Indian Journal of Experimental Biology
Vol. 51, January 2013, pp. 88-96

In vitro seed germination of economically important edible bamboo *Dendrocalamus membranaceus* Munro[†]

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Received 18 April 2012; revised 19 September 2012

An *in vitro* propagation protocol using mature seeds of *D. membranaceus* was successfully established. Scarcity of seeds in bamboos because of their long flowering periods and irregular seed set resulting in low viability and germination potential, motivated us to undertake the present study. The effects of sterilants, light conditions, exogenous application of plant growth regulators and temperature in overcoming germination barriers in ageing seeds of bamboo were studied. It was found that $HgCl_2$ (0.1%) along with bleach (15%) was more effective in raising aseptic cultures. Dark conditions, high temperatures around 30 °C and soaking of seeds in GA_3 solution (50 ppm) overnight stimulated high percent of seed germination with corresponding increase in shoot length (2.7±0.7 mm) and number of sprouts (2.1±0.7) per explants during culture initiation. 6-benzylaminopurine acted synergistically with kinetin to give optimum germination rate of 70±13.9% as compared to 63.13% when used individually. For prolonged maintenance of cultures, 2% sucrose was found to be suitable for promoting photomixotrophic micropropagation. Following this procedure, about 65% survival of plantlets could be achieved during hardening. Biochemically seeds consume starchy endosperm for emergence of radicle which is taken as a sign of germination as also evident from the present study. Loss of viability and vigour after a year was confirmed by Tetrazolium chloride test. Micropropagation protocol developed here will ensure regeneration of large number of plants in a relatively short time. Conclusively, *in vitro* propagation protocol developed in *D. membranaceus* using mature seeds as an explants is reported for the first time.

Keywords: Acclimatization, *Dendrocalamus membranaceus*, Germination, Gibberellic acid, Low viability, Seeds

Bamboos have been in demand due to their multifarious uses. India has abundant bamboo resources with about 24 genera and 138 species. About 2.5 billion people depend on or use bamboos to a value of US$7 billion per year and these figures have gone much beyond that as these provide alternative source for meeting many of our needs[1]. The current work focuses on the knowledge of seed viability, germination pattern and seedling behaviour of bamboo in the sub-Himalayan zone. Though bamboos are fast growing and early maturing, their peculiar monocarpic nature and the mass scale dying out after flowering has become a problem to the managers for programming a sustainable management. Demographic characteristics of bamboo regeneration from seeds remain largely unknown[2] and there is a great variation in their capacity to remain dormant and viable in soil. Intermast period being very long[3], it would be difficult to store large quantities of seeds for mass scale planting till next flowering cycle is repeated. Seed viability is generally very low in bamboos, as endogenous levels of auxins and abscisic acid (ABA) in seeds is found to be one of the major factors related to the loss in seed viability in stored bamboo seeds[4]. Bamboo seeds are short lived, germinate within 3-7 days, lose viability within 1-2 months[5] and germination potential being season dependent. Absence of seed set in bamboos due to cumulative effect of physical and physiological factors[6] have prompted researchers for devising methods for artificial seed production for propagation[7]. In some species as *Bambusa tuldoides*, viable seed production was reported for the first time in Argentina[8]. Potential application of tissue culture for propagation using seeds in bamboos is well documented[9,10]. Conventional methods of vegetative propagation pose problems for a number of reasons as

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cuttings, offsets and rhizomes are bulky and not available in desired numbers, are difficult to handle and transport and plantlet survival after natural regeneration is usually very low. Most of the forest covers of bamboos especially of *D. membranaceus* are fast declining because of poor harvesting methods, lack of knowledge on propagation techniques, flowering control and proper storage of seeds. Hence, inducing seed germination in a sterile environment in presence of appropriate phytohormones leads to an enhancement of productivity and also for conservation of this important species. Therefore, use of *in vitro* methods seems to be a good alternative to counter this problem.

The present research intrest in *D. membranaceus* Munro (Poaceae), native of Myanmar was due to the fact that it has been listed among 18 additional taxa under 'Important' category (www.unep-wcmc.org; www.inbar.int). *Dendrocalamus membranaceus* is considered an ecologically important species as it has an extensive root system which prevents soil erosion. Major threats to this species include logging of rainforests in which this bamboo resides, unsustainable land use by practices like slash – burn and shifting agriculture and harvesting for international exportation. The major threat documented for this species is the beetle *Crytotrachelus longimanus* which is a major forest invasive species occurring mainly in Thailand which proves detrimental to the natural population of this species. Hence the methods of conservation like micropropagation through seeds, nodal explants or somatic embryogenesis hold greater significance. It is fast becoming a high value crop. Besides innumerable other uses, it is edible, widely used for construction purposes in Myanmar and Thailand and one of the most promising species for pulp. After studying 27 bamboos belonging to 10 genera for the edibility of shoots, this bamboo was considered to be excellent from processing point of view, as the young shoots are smooth and easy to handle[11]. In China, it is used for making chopsticks, shreds and paper. Therefore, devising a protocol for their propagation by tissue culture is highly desirable and selection at the seedling stage is quite useful in multiplying only the fast growing ones as plus plants.

Conservation and propagation of the bamboos need attention as stressed upon by UNDEP, FAO, INBAR and the programs like FORTIP[12]. Chenopodiaceae, Liliaceae, Poaceae and Solanaceae often are included in list of plant families with seeds having physical dormancy[13,14]. The seeds show natural ageing in other species of Poaceae as well[15]. It has been seen several times that the seeds of significant number of species are sold and distributed by commercial seed companies in a dead on delivery (DOD) state[16], which is also among the major problems encountered. By the present study it is intended to improve the germination percentage by subjecting seeds to different physico-chemical environments under *in vitro* and *ex vitro* conditions so as to enable selection of plants which show better phenotypic traits (elite plants) and further micropropagate them to produce plants for large scale plantation. The protocol for *in vitro* propagation in bamboos provides a good alternative for propagation which is highly desirable due to its high demand and depletion in the natural forests due to unscientific large scale extraction by the rural population for meeting their increased requirements.

Materials and Methods

Plant material—Poaceae have caryopses as propagation units which are single seeded and where testa is fused with thin pericarp. Seeds for the present experimentation were procured from M/s Allen Green Seeds, Wuyang province, China. The seeds were approximately more than 6 months old i.e. ageing seeds. Seeds are broadly ovate, rounded at base with a pointed end, 5-7.5 mm long and dark brown in colour. The seeds were germinated and also preserved in the seed bank of the Institute. Once the plants grew big, these were got identified by the scientists of IHBT Herbarium. The mother plants are been grown and preserved in the campus.

Culture establishment—The explants (glumed seeds) were prepared carefully by floating in water. Debris and empty seeds were separated this way. The de-husked seeds were rinsed in 0.01% (v/v) Tween 20 for 10 min followed by antifungal treatment with bavistin 0.25% (w/v) and a broad spectrum antibiotic streptomycin sulphate 0.25% (w/v) for 45 min by gentle shaking. Final steps of surface sterilization were performed in laminar flow with 15% sodium hypochlorite for 10 min and 0.1% (w/v) $HgCl_2$ for 10 min. Each treatment was followed by repeated washings for a minimum of 3 times in autoclaved distilled water.

Culture establishment and germination—Explants were inoculated on sterile MS media medium[17] (*p*H 5.6-5.8) and supplemented with various

concentrations and combinations of plant growth regulators like 6-benzylaminopurine (BAP) and kinetin (KN). Depending upon the requirement for initiation media or proliferation media 2.0-3.0% (w/v) sucrose was added as a carbon source with 0.75% (w/v) agar. Three replicates were taken for each treatment having 10 seeds each.

Growth conditions—Explants were maintained at 25±2 °C at photoperiod of 16 h light and 8 h dark under light intensity of 70 µmol $m^{-2} s^{-1}$ with 75% RH. Seeds were kept in dark chambers initially under same *in vitro* conditions. The experiment was repeated thrice with 10 seeds in each treatment. Seeds were also maintained *ex vitro* in BOD incubators at 5,10,15,25,30 and 35 °C.

Transplantation and acclimatization—After washing gently with lukewarm water and a sable hair brush to remove the traces of any agar sticking to the roots, plantlets were treated with an aqueous solution of bavistin to reduce chances of fungal contamination. Plantlets were then transferred to a potting mix of sand:soil:FYM::(1:1:1) and kept in greenhouse in a polytunnel under low light intensity and high humidity for a week. Proper care was taken by watering them regularly and spraying with Hogland's solution at 7-10 days intervals. Growth performance and percentage survival were recorded after every week.

Starch test—Transverse hand sections were stained with weak solutions of iodine (I_2) for 20 min in a petriplate (3.0 cm) for observing the presence of starch. I_2 solution was prepared by dissolving 0.3 g I_2 and 1.5 g KI in water and final volume was made upto 100 mL. The presence of starch was confirmed by appearance of dark blue-black colour.

Viability test—Seeds were sectioned longitudinally and divided in three lots and subjected to 2,3,5 triphenyl tetrazolium chloride (TTZ) test. Seeds were dipped in an aqueous solution 0.1% of TTZ for 24 h at 30 °C in dark as outlined by ISTA (1985). Percentage viability (V) is taken as V= no of viable seeds / total no seeds tested × 100.

Statistical analysis—The data were statistically analyzed by one way ANOVA (main effects) and values are expressed as mean±SD. Significance of difference between means were tested by LSD (P<0.05).

Results

Initiation of cultures

Effect of sterilants—Presently, seeds could be effectively sterilized using two different sterilants. Out of the various combinations of microbial disinfectants tried, treatment of seeds with 0.1% $HgCl_2$ and 15% sodium hypochlorite (NaOCl) for 10 min each gave the best results with 77.8±9.6 aseptic cultures establishment (Fig. 1). A combination of two sterilants at a moderate concentration for a long duration during the treatment was effective rather than high doses for a short time.

Effect of light—Seeds were incubated in continuous dark initially for 1 week during initiation of sprouting (Fig. 2a). Protrusion of radicle > 2 mm was taken as scale for germination which was observed after 7.0±0.9 days (Fig. 2b). After initiation of germination, cultures were shifted to light in alternating photoperiod (16 h light/8 h dark) for plantlet development and normal growth (Fig. 2c and d). Presence of light significantly increased the time (17.0±2.0 days) for emergence of radicle during lag phase of growth.

In vitro germination studies and clonal propagation

Effect of Plant growth regulators (PGRs)—Petriplates (85) representing each of the treatment type including control were placed randomly on one of the shelves of culture room having uniform environmental conditions. These were tested for BAP and KN concentrations from 2.2 to 13.9 µM and their effect on rate of multiplication of cultures. Germinated seeds proliferated within 15 days of incubation. BAP when used alone at 8.8 µM gave the best proliferation rate of 63±13.94% (Fig. 3a). KN when used alone at low concentrations of 2.3 µM

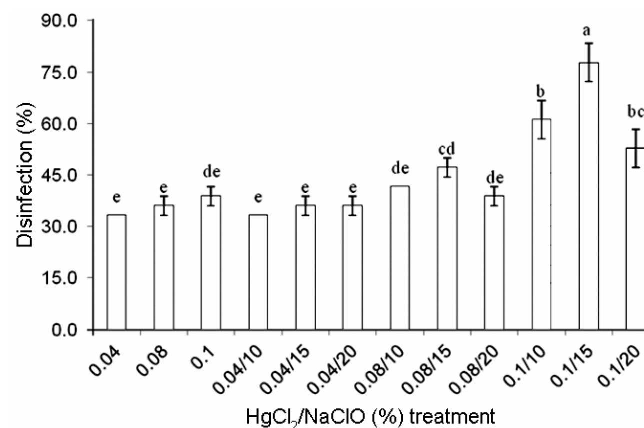

Fig. 1—The role of mercuric chloride ($HgCl_2$) alone and in combination with sodium hypochlorite (NaClO) in combination for disinfection of explants. [A treatment with 15% NaClO for 10 min and 0.1% $HgCl_2$ for 10 min proved to be the best combination. The results were repeated thrice with 10 seeds in each replicates]

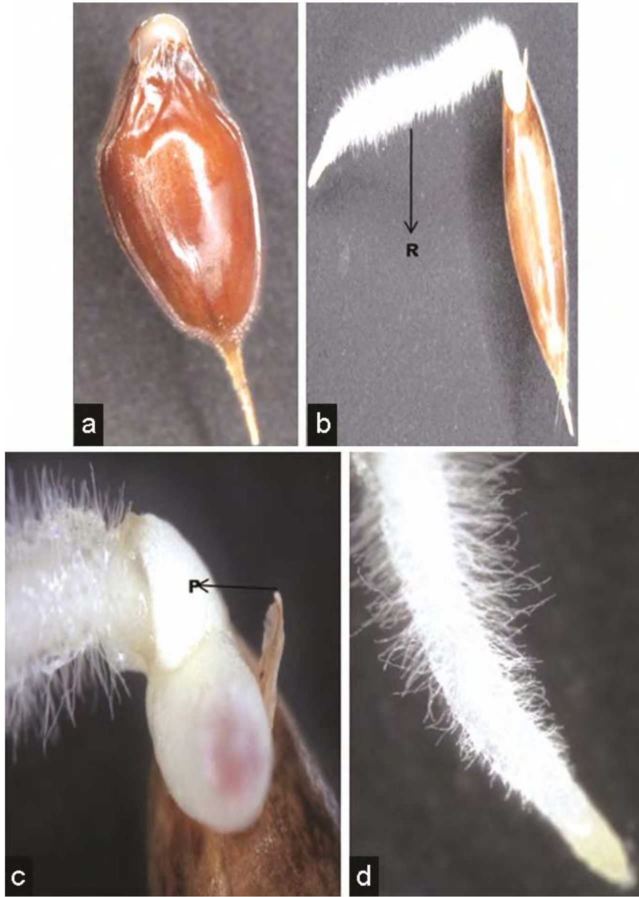

Fig. 2—(a) Morphology of seed showing rounded base with a pointed end, dark brown in colour about 5 mm long, (b) initiation of germination by emergence of radicle (R) about 2 mm long, (c) micropylar end showing emergence of radicle and plumule (P), (d) radicle showing numerous root hairs.

gave best results of (16.5±0%) proliferation. Results obtained were insignificant when KN was used alone. Multiplication rate was maximum when BAP/KN were used together. BAP/KN (8.8 µM/2.3 µM) gave highest proliferation percentage of 70.0±13.9. In all the treatments, rate of proliferation of germinated seedlings was significantly higher than the control except when high dose of KN was used. Germinated plantlets growing vigorously were selected and further micropropagated clonally by splitting and sub-culturing after every 20 days interval on BMS medium containing 2% sucrose; sucrose (2%) being the most suitable for long term proliferation of cultures with 19.1±1.5 shoots. The experiment was repeated thrice with 10 flasks having 3-4 clumps of shoots after sub-culturing. *In vitro* raised *D. membranaceus* plantlets can be multiplied by enhanced axillary proliferation upto many passages. More than 30 passages were worked out and cultures retained their multiplication potential reasonably well even after that. For rooting, no additional source of auxin was needed as the plantlets grew well on the media containing both the cytokinins. This was probably due to the synergistic effect of the two resulting in cell division and shoot morphogenesis by regulating the axillary bud growth. As seed (zygotic embryo) has a bipolar axis, roots were simultaneously formed. The two cytokinins were not inhibitory for the growth of roots. After about 30 days, seedlings thus produced were shifted to basal MS media (BMS) for their proper growth and development and these

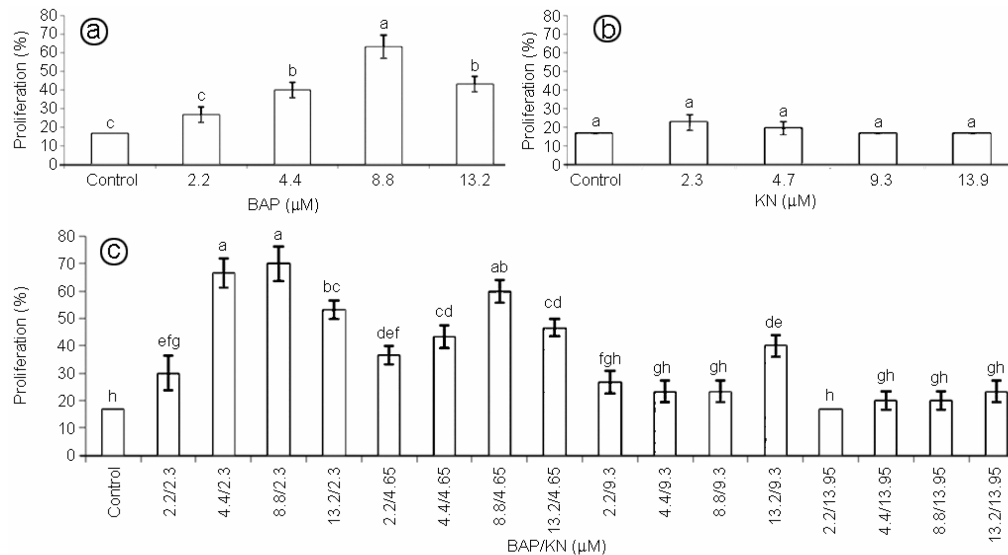

Fig. 3—(a) Effect of 6-Benzylaminopurine (BAP) on rate of proliferation showing best response on 8.8 µM BAP, (b) Kinetin (KN) at a low concentration of 2.3 µM shows best response as compared when used at high concentrationas, (c) combination of BAP/KN at 8.8 /2.3 µM respectively gave the best proliferation percentage. [Data were recorded by taking 5 replicates having 6 seeds in each flask]

survived well with no additional supplementation of PGRs.

Effect of gibberellic acid (GA_3)—When seeds absorb water, the hormone GA_3 appears in the embryo and is translocated in the aleurone layer, where it activates the metabolism to initiate sprouting. Three replicates having 10 seeds each for every treatment of GA_3 were kept overnight (10-70 ppm). The results obtained clearly indicate that GA_3 enhanced germination and the best concentration was found to be 50 ppm giving 73.3±5.7% germination (Fig. 4a). After about 2 weeks a corresponding increase in shoot length (2.7±0.7 mm) and number of sprouts (2.1±0.7) were also observed.

Ex vitro studies on ageing seeds

Effect of temperature—Presently, seed samples which either showed delayed germination or failed to germinate under normal growth conditions were surface sterilized and were placed on wetted Whatmann No 3 filter paper discs in petridishes. Eighteen petriplates having 6 seeds each were incubated at different temperatures (5,10,25,30,35 °C) in BOD incubator (Narang Scientific Works, New Delhi) chambers. The temperatures were selected on the basis of observation on the germination behaviour under *in situ* and *ex situ* conditions. Results show absolute dependence of germination on favourable temperature. Here, 30 °C was found optimum temperature at which 72±9.6 % germination could be achieved (Fig. 4b). Low temperature of (5 °C) was not suitable for the ageing seeds to regain their potential for germination and growth as also evident by the results of a low germination 22.0±9.6%

Hardening and acclimatization—After 30 days, the germinated plantlets procured from all the treatments *in vitro* (culture lab) and *ex vitro* (BOD incubators) were removed from culture bottles. About 65% survival after hardening was achieved. All the plants have expanded leaves and no albinism. Rainy season was found to be the best time for out planting of seedlings in the field. The growth data of 7 month old field established plants is given in Table 1. The complete protocol for micropropagation from ageing bamboo seeds of *D. membranaceus* was thus successfully established (Fig. 5 a-f).

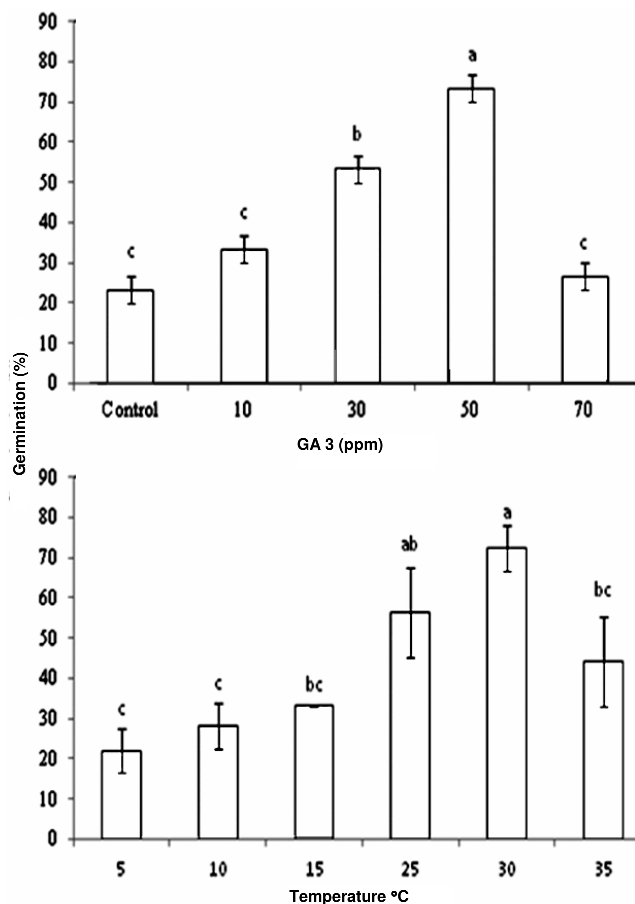

Fig. 4—(a) Effect of GA_3 on seed germination where best response was found at 50 ppm concentration, (b) temperature (30 ºC) gave best response of 73.3±9.6[a] germination percentage and was found to be the optimum for germination to occur in the ageing seeds rather than a standard of 25 ºC. Data were recorded using 3 replicates having 10 seeds each.

Table 1—Data recorded after hardening of the plants in the field for 7 months

Parameter	Average	Inference
Height	104.23 cm	Increase in height indicated that plants acclimatized well in the field conditions
No of shoots	26.13	Increased in number resulted in more Net Primary Productivity (NPP)
No of leaves	297.5	Increased in number and consequently increase in chlorophyll content in the *ex vitro* grown plants
No of axillary shoots/clump	13	Increased in number indicating increase in biomass and vigorous growth and axillary buds serve as potential plants
No of internodes/shoots	6.6	An idea about how long the plant can grow. Plant started responding to the field conditions and hence increasing in size

Starch test—The members of this family have highly developed embryos, triploid endosperm consisting mainly of starch which is dead storage tissue (Fig. 6a) and aleurone layer of living cells. Three replicates with 3 seeds each confirmed the presence of starch (Fig. 6b) by appearance of deep blue colour due to amylase. Emergence of a short plumule after an average of 2 days from the emergence of radicle and the growing radicle results in consumption of starchy endosperm. The appearance of hollow areas in the longitudinal sections of germinating seeds (Fig. 6c) confirms the consumption of starchy endosperm for growth and development of the embryo.

Viability test—All the seeds not responding to any of the above treatments were subjected to TTZ test. Only seeds showing strong red stained embryos at their swollen stalk ends were considered viable and counted. After a year, 99% seeds become non-viable as shown by TTC test (Fig. 6d).

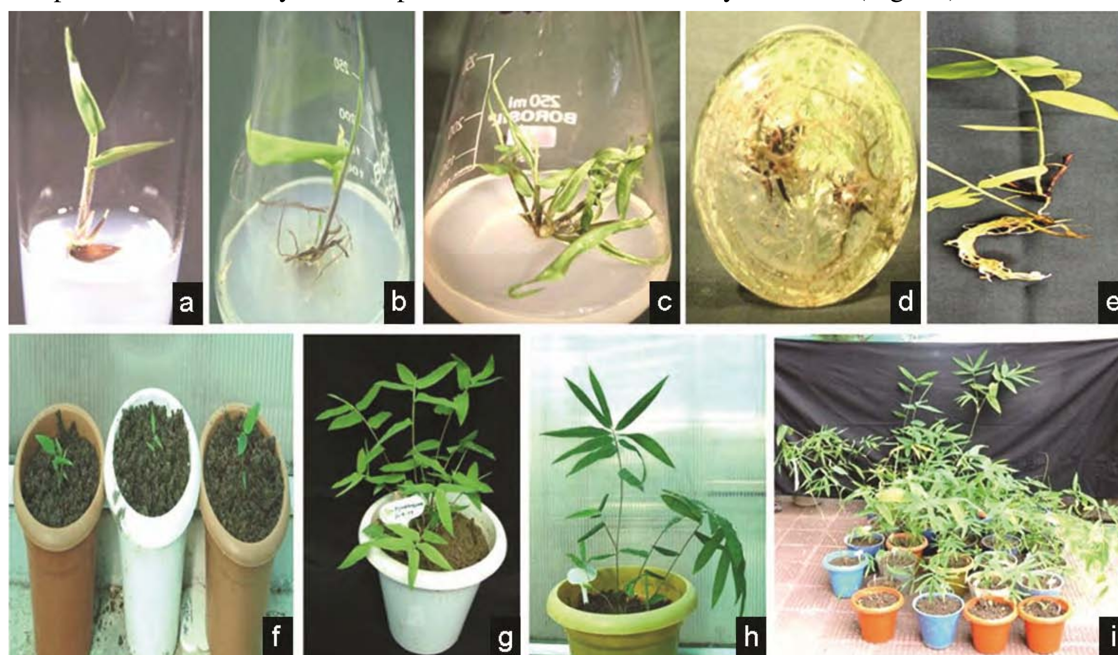

Fig. 5—(a) Sprouting on basal MS media (BMS) after 5 days, (b) growth of bud observed after 2 weeks, (c) proliferation of germinated seedling on Murashige & Skoog (MS) media supplemented with 8.8 µM BAP and 4.65 µM KN. Five replicates were taken per treatment and each experiment was repeated thrice, (d-e) rooting of the germinated seedling on the same media. Later on plantlets were shifted to basal MS media for healthy growth of roots, (f) acclimatized plantlets in green house after 30 days, (g) hardened plantlet after 2 months, (h) plants observed after 4 months, (i) micropropagated plants after 7 months after which they were shifted to field.

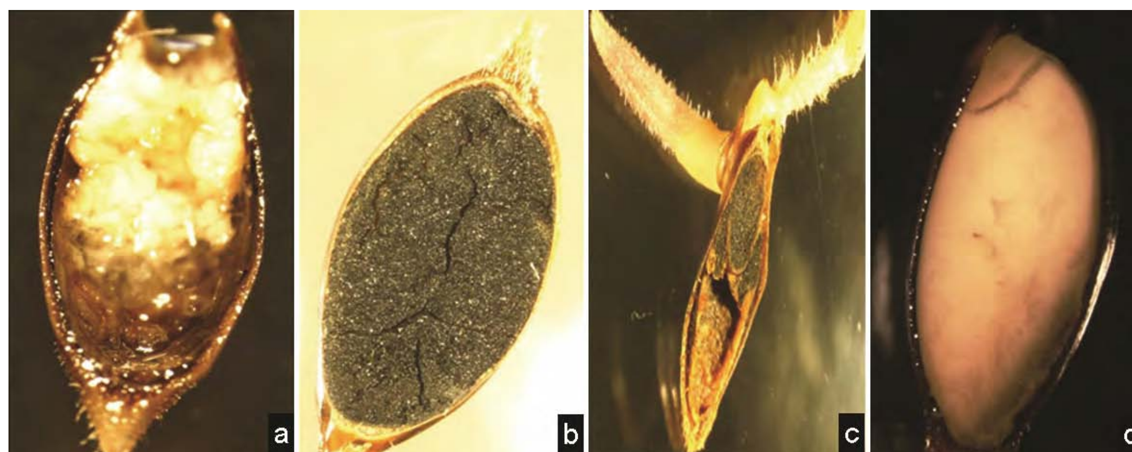

Fig. 6—(a) LS of seed showing starchy endosperm, (b) starch test showing blue black colour in areas of endosperm where starch was present, (c) hollow areas in starchy endosperm showing absorption of starch by the emerging radicle of germinating seeds, (d) absence of red stain by TTZ test showing non-viable embryo.

Discussion

Control of contamination and establishment of aseptic cultures is a priority requirement. The problem of fungal and bacterial contamination during culture establishment was not uncommon in bamboos especially when seeds were used as explants. Various protocols for surface sterilization have been developed to counter this problem[18]. Seeds require a longer duration of treatment with antimicrobial chemicals as compared to nodal explants. Use of ethanol, commercial bleach and $HgCl_2$ in raising aseptic cultures is quite a common practice[19,20]. Henceforth, various procedures are known to be followed depending upon the explants used. Different germination experiments were carried out to study the effect of light, plant growth regulators (PGRs) including role of gibberellic acid (GA_3) and effect of temperature in improving the germination percentage of ageing seeds. Bamboo seeds germinate at higher percentage under shade than in direct sunlight. Thus, bamboo seeds can be considered as negatively photoblastic[21]. Light is an important variable and is not a requirement for initiating germination in some species including bamboos. Moreover, germination in this species is hypogeous, for the kernel with the enclosed scutellum remains below the surface of the ground. Hence, dark conditions favour germination initially. Certain PGRs in minute quantities are known to enhance the germination percentage and reduce the germination time[22]. BAP is a potent cytokinin used in tissue culture for proliferation of shoots and also used for germination experiments. The role of PGR's and micropropagation of bamboos using seeds as explants were inter-related[23-26]. KN alone has been found to be unconducive and have minor morphoregulatory role in seed germination and growth in *D. membranaceus*. The combination of two cytokinins stimulated the growth of multiple shoots and allow large number of plants to be produced from a limited supply of original plant material. Hence, in the present study, the active role of phytohormones in the regulation of seed germination was well established. *In vitro* propagation also depends on exogenous source of carbon[27]. During long term maintenance of cultures sucrose concentration was reduced from 3 to 2% which promoted the photomixotrophic micropropagation under low Photosynthetic Photon Flux Density (PPFD) of culture lab compared to *ex vitro* conditions.

Ageing seeds are a difficult and complex system to be dealt with. Propagation by seeds generally show carryover effects[28] and it becomes difficult to obtain all the seeds in a healthy condition. In cereals, a certain degree of physiological dormancy (PD) generally exists[29]. Bamboo belonging to the same family does not exactly exhibit any dormancy but lose their viability very fast. Exogenous supply of GA_3 was effective in overcoming physical dormancy PD and PD dormancy to a certain degree in cereals as presoaking seeds in a solution of nutrients adds to the vigor of seeds. The present results showed that after a time lapse of 8-10 months, seeds did not respond to the above treatment of phytohormones as BAP, KN etc. The mean germination time (MGT) increased as the seeds became older particularly seeds of the year 2010 were not likely to give the same results in the year 2011, as older seeds contained a greater proportion of physiologically late germinating seeds[30]. GA_3 as an additive during culture initiation proved to be beneficial. Addition of GA_3 can increase the formation of rough endoplasmic reticulum and polyribosomes[31], besides its traditional role in increasing the embryo growth potential and endosperm weakening. Cell elongation, cell division and general growth and development of many plant species is affected by such treatments[32]. Many seeds have minimum, maximum, and optimum temperatures at which they germinate. For example, tomato seeds have a minimum germination temperature of 50 °F and a maximum temperature of 95 °F, but an optimum germination temperature of about 80 °F (http://pubs.ext.vt.edu/426/426-001/426-001.html) followed by germination at other temperatures. Regarding the role of temperature the present results corresponds with the above observations giving maximum germination at slightly higher temperatures. The optimum temperature after a time lapse of 10 months or so was found to be 30 °C in this species. Acclimatization is the final and critical step for the success of micropropagation protocol devised. After *ex vitro* transfer, these plantlets get easily impaired by sudden changes in environmental conditions and need a certain period for adaptations to increased irradiance, low relative humidity (RH) and changes in the water potential listing a few. There were no apparent abnormalities observed once plantlets get acclimatized after hardening of 5-6 months in a greenhouse. Hence, the protocol

devised here can be used for cultivation of large number of plants.

Biochemically, mobilization of food stored in the starchy endosperm is a post-germination event and gibberellins play an important role in the production and secretion of hydrolytic enzymes from the aleurone layer[33]. Activity of the starch degradative enzyme β-amylase increased in the cotyledon as starch started to decline when germinaton progresses[34]. Starch is the major reserve material present in the endosperm as visible from the starch test. Obtaining commercially useful seedlings is hindered by innumerable factors including uneven germination, low tolerance to desiccation reducing seed longevity and hence viability. Ageing of seeds is progressive and leads to death of the seed and hence non viability[35]. The present results using TTZ showed approximately all seeds to be non-viable after 1 year. Non-viability of seeds have been tested earlier by TTZ[36] and was considered an important factor in seed trade, crop production and also in germplasm conservation and management.

PGR treatments were found to be ineffective after a certain time period which indicated the shelf life of the seeds which turned out to be 6-8 months and to the maximum of 1 year in the present study. The possible reason stated for this is due to the depletion of hormones supplemented with lack of presence of an efficient antioxidant enzymatic system to protect membranes from oxidative stress during ageing. There is not much documentation on a good method for germination; much more insights are needed into the aspect of seed germination for large scale propagation of bamboos. GA_3 was found to be effective in increasing germination percentage at higher temperatures. Non availability, shortage of starting experimental material due to factors cited above, establishing a protocol for micropropagation in economically important bamboo *D. membranaceus* Munro can prove beneficial to meet the bamboo plantation targets in the country. Genetic gain can be maintained by selecting the best phenotypes as seed producers.

Acknowledgement

The authors are grateful to Dr. P S Ahuja, Director, CSIR-IHBT, Palampur for facilities.

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