CHAPTER 2

MATERIALS AND METHODS

2.1 MATERIALS

2.1.1 Chemicals

a) KC Medium  (Hi Media, India)

b) MS Medium (Hi Media, India)

c) Alcohol (Changshu Yangyan Chemkul, China)

d) Agar (Sisco Research Laboratories, Mumbai)

e) Sucrose (Thomas Baker Ltd, Mumbai)

d) Hypochlorite (Sisco Research Laboratories, Mumbai)

e) Primers (Synergy Scientific Services, Chennai)

f) PCR (RAPD) Master Mix (Synergy Scientific Services, Chennai)

g) Restriction Enzymes (Genie, Bangalore)

h) Agarose (Sisco Research Laboratory, Mumbai)

i) Silica gel (Sisco Research Laboratory, Mumbai)

j) Ethidium Bromide (Sisco Research Laboratory, Mumbai)

k) Gel loading Dye (Genie, Bangalore)

l) IAA, BAP (Glaxco Smith Kline Pharmaceuticals, Mumbai)

m) 2,4 - D and Kinetin (Loba Chemie Ltd, Mumbai)

All other chemicals used are of Analytical Reagent Grade.
2.1.2 Instruments Used

a) BioSpectrophotometer BL 198
   Make : ELICO, Hyderabad.
   Range : 190-1100 nm

b) Electronic Balance
   Make : Adair & Dutt, Calcutta
   Sensitivity : ±0.0001 gm

c) pH / mV meter
   Make : ELICO Ltd, Hyderabad
   Sensitivity : ±0.01

d) Electroporator 2510
   Make : Eppendorf, USA.

e) PCR
   Make : Thermo Hybraid, UK

f) Electrophoresis Unit
   Make : Medox, Chennai

g) UV Trans Illuminator
   Make : Biotech R&D, Salem

h) Centrifuge
   Make : Remi Instruments Pvt Ltd, Mumbai

i) Mini Fermentor
   Make : Wheaton, USA
   Volume : 4 Liters


2.2.1 COLLECTION OF SAMPLE

- *Dendrobium* pods were collected from the farm house of an Orchid Grower in Chennai, India.

- *Bacopa monnieri* plants were collected from “Aringar Anna Medicinal Farm” Anna Nagar, Chennai, India.

- *Nerium oleander* pods were collected from the Farm house of village Ariyapadi near Vandavasi, Tamilnadu, India.

2.2.2 STERILIZATION METHODS:

The protocols used for surface sterilization are as follows:

2.2.2.1 PROTOCOL 1

- The explants were washed in tap water thoroughly.

- The segments were then washed in 70% ethanol for 30 Seconds.

- Then the segments were washed in 25% sodium hypochlorite : 0.01% Tween 80 for 25 minutes.

- The segments were washed finally with distilled water (2 cycles).

2.2.2.2 PROTOCOL 2

- The explants were washed in running tap water for 30 minutes.

- In the next step, the explants were soaked in aqueous solution containing 0.2% Bavistin and 0.3% Streptomycin.

- This was followed by gentle wash in sterile double distilled water for 5 minutes for two cycles.
FIGURE 1.1

MATURE PODS OF DENDROBIUM

BACOPA MONNIERI IN FIELD

NERIUM OLEANDER POD
Then the explants were immersed in aqueous solutions of 5% Savlon for 10 minutes and were shaken regularly.

Then the explants were washed thoroughly with sterile double distilled water for 5 minutes (two cycles).

After this treatment, the explants were sterilized with 0.01% Mercuric chloride aqueous solution for 4 minutes. Then the explants were removed from the sterilizing solution and rinsed thoroughly for two times with sterile double distilled water.

2.2.2.3 PROTOCOL 3

The explants were washed in running tap water for 30 minutes.

In the next step, the explants were soaked in aqueous solution containing 5% Cetrimide for 10 minutes.

This was followed by gentle wash in sterile double distilled water for 5 minutes.

Then the explants were immersed in aqueous solutions of 10% Sodium Hypochlorite for 10 minutes and were shaked regularly.

After this treatment, the explants were sterilized with 0.1% Mercuric chloride aqueous solution for 5 minutes.

The segments are then washed in 70% ethanol for 2 minutes.

Then the explants were removed from the sterilizing solution and rinsed thoroughly for two times with sterile double distilled water.
2.3 MEDIA COMPOSITION

2.3.1 MURASHIGE AND SKOOG (1962) MEDIUM:

<table>
<thead>
<tr>
<th>Constituents</th>
<th>Amounts (mg/l)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Macronutrients</td>
<td></td>
</tr>
<tr>
<td>( \text{NH}_4\text{NO}_3 )</td>
<td>1650</td>
</tr>
<tr>
<td>( \text{KNO}_3 )</td>
<td>1900</td>
</tr>
<tr>
<td>( \text{CaCl}_2\cdot2\text{H}_2\text{O} )</td>
<td>440</td>
</tr>
<tr>
<td>( \text{MgSO}_4\cdot7\text{H}_2\text{O} )</td>
<td>370</td>
</tr>
<tr>
<td>( \text{KH}_2\text{PO}_4 )</td>
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<tr>
<td>2. Micronutrients</td>
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</tr>
<tr>
<td>( \text{MnSO}_4\cdot4\text{H}_2\text{O} )</td>
<td>16.90</td>
</tr>
<tr>
<td>( \text{FeSO}_4\cdot7\text{H}_2\text{O} )</td>
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</tr>
<tr>
<td>( \text{ZnSO}_4\cdot7\text{H}_2\text{O} )</td>
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</tr>
<tr>
<td>( \text{H}_3\text{BO}_3 )</td>
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<tr>
<td>( \text{KI} )</td>
<td>0.83</td>
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<tr>
<td>( \text{Na}_2\text{MoO}_4\cdot2\text{H}_2\text{O} )</td>
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<tr>
<td>( \text{CoCl}_2\cdot6\text{H}_2\text{O} )</td>
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</tr>
<tr>
<td>( \text{CuSO}_4\cdot7\text{H}_2\text{O} )</td>
<td>0.025</td>
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<tr>
<td>( \text{Na}_2\text{EDTA}\cdot2\text{H}_2\text{O} )</td>
<td>30.00</td>
</tr>
<tr>
<td>3. Vitamins (Hi Media)</td>
<td></td>
</tr>
<tr>
<td>Myoinositol</td>
<td>100</td>
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<tr>
<td>Glycine</td>
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<tr>
<td>Nicotinic acid</td>
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<tr>
<td>Pyridoxine Hcl</td>
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</tr>
<tr>
<td>Thiamine Hcl</td>
<td>0.1</td>
</tr>
<tr>
<td>4. Sucrose</td>
<td>30000</td>
</tr>
<tr>
<td>5. Agar</td>
<td>8000</td>
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2.3.2 KNUDSON C MODIFIED MEDIUM

<table>
<thead>
<tr>
<th>Constituents</th>
<th>Amount (mg/l)</th>
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</thead>
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<td>Ca(NO$_3$)$_2$</td>
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<tr>
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<tr>
<td>MgSO$_4$</td>
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<td>MnSO$_4$</td>
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<tr>
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<tr>
<td>H$_3$BO$_3$</td>
<td>0.056</td>
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<tr>
<td>Sucrose</td>
<td>30000</td>
</tr>
<tr>
<td>Agar</td>
<td>8000</td>
</tr>
</tbody>
</table>

2.3.3 GROWTH REGULATORS

AUXINS

2,4-dichlorophenoxyacetic acid (2,4-D)
Indole acetic acid (IAA)
Alpha-Naphthalene acetic acid (NAA)

CYTOKININS

6-Benzyl amino purine (BAP)
Kinetin (Kn)
Coconut water
Banana extract

2.4 INITIATION OF CULTURES:

Sterilized explants were transferred aseptically to sterilized glass plate in the laminar flow hood. Then a cut was given on both basal as well as the top portion of the explants to remove undesirable/dead portions after surface sterilization.
The forceps were earlier rinsed in the 70% ethanol and were flamed and cooled. Then the lid from one test tube was removed and test tube's mouth was flamed to avoid any chance of contamination.

Each nodal explant was then placed in an erect position in the test tube containing medium with the help of long forceps.

The lid was finally closed carefully, flamed lightly and sealed with Klin film. The forceps were then again rinsed with 70% alcohol to avoid any chance of cross contamination. The same procedure was undertaken for all the explants.

These jars were finally kept in the growth room with temperature conditions 25±2 °C, with a photoperiod of 16 hours daylight and 8 hrs night break under the cool white fluorescent light of average 2500 lux (cool white fluorescent tube light 40 W GE).

2.4.1 ESTABLISHMENT OF CULTURES

After approximately 9-10 days of inoculation, the axillary bud break was seen in some explants. When the explants attain bud proliferation, these cultures were then transferred to jars containing fresh medium.

After 21-25 days of incubation with a clean and sterilized forcep in the laminar flow hood, the initiated plants were taken out of the test tube, medium adhered to the plants was removed, undesirable/brownish leaves were removed from the plants and were taken to the culture bottles containing autoclaved semi-solid media having the same combinations as that of the culture initiation.
Then the bottles were placed in the culture room under the standard conditions of temperature (25± 2°C) for 16/8 hrs of day/night break under the cool white fluorescent light of average 2500 lux (cool white fluorescent tube light 40 W GE).

2.5 AXILLARY SHOOT PROLIFERATION
2.5.1 MULTIPLICATION OF SHOOTS BY REPEATED SUB-CULTURING IN MULTIPLICATION MEDIA

• The preparation and sterilization steps for the medium, instruments and chamber were repeated as before.

• Multiple shoots/cluster were transferred from the culture bottle to a sterile glass plate using flamed sterilized forceps, the brown leaves were removed from the primary shoots and sectioned into one node piece after removing the leaves.

• These nodal segments were transferred to the multiplication media. All this work was done with extreme care and inside the laminar flow hood to avoid any possible chance of contamination.

• These culture bottles were then incubated in the growth room. These steps were repeated every 25-30 days for the next sub-culturing.

2.6 ROOTING OF THE SHOOTS

Axillary shoots developed in cultures in the presence of cytokinin generally lack roots. To obtain full plants the shoots must be transferred to a rooting medium, which is different from the shoot multiplication medium, especially in its hormonal composition. A low salt medium is found satisfactory for rooting of shoots in large number of plant species.
2.6.1 ROOTING PROTOCOL

In the laminar flow, under sterile conditions the Klin film and cap was removed from the culture bottles in sterile condition (laminar flow hood) and with the help of sterile forceps, the multiplied shoots were removed from the medium and placed on the sterile glass plate.

With the help of sterile scalpel, elongated shoots up to 1-2 cm in length were cut and placed into the rooting medium. The culture bottles were then capped and placed in the growth room under the same condition. The time required for in vitro rooting of shoots may vary from 10 – 15 days.

2.7 TRANSPLANTATION AND ACCLIMATIZATION OF THE PLANTLETS

The transfer of plants from the culture vessel to the soil requires a careful, stepwise procedure. The roots of the plants are gently washed to remove the agar sticking to them. The most essential requirement for the successful transplantation is to maintain the plants under a very high humidity (90 – 100%). For the first 10 – 15 days by keeping them under mist or covering them with clear plastic. Some small holes may be poked in the plastic for air circulation. Inside the culture vessel the humidity is high and thus, the natural protective covering of cuticle is not fully developed. During this time plant attain ability to synthesize more food and develop cuticular covering. Plants are maintained under shade and are then ready to be used in open nursery.

2.7.1 PROTOCOL FOR TRANSFER TO SOIL:

After 10 – 14 days of culture on rooting media, the rooted plantlets were transplanted to pots or trays for hardening prior to their final transfer to soil.
Rooted plantlets were taken out of the culture bottles with the help of forceps and washed thoroughly with water to remove any remaining of the medium. 5% cetrimide treatment was given to the plants in order to protect them from the fungal attack in the near future.

After this the plants were carefully planted in the plastic cups containing different soil mixtures in different ratios.

2.8 PROTOCOL FOR CALLUS INDUCTION

Three types of explants: Leaf segments; internodal segments and nodal segments were used as a source of explant.

Explants used for callus induction were taken from established cultures of *Bacopa*. The medium employed was MS Basal with different concentration and combinations of phytohormones such as NAA, Kinetin and 2,4-D.

After inoculation of the culture, the bottles were properly capped and sealed.

After labeling, these were transferred to the incubation room where they are incubated at 25± 2°C in the rack covered with black paper.

2.9 ISOLATION OF DNA

2.9.1 REAGENT PREPARATION

2.9.1.1 EXTRACTION BUFFER [\(\text{pH} 7.8\)]

100mM Tris Hcl
100mM EDTA (Ethylene Diamine Tetra Acetic acid)
500mM Sodium Chloride.

2.9.1.2 20% SDS (SODIUM DODECYL SULPHATE)

20g of SDS was dissolved in 100ml of distilled water.
2.9.1.3 SUSPENSION BUFFER [pH 8.0]

50mM Tris HCl

10mM EDTA (Ethylene Diamine Tetra Acetic acid)

2.9.2 PROCEDURE FOR DNA ISOLATION

5g of Bacopa material (leaves) was weighed and freeze-dried quickly in liquid nitrogen and ground to a fine powder using mortar and pestle.

75ml of Extraction Buffer was added in a small volume and ground thoroughly.

The homogenate was transferred to 250ml conical flask and to the homogenate, 5ml of 20% SDS was added and mixed thoroughly using magnetic stirrer for 15-20 minutes.

Then the contents were incubated at 65°C for 10 minutes. 50ml of Potassium acetate solution was added, mixed and incubated at room temperature for 30 minutes, in order to precipitate proteins and polysaccharides.

The contents were centrifuged at 25,000rpm for 15 minutes. The pellet was discarded and supernatant was collected.

To the supernatant 1/6th volume of ice cold iso propanol was added and incubated at room temperature for 30 minutes.

Then the DNA pellet was collected by centrifuging at 20,000rpm for 15 minutes.

The pellet was suspended in 3ml of suspension buffer, 1.8ml ice cold iso propanol and 180µl of 3M Sodium acetate and incubated at room temperature for 1 hour.
The DNA was repelleted by centrifugation. Then the pellet was washed with 80% ice cold ethanol and air dried.

Finally pellet was suspended in TE buffer and stored at low temperature for further use.

The DNA was subjected to Agarose Gel Electrophoresis.

2.10 AGAROSE GEL ELECTROPHORESIS

2.10.1 REAGENT PREPARATION

2.10.1.1 AGAROSE

1g of agarose was weighed and dissolved in 2ml of 1x TAE(Tris, Acetic Acid, EDTA) buffer.

2.10.1.2 50X TAE BUFFER

24g of Tris base, 5.71ml of glacial acetic acid, 0.5M EDTA were mixed in 100ml of distilled water (pH-8).

2.10.1.3 GEL LOADING DYE

100µl of bromophenol blue (0.25%) was taken in a microfuge tube. To this, 600µl of TAE buffer was added. Later, 300µl of glycerol(30%) was mixed with it. From that, 5µl of solution was taken for loading the well.

2.10.1.4 STAINING SOLUTION

0.5µg of Ethidium bromide (EtBr) was dissolved in 1ml of double distilled water.

2.10.1.5 TE BUFFER[pH-8.0]

10mM Tris buffer, 1mM EDTA were dissolved in 100ml of distilled water.
2.10.2 PROCEDURE FOR AGAROSE GEL ELECTROPHORESIS

The open ends of the gel plate was sealed with cellophane tape. The gel plate was wiped with cotton piece saturated with absolute alcohol.

1g of agarose was dissolved in 100ml of 1x TAE buffer by warming the flask in water bath at 100°C. The solution was cooled to 50°C. The flask was constantly swirled during cooling. After cooling add 10-15µl of ethidium bromide. Then the solution was poured on to the notched end of the tray. The solution was allowed to set. After solidification, the comb as well as the cello tape were removed. The plate was placed on a raised platform in the apparatus.

The electrophoresis apparatus was filled with 300ml of 1x TAE buffer.

In a microfuge tube, 20µl of sample and 5µl of the gel loading buffer were added and mixed thoroughly.

25µl of this solution was added in the well and connected to the power supply at 50V.

Electrophoresis was terminated before the tracking dye reaches the end of the gel.

After the electrophoresis was completed, the gel was removed from the platform and placed under the UV transilluminator for visualization of band.

2.11 PROCEDURE FOR RESTRICTION DIGESTION OF DNA

3µl of the *Bacopa monnieri* DNA was taken in the microfuge tubes labeled 1,2,3,4&5.
To this 2.5µl of assay buffer, 2µl of restriction enzyme (EcoRI, BamHI, HindIII, Sau3A) was added and the mixture as made up to 20µl with sterile double distilled water. The mixture was mixed gently by tapping the tube. The tubes were incubated at 37°C for one hour. Simultaneously 1% agarose was prepared. After an hour 5µl of gel loading buffer was added to all the tubes. 10µl of *Bacopa monnieri* DNA with 1µl of gel loading buffer would serve as control. 10µl of marker with gel loading buffer was loaded in the wells of agarose gel. Then the digested samples, control was loaded in the wells. The samples were subjected to electrophoresis at 50-1000V for 1 hour. Then the restriction pattern was observed in the UV transilluminator.

### 2.12 RAPID AMPLIFIED POLYMORPHIC DNA (RAPD)

**Primers**

AAATCGGAGC, GTCCTACTCG, GTCCCTAGCG, TGCGCGATC, GAACGTACGCG, GCACGCCGGA, CACCCTGCGC, CTATCGCC, GCCGGGATCCGC, GCGAATTCCG, CCCTGCAGGC, CCAAGCTTGC, GTGCAATGAG, AGGATACGTG, AAGATAGCGG, GGATCTGAAC, TTGTCTCAGG, CATCCCGAAC, GGACTCCACG, AGCCTGACGC.
2.12.1 METHODS

Reaction mixture contained the following:

- DNA stock: 2.5µl
- RAPD master mix: 10µl
- Primer: 1µl

Sterile water added to make up 25µl

General cycling steps followed:

- Step 1: Initial denaturation at 94°C for 5 min
- Step 2: Denaturation at 94°C for 1 min
- Step 3: Primer annealing at 55°C for 1 min
- Step 4: Primer extension at 72°C for 2.00 min
- Step 5: Go to 2, 39 times
- Step 6: Final extension at 72°C for 10 min
- Step 7: 4°C for ever.

After the reaction, DNA was analysed through agarose gel electrophoresis.

The RAPD analysis was performed using 20 primers for mother and micropropagated plants.