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Micropropagation protocol of less thorny bamboo variety *Bambusa bambos* (L) Voss endemic to Maharashtra state of India

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Abstract

Bambusa bambos, locally known as *Katang* is a commercially important bamboo. It is used for house construction, scaffolding, and rafters. *B. bambos* is having problems in clump management and ultimately for harvesting due to its thorny nature. To overcome these problems less thorny variant of *B. bambos*, which are very rare, were selected as mother plants. Using these selected mother plants micropropagation protocol was developed. Nodal segments containing pre-existing axillary buds were surface sterilized and then inoculated on solid Murashige and Skoog's (MS) basal medium containing cytokinins 1.5 mg/l BAP and 0.25 mg/l Kn. For obtaining multiplication the explants were cultured alternately on MS liquid medium supplemented with 1.5 mg/l BAP and 0.25 mg/l Kn and MS solid medium supplemented with 5 mg/l BAP and 0.25 mg/l NAA. Good multiplication (12 shoots/ culture) was observed in 90% of the cultures. For root induction, the in vitro raised shoots were divided into clumps of 4-5 shoots each and transferred on gelled half-strength MS medium supplemented with 3 mg/l NAA. The rooting percentage observed was 85%. The rooted plantlets were then transferred to trays containing cocopeat. After six weeks the primary hardened plantlets were transferred to bags containing garden soil and compost in the ratio 1:1 for 4 weeks. The hardened plantlets gave 100% survival on field plantation.

Key words: Less thorny, *Bambusa bambos*, *Katang*

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1. Introduction

Bamboos are the fast growing non woody plants having 700 genera and more than 1250 species. The bamboo plant belongs to the sub family *Bambusoideae* of family *Poaceae*. They are economically important due to their various applications like construction material, in furniture industry, food, fodder and for bio energy. The largest forest area under bamboos is in India with 9.57 million hectares of bamboo forests or 12.8 per cent of the total forest area. The major bamboos are *Dendrocalamus strictus* and *Bambusa bambos* with an overall annual production of 5 million tons (N. Krishnakumar, 2017).Maharashtra has three native species of bamboo namely *Dendrocalamus strictus (Manvel*), *Bambusa bambos* (*Katang*) and *Oxytenenthara stocksii* (*Manga*). The particular species *B. bambos*is very popular due to its extensive use in house construction, scaffolding, rafting etc. It is also known as 'Giant Thorny Bamboo' or 'Indian Thorny Bamboo'. It prefers humid tropical climate, moist soil and grows along perennial rivers and valleys as well as in other moist sites. It is also used as raw material for pulp and paper industry. The spiny branches are used for fencing. It has been widely used in Indian folk medicine. In Ayurveda, the entire plant is used as astringent, laxative, for inflammatory conditions and as diuretic (Joshi 2000). It can also be useful in agroforestry, reforestation, stabilizing of eroding river banks and as wind breaks. Being a giant bamboo species it can be used for biomass production and as renewable source of energy. It can be directly burnt for heat and/or power generation to replace carbon intensive fossil fuels (Salam & Dube 2009). The thorny nature of this species makes it difficult to harvest and manage on commercial scale. For this particular reason we have selected less thorny mother plants of this species and procured them from local bamboo nursery.

B. bambos species prefers sandy loam and fertile soil with tropical to sub-tropical climatic conditions. The species is distributed throughout India up to the altitude of 1200m.*B. bambos*plants attain a height of about 30 m and reach a diameter of about 15-18 cm. The internodal length is 30-45 cm with wall thickness of 1.5-2.5 cm. The species flowers gregariously and the clumps die after flowering. The flowering cycle is of 30 to 45 years. The seeds are light in weight (about 60000 – 75000/kg). The viability of the seeds is about six months. This bamboo is propagated through seeds and through vegetative propagation or tissue culture. The *B. bambos* plantation requires about 204 plants/ha (7x7). The annual yield is 5 to 36 t/ha. (Duriyaprapan & Jansen 1995)

Current methods of propagation of this commercially important species basically consist of culm cutting, rhizome division, offset cuttings, branch cuttings, air layering or seed

propagation (Prutpongse and Gavinlertvatana 1992). Rhizome cutting and culm cuttings are the most widely used methods of vegetative propagation. There are number of problems with this type of propagation. The offsets used are bulky and heavy. Clumps are not always available due to seasonal specificity, so that large scale vegetative propagation is impossible (Saxena and Bhojwani 1993).

To overcome these problems and make quality bamboo planting material easily available tissue culture or micropropagation method has been tried for many economically important bamboo species. In this paper we present the tissue culture protocol for the select less thorny variant of *B. bambos.*

2. Materials and methods:

2.1. Selection of location and mother plants

The mother plants having less thorny nature were procured from The Bamboo Nursery at Bhor, Pune- Maharashtra, India.

2.2. Procedure for collection of buds

Nodal segments with pre-existing axillary buds were collected from the field growing *B. bambos* plants. The optimal season for bud collection was arrived at after conducting initiation trials for over a period of one year from the same location. The branches containing pre-existing axillary buds were collected early in the morning. They were wiped with 70% ethanol and stored in polythene bags for transporting to the tissue culture laboratory.

2.3. Initiation treatment

In the laboratory the branches were cut into nodal segments containing a single node (2-3 inches). The leaf sheath and nodal ring were removed without damaging the axillary bud. These segments were washed by RO water to remove the dust particles. They were then rinsed with sterile distilled water containing few drops of liquid detergent - Tween 80 for 15 minutes followed by 3-4 washes with sterile distilled water. This was followed by fungicide treatment (Bavistin 2 %) for 40 minutes followed by 3 times washing with sterile distilled water. The next treatment of mercuric chloride (HgCl2, 0.1% for 5 minutes) was carried out under the laminar air flow. This was also followed by three to four washes of sterile distilled water. Then both the ends of the surface sterilized nodal segments were trimmed to prepare the final explant of about 2-3 cm.

2.4 Media trials for each stage

The explants were cultured singly in MS medium (Murashige and Skoog 1962) supplemented with various concentrations and combinations of the cytokinins benzyl amino purine (BAP) as 1, 1.5, and 2.0 mg per litre and kinetin (Kn) at 0.25 mg per litre, referred to as initiation media. The pH of all the media was adjusted to 5.8 with the help of pH meter by adding 0.1N NaOH or 0.1N HCl accordingly prior to autoclaving at 15 lbs. pressure and 121°C temperature for 15 mins. After four weeks, the clean cultures were transferred to MS medium supplemented with various concentrations and combinations of BAP (3, 5 and 7) and naphthalene acetic acid (NAA) at 0.25 mg per litre, referred to as multiplication medium. The clean cultures were transferred alternatively to liquid initiation medium and solid multiplication medium every three weeks for total eight cycles of multiplication. Aseptic conditions were maintained in the tissue culture laboratory throughout the whole experiment. Cultures were incubated at 24±2°C temperature under illumination of 2500 lux and 16/8 hours of photoperiod. For induction of rooting the elongated shoots were transferred to half-strength MS medium with various concentrations of auxins IBA (Indole butyric acid) and NAA. The rooting percentage was recorded after 30-45 days.

2.5. Hardening and field trial

The rooted plantlets were gently washed under running tap water to remove any media adhering to the roots. Immediately after that they were transferred to seedling trays containing the cocopeat. The humidity around the plantlets was maintained at 80% by covering the trays with polythene bags. The plantlets were drenched with19:19:19 and humic acid and were sprayed with fungicide Bavistin (0.2%) once in week. The polythene bags covering the plantlets were gradually perforated to expose the plantlets to the outer normal environment. After 27 days, the primary hardened, rooted plantlets were transferred to polybags (4”X6”) containing a mixture of soil: cocopeat in the ratio 1:1 for secondary hardening. They were maintained in poly-houses for six weeks. The surviving plantlets were then transferred to field conditions. The survival percentage in the field was recorded after six weeks of transfer.

3. Results and Discussion

3.1. Results

To determine the most appropriate season for bud collection of *B. bambos* plant for initiation, the initiation experiment was conducted five times in one year by collecting the buds at five

different seasons as described in the following table. Each initiation trial was conducted with a batch size of 100 buds per batch. The percentage of clean and sprouting buds was calculated after each trial. The optimal season for bud collection of *B. bambos* was identified as between the months of March to May as the initiation trials in these months yielded more than eighty percent clean and sprouting cultures (Table 1).

Table 1. Trials for determining best season for bud collection.

Season	% of clean cultures	% of sprouting cultures
1. June- August	65	65
2. September- November	50	72
3. December- January	90	0
4. February-March	80	92
5. April – June	86	92

About 86% clean and sprouting cultures were obtained in the initiation stage using the surface sterilization procedure described above. The initiation medium, MSB with BAP (1.5 mg/L) and Kn (0.25 mg/L) resulted into good sprouting of the axillary buds at the rate of 86% in the initiation stage. (Table 2) These were maintained in the same medium for four weeks to get good established sprouting cultures for further multiplication.

Table 2. Initiation media trials

Growth hormones/L	% of responding explant	No. of regenerated Shoots / culture	Average shoot length in cm.
BAP 1 + Kn 0.25	50	2 ± 1	4
BAP 1.5 + Kn 0.25	86	6 ± 1	6
BAP 2 + Kn 0.25	79	4 ± 1	4.5

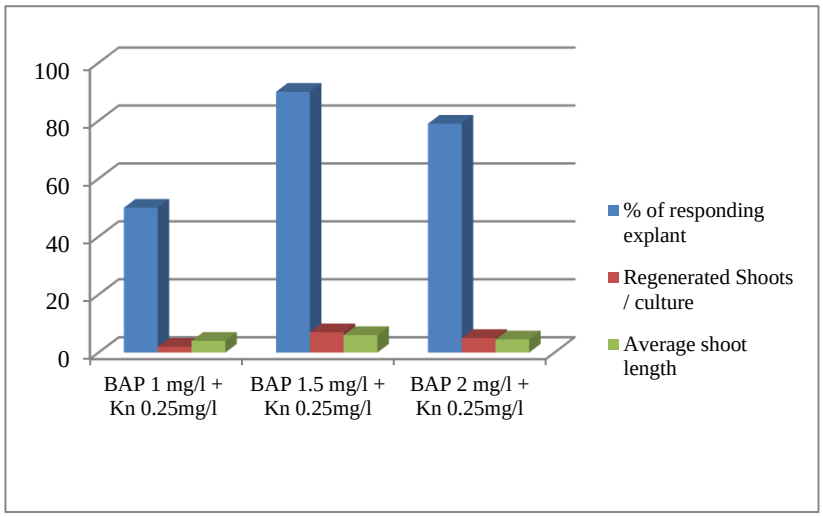

Fig.1. Media standardization for initiation

The well established, clean and sprouting explants were transferred to MS basal medium containing various combinations of hormones benzyl amino purine (BAP), kinetin (Kn) and naphthalene acetic acid (NAA) for getting further multiplication Among the various combinations tried we got better results as regards the percentage of multiplying buds and the number of shoots per bud with the media combination BAP (5 mg/L) and NAA (0.25 mg/L), referred to as multiplication medium (Table 3).

Continuous transfers on this multiplication medium had resulted in browning of cultures and in vitro flowering during our earlier work on this species (data not shown). For solving this issue we have experimented with alternate transfers on liquid initiation medium and semi-solid multiplication medium. This procedure resulted in good multiplication rate of 12 shoots/ culture in 90% of the cultures and also solved the problem of browning and flowering.

Table 3 Multiplication media trials

Auxin/Cytokinin(mg/l)	Alternate media	% of responding explant	Regenerated Shoots / culture	Average shoot length
BAP 3 + NAA 0.25	_	50	4	5.5
BAP 5 + NAA 0.25	BAP 1.5 + Kn 0.25 (liquid)	92	12	9.3
BAP 7 + NAA 0.25	_	72	10	7

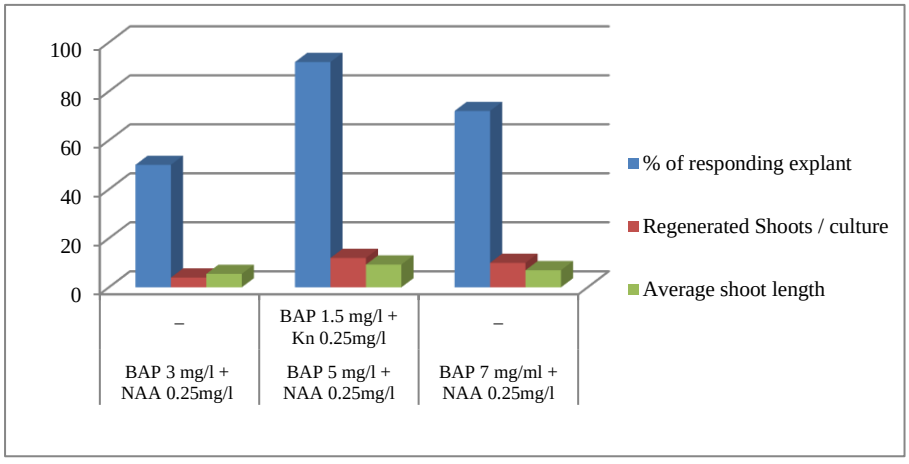

Fig. 2. Media standardization for Multiplication

In the trials conducted for inducing rooting the medium having combination of half strength MS basal medium supplemented with NAA (3 mg/L) resulted in good rooting in 85% of the cultures. (Table 4)

Table 4. Rooting media trials

Auxins in mg/L	% of cultures showing rooting	Number of roots / culture	Average Root length
IBA3	20	1 ± 1	1.75
NAA 3	87	7 ± 1	10.5
IBA 3 +NAA 3	66	3 ± 1	3.2

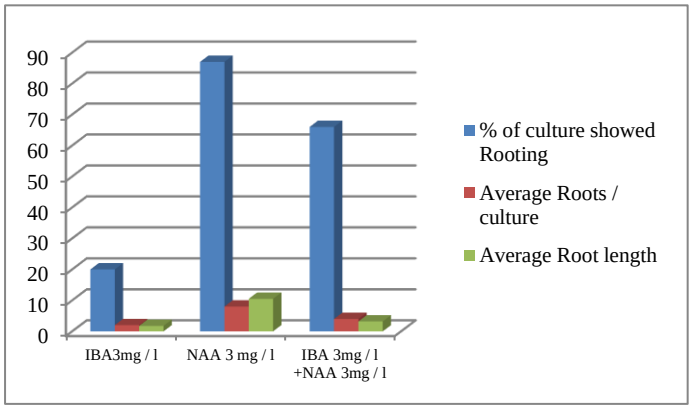

Fig. 3. Media standardization for Rooting

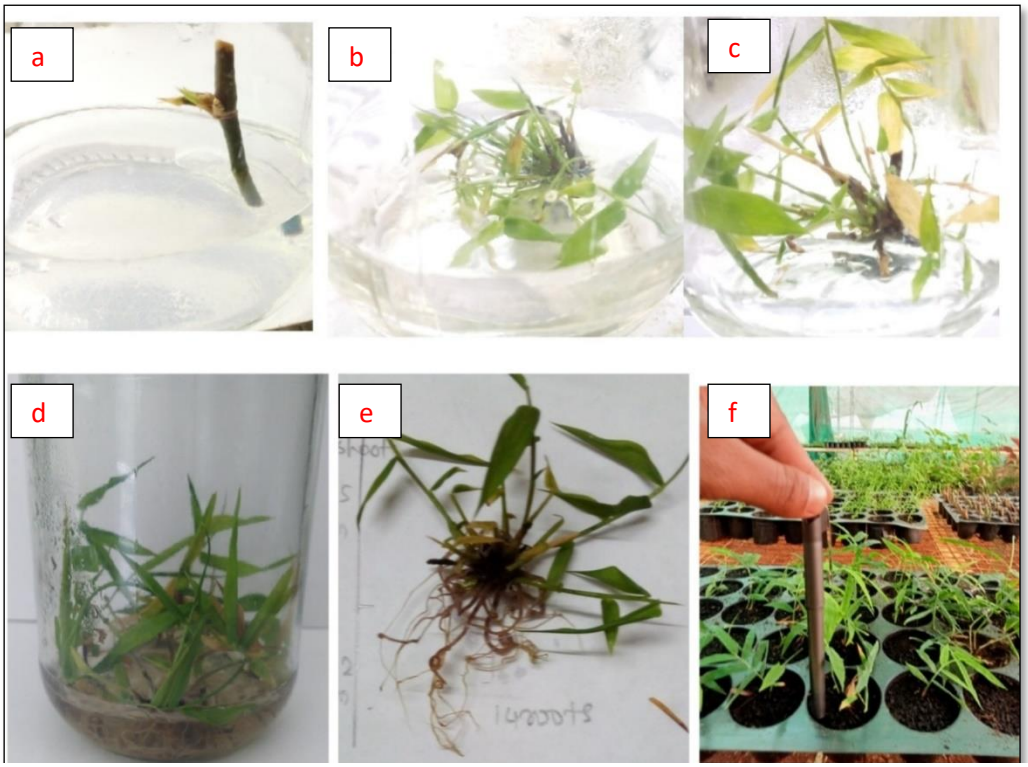

Fig. 4. a. *Bambusa bambos* Initiated bud, **b. &c**. – Multiplication stage**d**. – Rooting stage, **e**. - Harvested sapling, **f.**-Hardened Plants

The hardening procedure developed in this experiment has resulted in 100% survival of the tissue cultured plantlets in both primary and secondary hardening stages.

3.2. Discussion

In the process of establishing successful micropropagation protocol for any plant species obtaining aseptic cultures and getting bud break are the initial important events. This depends upon amount of contaminations and physiological status of the collected explants during various seasons of the year (Ramanayake and Yakandawala 1997). In our experiment axillary buds collected during rainy season resulted into heavy contamination percentage in the initiation stage. This is in contrast to (Mishra et al. 2008) who recorded maximum clean cultures from buds collected in rainy season for *B. tulda*. Mercuric chloride at 0.1% concentration for five minutes was effective in giving us 86% clean and sprouting explants in the imitation stage. This is in accordance with the findings of several researchers (Saxena and Bhojwani 1991; Arya et al. 2001; Mishra et al. 2001) who routinely employ 0.1% mercuric chloride as an effective sterilizing treatment for different bamboo species. The initiation medium, MSB with BAP (1.5 mg/L) and Kn (0.25 mg/L) resulted into good sprouting of the axillary buds at the rate of 86% in the initiation stage. Various reports of use of MS basal medium for micropropagation of *B. bambos* are available from earlier researchers (Arya and Sharma 1998; Nayak et al. 2010; Anand et al. 2013; Brar 2014). BAP has been shown to be effective for inducing shoot proliferation in several bamboos viz. *B. arundinacea*, *Bambusa vulgaris*, *Dendrocalamus. strictus* (Nadgir et al. 1984), and in *B. nutans* and *D. membranaceus (*Yasodha et al. 1997). Similarly, Kn is also reported to be used for induction of various responses in micropropagation of bamboos. For example (Rout & Das 1994) in *D. giganteus* for somatic embryogenesis, (Ravikumar et al. 1998) in *D. strictus* for plant regeneration, (Rout & Das 1994) in *D. strictus* for in vitro flowering. The synergistic effect of the two cytokinins BA and Kn was reported best for shoot multiplication in *D. giganteus* (Arya et al. 2006) and *B. glaucescens* (Shirin and Rana 2007).

The media combination BAP (5 mg/L) and NAA (0.25 mg/L) resulted in high percentage of multiplying buds and the higher number of shoots per bud. NAA has been effectively used in micropropagation of various bamboo species. Our results of stimulation of multiplication response with low concentration of NAA are in accordance with reports of earlier research on bamboo species such as (Arya and Sharma 1998) for *B. bambos* and (Islam and Rahman 2005) in *B. nutans*. The experiment of alternating the transfer cycles on liquid initiation medium and semi-solid multiplication medium resulted in good multiplication rate of 12 shoots/ culture in 90% of the cultures. This also solved the problem of browning and in vitro flowering. Several workers have reported higher rates of shoot multiplication and improved growth in liquid medium in comparison to semi-solid medium (Saxena and Bhojwani 1993;

Sood et al. 2002; Das and Pal 2005; Arya et al. 2006; Shirin and Rana 2007; Ogita et al. 2008). The slower growth or poor shoot multiplication on semi-solid medium vis-a-vis liquid medium may be attributed to the fact that solubilized agar binds water, absorbs nutrients and PGRs resulting in reduced uptake of nutrients, PGRs and other essential constituents by cultured tissues (Singh et al. 2013).

Inducing rooting in the elongated shoots obtained after the specific number of multiplication cycles is the most important step in micropropagation of any plant species. There are reports of induction of rooting without growth regulators (Shirgurkar et al. 1996; Watanable et al. 2000) but most tissue culture protocols require auxins alone or in combination with cytokinins for induction of rooting. The role of auxins in root development is well established and has been reviewed by (Scott 1972) and (Torrey 1976). The rooting medium namely half strength MS basal with NAA (3mg/L) resulted in good rooting response of 85% of rooted shoots. There are reports of getting rooting response in *Bambusa* species by using NAA alone or in combination with IBA. For example (Arya and Sharma 1998) in *Bambusa bambos* have used NAA alone while (Rathore et al. 2009) have used it in combination with IBA for *Bambusa bambos.* Our results are in agreement with these reports. Acclimatization that is the process of making the tissue culture raised plantlets to survive in the conditions outside the laboratory is a very important and crucial step in establishing the micropropagation protocol. The plantlets are accustomed to low light, high humidity and low temperature conditions which are in contrast to the outside conditions. This change has to be gradual and monitored in the hardening process. Our procedure of primary and secondary hardening has resulted in 100 % survival of the tissue culture raised plantlets. Other workers have experimented with various media combinations for primary and secondary hardening. For example (Mishra et al. 2011; Singh et al. 2011) tried different potting mixtures like soil, sand, soilrite, perlite, vermiculite, compost or farm yard manure either alone or in various ratios. Addition of vermi-compost to the sand was found to improve the survival of plants probably due to increased porosity of sand and better aeration of roots (Singh et al. 2011). Our results are consistent with these reports and the variations may be species specific.

Conclusion

Thus we conclude that the process described in this experiment will prove to be cost effective in producing large quantity of quality planting material of *Bambusa bambos*. When

commercialized this protocol will surely be helpful in providing farmers with quality planting material of this locally popular and economically important bamboo species.

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Conflict of Interest

It is hereby confirmed that the manuscript has been read and approved by all the named authors and there is no conflict of interest. All regulations of our institute including intellectual property rights have been followed and there are no impediments to publication.

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