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In vitro Seed Germination and Micropropagation of Edible Bamboo *Dendrocalamus giganteus* Munro using Seeds

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Abstract: Successful propagation from seeds of *Dendrocalamus giganteus* Munro was achieved by *in vitro* methods. Incidence of sporadic flowering was recorded. The descriptions of inflorescence and floral morphology are in agreement with the prior taxonomic descriptions but a more detailed description and illustrations are presented in this communication. *In vitro* seed germination was enhanced in gibberellic acid (GA_3) supplemented liquid Murashige and Skoog (MS) medium in the presence of light. Shoots multiplication was obtained directly from seeds inoculated on MS medium containing benzylaminopurine (BAP). In another method, callus was induced and proliferated on MS medium supplemented with 3 mg L^{-1} 2,4-dichlorophenoxy acetic acid (2,4-D) and 0.5 mg L^{-1} kinetin (Kn). *De novo* production of shoots took place from white, compact and nodular calli after transfer to MS medium containing 1 mg L^{-1} naphthalene acetic acid (NAA) and 0.5 mg L^{-1} Kn. Regenerated shoots were excised and induced to root on media with auxin. *In vitro* rooting percentage of shoots reached 86% with half-strength MS medium containing 5 mg L^{-1} indole-3-butyric acid (IBA). After the process of hardening and acclimatization, the plantlets established well when transferred to field during favourable season.

Key words: Bamboo, callus induction, *Dendrocalamus giganteus*, *in vitro* germination, shoot proliferation, organogenesis, root induction

INTRODUCTION

Dendrocalamus giganteus Munro is a giant bamboo, native to southern Myanmar and northwestern Thailand, which is cultivated in India, Sri Lanka, Bangladesh and Southern China (Louppe *et al*., 2008). It occurs naturally in humid areas at slightly higher altitudes (up to 1200 m). It can, also, be grown successfully at low altitudes on rich alluvial soils. In Manipur (23°47'-25°41' NL; 92°58'-94°47' EL), it grows luxirantly along the river banks on sandy and clay mixture of soils. The culms are erect, 14-25 m tall and 8-20 cm in diameter, grey-green and covered with white waxy scurf when young. It is used extensively in making different kinds of decorative pieces, house construction, water pipes, for making pulp etc. Besides, the young tender shoots are considered as the most popular succulent vegetables found in Manipur. The young succulent shoots are sold in the markets by vendors in fresh, pickle, salad or fermented form. Furthermore, the ethnic communities of Manipur prefer to consume the young shoots of this species as compared to other edible bamboo species found in the region due to its delicious taste and less irritant sensation in the mouth and throat. The irritability is caused due to the presence of cyanogenic glycoside-taxiphyllin which can be removed by cooking (Schwarzmaier, 1977; Ferreira *et al*., 1995; Haque and Bradbury, 2002). Bamboo shoots are rich in various nutrients, minerals, and also contain lignans and phenolic acids which have anticancer, antibacterial, antifungal and antiviral activity (Bhatt *et al*., 2005; David and Nirmala, 2007). The demand for this bamboo species is increasing much more than their availability, hence it is necessary to conserve this resource for sustainable development. Bamboo is known for its monocarpic habit, i.e., flowering once before culm death (McClure, 1966). Bamboo flowering is regarded as a bad omen in several states of India, especially where the flowering incidence is accompanied by an increase in rodent population. It is believed that flowering of bamboo brings disasters like famine and other natural calamities, which has compelled the rural people to destroy the clumps after or during blooming (Mohan-Ram and Hari Gopal, 1981; John and Nadgauda, 2002). Conventional propagation of bamboo through seeds is not reliable because the sporadic flowering that takes place annually in isolated clump rarely set seeds (Ramayana and Yakandawala, 1998) and so far, there is no report available on gregarious flowering of *Dendrocalamus giganteus.* Furthermore, vegetative propagation using culm cuttings and rhizomes is

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independable due to the bulky size of the propagules for plantation purposes. The vegetatively propagated clones will also have the same physiological state of their wild stock and will tend to flower leading to the entire culm death. Hence, the present study was undertaken to develop a method for *in vitro* propagation of *Dendrocalamus giganteus* Munro, an important edible bamboo using seeds.

MATERIALS AND METHODS

Plant material: Surveys were carried out to record the incidence of flowering. During June, 2005, incidence of sporadic flowering of *Dendrocalamus giganteus* Munro was noticed in 3 locations along riverbanks of the Arapti river (24°44'N, 93°56'E), Manipur. All culms were flowered in the three flowering clumps. Inflorescences were collected and key morphological characters of fresh spikelets were recorded. Measurements were taken from at least five different fertile florets from each inflorescence. Seeds were harvested from flowering spikes and stored at 4°C for 4 months.

***In vitro* germination and direct shoot organogenesis from seeds:** Seeds were dehusked and washed with 0.1% w/v Exalin (Merck, India) detergent solution for 10 min to remove foreign debris. The seeds were surface sterilized in 20% (v/v) of sodium hypochlorite (NaOCl) for 20 min, and rinsed five times with sterile distilled water. Single seeds were put into 100×10 mm test tubes containing MS culture medium supplemented with 2% sucrose (w/v) with or without 0.7% agar (v/w). Seeds incubated in liquid MS medium were supported with filter paper bridge. The experiments were repeated three times, each repetition comprising 25 seeds (tubes). All media were supplemented with different concentrations of PGRs i.e., BAP, Kn and GA_3 (0.1, 0.2, 0.3, 0.5, 1.5 and 3.0 mg L^{-1}) followed by incubation both in the dark and light. GA_3 was sterilized using 0.2 μM millipore filters and added after autoclaving. After 2 weeks of incubation, percentage of seeds germination was recorded. Shoot proliferation from intact seeds were carried out in MS medium supplemented with various concentrations of BAP (1-10 mg L^{-1}). Propagules of three shoots were excised and subcultured in MS medium containing different concentrations of BAP (1-10 mg L^{-1}) for further multiplication. Data for multiplication rate were recorded after third subcultures.

Culture conditions: The pH of the medium was adjusted with 1 N NaOH and 1 N HCl to 5.7±0 prior to the addition of 0.8% agar (Hi-Media). All culture tubes containing media were autoclaved at 121°C for 20 min. Cultures were maintained in a growth chamber at 25±2°C with a 16 h photoperiod at 90-95 μmol m^{-2} sec^{-1} provided by cool white fluorescent lamps.

Callus induction, growth and maintenance: Modified MS (Murashige and Skoog, 1962) medium containing B_5 vitamins (Gamborg *et al*., 1968) supplemented with various concentrations of 2, 4-D (0.5, 1, 2, 3 and 5 mg L^{-1}) either alone or in combination with BAP or Kn (0.2, 0.5 and 1 mg L^{-1}) was used for callus induction. Using a dissecting microscope, fully matured aseptic seeds were selected based on the completely filled endosperm and darked colored seeds without wrinkles on the outer surface. Zygotic embryos were rescued from the seeds using scapel with proper care and inoculated on the callus induction medium. Initially, the cultures were maintained in the dark at 25±2°C till callus induction and after 4 weeks, all cultures were maintained under 16 h photoperiod providing an approximate irradiance of 15 μmol m^{-2} sec^{-1} at the same temperature.

Root development and acclimatization of plantlets: *In vitro* raised shoots of about 1.5-2.5 cm were harvested for rooting experiment and cultured on half-strength MS medium supplemented with IBA or NAA at (1, 2, 3, 5 and 7 mg L^{-1}). Rooting percentage and roots per propagules were counted after 4 weeks of culture in rooting medium. The rooted plantlets were washed with sterilized water to remove adhered agar and transferred to bottle containing sand and soil (1:1) for hardening. After 1 month, the plantlets were transplanted to pots containing sand:soil: FYM (farm yard manure) and kept under shade house with proper watering twice a day for 3 months.

Statistical analysis: All experiments were set up in a completely randomized design. Data within each experiment was subjected to Analysis of Variance (ANOVA) using Statistical package. If the data were found significant at $p \leq 0.05$, Tukey's test was employed for comparison of treatment means.

RESULTS AND DISCUSSION

Description of inflorescence: Inflorescences were borne on leafless branches between the leafy branches at a node (Fig. 1a) and are up to 80 cm long. The branching pattern was similar to that of a vegetative culm. Numerous floral buds were arranged with 1-7 pseudospikelets in capitate-like clusters in the nodes (Fig. 1b). Spikelets were ovoid, laterally compressed, 1-2×0.8-1.2 cm, reddish to dark purple, comprising 6-8 florets. Florets were bisexual, 11-14 mm long, broad at the base; anthesis basipetal

(Fig. 1c); lemma broadly acuminate, concave, glabrous, pale green in fresh specimens and overlapped with the palea (Fig. 1d); palea subtending a bisexual floret, membranaceous, 2-keeled, ciliate on the keels, 2-3 veined between them, pale green (Fig. 1e); Stamens 6; anthers 4-7 mm, linear, acute, pale green, basifixed, with linear dehiscence; filaments thread-like, elongating to 10-12 mm during anthesis (Fig. 1f, g). Pollen released were yellow and powdery which were attractive and bees were seen visiting the flower. Ovary was ovoid, 1-2 mm long and had style long ending in 1-2 short purple stigma (Fig. 1h). Seeds caryopsis; cylindrical to ovoid, 8-12× 4-6 mm, light brown, hairy, thin pericarp and filled with large starchy endosperm (Fig. 1i). In this study, comprehensive observations of floral morphology at several stages of development were documented, in order to obtain seeds which would show genetic fidelity. The inflorescence characters and floral morphology of *D. giganteus* as described above were in agreement with descriptions given by Gamble (1896) but more comprehensive. In the present report, the flowering did not produce mass seeding but only few spikes set seeds which indicated sporadic flowering. Such a flowering behavior was reported in Sri Lanka by Ramayana and Yakandawala (1998).

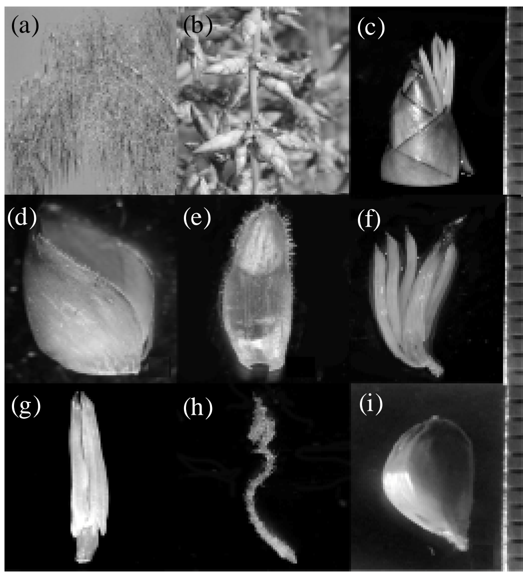

Fig. 1(a-i): Different floral parts of *Dendrocalamus giganteus*: (a) Flowering clump, (b) Capitate arrangement of spikelets on nodes, (c) A floret showing basipetal anthesis, (d) Lemma, (e) Palea, (f) Androecium, (g) Stamen, (h) Pistil and (i) Caryopsis

***In vitro* seeds germination:** Figure 2 illustrates the effect of BAP, Kn and GA_3 on *in vitro* seed germination. In controls, about 20% seed germinated in the first 6 days and the rest germinated evenly over the next 12 days. However, in the presence of optimum PGRs, between 50-85% seeds germinated during the first 6 days and reached almost the maximum by the 12th day both in dark and light culture. (GA_3) was found to be better than BAP and Kn for promoting the germination of seeds. (GA_3) was found to be more effective at 0.5 mg L^{-1} in the presence of light. In contrast, the cytokinins (BAP and Kn) increased germination at higher concentrations. Our results agree with other studies showing that application

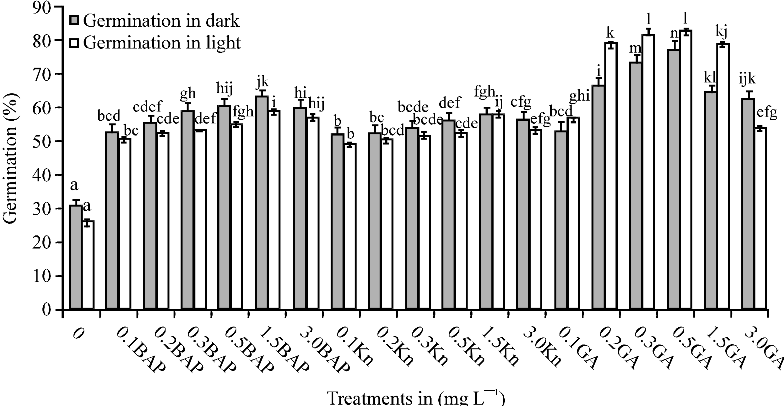

Fig. 2: Percentage *in vitro* germination of *D. giganteus* seeds in MS medium supplemented with BAP, GA_3 and Kn subjected to light and dark. Values are Mean±SE of ten replicates. Columns marked by a common letter do not differ significantly by Tukey's test ($p<0.05$). Bars denote the standard error (SE)

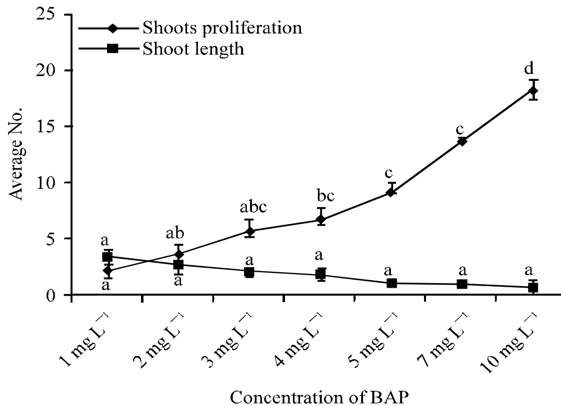

Fig. 3: Effect of BAP concentration in MS medium on direct shoot organogenesis from seeds of *Dendrocalamus giganteus*. Values are Mean±SE of ten replicates. Columns marked by the same letter do not differ significantly by Tukey's test ($p<0.05$). Bars denote the standard error (SE)

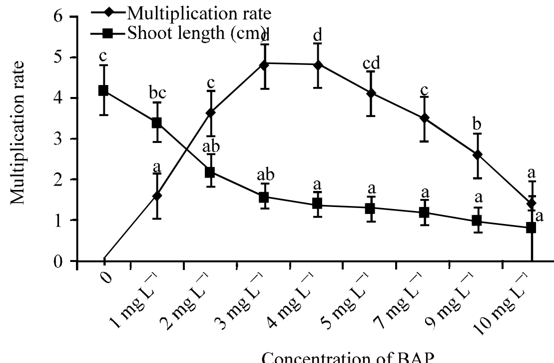

Fig. 4: Effect of BAP concentration in MS medium on shoot multiplication and shoot length of *Dendrocalamus giganteus*. Values are Mean±SE of ten replicates. Columns marked by a common letter do not differ significantly by Tukey's test ($p<0.05$). Bars denote the standard error (SE)

of GA_3 is an effective method to promote germination and to increase the germination capability of the seeds of several other species (Perez-Garcia and Duran, 1990; Upreti and Dhar, 1996; Khan and Ungar, 1998; Hernandez-Verdugo *et al*., 2001; Miransari and Smith, 2009; Prakash *et al*., 2011). Contrary to the present observation regarding the superiority of GA_3, earlier report indicated that exogenous cytokinins have various effects on seed germination in different species (Mahmoodzadeh *et al*., 2010; Mirabdulbaghi *et al*., 2011). The seedlings were transferred to MS medium without growth regulators for further elongation and growth (Fig. 6a).

The shoots proliferated from seeds on MS medium supplemented with various concentration of BAP within 4 weeks. Shoot proliferation per seed increased gradually with the high levels of BAP (Fig. 3). The mean number of shoots produced was 9.2 at 5 mg L^{-1} BAP (Fig. 6b). Although, the highest mean number of shoots was obtained at 10 mg L^{-1} BAP, the shoot length decreased insignificantly with the high levels of BAP. The shoots developed were excised into 3-5 shoot clusters and subcultured in different concentrations of BAP supplemented medium. The highest multiplication rate was obtained at 3 mg L^{-1} BAP which showed no significant difference with 4 mg L^{-1} BAP (Fig. 4, 6c). After the fifth subculture, the shoot multiplication rate declined drastically. The present investigation indicated that the number of shoots produced was directly correlated to BAP concentration. Cytokinin-induced direct shoot regeneration from intact seedlings had been reported in *Phaseolus* species (Malik and Saxena, 1992), *Arachis hypogaea* (Saxena *et al*., 1992), *Murraya koenigii* (Bhuyan *et al*., 1997), *Litchi chinensis* (Das *et al*., 1999), *Dendrocalamus asper* (Arya *et al*., 1999), *Cyclamen mirabile* (Yamaner and Erdag, 2008).

Table 1: Effect of Plant Growth Regulators (PGRs) on organogenic callus induction from zygotic embryos of *Dendrocalamus giganteus*

Treatments of PGRs (mg L^{-1})	Response (%)	Nature of callus obtained
0.5 2,4-D	42.3 ± 0.7^{a}	White, soft and friable
1.0 2,4-D	53.3 ± 0.8^{b}	"
2.0 2,4-D	74.5 ± 0.4^{d}	White, nodular and friable
3.0 2,4-D	81.7 ± 0.5^{ef}	White, nodular and compact
5.0 2,4-D	65.8 ± 0.7^{c}	Brownish, non-nodular and hard
3.0 2,4-D+0.2 BAP	86.0 ± 0.8^{h}	White, nodular and compact
3.0 2,4-D+0.5 BAP	90.2 ± 0.9^{i}	"
3.0 2,4-D+1.0 BAP	78.8 ± 0.5^{e}	White, hard and friable
3.0 2,4-D+0.2 Kn	82.3 ± 0.4^{fg}	White, nodular and compact
3.0 2,4-D+0.5 Kn	85.2 ± 0.7^{gh}	Greenish White, nodular and compact
3.0 2,4-D+1.0 Kn	63.8 ± 0.5^{c}	White, hard and friable

Values are means ± SE of ten replicates. Data scored after 4 weeks in culture. Means followed by the same letter do not differ significantly by Tukey's test ($p\leq0.05$)

Callus induction and adventitious shoots proliferation: Callus induction from the zygotic embryos took place in modified MS medium (containing B_5 vitamins) fortified with various concentrations of 2,4-D alone or in combination with BAP or Kn at different concentrations after 2 weeks of culture (Table 1). The optimum concentration of 2,4-D for obtaining organogenic calli was 3 mg L^{-1} (Fig. 6d). When 2,4-D concentrations went down from 3 to 0.5 mg L^{-1}, callus induction percentage decreased as well as non-organogenic texture were obtained. At the increased concentration of 2,4-D, the

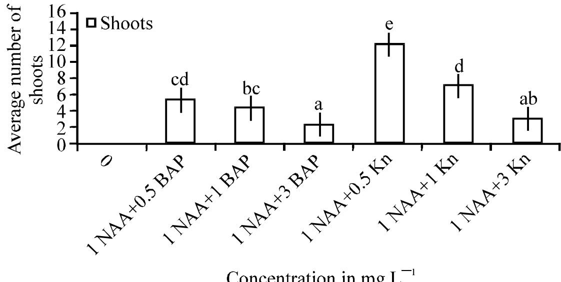

Fig. 5: Optimum combinations of NAA with various concentrations of BAP and Kn for shoot proliferation in callus cultures derived from zygotic embryos of *Dendrocalamus giganteus*. Values are Mean±SE of ten replicates. Columns marked by a common letter do not differ significantly by Tukey's test ($p \leq 0.05$). Bars denote the standard error (SE)

callus obtained was brownish and hard. The combination of 3 mg L^{-1} 2,4-D and 0.5 mg L^{-1} Kn produced maximum percentage of callus induction and organogenic calli proliferated after 40 days culture (Fig. 6e). During organogenic callus formation, calli with various textures was noticed and only the white, nodular and compact responded for shoot regeneration. Our experiments proved that the combined effect of 2,4-D and Kn showed best response for organogenic callus induction. Similar to our results, in most of the dicot plants, a combination of high amount of auxins (2,4-D or NAA) with low amount of cytokinins (BAP or Kn) was widely used for the initiation of organogenic callus (Caboni *et al*., 2000; Kumari *et al*., 2008; Valizadeh *et al*., 2007; Siwach *et al*., 2011; Sharma *et al*., 2012) and including some bamboo species (Rout and Das, 1994).

Adventitious shoots were proliferated from the organogenic callus when subcultured to modified MS medium supplemented with optimum levels of 1 mg L^{-1} NAA and various concentrations of BAP or Kn (0.5, 1, and 3 mg L^{-1}). After one month, maximum mean number of 12 shoots was regenerated from the callus in the media supplemented with 1 mg L^{-1} NAA and 0.5 mg L^{-1} Kn (Fig. 5, 6f). Comparatively, BAP produced a maximum of 4.3 shoots in 0.5 mg L^{-1} after 40 days culture. The present investigation confirmed that the combination of NAA with cytokinins promotes adventitious shoot proliferation from the callus cultures of *Dendrocalamus giganteus* using zygotic embryos as an explants. Similar to our findings, BAP or Kn was widely used for multiple shoot initiation from the callus cultures (Huang *et al*., 1989; Martin, 2002; Satyavani *et al*., 2011). In some plant species, shoot multiplication and regeneration was

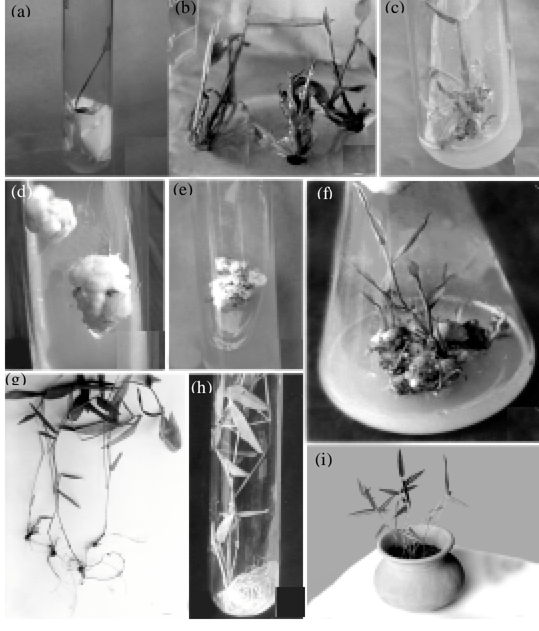

Fig. 6(a-i): Seed germination and propagation of *Dendrocalamus giganteus* using *in vitro* techniques: (a) Seedlings growth in MS medium, (b) Direct shoots organogenesis on MS+5 mg L^{-1} BAP, (c) Shoot multiplication on MS+3 mg L^{-1} BAP, (d) White, nodular and compact calli obtained in MS+3 mg L^{-1} 2,4-D, (e) Callus proliferation in MS+3 mg L^{-1} 2,4-D+0.5 mg L^{-1} Kn, (f) Clusters of adventitious shoots regenerated from green and nodular organogenic calli, (g) *In vitro* rooting of organogenic shoots, (h) Rooting of *in vitro* microshoots and (i) Hardening

efficiently achieved by thidiazuron (TDZ) (Malik and Saxena, 1992; Kumari *et al*., 2008; Kumar and Kumari, 2010) and 2-isopentenyladenine (2iP) (Chang and Chang 2000; Tonon *et al*., 2001).

***In vitro* rooting of shoots:** Microshoots rooted on a PGR-free ½ strength MS medium following initial incubation for 30-40 days on medium containing IBA. Initial exposure to IBA and subsequent withdrawn of auxin from the medium was found very effective for overall root development. This rooting procedure resulted best root growth within 7-10 days after transfer to auxin-free medium. Among the various concentrations of IBA tested, significant rooting was obtained at 5 mg L^{-1} producing 13 average root numbers with 86% rooting in 40 days (Fig. 6h, Table 2). In

Table 2: Effect of auxins on rooting of *Dendrocalamus giganteus* on ½ strength MS medium

Auxins concentration (mg L^{-1})	Rooting (%)	Average root No.
1 NAA	18.8 ± 0.5^{a}	3.2 ± 0.5^{a}
2 NAA	33.3 ± 0.8^{b}	4.3 ± 0.4^{abc}
3 NAA	62.3 ± 0.7^{e}	5.3 ± 0.3^{bcde}
5 NAA	73.0 ± 0.4^{f}	6.2 ± 0.3^{cde}
7 NAA	58.7 ± 0.4^{d}	4.0 ± 0.4^{ab}
1 IBA	40.5 ± 0.4^{c}	5.2 ± 0.5^{abcd}
2 IBA	79.0 ± 0.5^{g}	7.3 ± 0.4^{ef}
3 IBA	84.0 ± 0.5^{h}	8.5 ± 0.4^{f}
5 IBA	86.0 ± 0.4^{h}	13.0 ± 0.6^{g}
7 IBA	63.0 ± 0.7^{e}	6.7 ± 0.5^{def}

Values are Mean±SE of ten replicates. Means followed by the same letter do not differ significantly by Tukey's test ($p \leq 0.05$)

the case of NAA also root induction was noticed but in the concentrations of NAA tested rooting percentage was low as compared to IBA. The *in vitro* raised shoots from embryogenic calli failed to root on a hormone-free MS medium. Of the various auxin treatments, the best results were obtained with 1-3 mg L^{-1} IBA supplemented ½ strength MS medium (Fig. 6g). Such similar stimulatory effect of IBA on *in vitro* root induction from seedling has been reported in some bamboo species (Arya *et al*., 1999; Bag *et al*., 2000) and other important crops (Molla *et al*., 2004; Mirabdulbaghi *et al*., 2011). On the contrary, the synergistic effect of NAA and IBA proved to be beneficial for rapid root initiation of orchid via seed culture (Nongdam and Chongtham, 2011). In the present case, no auxin protector coumarin, additives and amino acids were required though these have been reported to enhance *in vitro* root induction (Saxena, 1990; Agnihotri and Nandi, 2009).

Hardening and acclimatization: After following the process of hardening and acclimatization (Fig. 6i), the newly transplanted plantlets survived up to 80-90% exhibiting normal growth. As observed, these plantlets established well and grew favorably during monsoon.

CONCLUSION

These reports present the evidence and importance of observing the incidence of sporadic flowering and behaviors for this important bamboo species, which will allow successful micropropagation using seeds. Furthermore, an efficient and simple protocol for *in vitro* seed germination and micropropagation through direct shoot organogenesis and indirect adventitious shoot multiplication from callus cultures have been described.

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