

Micropropagation of *Nerium Oleander* through the Immature Pods

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Abstract

Oleander (*Nerium oleander* L.) is a vegetatively propagated ornamental plant valued for its evergreen foliage and showy terminal flower clusters that are available in different colours. *Oleander* is cultivated recently as a flowering pot plant and therefore abundant propagation plant material for commercial use is of great importance. This species also produces secondary metabolites (Paper & Franz 1989), some of which are of pharmacological interest. In vitro culture of plants has gained importance during recent years because, besides other application, this technique can be used for the rapid multiplication of some plants (Tisserat 1987). As far as we are aware, there are no published reports about micropropagation of *Nerium oleander* and the aim of the present work was to determine the culture conditions for micropropagation of this plant.

To induce callus formation from pods of *Nerium oleander*, and study the *in vitro* growth conditions of calli. Study the specific growth factor for root and shoot induction, and transplantation and acclimatization of explants and isolation of the DNA from explants. Standardization of micropropagation protocol of *Nerium oleander*.

In the case of *Nerium oleander*, the micropropagation technique from pods were established. We also raised plantlets from the callus and also subjected the DNA for analysis and done RAPD to find the difference between the normal and *in vitro* plants, the result is no difference-this shows that the plants are identical. Standardization of surface sterilization protocol, aseptic culture initiation, establishment and multiplication, suitable media for rooting and a suitable protocol for hardening in order to achieve quality transplant. Optimization of medium for callus induction, maintenance and regeneration.

Keywords: Micropropagation, *Nerium Oleander*, Immature Pods

1. Introduction

Oleander grows well in warm subtropical regions, where it is extensively used as an ornamental plant in landscapes, parks, and along roadsides. It is drought tolerant and will tolerate occasional light frost down to -10° C, 14° F. It is commonly used as a decorative freeway median in California and other mild-winter states in the Continental United States because deer will not eat it due to its high toxicity, it is tolerant of a variety of poor soils, and drought tolerant. It can also be grown in cooler climates in greenhouses and conservatories, or as indoor plants that can be kept outside in the summer. *Oleander* flowers are showy and fragrant and are grown for these reasons. Over 400 cultivars have been named, with several additional flower colours not found in wild plants having been selected, including red, purple, pink and orange; white and a variety of pinks are the most common. Many cultivars also have double flowers. Young plants grow best in spaces where they do not have to compete with other plants for nutrients (Isabel Santos et.al, 1994).

Oleander is one of the most poisonous plants in the world and contains numerous toxic compounds, many of which can be deadly to people, especially young children. The toxicity of *Oleander* is considered extremely high and it has been reported that in some cases only a small amount had lethal or near lethal effects. The most significant of these toxins are oleandrin and nerine, which are cardiac glycosides. They are present in all parts of the plant, but are most concentrated in the sap, which can block out receptors in the skin causing numbness. It is thought that *Oleander* may contain many other unknown or un-researched compounds that may have dangerous effects. *Oleander* bark contains rosagenin which is known for its strychnine-like effects. The entire plant,

including the milky white sap, is toxic, and any part can cause an adverse reaction. *Oleander* is also known to hold its toxicity even after drying. It is thought that a handful or 10-20 leaves consumed by an adult can cause an adverse reaction, and a single leaf could be lethal to an infant or child.

2. Materials and Methods

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

2.1 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).

2.2 Establishment of cultures

After approximately 9-10 days of inoculation, the axillary bud break were 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 were 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).

2.3 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 were 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.

2.4 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.

2.5 Protocol for Rooting

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.

2.6 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 were 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 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 *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 of the culture, the bottles were properly capped and sealed. After labeling, these were transferred to the incubation room where they are incubated at $25 \pm 2^\circ\text{C}$ in the rack covered with black paper.

2.9 Procedure for DNA isolation

Nerium oleander material (leaves) of 5gm was weighed and frozen 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 25,000rpm for 15 minutes. The pellet was discarded and supernatant was collected. To the supernatant $1/6^{\text{th}}$ 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 180 μl 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.

3. Results and Discussion

The surface sterilization procedure was optimized and this helped in preventing blackening of tissues and establishment of clean cultures. The sterilization procedure initially followed does not include any step with antibacterial treatment and large number of explants were found contaminated. Contamination was controlled after the addition of antibiotic, 5% Cetrimide and there was no adverse effect on bud sprouting and shoot multiplication.

Shoot initiation and establishment from *Nerium oleander* pods cultured on MS basal and MS medium supplemented with various combinations of growth regulators i.e. BAP in combination with NAA and Kn is described in Table. Most of the other research studies for other medicinal plant species have shown the use of cytokinin alone or in combination with other in different concentrations. For example for *Paederia foetida* and *Centella asiatica* multiple shoots were obtained in MS medium supplemented with BAP 1.0 mg /litre (Singh *et al*, 1999), and in *Rauwolfia serpentina* on MS medium supplemented with benzyladenine and NAA (Sehrawat *et al*, 2001), whereas for *Nerium* optimum shoot proliferation was achieved in different combination of hormones in different concentrations. We have included coconut water and banana extract as growth regulators which showed good results. During initial week after inoculation, bud initiation was very low. However, bud initiation was found to be started in most of the cultures initiated from 9-10 days by showing a small newly sprouted bud, which proliferate into shoot buds with leaves during 21-25 days which were placed in the culture room under the standard conditions of temperature ($25 \pm 2^\circ\text{C}$). All the experiments were performed thrice with 3 replicates per treatment.

Isabel Santos, Isabel Guimaraes and Roberto Salema, 1994, also reported that shoot bud initiation was observed visually on the ninth day of incubation in all replicates in the media having different concentrations of BAP, Kn, coconut water and banana extract. After 3-4 weeks thick mat of shoot buds spread over 90-100% of the surface of explant in the presence of 2mM BAP, 4mM Kn, coconut water and banana extract. Tiwari *et al*, 2001, reported use of range of cytokinins. Of the four cytokinins (6- benzyladenine, thidiazuron, kinetin and 2-isopentenyladenine) he reported that thidiazuron (6.8 μ M) and 6- benzyladenine (8.9 μ M) proved superior to other treatments. Optimum adventitious shoots buds induction occurred at 6.8 μ M thidiazuron (TDZ). However, in our case, we have not used TDZ/2-ip, to ensure that the protocol standardized be cost effective.

It was observed that although bud break occurred in all the medium under study, following mediums were compared in terms of bud breakage percentage, average shoot length and percentage cluster formation. NOM4 (0.4 mg/l BAP + 0.5 mg/l kinetin) was found to be the best initiation medium in terms of bud breakage, and lower contamination percentage, however cluster formation was observed to be very low in this media in the initiation stage but was increased after establishment stage and had become highest during later stages. NOM (0.1 mg/l BAP + 0.1 mg/l NAA) was observed to be having highest shoot length with higher percentage of cluster formation which was not observed in case of NOM4. In the other three mediums: NOM2 (1 mg/l BAP + 0.2 mg/l NAA), NOM5 (1.0mg/l BAP + 1.0mg/l Kn) and NOM3 (4mg/l BAP, 0.4 mg/l NAA), average shoot length and cluster formation was less as compared to above defined mediums.

3.1 Multiple shoot proliferation

After 25-30 days of first subculture, established cultures were transferred to culture jars having respective media combinations. Multiplication of shoot cultures was carried out by culturing nodal segments/clusters excised from in vitro-raised plants. S. Hatzilazarou *et al* 2000, also found that maximum numbers of plants were obtained on medium containing Kin/2-ip (0.1 mg/l) and Kin (1mg/l) in shoot tip and nodal cultures of *nerium* respectively.

Isabel Santos *et al* 1994, reported that out of two cytokinins used BAP was found to be more suitable than Kn as BAP resulted in quicker and better response than the latter while addition of NAA (0.2mg/l) proved synergistic. Isabel Santos *et al* 1994, Hatzilazarou *et al* 2000 also reported that addition of BAP resulted in the increase in number of shoots, mean shoot length and number of roots/explant. Hatzilazarou *et al* 2000, observed direct regeneration of shoots and roots occurred in nodal explants in *Nerium* on MS medium containing NAA (0.1 mg/l) and BAP (0.5 mg/l). Addition of higher levels (2.0 and 3.0 mg/l) of BAP in the media, helps in differentiation of shoots in 63 and 93% cultures from the nodal portion and shoots attained height of 5 cm in the ninth week in the above medium. Addition of 0.2 mg/l NAA to the above medium caused shoot regeneration in 95% of the cultures and shoots attained height of 5 cm in the eighth week. There is also report for use of high concentration of BAP (9- 10mM) for shoot regeneration (Isabel Santos *et al* 1994, Hatzilazarou *et al* 2000). Both these reports clearly supported our observations as well.

Observations were taken for evaluating the growth of explants by taking parameters like average shoot length and number of nodes (15 shoots randomly selected per medium). The experiment was carried out in seven mediums having different concentrations of growth regulators each with 3 replications, only results of best medium are given. The medium showing best results was NSM4 and NSM5 with highest average shoot length of 5.8 and 4.9 respectively with 100% cluster formation. Sub culturing was carried out after 25-30 days using the same medium combinations as for initiation and establishment stages. Shoot clusters obtained in each subculture was divided in approximately 1cm² size with approximately 4-5 small shoots in each cluster and inoculated in each bottle.

Final observation was taken after 3rd subculture stage after initiation with 3 replications per treatment and summarized in the following Table. Cluster formation initiated from the basal node; progressively increases in size from each subculture and number of shoots initiated from the nodal portion vary from medium to medium. Isabel Santos *et al* 1994 also observed that each explant was transformed into a dense mass of profusely regenerating shoot buds (as "cluster formation" in our case).

It was observed that in case of NSM4 highest number of shoots (>3 cm) originated from the basal node and average number of shoots formed in case of NSM4 is 5.87; 5.2 in NSM1; 2.99 in NSM7 and lowest no of shoot regenerated in case of NSM3 is 2.87. In term of number of nodes/explant, same order is followed i.e. NSM5 having 7.56, NSM4 having 7.13, 5.12 in NSM7 and in case of NSM3 is 3.52. It was also observed that number of clusters obtained by dividing each mother culture (cluster) is ranging from 1.0 (NSM3) to 4.1-4.4 (NSM4 and NSM5) and hence can be ascribed as suggested multiplication rate for each medium.

It was observed that during each passage, the number of leaves/shoots has increased substantially along with the height of shoots. The leaf size was approximately 0.4-0.6 cm. It was observed that morphogenic responses

exhibited in the form of shoots or roots are correlative to a specific auxin /cytokinin ratio and banana extract. In comparison to NOM3 media, NOM4 has shown a significant increase in no of shoots and average shoot length.

3.2 Rooting

After two cycles of multiplication subculture, elongated shoots of 2-3 cm in length were excised and cultured on MS basal medium having different combinations of MS media with plant growth regulators (BAP,NAA,IAA, Kinetin, Banana extract and coconut water).

The experiments were conducted twice, with 3 replications (with 3 shoots per bottle). Rooted shoots were taken after 2 weeks, shoot length, root length and no of roots per explant (total 9 explants per treatment each time), fresh weight and dry weight (keeping them in an oven with 50°C for 24 hrs) were measured.

Initiation of rooting took place after 10-15 days of inoculation. Single and multiple roots were formed from the base and the nodal portions and the length of the roots were 2-3 cm within 25-30 days.

It was observed that NRM6(MS+ 100ml/coconut water)recorded highest S/R (3.02) and 2.84 for NRM7(MS+100ml/ibanana extract) with considerable higher no of roots (9.89 and 8.76 respectively). Similar response was also observed in case of NRM4 and NRM5 in terms of S/R of 1.27 and 1.32 and root number of 3.94 and 3.39 respectively. Our results indicate 100% root formation in 4 mediums.(NRM4,NRM5,NRM6,NRM7) Isabel Santos et.al 1994 observed highest rate of rooting (90%) for *Nerium* on full-strength MS medium containing 2.46 mM IBA.

Ex-vitro rooting was also carried out using single shoots of 1-2 cm in height derived from MS medium and then transplanted in Soil: Vermicompost mixture in the ratio of 4:1 and Soil: Vermicompost mixture in the ratio of 2:1 which gave 100% survival rate.

3.3 Callus induction

Callus induction requires the presence of auxins or cytokinins or both or 2,4,D in the nutrient media depending on the source of explant. Callus initiation was carried out by using leaf segment as source of explant. Initiation was carried out using different growth regulators such as BAP, IAA and 2,4-D containing media.

The explants enlarged with in 12-14 days of inoculation; however callus formation started after 20-25 days at the ends of the explant. Appearance of callus were globular and were of pale green in colour. In the medium NCM6 (MS +100ml/l coconut water), rapid callus growth were observed as pale yellow and of globular appearance.

The addition of 1mM BAP along with 2,4- D, induced a thin layer of granular callus after 4 weeks of culture. However, all the explants turned brown to black at the base after 6 weeks of culture. In case of NCM1 (MS +1 mg/l BAP + 1 mg/l IAA) initial small globular callus were formed, from where small shoot buds have been regenerated.

IAA induced callus at the cut ends of both stem and leaf explants along with 1-5 shoots per explant, while in combination with BAP, it induced only shoots (1-10 per explant) within 2 weeks after incubation. These shoots attained average height of 4-5 cm after placing in light for 8-9 days when transferred into multiplication medium i.e. NCM5 (MS +1 mg/l BAP + 1 mg/l kinetin). The response shown by regenerated shoots in multiplication medium was similar to the response shown by explants inoculated in multiplication medium in light. Loose jelly type callus formation was observed in NCM2 (MS+ 0.5mg/l 2,4-D), NCM7 (MS+ 100ml/l BananaExtract), NCM6 (MS+100ml/l coconutwater) and NCM1(MS+ 0.5mg/lBAP+0.5mg/lNAA) which however turned brown after few days.

Response of callus induction varies with the type of explant. When the same were transferred to NCM4 and NCM5 then callus again started showing regeneration threads after 10-20 days in dark. Callus formation from nodal explants of *Nerium* cultured on MS medium containing 0.5 mg/l 2,4-dichlorophenoxyacetic acid (2,4-D) Callus induction was observed from hypocotyl, root, and cotyledonary leaf segments, grown on Murashige and Skoog (MS) medium supplemented with various concentrations and combinations of 2,4-dichlorophenoxyacetic acid (2,4-D), kinetin (Kn),coconut water, Banana Extract.

Transplantation

Explants used for carrying out hardening experiment using different soil mixture were grown in MS basal medium as reported before. After sufficient rooting, *Nerium* plantlets were transferred to the protrays and polybags having different soil mixtures in different concentrations for hardening. Agar was removed from the rooted plantlets and then plantlets were washed with tap water. Potting mixtures of desired combinations were prepared and mixed properly and filled in the protrays and polybags. The polybags and protrays were sprinkled with water. The roots of each plant were dipped in 5% cetrimide solution to avoid future fungal attack and then

roots of explants were inserted carefully into the soil mixture. The plants were then again sprinkled with water and kept inside the polyhouse at tissue culture.

Then plants were transferred to the polyhouse with misting/pad fan cooling system where humidity maintained is approximately 80% and temperature was 28 –30°C and kept there for 15 days. In the next step they were transferred to the shade house (75%) with overhead sprinkler system for irrigation for next 10 days. Finally they were transferred to the open area for 9-10 days before transferring them to the field. Success of hardening protocol was determined by calculating survival %age. Other parameters included were Fresh Weight, Dry Weight (measured as discussed previously), Root Length, Shoot Length, Shoot/Root ratio and number of roots.

Survival %age for all the mixtures used was >95 The observation was taken 30 days after transplantation. However in terms of root length (7.09 cm) and shoot length (8.79 cm), NVM2 potting mixture (2 soil: 1 vermicompost) was found to be the best combination. Number of roots were found to be highest in case of NVM2, soil and vermicompost mixture (2:1), however in terms of fresh and dry weight, Soil+ vermicompost (4:1) (NVM2) were found to be the better option with significantly higher number of roots and high shoot/root ratio.

It is observed that across five parameters two best hardening mixtures were NVM2 and NVM3. High shoot length is desired in case of *Nerium* as once shoots are long, they fall down and new shoot buds form from each node as runner and further multiply once they touches soil and form roots. 1-2 side branches were observed in case of *Nerium* during hardening process. Since the average survival of the *Nerium* rooted plantlets were >95% and hence assumed that the different potting mixture did not have significant differences. However, for number of roots per plant, the treatment combinations did not show consistent results and hence it is observed that further experiments are necessary to ascertain the results.

For further experimentation, few plantlets (approximately 25) randomly selected from the hardening stage after 35 days. It was observed that during the course of preparation of this thesis work of transplantation, the plants are surviving (100%) and started growing vigorously. Thus established the successful protocol of *Nerium oleander* micropropagation as undertaken under the present study.

Isolation of DNA

5g *Nerium oleander* leaves were subjected various mechanical (mortar and pestle, magnetic stirrer, heat treatment)treatment and chemical treatment (SDS, iso propanol, potassium acetate, sodium acetate, ice cold ethanol). Genomic DNA was isolated with yield of 250-300mg/mg of *Nerium oleander* leaves.

The isolated genomic DNA from plant was observed as Orange red bands in agarose gel under the UV transilluminator and the molecular weight was determined as ≥ 2000 Kb.

Isolation of DNA: DNA was successfully isolated by mechanical and chemical treatment and molecular weight was determined as ≥ 2000 Kb.

RAPD analysis

Polymerase chain reactions (PCRs) were carried out in 25 J.l volume. A reaction tube contained 25 ng of DNA, 0.2 unit of Taq DNA polymerase, 100 J.l each of dNTPs, 1.5 mM MgCl₂ and 5 pmol of decanucleotide primers. The amplifications were carried out using a thermal cycler (MJ Research, USA) following the protocol of Shoyarna *et al* (1997). The amplified products were loaded in 1.2% agarose gel containing 0.51J.g ml⁻¹ of ethidium bromide and photographed by Polaroid system. Custom-made decanucleotide primers were obtained from M/s Bangalore Genie, India. Twelve decamer primers having the sequences AAATCGGAGC, GTCCTACTCG, GTCCTTAGCG, TGCGCGATCG, AACGTACGCG, GCACGCCGGA, CACCCTGCGC, CTATCGCCGC, CGGGATCCGC, GCGAATCCG, CCCTGCAGGC, CCAAGCTTGC were used to analyse 100 in vitro regenerated plantlets from each cultivar.

RAPD Analysis

The RAPD analysis was performed to compare the mother and micro-propagated plant and 99.9% homogeneity was observed.

5. Conclusion

Oleander (*Nerium oleander* L.) is a vegetatively propagated ornamental plant valued for its evergreen foliage and showy terminal flower clusters that are available in different colours. Oleander is cultivated recently as a flowering pot plant and therefore abundant propagation plant material for commercial use is of great importance. This species also produces secondary metabolites (Paper & Franz 1989), some of which are of pharmacological interest. In vitro culture of plants has gained importance during recent years because, besides other application, this

technique can be used for the rapid multiplication of some plants (Tisserat 1987). As far as we are aware, there are no published reports about micropropagation of *Nerium oleander* and the aim of the present work was to determine the culture conditions for micropropagation of this plant.

In the case of *Nerium oleander*, the micropropagation technique were established using green immature pods as the explants source. We have established a protocol for sterilization of pods, studied the medium for the initiation of seeds germination. We developed the method for the callus initiation, multiple shoots development and also raised plantlets from the callus. The plantlets were successfully transplanted to the field and achieved > 90% survival. We done the DNA for analysis and found the molecular weight of *Nerium oleander* were > 2000Kb. The RAPD analysis were done to find the difference between the normal and *in vitro* plants; but the result is no difference, this shows that the plants are identical. ie 99.9 % homogeneity.

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Table 1. Observation for auxillary bud induction

MEDIUM	CONTAMINATION	BUD BREAKAGE	AVG.SHOOT LENGTH (CM)	CLUSTER FORMATION
NOM	0.37±0.19	0.98±0.06	6.7±0.49	No cluster
NOM2	0.27±0.04	0.64±0.06	3.2±0.034	0.69±.08
NOM3	0.29±0.08	0.46±0.14	3.9±0.491	0.88±0.06
NOM4	0.18±0.23	0.92±0.14	6.4±0.46	1.04±0.17
NOM5	0.29±0.06	0.75±0.32	5.3±.04	0.62±0.11

NOM = MS + 0.1 mg/l BAP +0.1 mg/l NAA
 NOM2= MS + 1.0 mg/l BAP + 0.2 mg/l NAA
 NOM3 =MS + 4.0 mg/l BAP + 0.4 mg/l NAA
 NOM4 =MS +0.5 mg/lBAP + 0.5 mg/l Kn
 NOM5 =MS +1.0mg/lBAP + 1.0mg/l Kn

Table 2. Observation of multiple shoot proliferation

MEDIUM	NO. OF SHOOT >3CM	NO.OF NODES	AVERAGE SHOOT LENGTH
NSM3	3.36±0.17	3.52±0.12	2.89±0.62
NSM4	9.98±0.12	7.13±0.16	5.87±0.54
NSM5	8.04±0.27	7.56±0.25	5.2±0.64
NSM7	6.12±0.27	5.12±0.38	2.99±0.34

NSM3 = MS +4.0 mg/l BAP + 0.4 mg/l NAA

NSM4 = MS + 0.5 mg/l BAP + 0.5 mg/l Kn

NSM5 = MS + 1.0 mg/l BAP + 1.0 mg/l Kn

NSM7 = MS + 100 ml/l banana extract

Table 3. Observation for rooting

MEDIUM	ROOT LENGTH (CM)	SHOOT LENGTH (CM)	SHOOT/ROOT	No. OF ROOTS	FRESH WT (gms)	DRY WT(gms)
NRM4	2.57±0.11	6.24±0.25	1.74±0.14	3.94±0.09	1.27±0.07	2.45±0.05
NRM5	1.98±0.29	6.85±0.39	1.23±0.69	3.39±0.09	1.32±0.18	2.77±0.08
NRM6	3.79±0.19	8.56±0.23	3.02±0.32	9.89±0.18	1.08±0.18	1.96±0.21
NRM7	2.89±0.34	4.89±0.12	2.84±0.34	8.76±0.23	1.25±0.02	2.32±0.42

NRM4 = MS + Sucrose (10 gm/l) + Agar (8 gm/l)

NRM5 = MS + Sucrose(20 gm/l) + Agar (7 gm/l)

NRM6 = MS + Sucrose(20gm/l) + Agar(8gm/l)

NRM7 = MS + Sucrose(30 gm/l) + Agar (7 gm/l)

Table 4. Observation for the callus induction

MEDIUM	CONTAMINATION	REGENERATION	APPEARANCE
NCM1	No Contamination	Regeneration	Globular, Pale green
NCM2	No Contamination	No Regeneration	Jelly type brown
NCM6	No Contamination	Regeneration	Globular, Pale green
NCM7	Contamination	No Regeneration	Jelly type green

NCM1 = MS + 0.5 mg/lBAP + 1mg/l 2,4 – D

NCM2 = MS + 0.5 mg/l 2,4 –D

NCM6 = MS + 100 ml/l coconut water

NCM7 = MS + 100ml/l banana extract

Table 5. Observation for the different soil mixtures

TREATMENT COMBINATION	SHOOT LENGTH (CM)	ROOT LENGTH (CM)	SHOOT/ROOT	NO. OF ROOTS	FRESH WT (gms)	DRY WT (gms)
NVM1	7.25±0.96	5.21±0.72	1.76±1.33	2.5±0.64	0.0043	0.0589
NVM2	8.79±0.74	7.09±0.62	2.78±0.45	3±0.31	0.0042	0.0742
NVM3	7.28±1.53	5.29±0.52	1.56±1.06	3.5±0.25	0.0052	0.0712

NVM1 = Soil + Farmyard manure(4:1)

NVM2 = Soil + Vermicompost (2:1)

NVM3 = Soil + Vermicompost (4:1)

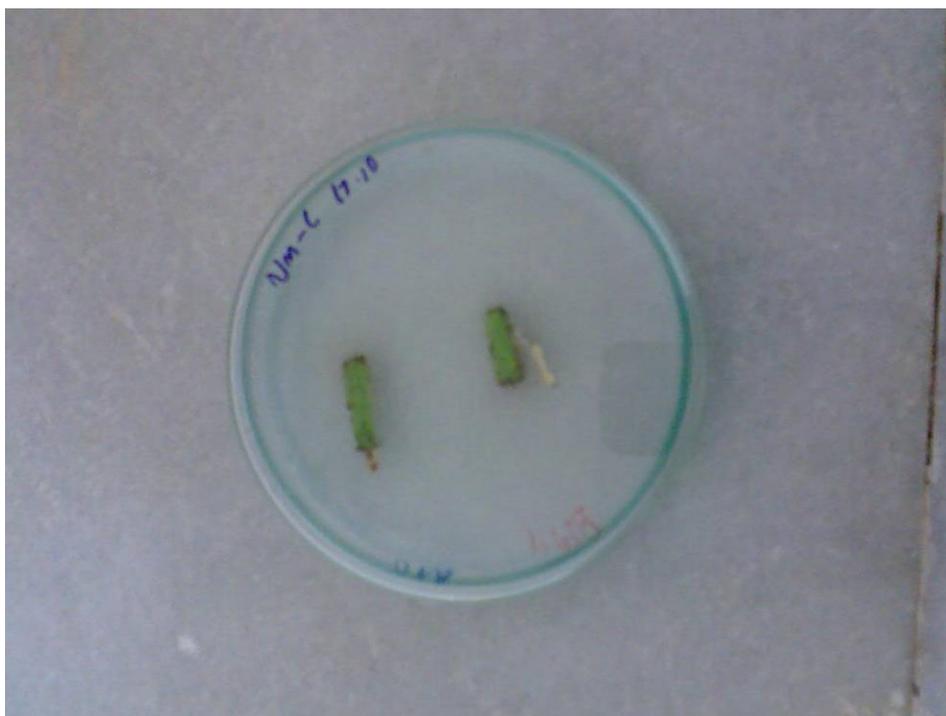


Figure 1. Initiation of pods of *nerium oleander*



Figure 2. Proliferation of calli



Figure 3. *Nerium oleander* proliferation of shoots from pod



Figure 4. *Nerium oleander* *in vitro* shoots and roots development

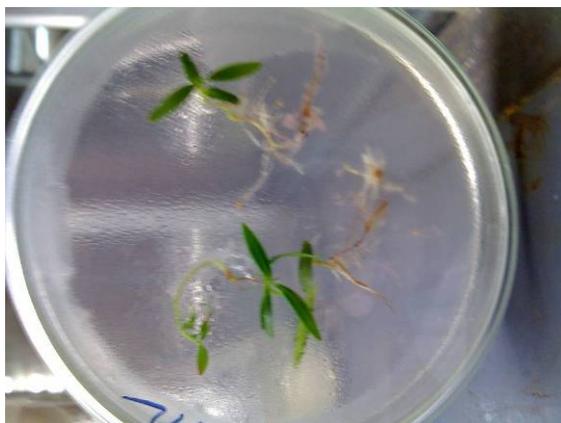


Figure 5. *Nerium oleander* *in vitro* shoots and roots development

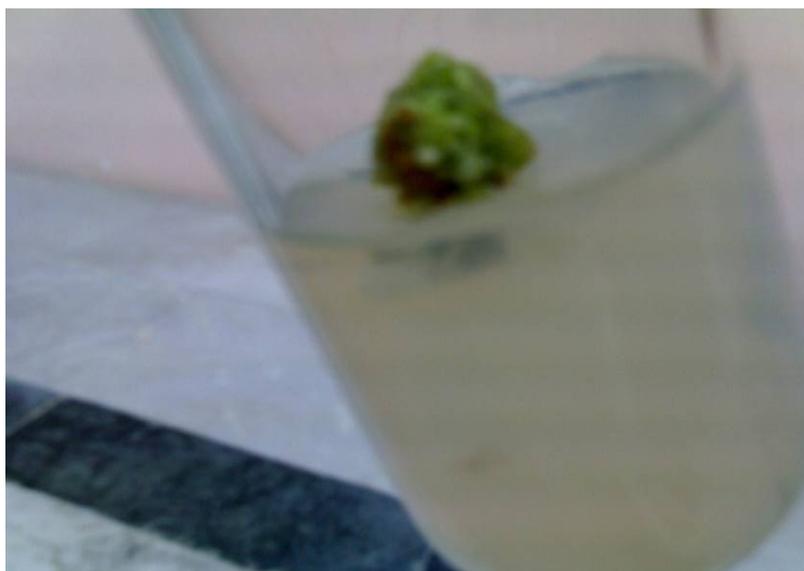


Figure 6. Induction of calli in *nerium oleander*

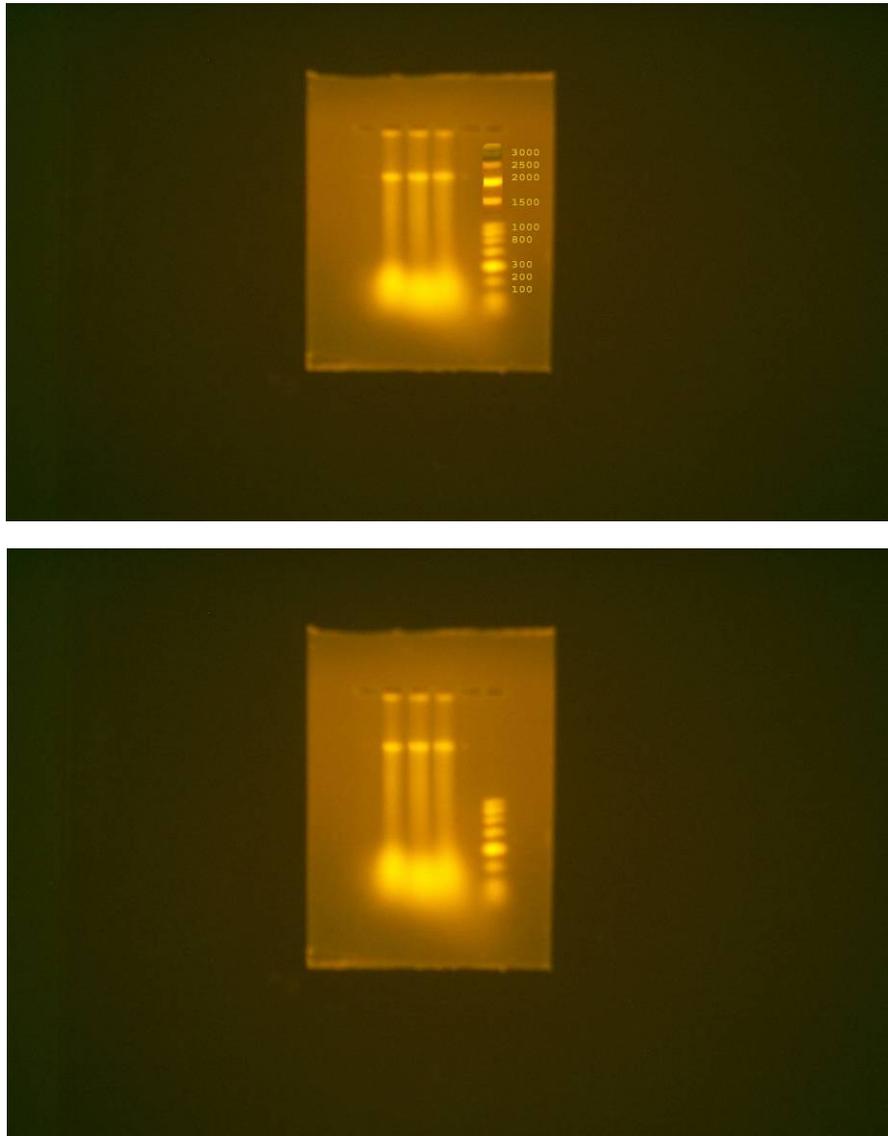


Figure 7. Isolation of dna and molecular weight determination

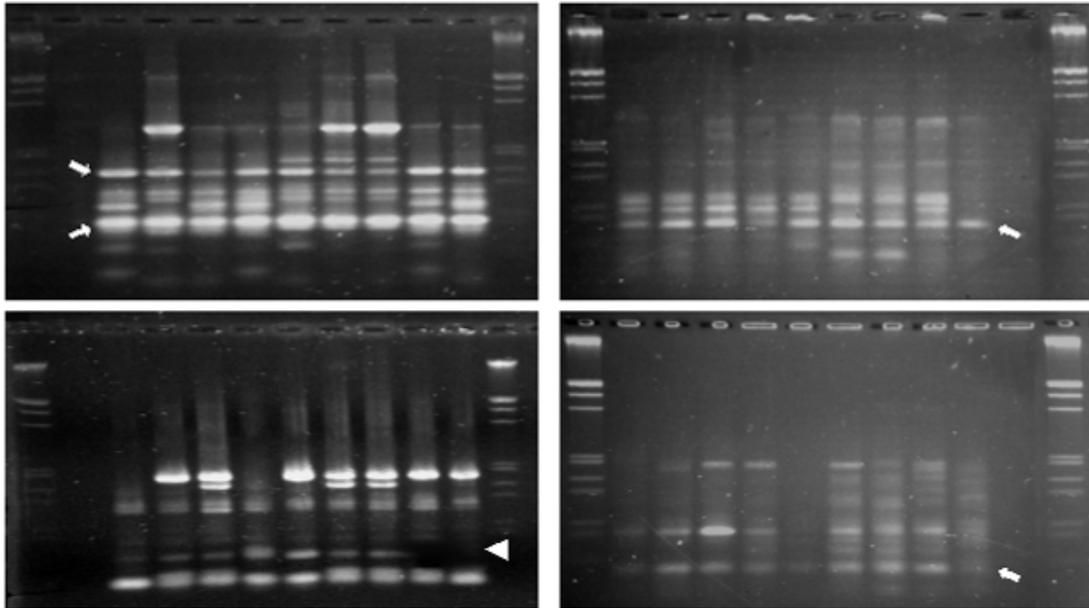


Figure 8. Rapd analysis of mother and micropropagated plant of *nerium oleander*