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# Effect of nutrient media and phytohormones on *in vitro* establishment of *Bambusa balcooa. Roxb.*

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**ABSTRACT**

*Bambusa balcooa.Roxb.* is one of the most commercially important bamboo species. Some varieties of bamboo are being grown in the company's green house. However this species is very rare and it was not cultured until now due to lack of demand and maintenance problems. In this work *B. balcooa* ex-plants have been established and propagated by the axillary shoot bud proliferation method. Earlier several works have been done on this plant and a protocol has been. This work emphasizes on effects of phytohormones at different concentrations and combinations upon the novel propagules that develop from the bamboo explants on placing them in multiplication media. The propagules placed in media containing cytokinins BAP (2 mg/L) and Kinetin (1 mg/L) exhibited best results of linear as well as radial growth. The other propagules in media with combinations of BAP, Kinetin and NAA were found to be dried out and did not depict noticeable growth. Comparatively next best growth pattern to the former was observed in control media that lacked phytohormones. This experiment was helpful in estimating the quantity and effect of particular plant growth regulating hormones on the *Bambusa balcooa.Roxb.*

***Keywords*:** BAP; Kinetin; NAA; propagules

## 1. INTRODUCTION

From time immemorial there have been natural selections as well as measures taken by cultivators for improving and culturing plants. About 250 years ago Henri-Louis Duhamel du monceau demonstrated callus formation on the decorticated regions of the elm plants. Plant tissue culture or the aseptic culture of tissues and their components under defined conditions in vitro, is an important tool in commercial application manifesting into environmental as well as individual benefits.

Later a German botanist Gottlieb Haberlandt, who is considered as the father of plant tissue culture, first developed the concept of *in-vitro* explants culture. The early studies led to root cultures, embryo cultures, and the first true callus/tissue cultures. The period between the 1940s and the 1960s was marked by the development of new techniques and the improvement of those that were already in use. The availability of these techniques led to the application of tissue culture to areas of cell behavior including cytology, nutrition,

metabolism, morphogenesis, embryogenesis and pathology. In 1964 Maheshwari and Guha were first to develop anther and pollen culture for production of haploids.

Plant Tissue Culture is a subject that deals with culturing and multiplying plant parts. It is well known that, unlike animal cells, plant cells retain their totipotency even after differentiation. This is a rapidly growing field that emphasizes on not only producing pathogen free plants but also large number of plantlets in a short time. Further improvements in this field have led to production of several beneficial phytocompounds. The property of stem and root, comprising of meristems, typically to proliferate by indefinite growth is being exploited for the purpose of developing clones of a desired species. Plant Tissue Culture also allows us to perform genetic modifications to avail distinct characteristic traits in the offspring generation.

The potential of micropropagation for mass scale production of plants has raised hopes of culturing and maintaining an appreciable bulk of plants that take generally several years to perpetuate. As bamboo is a prime renewable resource for biomass production, a lot of research has been focused on development of standard protocol for obtaining healthy plantlets *en masse.* The issue of global climatic variations and the appraisal about improving sustainable development has simulated efficient protocol for *in-vitro* culture and field cultivation of *Bambusa balcooa.Roxb.* It is generally found in agency tracts in central delta region of East Godavari basin drained by the Godavari and the Pampa riverlets of India.

The shoots of certain species of bamboo contain a toxin taxiphyllin (a cyanogenic glycoside) that produces cyanide in the gut on consumption but some of the primates ingest this bamboo to concentration of several fold that can kill a human. Soft bamboo shoots, stems, and leaves are the major food source of the giant pandas in china and the red pandas in Nepal. Bamboo can be used in desalination of sea water. A bamboo filter is used to remove the salt from seawater due to its special property Larvae of the bamboo borer (the moth *Omphisa fusidentalis)* of Laos, Myanmar feeds off the pulp of live bamboo. In turn, these caterpillars are considered a local delicacy.

## 2. PROTOCOL FOR MICROPROPAGATION

### Preparation of media

Glassware with stoppers was prepared and placed on preparation table. Fresh stocks were prepared from the required chemicals as described in the table below:

**Table 1.** MS media composition.

| Chemical | Concentration of stock (g/L) | Required concentration (mg/L) |
|---|---|---|
| Ammonium Nitrate | 33 | 1650 |
| Calcium Chloride | 8.8 | 440 |
| Magnesium Sulfate | 7.4 | 370 |
| Potassium Nitrate | 38 | 1900 |
| Potassium dihydrogen Phosphate | 3.4 | 170 |
| Boric Acid | 1.24 | 6.2 |

| | | |
|---|---|---|
| Cobalt Chloride | 0.005 | 0.025 |
| Copper Sulfate | 0.005 | 0.025 |
| Manganese Sulfate | 3.38 | 22.3 |
| Potassium Iodide | 0.166 | 0.83 |
| Sodium Molybdate | 0.05 | 0.25 |
| Zinc Sulfate | 1.72 | 8.6 |
| Ethylene diamine tetra aceticacid(EDTA) | 7.46 | 37.35 |
| Ferrous Sulfate | 5.57 | 27.85 |
| Myoinositol | 20 | 100 |
| Nicotinic acid | 0.1 | 0.5 |
| Pyridoxine Hcl | 0.1 | 0.5 |
| Thiamine Hcl | 0.02 | 0.1 |
| Glycine | 0.4 | 2 |
| Sucrose | - | 3000 |
| Agar | - | 8000 |

Using the above table L of MS agar solid medium was prepared. Once the stocks were ready, sufficient amount of water that was kept for boiling was mixed with agar. Finally the stock mixture was added to the boiling solution in the steel container. Consequently pH of the solution was adjusted to 5.8 using conc. HCl and 1 M NaOH accordingly. The media was divided into 5 (1 L) partitions and phytohormones were added as described below:

**Table 2.** Phytohormones concentrations in MS media.

| Serial | BAP Mg/L | Kinetin Mg/L | 2iP Mg/L | NAA Mg/L |
|---|---|---|---|---|
| 1 | 2 | - | - | 1 |
| 2 | 2 | 1 | - | - |
| 3 | 2 | 1 | - | 1 |
| 4 | 2 | 1 | 1 | - |
| C | - | - | - | - |

*C = Control

Phytohormones stocks were prepared by dissolving 10 mg of each in few drops of 1M NaOH and then making upto10 ml. Finally the required amount was added to MS media. Media after preparation should be checked for clarity. Media had been poured into the set

glass bottles and capped. Each bottle occupies about 40 ml of media that accounts to $1/6^{th}$ of total volume. The head volume results to be 5 times of the media volume. These bottles were arranged and transferred into autoclave. Proportion of media volume to the head volume is supposed to be 1:5.

**Sterilization of media**

Media had been sterilized at 121 °C T and 15 lb P for 20 min along with other requirements like cotton, paper, instruments etc. The Ph of medium drops by 0.03-0.05 units after autoclaving. The autoclaved media was stored along with other media and requirements in the *storage room* with proper labeling.

**Preparation of explants (Trial -1)**

The explants were washed with sterile water, soaked in fungicide solution for about an hour and a half. The composition of fungicide solution is: Bavistin 2.5 g, Streptomycin 300 mg, Tetracycline 300 mg and 2-3 drops of Tween 20. Then the outer layers were removed and the required shoot pieces for initiation were excised using a scalpel and forceps.

**Sterilization of work area**

Rest of the sterilization was done before initiation in the LAF. The *room* is disinfected by vacuuming and fumigation. The work bench of LAF was wiped with absolute alcohol. The instruments, cotton, paper, burners, media bottles and sterilizing agents were also wiped and placed in the chamber. UV was turned on for 15 min. When the work bench was ready to use the pretreated ex-plants were placed inside. Proper cleansing of hands, feet and face is advised before entering into the inoculation room with sterile dress.

**Inoculation of explants (Trial -1)**

During the course of transfer of the explants to culture bottles, the instruments that were in use were dipped in 70 % alcohol and kept in the glass bead sterilizer maintained at 200 °C for 15-20 sec. Explants were taken in sterile water first and rinsed. Then they were washed with 70 % alcohol for 30 sec, sequentially with sterile water again. After rinsing in 240 mg $HgCl_2$ for 8 min they were washed in A+C water serially for 5,3,1 mins. Then explants were rinsed with 120 mg $HgCl_2$ containing Tween 20.

Subsequently explants were washed in sterile water thrice until the foam disappeared. The explants were removed of the thin hairy outer layer and further trimmed pieces were transferred into *PF0.1 initiation media* after proper drying in order to prevent contamination. The bottles were clean wrapped and placed in *growth room*.

The conditions that were maintained at *growth room* were 25-29 °C T and 16/8 light-dark cycle. The moisture trapped in the bottles was sufficient for the humidity count. *Growth room* is fitted with mobile incubation chambers that have 40 W white florescent tube lights providing about 1600 flux of light intensity. The total capacity of a single growth room is to accommodate 1Lac bottles simultaneously.

**Re-sterilization of contaminated explants (Trial-1)**

Explants that suffered contamination were identified and taken for resterilization. These bottles were given out from the pass door in the *disposal room.* First step was to remove the infected part of the explant and wash it properly in sterile distilled water. Then they were soaked in fungicide solution for 30 min and washed with sterile water twice. In the LAF, after rinsing in 120mg $HgCl_2$ solution for 4 min they were washed with A+C water serially for 5,3,1 mins. Finally they were washed in sterile water, dried and re-inoculated into *PF0.1 initiation media.* These were incubated back in *growth room*.

**Inoculation of explants (Trial -2)**

Process for sterilization and inoculation of the explants the second time was as per the same protocol as mentioned sequentially above. The trial 2 explants needed only to be trimmed of leaves and were quite easy to handle. The explants that were placed in growth room were examined every 5 days and the conditions were maintained as prior.

**Re-sterilization of contaminated explants (Trial -2)**

Explants that were found to be contaminated by fungus were re-sterilized by the earlier described process. This process was carried out twice for culture bottles of the same batch. In the first resterilization rinsing with 120 mg $HgCl_2$ was done followed by washing with A+C water. When second time this process is repeated then only 120 mg $HgCl_2$ treatment was done as the plants cannot withstand intense chemical treatment repeatedly.

**Multiplication of plantlets**

Simultaneously few of the resterilized explants were placed in *AI2.5 multiplicative media,* under sterile conditions to achieve contamination-free propagules from these explants. The procedure for inoculation was followed as earlier described. These culm pieces were expected to give reliable shoots for further comparative analysis of plants at different concentrations of phytohormones.

**Inoculation in media with different phytohormones**

The well grown propagules that developed from the explants placed on *multiplication media* were selectively graded and inoculated onto labeled MS media with various compositions of cytokinins and auxin. The bottles were checked for cap contamination before unsealing them in the chamber. Transferring of propagules to media was done under sterile conditions as described above in the initiation protocol.

## 3. RESULTS

**Explants (Trial-1)**

| | |
|---|---|
| Day 5 | No response |
| Day 20 | Contamination |

Day-5

Day-20

**Resterilized explants (Trial-1)**

| | |
|---|---|
| Day 30 | Contamination |
| Day 32 | Explants discarded |

**Explants (Trial-2)**

Day-5

Day-10

Day-15

Day-20

Day-25

| Day 5 | Response |
|---|---|
| Day 10 | Tiny propagules |
| Day 15 | Propagules growing |
| Day 20 | Well grown propagules |
| Day 25 | Developed plantlets |

**Resterilized explants (Trial-2)**

| Day 16 | Propagules grow |
|---|---|
| Day 20 | Contamination |

Day-16

Day-20

**Sub cultured healthy explants (Trial-2)**

| Day 20 | propagules arise |
|---|---|
| Day 30 | Well developed shoots |

**Explants (Trials-2) in multiplication media**

| Day 20 | Multiple propagules developed |
|---|---|
| Day 35 | Propagules grow |

**Propagules of explants (Trial-2) in media with different compositions of phytohormones**

| | |
|---|---|
| Day 50 | Propagules show varied responses in various media |

Propagules in MS Media 1

Propagules in MS Media 2

Propagules in MS Media 3

Propagules in MS Media 4

Propagules in MS Control Media

## 4. DISCUSSION

Regeneration of *Bambusa balcooa.Roxb.* species by sexual means is impossible because no seeds are set after flowering. Vegetative propagation is hindered due to bulky propagules, low rooting ability of the culm and branch cuttings, and seasonal specificity. This makes *in vitro*-based methods of regeneration important. This project works on efficient micropropagation protocol for multiplication of *Bambusa balcooa.Roxb.* from nodal explants. Initiation of the well sterilized explants (trial 1) was carried out in PF 0.1 media. There was no response for weeks moreover the bottles suffered stubborn contamination. After a resterilization these were placed back into medium but there was no result. Hence they were discarded. The next explants (trial 2) were excised of leaves and nodal segments were collected. Once the shoots showed response in 10 days of inoculation, the explants were placed in *AI 2.5 multiplication media.* Although some of the ex-plants were discarded in the middle due to contamination, sufficient number of propagules was obtained for the analysis. These showed positive response by producing novel shoots from axillary bud. The propagules that developed from these ex-plants were placed onto MS media with different compositions and combinations of phytohormones.

In MS media 2, containing BAP 2 mg/L and Kinetin 1 mg/L propagules showed efficient linear and radial growth within a time period of 15 days. As the ratio of contaminated to healthy plants is 1:2, best of the three inoculated was considered. According

to previous reports on this experiment, the best concentration ratio of BAP: Kinetin for development of propagules is 3:1. At all other concentrations it was found that they exhibited negligible growth. In this project the next best result was provided by control media astonishingly. In media 3, one of the three showed drying while in case of media 1, two of them were drying. Very poor results were associated with media 1 containing only BAP 2 mg/L.

The drying out and absence of growth in the propagules can be attributed to the presence of auxin in media 1 and 3 but the exact reason for drying of the propagules in media 4 containing BAP 2 mg/L, Kinetin 1 mg/L and 2i P 1 mg/L could not be deciphered. The comparative analysis of growth pattern in propagules of various media is given below:

Graph: line graph representation of variations of propagule height

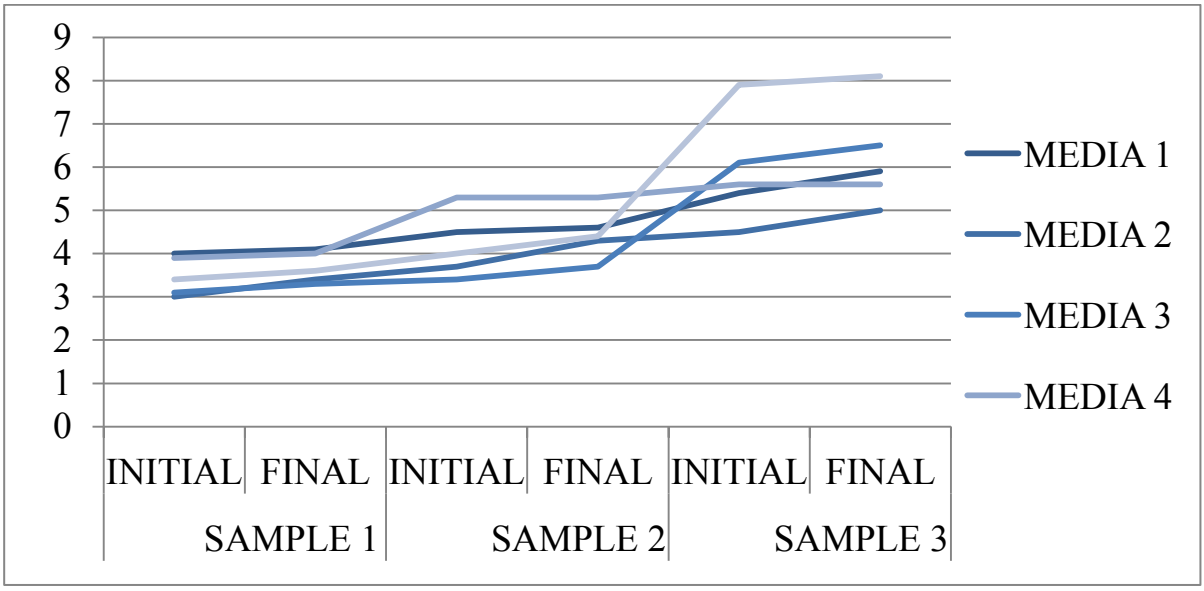
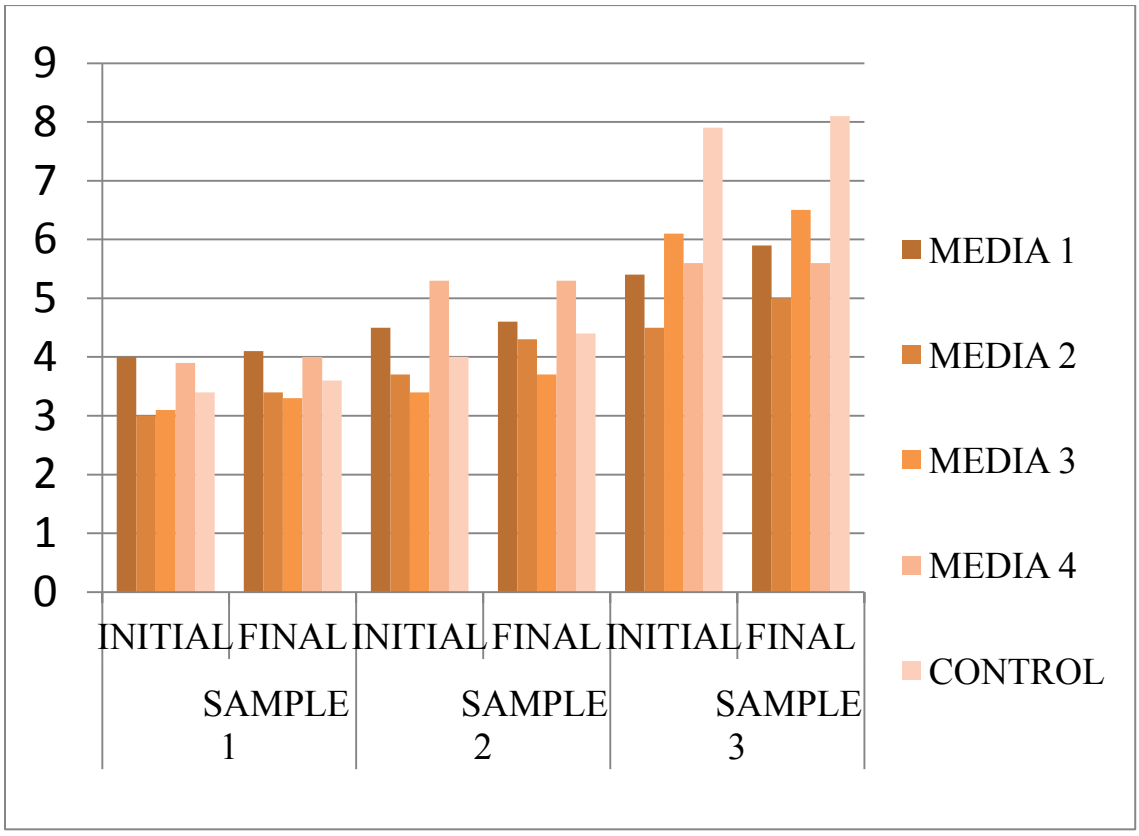



**Table 3.** Prominent phenotypic expressions of the Propagules placed on phytohormone containing media.

| Media | Sample1 | Sample2 | Sample3 |
|---|---|---|---|
| Media 1 | Dried<br>No growth | Dried<br>No growth | Green<br>Linear growth |
| Media 2 | Green<br>Radial growth | Green<br>Radial growth | Green<br>Linear growth |
| Media3 | Dried<br>No growth | Dried<br>No growth | Green<br>Radial growth |
| Media4 | Dried<br>No growth | Dried<br>No growth | Dried<br>No growth |
| Control | Green<br>Linear growth | Green<br>Linear growth | Green<br>Linear growth |




## 5. CONCLUSION

Bambusa balcoa is an ethno botanical plant. As this species is an economically important plant, there is much research going on this plant to make it available at low cost to people. Using axillary shoot propagation method this plant can be grown 5 fold faster and also produce durable culms for consequent utilization.

In this work the effect of phytohormones was studied on the explants and this culture has been established at the laboratory. The well grown propagules did not show successful results but experiment concluded with best growth pattern observed in media containing cytokinins BAP and Kinetin. The leftover cultures of this species would be sub cultured and the developed propagules would be shifted to rooting media.

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