^0C for hardening (Fig.1C). These were regularly fed with half strength MS medium solution without organics twice a week. After 15 days in the mist chamber these plants were shifted to agro net shade house to protect plants from strong sunlight. Under shade house these plants were acclimatized for 1-2 months and were later transferred to the field (Fig.1F).

Successful reports on somatic embryogenesis are available in many bamboos. Most of the reports are based on reproductive tissues like seeds, embryo, inflorescence or anthers, etc (Rout and Das, 1994; Saxena and Dhawan, 1999; Gillis *et al.*, 2007). Only few reported induction of somatic embryogenesis from vegetative tissues (Hassan and Debergh, 1987; Saxena and Bhojwani, 1993; Jullien and Tran Thanh, 1994; Godbole *et al.,* 2002; Ramanayake and Wanniarachchi, 2003; Satsangi, 2003; Lin *et al.,* 2004; Arya *et al.*, 2008). Use of vegetative tissue will facilitate the induction and establishment of somatic embryogenesis, as explants will be available throughout the year, which is limited in case of reproductive tissue as bamboo being monocarpic with long durations of flowering cycle. The present study describes the development of a protocol to induce somatic embryos on callus derived from the severed segments of the sprouted buds of the mature nodal explants from bamboo of a known physiological age. Nodal segments as explant for induction of callus was used in a number of bamboo species like *Bambusa vulgaris, Dendrocalamus giganteus* and *D. strictus* (Rout and Das, 1994), *D. giganteus* (Ramanayake and Wanniarachchi, 2003), *Bambusa edulis* (Lin *et al.,* 2004), *Dendrocalamus asper* (Arya *et al.*, 2008). Leaf sheath or leaf tissue was utilized for the initiation of somatic callus in *Bambusa flexicosa, Phyllostachys viridis, Bambusa glaucescens, Dendrocalamus asper* (Hassan and Debergh, 1987; Jullien and Tran Thanh Van, 1994; Arya *et al.*, 2008).Pseudospikelets were taken as explants for induction of somatic embryogenesis in *Bambusa balcooa* (Gillis *et al.*, 2007).

In the present case callus initiation and multiplication required dark condition, which is in accordance with reports of Yeh and Chang (1986 ; 1987), Rout and Das (1994) in *Bambusa vulgaris, Dendrocalamus giganteus* and *D. strictus*, Arya *et al.*, (2008) in *Dendrocalamu asper*.

Presently in *D. hamiltonii,* callus compactness and high embryogenic potential was consistently accomplished with cytokinin addition in multiplication medium. Highest frequency of embryogenesis and somatic embryos were seen on agarified MS medium supplemented with 5 µM 2, 4-D and 2.5µM BAP.

To maintain maximum embryogenic capacity in callus four weeks subculture duration was found to be the best. Callus subculturing is required frequently for further proliferation and embryo development as frequency of embryogenic callus increases on subculturing. In most of the bamboos subculture interval of 4-6 weeks is reported to be beneficial for maintenance of embryogenic potential. Dark conditions were found to be optimum for callus proliferation and embryo development.

In the present study 6% sucrose showed maximum scutellar and coleoptillar embryos and higher concentrations beyond 8% is found to be inhibitory. Sucrose has been found effective in increasing the medium osmoticum and having beneficial effect on embryo maturation. In gymnosperms sucrose is found to be effective for maturation of embryos (Hakman *et al.,* 1985; Finer *et al.,* 1989). Lelu *et al.* (1994) and Arya *et al.* (2008) reported that on 6% sucrose supplemented medium much improved organization of embryoids was observed. Godbole *et al.* (2002) also found sucrose to be effective for embryo maturation.

Table 1. Effect of BAP concentration in MS medium on shoots proliferation rate of *D. asper.* (propagule of 3 shoots taken). Data recorded after 4 weeks.

BAP (mg/l)	No. of Shoots	Multiplication rate
1.0	14.1± 2.9	5.6
2.0	33.4 ± 3.2	12.2
2.5	41.0 ± 5.1	15.3
3.0	46.5 ± 5.1	17.2
4.0	36.5 ± 3.6	13.3
5.0	30.2 ± 6.5	12.2
7.5	16.3 ± 2.5	6.2

Mean ± SD

Table 2: Effect of 2, 4-D + BAP on multiplication of embryogenic callus in *D. hamiltonii* on MS medium. 50 mg of fresh wt. of callus was inoculated initially. Data recorded after 4 weeks. Values are mean of 6 replicates with SE ±

2,4-D (µM)	BAP (µM)	Embryogenic response (%)	Fresh callus wt. (gm)	Callus multiplication rate	Mean no. of embryos
	2.5	67.99 ± 1.33	0.08 ± 0.00	1.65 ± 0.05	08.77 ± 0.15
5	5.0	68.89 ± 1.11	0.10 ± 0.00	2.05 ± 0.08	09.33 ± 0.24
	10.0	63.77 ± 2.89	0.07 ± 0.00	1.39 ± 0.06	08.23 ± 0.15
	2.5	73.33 ± 1.67	1.06 ± 0.01	1.99 ± 0.06	12.10 ± 0.21
10	5.0	76.67 ± 1.67	0.12 ± 0.00	2.45 ± 0.03	12.67 ± 0.33
	10.0	68.89 ± 1.11	0.09 ± 0.00	1.83 ± 0.04	09.97 ± 0.32
	2.5	54.33 ± 2.33	1.28 ± 0.08	08.37± 0.06	06.07 ± 0.23
15	5.0	63.89 ± 2.00	2.08 ± 0.04	07.85± 0.07	07.10 ± 0.21
	10.0	48.00 ± 1.53	1.68 ± 0.06	07.12± 0.07	04.60 ± 0.21
One Way Anova		F= 24.44**	F= 3070.45**	F= 40.10**	F= 126.38**

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

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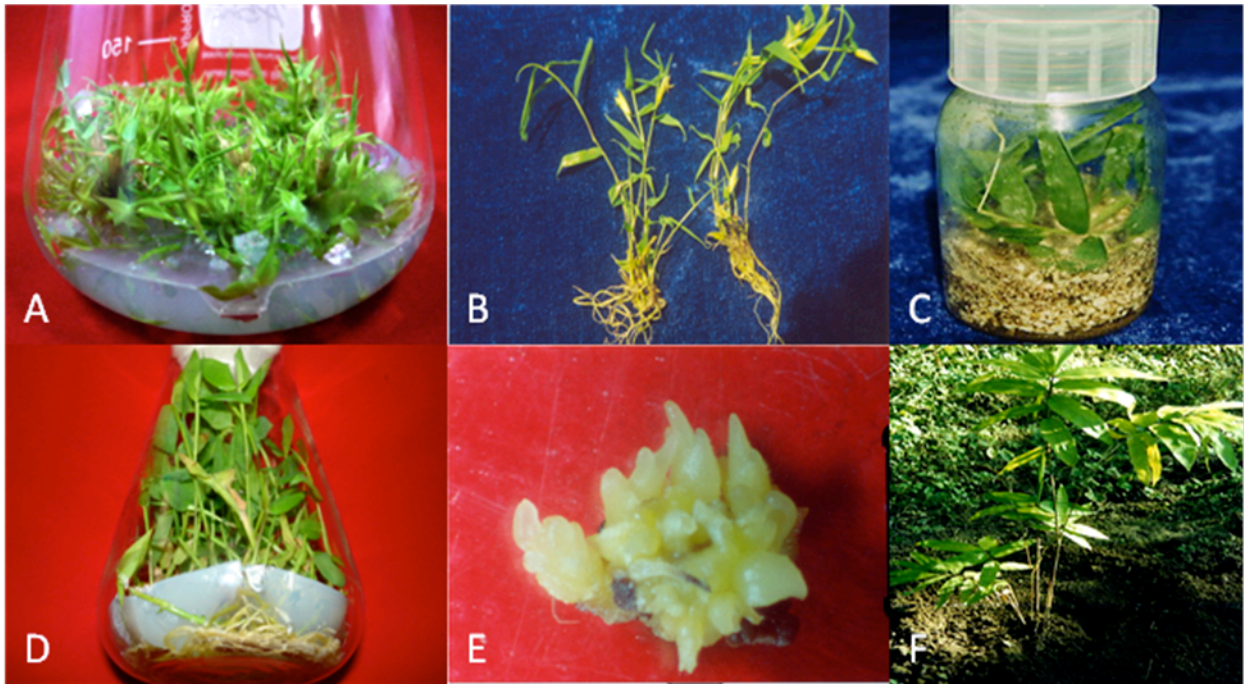

Figure 1. A to F: *In vitro* shoot proliferation and somatic embryogenesis in Bamboo.

A: *In vitro* shoot proliferation in *D. asper* (MS+ 3.0 mg/l BAP)

B: *In vitro* rooting in *D. falcatum* (MS+10.0 mg/l IBA)

C: *In vitro* hardening of TC plants

D: *In vitro* TC plants of *D. asper*

E: Somatic embryos of *D. falcatum*

F: TC plant in Field