Chapter III

Materials and Methods
3.0 Materials and methods

The present study “Factors affecting somatic embryogenesis and plant regeneration in Dendrocalamus asper and Dendrocalamus tulda (Edible bamboo) sp.” was carried out at Department of Biotechnology, Sant Gadge Baba Amravati University, Amravati. The original species of D. asper and D. tulda were procured from Central Nursery, Maharashtra Forest Department, Wadali, Amravati. These plants were maintained in greenhouse and used as source of explants. The mother source plants D. asper and D. tulda were also used as a source of explants from Wadali nursery.

3.1. Requirements

3.1.1 Chemicals

The details of different chemicals used and collected from different agencies to perform experiments are as under.

Salts of macro and microelements of analytical grade were obtained from Loba chemie, Himedia, Sigma, Qualigens, Sdfine, Merck. Etc.

Vitamins, Amino acids, Sucrose were obtained from Hi media Laboratories Pvt. Ltd., Bombay.

Myo inositol, Thiamine HCl, Plant growth regulators (Auxin, e.g. 2,4-D, NAA, IBA, IAA etc. and cytokinin e.g. Kinetin, BAP) of cell culture tested grade were purchased from M/S Sigma Chemicals, Saint Louis, Missouri 63178 USA. and Hi media Laboratories Pvt. Ltd., Bombay.

Phytagel was purchased from (Hi media).

Bavistin (Carbandezim: A systemic Fungicide) were obtained from M/s BASF India Limited, Bombay.
Streptomycium Sulphate (an Antibiotic) was obtained from M/S Hindusthan Pharmaceuticals, Pune.
Teepol (Detergent liquid soap- Laboline) was procured from M/S BDH Glaxo chemicals Limited, Bombay.
HgCl\textsubscript{2}, NaOCl, an explant sterilant were used from Hi media.

3.1.2 Glass wares
All the glasswares used for the experiment during the work were ‘Borosilicate Laboratory grade’ and were procured from M/s. Borosil India Limited, Bombay.

3.2 Washing of Glass wares
Glasswares were soaked in 1:10 Laboline (detergent) solution in distilled water for two hours followed by rinsing with tap water. Then they were soaked in 1N HNO\textsubscript{3} for overnight. On the next day the glasswares were washed 2-3 times with tap water and followed by double distilled water. After washing they were dried in oven at 110\degree C. Finally the glasswares were stored in dust proof cupboards until further use.

3.3 Sterilization of glass wares
Test tubes and conical flasks were plugged with non-absorbent cotton wrapped in muslin cloth. Prior to use all glasswares were autoclaved at 1.06 kg/Sq.cm pressure at approximately 121\degree C temperature for one hour. Autoclaved glasswares were then dried in oven at 80-100\degree C for two hours. Forceps, scalpels, blades and scissors were first autoclaved and then during inoculation sterilized by dipping in absolute alcohol and holding on flame alternately.
3.4 Selection of culture media

The basal media developed by Murashige and Skoog's (1962) was standardized using different concentrations and combinations of growth hormones for in vitro callus induction and regeneration from tissues of *D. asper* and *D. tulda*.

3.5 Adjuvant to the basal medium

The MS medium was supplemented with following adjuvant for initiation of growth, establishment of cultures, maintenance and multiplication of cultures and morphogenesis of the explant.

- 2,4-Dichlorophenoxy acetic acid (Auxin)
- Naphthalene acetic acid (Auxin)
- Indol butyric acid (Auxin)
- 3-Indol acetic acid (Auxin)
- 6- Benzyl amino purine (Cytokinin)
- Kinetin (Cytokinin)
- Sucrose (Carbon source)
- Thiamine HCl (Vitamin)
- Myoinositol (Vitamin)
- Ascorbic Acid (Antioxidant)

3.6 Composition of Media

The composition of MS basal medium used for the present study is given in Table 1. Concentrated solution (stock solutions) containing different ingredients were prepared in sterile double distilled water and stored in reagent bottles at 4°C not more than 15 days after preparation.
Separate stock solutions were prepared for each growth regulators by dissolving it in a minimal quantity of the appropriate solvents. The stock solutions of auxin (1mg/ml) were prepared by adding a few drops of 0.1N NaOH (3ml for every 10 mg of auxin) to a weighed quantity of auxin and then making up the required volume with sterile double glass distilled water. The stock solutions of cytokinin were prepared by adding few drops of 0.1 N HCl (3ml for every 10 mg of cytokinin) to a weighed quantity of cytokinin and then making up the required volume with sterile double glass distilled water.

3.7 Preparation of culture media
A more convenient and popular method, were to prepare a series of stock solutions. To prepare Murashige and Skoog’s basal medium, four different stock solutions were prepared.

a) Major salts (20X concentrated)
b) Minor salts (200X concentrated);
c) Iron (200X concentrated);
d) Organic nutrients except sucrose (200X concentrated).

For the preparation of stock solutions (a)-(d) each component were separately dissolved to the last particle and then mixed with the others.

The sequence of steps involved in preparing a medium is as follows:

1) Required quantities of Phytagel and sucrose were weighed and dissolved in double distilled water, about ¾ volume of the medium, by heating them in water bath.
2) Appropriate quantities of the various stock solutions, including growth regulators and other special supplements were added.

3) The final volume of the medium was made up with double glass distilled water.

4) After mixing well, the pH of the medium was adjusted using 0.1 N NaOH and/or 0.1 N HCl.

5) Then the medium was poured into the desired cultured vessel. 15 ml of the medium was dispensed in a 25 X 150 mm culture tube, and about 50 ml in 150 ml flask.

6) Tubes and conical flasks were plugged using cotton plugs wrapped with muslin cloth and finally tubes were wrapped with aluminum foils and autoclaved at 121°C temperature and 1.06 Kg/Sq.cm pressure for 30 min. After the steam was completely released, tubes and conical flasks were removed.

7) The test tube slants were prepared. The slant tubes and flasks were stored in dust free area until further use.
Table 1: Stock solution for MS basal medium

<table>
<thead>
<tr>
<th>Stock solution I</th>
<th>Constituents</th>
<th>Amount (Mg/l)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>MgSO$_4$.7H$_2$O</td>
<td>7400</td>
</tr>
<tr>
<td></td>
<td>KH$_2$PO$_4$</td>
<td>3400</td>
</tr>
<tr>
<td></td>
<td>KNO$_3$</td>
<td>38000</td>
</tr>
<tr>
<td></td>
<td>NH$_4$NO$_3$</td>
<td>33000</td>
</tr>
<tr>
<td></td>
<td>CaCl$_2$.2H$_2$O</td>
<td>8800</td>
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</table>

<table>
<thead>
<tr>
<th>Stock solution II</th>
<th>Constituents</th>
<th>Amount (Mg/l)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>H$_3$BO$_3$</td>
<td>1240</td>
</tr>
<tr>
<td></td>
<td>MnSO$_4$.4H$_2$O</td>
<td>4460</td>
</tr>
<tr>
<td></td>
<td>ZnSO$_4$.7H$_2$O</td>
<td>1720</td>
</tr>
<tr>
<td></td>
<td>Na$_2$MoO$_4$.2H$_2$O</td>
<td>50</td>
</tr>
<tr>
<td></td>
<td>CuSO$_4$.5H$_2$O</td>
<td>5</td>
</tr>
<tr>
<td></td>
<td>CoCl$_2$.6H$_2$O</td>
<td>5</td>
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</table>

<table>
<thead>
<tr>
<th>Stock solution III</th>
<th>Constituents</th>
<th>Amount (Mg/l)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>FeSO$_4$.7H$_2$O</td>
<td>5560</td>
</tr>
<tr>
<td></td>
<td>Na$_2$EDTA.2H$_2$O</td>
<td>7460</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Stock solution IV</th>
<th>Constituents</th>
<th>Amount (Mg/l)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Inositol</td>
<td>20000</td>
</tr>
<tr>
<td></td>
<td>Thiamine HCl</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td>Pyridoxine HCl</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td>Nicotinic Acid</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td>Glycine</td>
<td>400</td>
</tr>
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</table>

For preparation of stock solution III, Dissolved FeSO$_4$.7H$_2$O and Na$_2$EDTA.2H$_2$O separately in 450 ml distilled water by heating and constant stirring. Mixed the two solutions, adjusted the pH to 5.5 and added double distilled water to make up the final volume to one liter and stored in amber color bottle. To prepare one liter of media 50 ml of stock solution I, 5 ml each of stock solution II, III, IV were taken.
Table 2: Disinfectants used for sterilizing plant material.

<table>
<thead>
<tr>
<th>Sr.No.</th>
<th>Disinfectants</th>
<th>Concentration (%)</th>
<th>Duration of treatment (Min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Benzalkonium Chloride</td>
<td>0.01-0.1</td>
<td>5-20.</td>
</tr>
<tr>
<td>2</td>
<td>Bromine water</td>
<td>1-2</td>
<td>2-20</td>
</tr>
<tr>
<td>3</td>
<td>Calcium hypochloride</td>
<td>9-10</td>
<td>5-30</td>
</tr>
<tr>
<td>4</td>
<td>Ethyl alcohol</td>
<td>75-95</td>
<td>30 sec</td>
</tr>
<tr>
<td>5</td>
<td>Hydrogen peroxide</td>
<td>3-12</td>
<td>5-15</td>
</tr>
<tr>
<td>6</td>
<td>Mercuric chloride</td>
<td>0.1-1.0</td>
<td>2-10</td>
</tr>
<tr>
<td>7</td>
<td>Silver nitrate</td>
<td>1</td>
<td>5-30</td>
</tr>
<tr>
<td>8</td>
<td>Sodium hypochloride</td>
<td>0.5-5</td>
<td>5-30</td>
</tr>
</tbody>
</table>
3.8 Transfer area for aseptic manipulation

All the aseptic manipulations like explant preparation, surface sterilization, inoculation as well as their subculturing operations were carried out in the laminar airflow hood. The working surface was cleaned using 0.5 % Dettol solution (V/V) and finally wiped with 90% alcohol. The door of the laminar flow bench was closed and the working area was exposed to ultra violet light for 30 minutes before each experiments.

The surgical instruments were cleaned and wrapped in brown paper /aluminum foil and autoclaved. Filter paper of required sizes were cut and kept in the petriplates and wrapped in brown paper. All these materials were autoclaved following usual procedure and stored in dust free area. During the course of explant preparation, surface sterilization, inoculation and other aseptic manipulation, all the surgical instruments viz. forceps, scalpel, needle and blade were deeped into the 90% alcohol and flamed to sterilize before every operation. The standard sterile techniques were followed as suggested by Street (1977) for inoculation and subculturing of explants.

3.9 Selection, isolation and preparation of explants

*Dendrocalamus asper* and *Dendrocalamus tulida* plants were regularly irrigated and maintained in the green house. The plant tissues such as leaf bid, shoots tip, node segments and root segments were selected from healthy plants. The isolated plant tissues were washed with tap water and then double glass distilled water. The explants were cut in proper
size in the petriplate and were washed with double glass distilled water. These explants were ready for surface sterilization.

3.10 Sterilization of Explant
The surface sterilization of explants was performed on laminar airflow. The leaf, shoot tip, node bud segments and roots were washed three times with tap water and then with double glass distilled water. Then the explants were ready for surface sterilization. The different concentrations with different incubation period of HgCl\textsubscript{2} were chosen for the surface sterilization of leaf bid, shoot tip, node bud segments and root segments.

A. Surface sterilization of Leaf bid, shoot tip, node bud segments and root segments.
The different concentrations of mercuric chloride were used for the surface sterilization of explants, leaf bid, shoot tip, node bud segments and root segments. The leaf bid, shoot tip, node bud segments and root segments were suspended in 0.1 to 1.0 % HgCl\textsubscript{2} (W/V) solution for 1 min to 10 min time duration. These treated explants washed thrice in beaker containing sterile double distilled water. After washing, these explants were blotted by sterile blotting paper. Then explants inoculated aseptically on MS medium supplemented with various concentrations of auxin and cytokinin. The observations were recorded after 3-4 days, of inoculation in terms of their survival and occurrence of contamination.

3.11 Incubation room
All the inoculated cultures of D. asper and D. tulda were incubated in culture room. The temperature was maintained
at 27 $\pm$ 2°C. The cultures were kept in light for 16 hours (1000-4000 lux) and 8 hour dark respectively.

Out of all the four different explants selected and tested for their growth responses in various combinations of MS media only new sprout nodal explants showed the response. Therefore it was continued for further study.

3.12 Induction and growth of callus

The explant new sprout bud segment were cultured on MS media fortified with different concentrations of auxin and cytokinin. Ten explants and slants per treatment were taken, which were replicated thrice. Days required for induction of callus were observed.

Physical appearances of callus were noted after 30 days. Callus growth was measured in terms of fresh weight and dry weight after 30 days. The new sprout node bud segment showed the better response for callus induction. Callus was taken on filter paper and washed 2-3 times with sterile double glass distilled water, to remove the remaining media from callus. The calli were soaked on butter paper and fresh weights were determined after harvesting the tissue on butter paper. This calli were again used for dry weight determination. For dry weight, callus tissue were dried for overnight in oven at 65°C for 12-18 hours to remove complete water. Then the dry weight were taken. The data was recorded and analyzed statistically for all the observations.
3.13 Somatic embryogenesis and multiple shoot induction

The fresh calli were transferred to different modified MS media fortified with different concentrations of auxin and cytokinin either single or in combination, for the induction of somatic embryos. The different physical and chemical factors were studied responsible for somatic embryogenesis. The cultures were kept in 16 hours light (1000-4000 lux) and 8 hours dark at the temperature 23°C to 27°C ± 2°C. The embryo formations were observed after incubation of callus. Noted down the days required for embryo formation as well as the induction of multiple shoots. The subculturing of the multiple shoots were performed on the same medium. The average number of shoots were recorded and analyzed statically.

When the height of multiple shoots attained 5-7 cm, the shoots were isolated and transferred on rooting medium. The whole experiment was repeated thrice.

3.14 Isolation of shoots for root induction

The individual shoots were inoculated on rooting media. The isolated shoots were washed thoroughly with sterile glass double distilled water and transferred for rooting on full and half strength MS medium supplemented with different concentrations of auxin and cytokinin. The cultures were kept under 16 hours light (3000-3200 lux) and 8 hours dark. In individual shoot, root initiation was observed after 10 days of shoot transfer. Each treatment was repeated thrice.
and the observations for average numbers of roots were recorded after 15 days.

3.15 Hardening of *in vitro* raised bamboo

The *in vitro* mature plantlets were about 7 cm tall, which were ready for the hardening. The plantlets were removed from the medium and roots were washed gently with sterile double glass distilled water to remove the excess media from the roots. *In vitro* grown root systems appeared to be very delicate, therefore care was taken to retain all the fiber roots intact to the plantlets. These plantlets were transferred into different hardening media constituted such as 1) Soil: Sand, 2) Soil: Sand: Cow dung: 3) Soil: Sand: Cow dung: Vermiculite.

The temperature, humidity and photoperiod were maintained during hardening the *in vitro* grown plantlets. After 10-15 days, the plantlets were transferred into polythene bags with harden media. Then the plants were transferred to green house. Through out the hardening procedure the survival rate of the plant was measured. Observations of successfully grown plantlets were taken after 15 and 30 days and data were statistically analyzed.
3.16 Statistical analysis

Ten aliquots of each treatment were used for recording observations.

The data obtained was statistically analyzed as per procedure by Panse and Sukhatme (1958) using the formula as follows.

\[
\bar{X} = \frac{\sum X_i}{n}
\]

Where \( \bar{X} = \text{mean} \)

\[\sum X_i = \text{Sum of 'i' observations}\]

\[
\text{S. D.} = \sqrt{\frac{\sum (X_i - \bar{X})^2}{n - 1}}
\]

Where \( X_i = \text{observations (} i = 1, 2, 3, \ldots, n \) \)

\( \text{S. D.} = \text{Standard Deviation.} \)

\[
\text{S. E.} = \frac{\text{S. D.}}{\sqrt{n}}
\]

Where \( \text{S. E.} = \text{Standard Error.} \)