CHAPTER 5

5 MICROPROPAGATION STUDIES ON NERIUM OLEANDER

5.1 INITIATION OF CULTURES

Sterilized explants were transferred aseptically to sterilized glass plate under 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).

5.2 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 laminar flow hood, the initiated plants were taken out 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).

5.3 AXILLARY SHOOT PROLIFERATION

5.3.1 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.
5.3.2 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.

5.3.2.1 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.

Table 5.1 MEDIUM CODE FOR *NERIUM OLEANDER* (NOM-NOM7)

<table>
<thead>
<tr>
<th>MEDIUM CODE</th>
<th>MEDIUM DETAILS</th>
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<tbody>
<tr>
<td>MS</td>
<td>MS basal medium as control</td>
</tr>
<tr>
<td>NOM</td>
<td>MS+0.1%mg/lBAP+0.1%mg/lNAA</td>
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<tr>
<td>NOM1</td>
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<td>NOM5</td>
<td>MS+1%mg/lBAP+1%mg/lKinetin</td>
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<tr>
<td>NOM6</td>
<td>MS+ Coconut Water 100ml/l</td>
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<tr>
<td>NOM7</td>
<td>MS + Banana Extract 100ml/l</td>
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</tbody>
</table>
5.4 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.

5.4.1 PROTOCOL FOLLOWED 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 are carefully planted in the plastic cups containing different soil mixtures in different ratios as shown in the table.
Table 5.2  SOIL MIXTURE CODE FOR *NERIUM OLEANDER*

(NVM – NVM3)

<p>| | |</p>
<table>
<thead>
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<tbody>
<tr>
<td>NVM1</td>
<td>SOIL</td>
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<tr>
<td>NVM2</td>
<td>SOIL: VERMICOMPOST (2:1) *</td>
</tr>
<tr>
<td>NVM3</td>
<td>SOIL: VERMICOMPOST (4:1) *</td>
</tr>
</tbody>
</table>

*parameter measured in v/v.

5.5 CALLUS INDUCTION

Callus is a mass of unorganized cells resulting either as a consequence of wounding in plants or in tissue culture. Callus is either homogeneous, parenchymatous mass or trachery elements or sieve elements or submerged cells or trichomes. Callus formation has been found in angiosperms, gymnosperms, pteridophytes and bryophytes. Callus is somewhat an abnormal tissue, which has the potentiality to produce normal roots and embryoids, and in turn develops into plantlets. Under the stimulus of endogenous growth substances or hormones added to the medium, the metabolism of cells, which were in quiescent state, is changed and they began active division. During this period, cell differentiation, which may have been occurring in the intact plant, is reversed and this give rise to new tissue, which is composed of meristmatic and unspecialized cell types.

Although callus remains unorganized, as growth proceeds, some kinds of specialized cells may be again formed and which can give rise to organs such as roots, shoots and embryos. For plant cells to develop into a callus it is essential that the nutrient medium contain plant hormones, i.e. an auxin, a cytokinin and gibberellins. The absolute amounts of these, which are required, vary for different tissue explants from different parts of the same plant and for the same explant from different genera of plants. Callus tissue
originating from herbaceous species material regenerates much better than material from woody plants. When subculture regularly on nutrient medium callus culture will exhibit a s-shaped or sigmoidal pattern of growth.

Callus growth can be monitored by fresh weight and dry weight measurements but dry weight measurements are more accurate but this requires sacrifice of the sample.

5.5.1 USES OF CALLUS CULTURES

For the synthesis of starting compounds that are subsequently modified to yield the desired product.

Use as starting material for the vegetative propagation of plants.

Their reverting to tissue culture allows the conservation of virus or fungi free and resistant cell lineages.

5.5.2 PROTOCOL FOLLOWED 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 *Nerium oleander*. The medium employed was MS Basal with different concentration and combinations of phytohormones such as NAA, Kinetin and 2,4-D.

After inoculation the culture bottles were properly capped and sealed. After labelling these were transferred to the incubation room where they are incubated at 25± 2°C in the rack covered with black paper.
Table 5.3   DIFFERENT COMBINATIONS OF CALLUS MEDIUM

(MEDIUM CODE: NCM – NCM4)

<table>
<thead>
<tr>
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<td>MS</td>
<td>MS basal medium as control</td>
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<tr>
<td>NCM</td>
<td>MS+0.5%mg/l BAP+1%mg/l 2,4-D</td>
</tr>
<tr>
<td>NCM1</td>
<td>MS+1%mg/l BAP+1%mg/l IAA</td>
</tr>
<tr>
<td>NCM2</td>
<td>MS+0.5% mg/l 2,4-D</td>
</tr>
<tr>
<td>NCM3</td>
<td>MS+1%mg/l 2,4-D</td>
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<tr>
<td>NCM4</td>
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</tr>
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<td>MS+ Coconut Water 100ml/l</td>
</tr>
<tr>
<td>NCM6</td>
<td>MS+ Banana Extract 100ml/l</td>
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</tbody>
</table>

5.6   ISOLATION OF DNA

5.6.1   REAGENT PREPARATION

5.6.1.1 EXTRACTION BUFFER [pH -7.8]

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

5.6.1.2 20% SDS (SODIUM DODECYL SULPHATE)

20g of SDS was dissolved in 100ml of distilled water.

5.6.1.3 SUSPENSION BUFFER[pH-8.0]

50mM Tris HCL
10mM EDTA. (Ethylene Diamine Tetra Acetic acid)
5.6.1.4 PROCEDURE FOLLOWED FOR DNA ISOLATION

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

75ml of Extraction Buffer was added in a small volume and grinded 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 25,000 rpm 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 20,000 rpm 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.
5.6.2 AGAROSE GEL ELECTROPHORESIS

5.6.2.1 REAGENT PREPARATION

5.6.2.1.1 AGAROSE

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

5.6.2.1.2 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).

5.6.2.1.3 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.

5.6.2.1.4 STAINING SOLUTION

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

5.6.2.1.5 TE BUFFER[pH-8.0]

10mM Tris buffer, 1mM EDTA were dissolved in 100ml of distilled water.

5.6.2.1.6 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.

1a 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.