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# Micropropagation of *Bambusa balcooa* and *Dendrocalamus giganteus*

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## Abstract of Project Proposal

1. Title of the Project : Micropropagation of *Bambusa balcooa* and *Dendrocalamus giganteus*

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5. Funding agency : Department of Biotechnology (GOI)

6. Objectives:

   To develop *in vitro* culture techniques for micropropagation of *Bambusa balcooa* and *Dendrocalamus giganteus* using tissues collected from culms of mature clumps

# Contents

# ABSTRACT

Micropropagation was attempted in the two commercially important bamboo species, *Bambusa balcooa* and *Dendrocalamus giganteus.* Two modes of plantlet regeneration was experimented upon using explants of adult field grown clumps of the two species- viz. induction of multiple shoots in axillary buds followed by rooting of micro shoots and plantlet regeneration through somatic embryogenesis.

In *B. balcooa* a complete micropropagation protocol was developed using nodal explants collected from adult clumps growing in the field. Multiplication was achieved through multiple shoot formation in liquid media without shaking. Plantlets were regenerated through in vitro rooting at 94.4 % and 100 % survival. Regeneration of B. *balcooa* through somatic embryogenesis was also obtained during the study. Embryogenic calli was induced from nodes of in vitro shoots on auxin containing media and conversion to plantlets obtained on a hormone free medium. Most of the plantlets were found to be albinos.

In *D. giganteus* successful induction of multiple shoot formation and embryogenic calli was possible but regeneration of plantlets was not feasible.

A pretreatment procedure with systemic fungicide and antibiotics along with surface sterilization wit mercuric chloride was required to control contamination in *B. balcooa*. Addition of lactic acid and reduced pH (3.0) in the initiation media further reduced the fungal and bacterial contamination including endogenous contamination and also improved the sprouting of axillary buds.

Nodes collected from upper part of the branches gave a better sprouting response than the lower buds. Nodes from the upper branches of the culm gave a better response than those from the lower branches.

In *D. giganteus* up to 86 % sprouting of axillary buds was obtained in December – January but not in the other seasons. The highest percentage of sterile cultures was also obtained in this season.

The results obtained in this study points to the need for elaborate pretreatment of mother plants or explants with antimicrobial compounds including systemic fungicides

Multiple shoot induction from sprouted axillary buds could be achieved in both the species by culture in medium containing low levels of the cytokinins - BAP and Kinetin. Higher levels of BAP gave better multiplication but the risk of *in vitro* flowering and senescence of cultures was greater.

Liquid media with out shaking was found to be better suited than solid media for shoot multiplication. Not only where the multiplication rates higher, were the shoots more vigorous and longer. This has implications in the cost of plant production with savings in cost of agar and effort required for cleaning of glassware. The advantage of using liquid media was further increased with the successful use of polypropylene bags as culture vessels. These containers are inexpensive and being clear permit better illumination of the cultures stacked together.

*In vitro* rooting was successful in *B. balcooa* with up to 94.4 % shoot rooting on a liquid rooting medium with filter paper support. The rooting treatment was for 5 days followed by transfer to a liquid basal media that permitted shoot and root growth. This two phase rooting however will add to the costs and should be replaced by ex vitro rooting. These will the next step to be investigated.

In *D. giganteus* shoots could not be rooted with any of the treatments attempted. Supplements of phenolic compounds like coumarin and phlorogluicinol to the rooting media have been tried but no success has been obtained so far.

Embryogenic calli was induced and maintained from nodes collected from *in vitro* shoot cultures of both the species. This method has the advantage of overcoming the problem of contamination that occurs when explants collected directly from the plant are used to initiate cultures. Since shoot cultures in the multiplication stage of both the bamboo species was very prolific it can form a good source of explants for somatic embryogenesis. In *B. balcooa* although plantlets could be regenerated most of them were albinos thereby indicating that the procedure required modifications and the risk of genetic variability needs to be ascertained. Further work is required to develop regeneration of plantlets from the embryogenic callus of *D. giganteus.*

# INTRODUCTION

## Introduction

There has been increased interest in planting of bamboo around the world after the realization that this fast growing group of perennial grasses can be a good source of renewable biomass for paper pulp, as feedstock for biofuel, as an alternative to conventional timber and as a raw material for several industries. Several new industrial products using bamboo has been developed in recent years. The growth of the bamboo edible shoot industry has also grown. All this has created the demand for planting material for raising plantations. In India the efforts of the National Bamboo Mission and the National Mission on Bamboo Applications (NMBA) in popularizing bamboo cultivation and utilization has resulted in great demand for quality propagules of several species in the priority list.

Propagation in bamboo is naturally through seeds and when seeds are a limitation through simple vegetative methods of rhizome transfer or rooting of culm cuttings. Many of the economically important bamboo species flower in long cycles of several decades and often with wild populations the flowering is unpredictable, seed as a means of propagation is undependable. When gregarious flowering occurs the clumps die and for several years there is a shortage of the bamboo resource for the users. Vegetative means of clonal propagation is limited by the number of propagules that can be taken form a clump and the rootability of the species. There are many important bamboos that give poor propagation rates but in others vegetative means is used widely to generate planting material. In recent times the requirement for bamboo planting material has increased by several folds around the world and there is a need for a method that can meet this demand. Micropropagation or the use of plant tissue culture methods for large scale clonal propagation is a viable alternative. Micropropagation has been successfully used in

the ornamental plant and horticultural industry for mass clonal propagation. Very high multiplication rates are possible and uniform and pest and disease free plantlets can be produced.

Tissue culture is carried out under sterile conditions on synthetic nutrient media in which plant growth regulators play an important role. The capital and recurring costs of the micropropagation lab include wages of skilled technicians, energy intensive specialized facilities for maintaining clean and controlled environment especially in the culture growth room which has controlled light and temperature.

Alexander and Rao (1968) reported for the first time the results bamboo in culture and in last two decades several new species have been added to the list. Several bamboo species however are still not amenable to micropropagation because of inherent problem in the technique, the intractable problem of contamination, poor rootability etc. A majority of the earlier reports of successful micropropagation is from seeds or seedlings.

The disadvantage of procedures where propagation is carried out from seed or explants taken from seedlings is that application is limited to situations where at least a limited number of seeds/seedlings are available which in the majority of bamboo species is after a long flowering cycle. Since through macroproliferation (Adaresh Kumar, 1991) successive cycles of plantlets could be produced tissue culture does not have an any great advantage unless it is in the scale of operation. Clonal propagation of adult clumps using rhizome offset and culm cuttings have been used very successfully with a number of bamboos species( Surendran and Seethalakshmi, 1985) the multiplication rates are very low and there is a limitation to the maximum number of culm cuttings or rhizome offsets that can be taken from a clump. Development of methods for micropropagation of bamboo from explants taken from mature clumps is therefore very much desirable and decades of research have resulted in successful procedures for a few species of economically important bamboo.

The difficulty in establishing sterile cultures from explants of adult field grown clumps is the lack of response of all axillary buds, the presence of endogenous contaminants that stubbornly persist as latent contamination in later stages of culture. Rooting of the shoots is another serious hurdle in obtaining a complete protocol. In this context further studies need to be taken up especially to ensure that cultures consisting of multiple shoots can be maintained and multiplied for longer periods so that conditions required for rooting of shoots can be attempted.

Somatic embryogenesis has been reported earlier in several species of bamboo both from seed or seedling explants as well as from explants taken from adult plants (Godbole *et al.*, 2002, 2004; Saxena and Dhawan, 1999, Sood et al., 2002). This can be an alternative strategy for micropropagation to overcome or avoid some of the problems which are encountered when dormant axillary buds are used as explants such as latent contamination or poor multiplication or root ability.

*Bambusa balcooa* and *Dendrocalamus giganteus* are two of the priority species identified by the National Bamboo Mission and the Department of Biotechnology for mass clonal propagation. Both the species are suitable for cultivation for timber and biomass for industrial applications as well as for building material. *B. balcooa* is a common species found in homesteads of eastern and northeastern states of India and is a thornless species with good wall thickness. Culms are used for paper and pulp, as scaffolding for construction and for industrial boards, composites and laminates. Young shoots are edible. *D. giganteus* is among the bamboo species with the largest culm diameter and height and culms of which are very useful for handicrafts, building and household items like containers besides the industrial products. Tender shoots are also edible.

This study was undertaken to standardize the micropropagation protocols for the two important species using axillary bud proliferation as well as somatic embryogenesis.

## MATERIALS AND METHODS

### A. Stock solutions and nutrient media preparation

The minerals and vitamins of the Murashige and Skoog's (1962) medium (MS) formed the basal medium were used in these studies. Supplements of plant growth regulators and other adjuvants were made as and when found necessary for different stages of culture. Sucrose (2 % w/v) was used as the standard carbon source and the media either solidified with 0.7 % (w/v) of agar- agar (Hi-Media, Laboratories, Mumbai) and dispensed into test tubes or bottles or used as liquid media.

Stock solutions prepared in various concentrations and stored in the refrigerator or freezer were used to formulate the different media. The required aliquots were added and volume made up with double distilled water. pH of all media were adjusted to the required level through addition of KOH or HCl (1N or 0.1 N). All media were autoclaved for 15 minutes.

### B. Experiments:

Micropropagation of the two species was attempted through two different routes viz. (a) Through axillary bud proliferation and multiple shoot formation followed by *in vitro* rooting b). Through somatic embryogenesis via callus induced from nodes of *in vitro* shoot cultures.

### a. Micropropagation through axillary bud proliferation:

i. Explants: Nodal explants were collected from adult clumps of *Bambusa balcooa* and *Dendrocalamus giganteus* growing in the bamboo collection of the Kerala Forest Research Institute, Peechi, Kerala. All clumps were greater than 15 years of age. Branches from the current year's culms were collected and transported to the

laboratory for preparing the explants. The dead sheath was removed and the dead tissue at the nodes scrapped off with a blade followed by a wipe with a solvent to remove the wax. The cleaned branches were washed with detergent and rinsed in distilled water. Explants were cut into segments of 3-4 cm with the axillary bud in the upper half.

ii. Pretreatment with antimicrobial and antifungal solution: Since preliminary experiments has shown the high incidence of contamination due to bacteria and fungi, a pretreatment with a systemic fungicide (1.5 % Bavistin, BASF) and combinations of the broad spectrum antibiotics Cefotaxime, Gentamycin and Tetracycline ( 200 or 400 mg/l each ) for 45 min. was given before surface sterilization.

iii. Surface sterilization: Cleaned explants after the pretreatment was transferred to a sterile container and treated under the LAF bench with 0.1 % mercuric chloride solution for various durations. After the treatment the mercuric chloride was poured away and three rinses with sterile distilled water were given and the explants inoculated in induction media to induce axillary bud sprouting.

iv. Nutrient media: The minerals and vitamins of the Murashige and Skoog's (1962) medium was used as the basal medium (MS) in all the experiments on both the bamboo species. A comparison of liquid unagitated media with media solidified with Agar /Gelrite (Gellan Gum; Phytagel) was carried out at the initiation, multiplication and rooting stages. The following media were used at the different stages of culture in the two species:

1. Shoot initiation media: Minerals and vitamins of Murashige and Skoog's (1962) (MS) media supplemented with BAP (0.5- 3 mg/l), + Kinetin (1 mg/l) with or without NAA (0.1 mg/l and sucrose (2 %). (Liquid media in test tubes ; pH : 5.7)

2. Shoot multiplication media : MS supplemented with BAP (1.75, 2, 3 or 6 mg/l) + Kinetin ( 0.5 1, or 2 mg/l ) with or without NAA (0.1 mg/l)) and sucrose( 2 % or 3 % ) ( Liquid media in flasks/plastic containers or PP bags ; pH 5.7)

3. Shoot multiplication medium for D. giganteus: MS + NH4 NO3 (1650 mg/l) + BAP (1.75, 2, 3 or 6 mg/l) + Kinetin (0.5 1, or 2 mg/l) with or without NAA (0.1 mg/l)) Medium solidified with Gelrite (0.2 %) for the first two subcultures and liquid medium for the later subcultures.

4. Rooting media: MS + IBA ( 1, 3, 5, 10 mg/l) alone or with NAA ( 1, 3, 5 mg/l) + sucrose 2 %

v. Effect of Lactic acid and pH of media on sprouting and contamination of nodal explants:

Lactic acid at 0.15 % was added to the initiation media and tested at two different pH (3.0 and 5.7) along with media without lactic acid (at pH 3.0 and 5.7) and tested for effect in controlling contamination.

vi. Effect of position of bud on establishment of cultures:

Buds collected from the lower branches ( lowest 3 ) were compared to those from the upper branches (6-7$^{th}$ branch from base) and the position of the basal nodes in a branch compared to the distal nodes for the influence of position on contamination rates as well as sprouting of axillary buds.

vii. Effect of liquid ( without shaking) vs. Solid media for shoot multiplication:

Multiple shoots derived from the nodal explants of the two species were separated into cluster of about 10-15 shoots and transferred to 30 ml of multiplication medium in liquid form or solidified with Gelrite (Phytagel 0.2 %) to test the effect on

multiplication rates and subculture duration. Evaluation was done at the end of three subcultures.

viii. Comparison of shoot multiplication in polypropylene bags with glass bottle and plastic containers:

Clear Polypropylene bags ( of the commercially type commonly available) used for packing grocery (size 23 X 30 cms ) were prepared for culture by resealing the edges with a heat sealer and folding down the mouth (a double fold) and retaining the fold with paper clips. 30 ml of media was poured into the bags before autoclaving. Comparison was made with cultures in bottles of 250 ml with PP caps and in Phytocon (PP culture containers, Sigma corp.) each with 50 ml of media. Shoots clusters of 10- 15 shoots were subcultured into each container and the shoot multiplication and growth of cultures evaluated for three subcultures.

ix. *In vitro* rooting

Shoot clusters of 3- 4 shoots from the $10^{th}$ subculture ( S10 ) onwards in the multiplication media was transferred to the rooting media and maintained in the dark for 3 days followed by shifting to light. MS basal media supplemented with IBA (1, 3, 5, 10 mg/l) alone or with NAA (1, 3, 5 mg/l) + sucrose 2 % was tested. In later experiments the shoots from the root imitation media were shifted to hormone free basal media after 5 days to permit development of rooted shoots. One experiment was carried out with *Luffa* sponge as the support for rooting instead of the filterpaper support.

The effect of a pre rooting subculture on solid multiplication medium was compared to liquid medium followed by root induction on liquid or solid medium. After root induction the shoots were transferred to liquid basal medium for further development.

*Ex vitro* rooting was attempted by giving clusters of shoots with 2-3 shoots a brief dip of IBA at 10, 50 and 100 mg/l and transfer to vermiculite in polybags or portrays and maintenance under mist for a week.

x. Hardening of plantlets

Rooted shoots were transferred to a mixture of vermiculite and sand (1:1) in poly bags (8 X 16 cm) or portrays (50 cc cavities; Velkan Industries, Bangalore) and maintained under mist for periods up to three weeks. Hardened plantlets were transferred to soil in polybags and maintained in the nursery for further growth.

**b. Somatic embryogenesis:**

i. Explants:

Nodes were excised from freshly growing shoot cultures of *B. balcooa* and *D. giganteus* described above. Each explant was about a cm long with the leaf sheath removed. Unopened spikelets that were seen in shoot cultures of *B. balcooa* maintained on high levels of BAP were also used as explants when they were available.

ii. Nutrient media:

For induction of embryogenic calli minerals and vitamins of the Murashige and Skoog's media supplemented with various combinations of the auxins 2, 4-D, NAA (1, 2, 3, 5 mg/l) with or without BAP (0.5 or 1 mg/l) and sucrose 3 % were attempted. Cultured were carried out in petridishes with 20 ml of media.

iii. Induction and maintenance of embryogenic callus

Nodes of both the species and spikelets of *B. balcooa* cultured in dark on 2, 4 -D containing media gave rise to yellowish white callus in about two weeks time which was maintained through subculture every 3-4 weeks into test tubes containing the embryogenesis media.

iv. Regeneration of somatic embryos.

Embryogenic calli that showed development of somatic embryos were transferred to solid hormone free basal medium or media with only a low level of BAP and shifted to light to induce conversion of embryos to plantlets.

# RESULTS AND DISCUSSION

## A. Plantlet regeneration through multiple shoot formation and in vitro rooting:

### *1. Establishment of shoot cultures*

Sprouting of axillary buds in nodal explants occurred within 2-3 days of culture on the initiation media but establishment of sterile shoot cultures was hampered by contamination especially endogenous contamination. This had a pronounced seasonal effect in the two species. To overcome the problem surface sterilization procedure was modified with an additional pre treatment step with systemic fungicides and antibiotics after the cleaning procedure and prior to the treatment with mercuric chloride solution.

Best sprouting of buds was obtained on MS + BAP (2mg/l) +Kin (1mg/l) +Sucrose (2%) for *B. balcooa* and MS +BAP (3mg/l) + NAA (0.1mg/l) + Sucrose 2% for *D. giganteus.*

The pretreatment were in general beneficial particularly the combination of systemic fungicide with antibiotics (Table1 and 2). In *B. balcooa* sprouting of axillary buds was 100 % but cultures lost to bacterial and fungal contamination were high in number. Pretreatment with a combination of 1.5 % Bavistin and a mixture of Cefotaxime and Tetracycline at 200 mg/l each for 45 min was effective in reducing the contamination significantly when compared to the chemicals individually or the control without any pretreatment.

**Table 1 – Effect of different pre-treatments on establishment of nodal explants of *B. balcooa***

| Sl. No. | Treatments | % of BC* | % of FC* | % of sterile sprouting | % of contamination |
|---|---|---|---|---|---|
| 1 | $HgCl_2$ (0.1%) | 20 | 12 | 68 | 32 |
| 2 | Bavistin (1.5%); $HgCl_2$ (0.1%) | 19 | 9 | 72 | 28 |
| 3 | Tetracycline (200 mg/L) +Cefotaxime (200 mg/L); $HgCl_2$ (0.1%) | 11 | 21 | 68 | 32 |
| 4 | Bavistin (1.5%) + Tetracycline (200 mg/L) + Cefotaxime (200 mg/L); $HgCl_2$ (0.1%) | 6 | 4 | 90 | 10 |

*__BC__- Bacterial Contamination; FC- Fungal Contamination.

Fig. 1. Sprouted nodes of *B. balcooa*

Fig 2. Sprouted nodes of *D. giganteus*

Fig 3. Multiple shoot formation in *B. balcooa* on liquid multiplication medium

Fig 4. Multiple shoot formation in *D. giganteus*

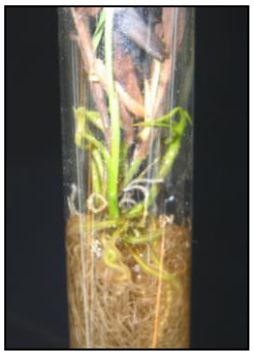

Fig. 6 *In vitro* rooting in *B. balcooa* on filter paper and on Luffa sponge ( right)

Fig. 7. Hardening of *B. balcooa* plantlets

Fig.8.Hardened plantlet of *B. balcooa* after one month in soil

Fig 9. Plantlets of *B. balcooa* in the Nursery.

When nodal explants of *D. giganteus* were pretreated with various combinations of fungicide and antibiotics, significant reduction of contamination particularly of fungus was achieved with Bavistin 1.5 % with Gentamycin and cefotaxime (400 mg/l) but this was at the cost of a reduction in sprouting percentage of the buds (Table 2). Whereas controls without any treatment gave 100 percent contamination in the experiment the sprouting of buds was as high a 90 %. These points to the need to have a more critical evaluation of different pretreatments with possible additional antibiotics with broad spectrum activity to be tested. Isolation of bacteria and test with sensitivity discs to select the appropriate antibiotic may yield results. Das and Pal (2005) have also noted the systemic fungal contamination that appeared even in 3$^{rd}$ or 4$^{th}$ subculture as a major problem in micropropagation of *B. balcooa*. Ramanayake *et al.* (2001) had reported the sprouting of axillary buds form dormant nodes in *D. giganteus* in the presence of high levels ( 6 mg/l) of BA but also found the induction of in vitro flowering at this level. This indicates that maintaining relative lower levels of cytokinin is desirable for shoot multiplication

**Table 2. Effect of pretreatment and surface sterilizations treatments on establishment of cultures from nodal explants of *D. giganteus***

| Sl No. | Experiment | % of sprouting | % of BC | % of FC | Total % contamination |
|---|---|---|---|---|---|
| 1 | 1.5 % Bavistin + 400 mg/l Gentamycin-1hr | 86 | 53.4 | 6.8 | 60.2 |
| 2 | 1.5 % Bavistin + 400 mg/l Streptopenicillin-1hr | 46.4 | 50 | 1.7 | 51.7 |
| 3 | 1.5 % Bavistin + 200 mg/l Cefotaxime-1hr | 60.8 | 60.8 | 2.1 | 62.9 |
| 4 | 1.5 % Bavistin + 400 mg/l gentamycin-30 min; | 30 | 40.8 | 0 | **40.8** |
| 5 | 1.5 % Bavistin + 400 mg/l Streptopenicillin- 30 min | 52 | 60 | 2 | 62.0 |
| 6 | 1.5 % Bavistin +200 mg/l Cefotaxime-30 min | 25 | 50 | 12.5 | 62.5 |
| 7 | Control | 90 | 80 | 100 | 100 |

**BC**- Bacterial Contamination; FC- Fungal Contamination

**2.** *Effect of lactic acid on sprouting and contamination rates in nodal explants*

Because of the heavy incidence of contamination other means of controlling has to be envisaged without compromising on the spouting of xaillary buds so as to obtain sterile cultures in sufficient numbers. Lactic acid (LA) an organic acid which is known to have antimicrobial properties was tested and found to give good results and also surprisingly improved the sprouting of buds over that of the control (Table 3). There was significant improvement with use of LA at pH 3.0, while LA at

higher pH was not very effective. Media at pH 3 without addition of LA was also found to give this surprising result since such a growth enhancing effect of LA or any organic acid is not reported for any species. The antimicrobial effect is taken advantage of in food preservation. These mild acids are non toxic to plants and animals compared to the typical surface sterilizing agents. It needs to be ascertained if such compounds can be used as exclusive surface sterilizing agents and mercuric chloride can be totally avoided.

**Table 3. Effect of Lactic acid on sprouting and contamination rates in nodal Explants of *B. balcooa* and *D. giganteus***

| | Treatment | % of sprouting | % of contamination |
|---|---|---|---|
| ***B. balcooa*** | | | |
| 1 | Control | 68 | 44.889 |
| 2 | LA* + pH 3.0 | **96.8** | **8.6** |
| 3 | pH 3.0 | 86.9 | 20 |
| 4 | LA + pH 5.7 | 72 | 33.5 |
| ***D. giganteus*** | | | |
| 1 | Control | 22 | 53 |
| 2 | LA+ pH 3.0 | **74** | **20** |
| 3 | pH 3.0 | 50 | 34 |
| 4 | LA+ pH5.7 | 29 | 46 |

* LA: Lactic Acid (0.15 % v/v)

3. *Effect of position of nodes on mother plant on sprouting and contamination rates on initiation media in B. balcooa*

The effect of position of collection of nodal explants from the mother plant was remarkable in both their sprouting response as well as in the incidence of contamination in the initiation media (Table 4). The nodes collected form upper branches gave better results in both the parameters and the basal buds were better than those towards the distal end of the branches. The contamination rates may be correlated to the proximity to the soil as well as to the fact that these branches form earlier than those at the top.

**Table 4. Effect of position of nodes on sprouting and contamination rates in *B. balcooa***

| Position of branch | Position of Bud on branch | % of contamination | % of sprouting |
|---|---|---|---|
| Upper branches | Distal | 17.4 | 86.9 |
| | Basal | **0** | **100** |
| Lower branches | Distal | 44.8 | 89.6 |
| | Basal | **12.5** | **100** |

4. *Shoot multiplication*

Multiple shoots of *B. balcooa* were induced and prolific multiplication of shoots took place when sprouted nodes along with the original explants were transferred to liquid MS basal media supplemented with cytokinins. Best results were obtained in 2 mg/l each of BAP and Kinetin and Sucrose 3%. Subcultures were carried out at 10 day intervals with splitting of the grown shoot clusters into 2-3 smaller clusters of and inoculation into fresh media in separate bottles. Keeping the cultures for longer periods resulted in media or leaves turning brown.

In *D. giganteus* best results were obtained with MS + BAP (3mg/l) + Kinetin (3mg/l) + $NH_4NO_3$ 1650mg/l + Sucrose 2% solidified with Phytagel (0.2%) in the early stages but liquid media was better for later stages.

Shoot multiplication in both the species was better on liquid media without shaking when compared to media solidified with Phytagel. A quantitative estimation of the shoot number was not feasible without sacrificing the cultures and therefore effects of different treatments was done through visual estimation.This could be attributed to the better contact tissues have with media being partially submerged in it. Although hyperhydricity is often observed in cultures maintained on liquid media in many species this appears to be not the case with bamboo shoots cultures. Considerable savings in the cost of propagation can thus be obtained by avoiding the use of agar or gelrite and also the concomitant costs involved in cleaning of glassware with solid media.

5. *Comparison of shoot multiplication in polypropylene bags with glass bottle and plastic containers*

Multiplying shoot cultures in glass bottles required subculture every 10 days to maintain them in healthy condition without browning. This was cumbersome since the spreading and leafy shoots are difficult to manipulate during transfers. Larger rigid plastic containers were therefore preferred and it was possible to maintain the shoot cultures for beyond 10 days. If media volume was increased to 200 ml and culture carried out in large containers the subculture period could be increased to beyond 20 days.

When shoot cultures were transferred to polypropylene bags shoot multiplication was still as good as in rigid plastic containers ( Fig. 5.). Increased availability of light through the clear thin walls of the bags enabled several cultures to be stacked side to side without light availability getting reduced substantially. Since the bags are inexpensive when compared with rigid glass or plastic containers considerable savings in cost of production could be achieved. This has not been quantified in this study since the life of the rigid containers could not be ascertained. Besides the time required carrying out transfers in the two types of containers depends on the skill levels of the technicians. Plastic bags, due to their flexible nature require some skill to remove and introduce tissues like a cluster of bamboo shoots which themselves are quite flexible.

**Fig. 5.** Shoot cultures of *B. balcooa*
in liquid media in polypropylene bags

6. *Comparison of solid and liquid media on in vitro root induction*

When sufficient shoots were produced *in vitro* rooting was attempted. A pre rooting step to ascertain the effect of solid media vs. liquid media on subsequent rooting was carried out and solidified with Phytagel 0.2% prior to the rooting step and the multiple shoots transferred to liquid MS basal media of same composition (control). After 10 days of incubation clusters of 2-3 shoots were transferred to root induction media. Root induction was observed within 5 days. Then the shoots were shifted after 5 days to modified MS basal media supplemented with sucrose 2% with out any plant growth regulators. Highest percentage of rooting was obtained in liquid media (94.4%)(Fig. 4). Percentages of rooting were lower in solid root induction media in both the control (66.6%) and in the experiment (45%). Results of the *in vitro* rooting of *B. balcooa* were given in the Table 5. Shoots maintained on solid medium showed browning and senescence but on transfer to basal media rooted shoots showed development of leaves in one week.

Rooted shoots of *B. balcooa* were then permitted to grow in the basal media until fresh leaves and a more robust root system had developed. In about a week the plantlet begins to develop a healthy root and shoot system and after two weeks the plantlet is ready for hardening and planting out. Hardening was successfully carried out in two weeks in polybags under humidity maintained by the cover provided by the inverted polybag (Fig. 5 and 6). Plantlets were then ready for transfer to soil and nursery for maintenance and further development before field planting.

No success was obtained with the experiments in *ex vitro* rooting. The shoots turned brown within 4-5 days and no rooting was observed in any of the treatments. It has to be ascertained if rooting could be obtained with a more appropriate choice of plant growth regulators and supplements

**Table 5. Comparison of *in vitro* rooting treatments of *B. balcooa***

| **Pre- rooting media** | **No. of replicates** | **Media used** | **Shoot status** | **Subculture media ( root development)** | **Shoot status** | **% of Rooting** |
|---|---|---|---|---|---|---|
| MS+ BAP (2 mg/l) +Kin (2 mg/l) + Sucrose 2% (Solid) | 20 | 1/2MS + IBA( 3 mg/l) + NAA ( 3 mg/l) + Suc 2% (Solid ) | Dried | ½ MS + Sucrose 2%+ Phytagel 0.2% | Green | 45 |
| | 20 | 1/2MS+IBA( 3mg/l ) + NAA ( 3 mg/l) +Suc 2% (Liquid ) | Green | ½ MS + Sucrose 2% | Green | 85 |
| MS+BAP (2 mg/l) + Kin (2 mg/l) + Sucrose 2% ( Liquid ) | 20 | 1/2MS + IBA(3 mg/l) + NAA (3 mg/l) +Suc 2 % (Solid) | Dried | ½ MS + Sucrose 2% | Green | 66.6 |
| | 20 | 1/2MS+IBA(3 mg/l) + NAA( 3 mg/l) + Suc 2% (Liquid ) | Green | ½ MS + Sucrose 2% | Green | 94.4 |

**B. Somatic embryogenesis:**

1. *Initiation and maintenance of embryogenic callus :*

Nodal explants of both the species showed signs of swelling in the axillary bud area within one week of culture (Fig. 10.). The best results were obtained with MS supplemented with 1 mg/l each of 2,4, D, BAP and NAA and 3 % sucrose Some signs of browning in the media adjacent to the explants were seen in many cases but this did not effect the callus development. Calli grew slowly and only after subculture was good growth seen to occur. Calli were yellowish to translucent white in colour ( Fig. 11 and 12) and granular in both the species.

Maintenance of embryogenic calli was done by subculture to fresh medium every 3-4 weeks. Somatic embryos were seen to develop spontaneously on the surface of the callus but no conversion occurred in the dark.

2. *Regeneration of somatic embryos:*

Embryogenic calli with somatic embryos visible on the surface were observed after transfer to various regeneration media. On all the media some parts of the callus turned dark green but only occasional formation of shoots was observed. In *B. balcooa*. On solid hormone free basal medium or media with a low level of BAP (0.1, 0.5 mg/l) and incubation in light, conversion of embryos was observed. The earliest indication of conversion was the development of green colour in the embryogenic areas of the calli (Fig. 13). Root and shoot development was simultaneous (Fig. 14). The presence of a large number of albino plantlets was notable. This might be a consequence of stress in the culture or due to somaclonal variation.

Fig 10. Callus induction in node of *B. balcooa*

Fig 11. Embryogenic callus of *B. balcooa*

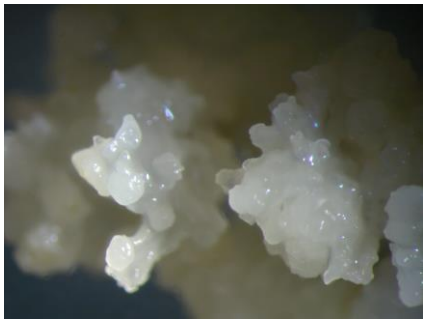

Fig 12. Embryogenic callus of *D. giganteus*

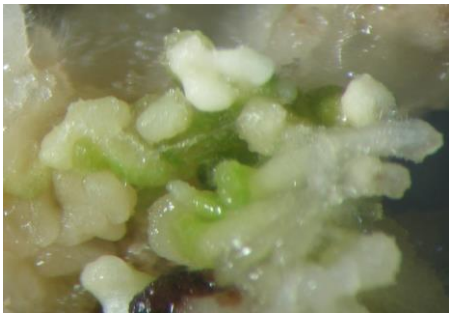

Fig. 13. Mature somatic embryos of *B. balcooa*

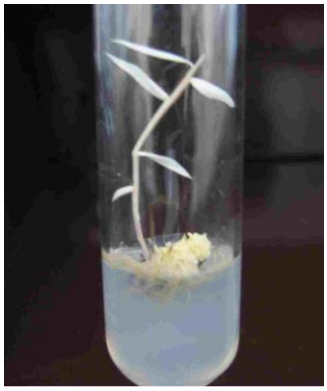

Fig. 14. Albino plantlet derived from somatic embryo of *B. balcooa*

## C. Conclusions

In *B. balcooa* a complete micropropagation protocol was developed using nodal explants collected from adult clumps growing in the field.

Addition of lactic acid and reduced pH (3.0) in the initiation media reduced the fungal and bacterial contamination including endogenous contamination. Improved sprouting was also seen on the media.

Nodes collected from upper part of the branches gave a better sprouting response than the lower buds. Nodes from the upper branches of the culm gave a better response than those from the lower branches.

In *D. giganteus* upto 86 % sprouting of axillary buds was obtained in December – January but the effect of season was pronounced. The highest percentage of sterile cultures was also obtained in this season. The results obtained in this study points to the need for elaborate pretreatment of mother plants or explants with antimicrobial compounds including systemic fungicides

Multiple shoot induction from sprouted axillary buds could be achieved in both the species by culture in medium containing low levels of the cytokinins - BAP and Kinetin. Higher levels of BAP gave better multiplication but the risk of *in vitro* flowering and senescence of cultures was greater.

Liquid media with out shaking was found to be better suited than solid media for shoot multiplication. Not only where the multiplication rates higher, the shoots were more vigorous and longer. This has implications in the cost of plant production with savings in cost of agar and effort required for cleaning of glassware. The advantage

of using liquid media was further increased with the successful use of polypropylene bags as culture vessels. These containers are inexpensive and being clear permit better illumination of the cultures stacked together.

*In vitro* rooting was successful in *B. balcooa* with upto 94.4 % shoot rooting on a liquid rooting medium with filter paper support. The rooting treatment was for 5 days followed by transfer to a liquid basal media that permitted shoot and root growth. This two phase rooting however will add to the costs and should be replaced by *ex vitro* rooting. Further experimentation is necessary to standardize ex vitro rooting and

In *D. giganteus* shoots could not be rooted with any of the treatments attempted. Supplements of phenolic compounds like coumarin and phlorogluicinol to the rooting media have been tried but no success has been obtained so far.

Somatic embryogenesis is expected to be more prolific means of micropropagation in bamboo. In the present study embryogenic calli was induced and maintained from tissues collected from *in vitro* cultures. This method has the advantage of overcoming the problem of contamination that occurs when explants collected directly from the plant are used to initiate cultures. Since shoot cultures in the multiplication stage of both the bamboo species was very prolific it can form a good source of explants for somatic embryogenesis. In *B. balcooa* although plantlets could be regenerated most of them were albinos thereby indicating that the procedure required modifications and the risk of genetic variability needs to be ascertained. Further work is required to develop regeneration of plantlets from the embryogenic callus of *D. giganteus.*

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