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Micropropagation of three selected species of bamboo
(Final Report of Project No. KFRI 392/06)

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Abstract of project proposal

1. Title of the Project : **Micropropagation of three selected species of bamboo** (Project No. KFRI **392/2003** (Plan)

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6. Objective **:** To develop efficient tissue culture procedures for mass clonal propagation of three species of bamboo viz. *B. balcooa, D. brandisii* and *Thyrsostachys oliverii* using material collected from mature field growing clumps.

Contents

Abstract

Micropropagation procedures for three important bamboo species was attempted in this study using nodal explants collected from mature clumps growing in the field. Propagation was induction of axillary bud proliferation and multiple shoots and *in vitro* rooting or through somatic embryogenesis.

In *Bambusa tulda*, nodes with dormant buds were treated with a antimicrobial solution consisting of Bavistin (1.5 % w/v) and the antibiotics Cefotaxim and Tetracyclin (100 ppm each) for 45 min before surface sterilization with $HgCl_2$ (0.1 % w/v) for 10 minutes followed with three rinses with sterile water. Sprouting of buds was obtained on a medium consisting of MS salts and vitamins, BAP 4.44 µM (1 mg/l), NAA 0.53 µM (0.1 mg/l) and sucrose 2 % (w/v) and Lactic acid (0.1%) with the pH adjusted to 3.0. Multiple shoots were induced when sprouted nodes were maintained through subcultures every 10 days on a multiplication medium consisting of MS salts and vitamins supplemented with BAP 7.77 µM (1.75 mg/l) and NAA 0.53 µM (0.1 mg/l) and sucrose 2 %. Rooting was obtained when clusters of 3-4 shoots were transferred to a rooting medium consisting of ½ MS salts and vitamins supplemented with IBA (3 mg/l) and Coumarin (10 mg/l)

In *Dendrocalamus brandisii*, similarly, nodal explants were induced to sprout and form multiple shoots on a liquid medium consisting of MS salts and vitamins supplemented with 8.88 µM BAP (2 mg/l) and 4.65 µM Kinetin (1 mg/l) + Lactic acid (0.1%) and sucrose 2 % with pH adjusted to pH 3.0 . The same medium was suitable for maintaining good shoot multiplication. Rooting was not successful in any of the treatments. The shoots turned senescent and leaves brown after a week of culture. Indirect somatic embryogenesis was induced in callus derived from nodes excised form *in vitro* shoot cultures and placed on solid medium consisting of MS salts and vitamins, 13.32 µM BAP (3 mg/l), 2.06 µM 2,4-D (0.5 mg/l) and sucrose 3 % and pH adjusted to 5.7 and solidified with Gelrite (0.2 %) and culture in the dark. Conversion of somatic embryos were obtained by subculture of callus to ½ MS salts and vitamins + 4.44 µM BAP (1 mg/l), sucrose 2 % and + Agar 0.75 %. Hardening of the plantlets was done under mist on vermiculite + sand (1:1) for three weeks.

Sprouting of axillary buds and multiple shoots were obtained from nodal explants of *Thrysostachys oliveri* cultured on a liquid medium consisting of MS salts and vitamins, Sucrose 2 % supplemented with BAP (2 mg/l) (8.88 µM), Kinetin (0.1 mg/l) (0.46 µM) and Lactic acid (0.1% v/v) with the pH adjusted to 3.0. High rates of multiplication of upto 5 fold were achieved on a liquid medium supplemented with 11.1 µM (3 mg/l) of BAP and 0.53 µM (0.1 mg/l) of NAA. Shoot multiplication was carried out in PP bags. Rooting was not achieved in any of the *in vitro* rooting experiments.

INTRODUCTION

Most of the published literature on tissue culture of bamboo species deals with cultures originating in seed or explants taken from seedlings. Obviously the application of this technique is limited to situations where at least a limited number of seeds/seedlings are available. On the other hand in the majority of bamboo species with long flowering cycles the availability of seed is a limitation in their mass propagation. Seeds even when stored under the best conditions lose viability after a few years and for rest of the flowering cycle of the species propagation is dependent on vegetative means. Macroproliferation, the method in which tillers of seedlings with a rhizome attached to them are separated at regular intervals to obtain successive cycles of plantlets (Adaresh Kumar, 1991). 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) but there are limitations when a large scale propagation is required since the multiplication rates are very low and the number of cuttings or rhizome offsets that can be extracted is a limitation. Thus the development of methods for micropropagation of bamboo from explants taken from mature clumps is urgently required. Such techniques have been reported in a few species like *D. strictus, D. giganteus, B. bambos* and *B. vulgaris.*

A serious problem in carrying out experiments is the difficulty in establishing sterile cultures from explants taken from field grown clumps. Endogenous contaminants are difficult to remove particularly during and immediately after monsoons thereby resulting in loss of a large proportion of the explants. When sufficient plant material is not available this is a serious constraint and necessitates the pooling together of all the available explants ignoring the effect of position on the plant. Rooting of the shoots obtained after multiplication currently appears to be the biggest hurdle in obtaining plantlets from mature bamboo clumps. This has been achieved by conventional means of treatment with auxins both *in vitro* as well as *ex vitro*. There appears to be a correlation between the rootability of shoots in tissue culture with that of culm cuttings used in vegetative propagation.

Somatic embryogenesis is the alternative route to plantlet regeneration from explants without the need for a meristem as in the axillary bud proliferation methods commonly used for micropropagation. 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 (Saxena and Dhawan, 1999; Rao *et al.*, 1985). The potential for scaling up is greatest for somatic embryogenesis since

Several hurdles in establishment of cultures from explants collected from the field grown clumps, obtaining good multiplication and plantlet regeneration have to be overcome before a protocol for bamboo can be considered good for large scale propagation. Several bamboo species of economic importance are still not amenable to propagation by conventional means. *Dendrocalamus brandisii* and *Thrysostachys oliverii* are two such examples. Both are species that are in great demand for cultivation. *D. brandisii* is a tall bamboo with large diameter culms that are useful in construction and as alternatives for

timber. *T. oliverii* is a popular bamboo species with farmers because of the culms with smaller diameter and thicker culm wall and a graceful appearance of the clump and foliage. Conventional vegetative propagation is not very successful the two species especially with *T. oliverii* in which only the lowest nodes give rooting. An alternative cloning method is therefore of great importance for large scale propagation of the two species.

It was with this background that this study was undertaken with the following objective:

Objectives

i. To develop efficient tissue culture procedures for mass clonal propagation of three species of bamboo viz. *B. balcooa, D. brandisii* and *Thyrsostachys oliverii* using material collected from mature field growing clumps.

MATERIALS AND METHODS

Standard plant tissue culture methods with regard to sterile technique, media preparation, stock solutions and culture incubation were followed with modifications carried out whenever found necessary.

1. 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/bottles/polypropylene containers 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 using either a domestic pressure cooker or a horizontal autoclave.

2. 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 composition of the media used at the different stages of culture in the two species is given below in the relevant section.

3. Explant collection

Branches form current year's culms with dormant axillary buds at the nodes were collected from the adult clumps of the different species growing in the KFRI Bamboosetum at Velupadam, Thrissur District, Kerala, India were collected, transported wrapped in moist newspaper to the laboratory and in most cases processed for culture the same day or else kept moist and stored at room temperature until the next day.

4. Surface sterilization:

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

Cleaned explants after the pre-treatment were 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 was given and the explants inoculated in induction media to induce axillary bud sprouting.

5. **Establishment of cultures**

Two different routes to plantlet regeneration from different explants were attempted:

a) Through axillary bud proliferation, multiple shoot formation and *in vitro* rooting.
b) Through somatic embryogenesis via callus induced from nodes of *in vitro* shoot cultures.

a. Micropropagation through axillary bud proliferation:

The following nutrient media were used for the different stages:

Initiation of shoot cultures:

MS salts and vitamins + BAP (1 mg/l) + NAA (0.1 mg/l) + Suc. 2 % + Lactic acid (0.1%); pH: 3.0 (Liquid medium)

Shoot multiplication:

MS salts and vitamins + BAP (1.75 mg/l) + NAA (0.1 mg/l) + Suc. 2 %+ Lactic acid (0.1%); pH: 3.0(Liquid medium)

Rooting:

Liquid or solid media with ½ MS salts and vitamins supplemented with a auxin (NAA/ IBA (3 mg/l) with or without Coumarin (10 mg/l)

RESULTS AND DISCUSSION

1. Bambusa tulda

Sprouting of axillary buds was obtained in 4-7 days of culture and completed in about 10 days (Figure 1). Each node gave rise to sprouts that varied from 1-4 in number. Sprouting was observed in 70 to 90 % of the explants in various batches and depended on the season, the size and position of the nodal explants and the presence or absence of microbial contamination. Sprouting was obtained even when cultures were contaminated but the influence on sprouting, if any, was not clear. The effect of season and position of the explants therefore could not be ascertained due to the loss of explants due to contamination in the establishment phase.

Fig.1. Sprouting of axillary buds in nodal explants of *B. tulda*

Fig. 2 Multiple shoots of B. tulda

Fig. 3 Rooting of shoots

Fig. 4. Hardened plantlets of *B. tulda*

Even in cultures that had undergone 2-4 subcultures contamination appeared all of a sudden. This could be explained either as delayed expression of deep seated but possibly surface microflora or the presence of true endophytes. Endophytes are bacteria and

fungus which are endosymbiotic and do not cause any disease. In the context of plant tissue culture however, endophytes cause a serious problem since they proliferate to the level that they effect proper development of plant tissues either by overgrowing the explants or by producing toxic products.

Control of endogenous microbial contamination was achieved during the initiation stage through pretreatment of explants with antimicrobial compounds and use of lactic acid and low pH (3.0)

Use of liquid media for culture of nodal explants and later shoot cultures without shaking was found to be better than solid media. This is apparently due to the improved contact of tissues with liquid media and the better uptake of the media components especially the mineral nutrients, vitamins and sugar when compared to solid media. The liquid media permitted the growth of the shoot clusters with shoots spread in a manner that filled the culture vessels. Shoots in solidified media on the contrary were short and densely clustered, with small leaves.

Rooting was obtained in shoot clusters consisting of 2-4 shoots of about 3-5 cm length when transferred to the rooting media. Root initials could be observed to form within a week in 70 % of the tubes. As soon as root emerged in the cultures the shoots were transferred to the shoot multiplication media or hormone free media to permit recovery of the shoots which shows signs of browning. Shoot s with root initiation could also be transferred for hardening into poly bags with a mixture of sand and vermiculite (1:1) and kept under intermittent mist.

Use of polypropylene (PP) bags as culture containers was also tested and found to be suitable for maintaining shoot cultures in the multiplication phase. For initiation it was essential that the explants were kept in erect position with only the lower part immersed in media and therefore only liquid media in test tubes were used. The use of PP bags as containers had advantages. Cost savings are considerable when compared to the glassware or the rigid plastic vessels conventionally used. Saving of space was another advantage since several bags could be kept hanging side by side with no loss of light availability due to the transparency of the PP bags. The rigid PP containers used in this study was sourced from domestic supplies shops and of the type used in storing in kitchens. Compared to labware these are inexpensive and have no significant differences in it utility as culture containers. These are routinely used for maintaining bamboo shoot cultures including the other species propagated in this study. PP bags although having the advantages described above are difficult to handle while carrying out transfers since the neck of the bags are not easily opened and sealed without compromising the sterility. Further modifications to the bags like fabricating a rigid neck would enable a wider used of PP bags in micropropagation.

Nutrient media:
The following nutrient media were found to be optimum for the various stages of culture of *B. tulda*:

Initiation: MS salts and vitamins + BAP (1 mg/l) + NAA (0.1 mg/l) + Suc. 2 %
Lactic acid (0.1%); pH: 3.0 (Liquid)
Shoot multiplication: MS salts and vitamins + BAP (1.75 mg/l) + NAA (0.1 mg/l)
+ Suc. 2 % (Liquid)
Rooting: ½ MS salts and vitamins + IBA (3) + Coumarin (10 mg/l) (Liquid)

2. Dendrocalamus brandisii

Multiple shoot formation

Multiple shoot induction from nodal explants was successful in this species as in the above case. Contamination through endophytes was a problem which was solved to an extend with the used of Lactic acid and lowering the pH.

Induction and multiplication of shoots was obtained on a liquid medium with MS salts and vitamins + BAP (2 mg/l) + Kinetin (1 mg/l) + Lactic acid (0.1%) adjusted to pH 3.0 As in *B. tulda* liquid media was found to give rapid and better growth of multiple shoots.

Rooting of shoots taken from subcultures beyond 4th subcultures was attempted on the rooting media. However no rooting was obtained in any of the treatments. The shoots turned senescent and leaves brown after a week of culture.

The possibility of cultures habituated to cytokinins and turning recalcitrant to rooting treatment needs to be considered. Changing the hormone levels during multiplication or change of the type of hormones during the multiplication phase or a separate culture in hormone free or low hormone media for a pretreatment before rooting treatment are the possible solutions. This could not be carried out in the duration of this study.

Somatic embryogenesis

A complete protocol was developed for cloning of adult clump through somatic embryogenesis

Explants used to initiate a callus were nodes from fast growing *in vitro* shoot cultures. This gave the advantage of overcoming the problem of loss of cultures due to contamination that was typical of explants collected directly from the plants.

The nutrient media used for induction of embryogenic calli consisted of MS salts and vitamins + BAP (3 mg/l) + 2,4-D (0.5 mg/l) + Suc. 3 % solidified with Gelrite (0.2 %) and with a pH 5.7. The same media was used for maintaining the callus in embryogenic state through at least 5 subcultures of 3-5 weeks.

Plantlet regeneration was achieved by transfer of embryogenic callus to a regeneration media consisting of ½ MS salts and vitamins + BAP (1 mg/l) + Suc. 2 %. + Agar 0.75 %; pH 5.7. Development of embryos was non synchronized and conversion to plantlets

occurred on the same media in the presence of light. Efficiency of conversion however was low.

Hardening of regenerated embryos was successful when plants were cleared of media under tap water and transferred to a media consisting of vermiculite + sand (1:1) and maintenance under a mist chamber for three weeks

Stages in regeneration of plantlets in *D. brandisii* through somatic embryogenesis

3. Thrysostachys oliveri

Induction of shoots from nodes and high rates of shoot multiplication was achieved in the species when nodal explants (2.5 – 4 cm) collected from branches of current year's culms were used. The buds were prepared by removing the sheath removed and wiping with acetone to remove wax and then treated for 1 hr with Bavistin (1 %) + antibiotics (Cefotaxime+ Tetracyclin (200 ppm) as a prophylactic measure against microbial contamination. Surface sterilization was done through a treatment with $HgCl_2$ (0.1 %) for 10 minutes before transfer to the initiation media.

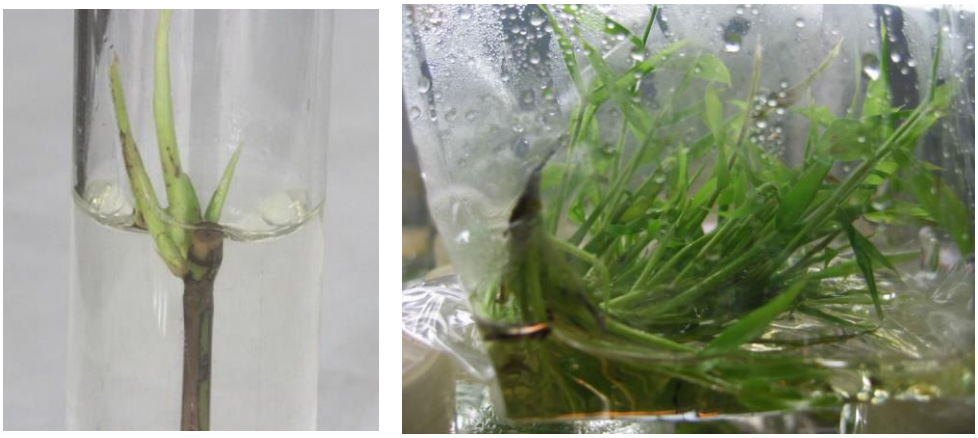
For initiation of shoot cultures: the media consisted of MS salts and vitamins + BAP (2 mg/l) + Kinetin (0.1 mg/l) + Sucrose. 2 % supplemented with Lactic acid (0.1%) and with adjusted to pH 3.0

Shoot multiplication was achieved on transfer of sprouted explants with shoots on a media with MS salts and vitamins + BAP (2.5 mg/l) + NAA (0.1 mg/l) + Sucrose 2 %

Upto 5 folds multiplication achieved in liquid media without the need for shaking. This was of considerable significance since typically shoot cultures are maintained in media with agitation on orbital shakers thereby incurring a big energy cost.

Shoot multiplication was aslp carried out in polypropylene bags as in the case of *B. tulda* described above.

Rooting was attempted on a series of media with combinations of auxins and phenolic compounds but no success was achieved. Further experiments required for induction of rooting in this difficult to root species.



Initiation of shoot cultures from nodal explants in *T. oliverii*

CONCLUSIONS

High rates of shoot multiplication were possible in all the three species studied in spite of the initial difficulty in establishing cultures due to the problem of contamination. It was interesting to observe a measure of control of contamination through the use of lactic acid and reducing the pH of the media. This approach requires closer study as a potential means of maintaining bamboo shoot cultures. It appears to be a case of suppression of microbes rather that eradication of contamination. High incidence of contamination is a hurdle that needs to be overcome through concerted research efforts since it is more common that is reported in literature.

Difficulty in rooting is a common problem faced in bamboo micropropagation of several important bamboo species. Regeneration through somatic embryogenesis as an alternative seems to show promise although the presence of a callus stage means that the danger of somaclonal variation exists and screening of plantlets for genetic fidelity should be carried out.

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