Chapter 8

Summary
8. SUMMARY

*Naringi crenulata*, belonging to the family Rutaceae is a small handsome tree with light green foliage. It has got several medicinal properties including purgative and sudorific. The dried fruit is a tonic, stomachic and used to cure malignant and pestilent fever. It is an antidote to various poisons. Moreover, it is considered to be a sacred tree, found in the premises of temples.

*Aegle marmelos* belonging to the family Rutaceae is a deciduous and medium sized tree having characteristic trifoliate leaves, which are divided into three leaflets. Bael tree has both mythological and medicinal significance (good for stomach ailments, diarrhoea and dysentery). Ripe fruit is a tonic, laxative and good for heart and brain. The fruit has a high nutritive value having riboflavin, carotene, niacin and ascorbic acid.

### 8.1 In vitro propagation of two medicinally important plants *Naringi crenulata* (Roxb.) Nicolson and *Aegle marmelos* (L.) Corr.

Methods for mass clonal propagation are essential to fulfill cultivars demand. The conventional methods for field propagation are unsuitable for generating large quantities needed. *In vitro* technique has found application in number of areas impacting on the conservation and management of rare and endangered plant species. The development of micropropagation method had allowed efficient exploitation of medicinal plant species. The present investigation was carried out to develop a successful *in vitro* regeneration of two medicinally important plants *Naringi crenulata* (Roxb.) Nicolson and *Aegle marmelos* L. Corr. belonging to family Rutaceae.

#### 8.1.1 *Naringi crenulata* (Roxb.) Nicolson

The actively growing young shoots were collected, defoliated and cut into pieces of 5-7 cm in length. The explants were surface sterilized by keeping under running tap water for 90 min. followed by treating with sodium hypo
chlorite and tween 20 for 5 min; 70% ethanol for 1 min; 0.1% fytolan with
tween 20 for 8 min and 0.1% mercuric chloride with tween 20 for 3 min and
the explants were rinsed thoroughly with sterile distilled water for 4-5 times.
Following disinfection, the nodes and internodes were cut aseptically and
placed in vitro both horizontally as well as vertically on MS medium
supplemented with different concentration and combination of BA, KN, NAA,
IAA and 2,4-D. The explants were subcultured to fresh media after two days of
inoculation. Among the different basal media tried, MS medium was
identified as the best. The nodal segments inoculated onto MS medium
supplemented with BA (1.5 mg/l) in combination with IAA (0.2mg/l) produced
maximum number of multiple shoots (11.8± 1.02) and maximum shoot length
(9.3±1.27).

Leaf and internode explants were inoculated on MS medium fortified
with various concentrations of 2,4-D and IAA for callus induction. Of the
various concentration of auxin used, the maximum percentage of callus
proliferation (61.1%) was observed from the internode explants when they
were inoculated onto medium containing 2,4-D (1.0mg/l). Callus proliferation
was also observed in the MS medium augmented with IAA (1.0 mg/l). Leaf
explants did not show any response.

The in vitro raised shootlets were inoculated onto full strength as well as
half strength MS medium containing 2% (w/v) sucrose with different
concentration of IBA and IAA for root induction. The maximum percentage of
root formation (75%) was observed in half strength MS medium supplemented
with IAA (0.5 mg/l)(T90). The maximum number of roots per shootlet (1.95±
0.11) was observed in the same medium and the maximum root length (2.48±
0.16) was observed in MS medium supplemented with IBA 2.0 mg/l (T73).

The 6-8 week old regenerated plantlets were washed thoroughly in the
tap water to remove traces of agar and transferred to polycups containing the
mixture of the garden soil and sand. The plants were irrigated with 10X diluted
MS liquid medium once in a week. The plantlets were kept in the culture room
for the first fifteen days and 73% of the plants succeeded in hardening process.
Those plants were transferred to pots under green house. 75% of them got established in the field.


The most suitable surface sterilization treatment was achieved by treating the explants under running tap water for 45 minutes followed by 70% alcohol for 1 min; then by 0.1% fytolan containing tween 20 for 5 min; and 0.1% mercuric chloride solution containing tween 20 for 3 min. the explants needed subculturing to fresh medium within two days for their survival. Following disinfection, the explants were inoculated onto MS medium with BA, KN, 2,4-D, IAA and NAA. Among the different media combinations tried, the maximum number of multiple shoot (7.33±0.47) was observed in BA (0.5mg/l) (T15) and shoot length (7.61± 0.25) was observed in MS medium supplemented with BA (0.5 mg/l) in combination with NAA (0.2 mg/l).

The sterilized internodal segments were inoculated onto MS medium augmented with various concentration of 2,4-D, IAA and combination of NAA and KN. The maximum percentage (83.4%) of callus formation was observed in MS medium augmented with KN (1.0mg/l) and NAA (2.0 mg/l).

The in vitro raised shoots were transferred to MS medium containing IAA and IBA for root induction. Highest percentage of root formation (62.0%) was observed in MS medium containing IBA (1.0mg/l) (T52). The maximum number of roots per shoot (1.6± 0.31) was observed in IBA 1.0mg/l (T51) and maximum root length (3.3± 0.30) was observed in IAA (2.0 mg/l) (T58).

The 6 week old regenerated plantlets were washed thoroughly in the tap water to remove the traces of agar and transferred to polycups containing the mixture of the garden soil and sand. The plants were irrigated with 10X diluted MS liquid medium once in a week. The plantlets were kept in the culture room for the first fifteen days and 66% of the plants succeeded in hardening process. Those plants were transferred to pots under green house. 70% of them got established in the field.
Explants cultivated under our protocol developed into normal plants in a period of 40 to 60 days showing enough elongation to identify 3 to 5 internodes. This plant morphology would also provide a source of new explants.

Our micropropagation protocols proved to be simple, reliable and it allows cloning of juvenile or adult trees of *Naringi crenulata* and *Aegle marmelos*. Healthy plants obtained by this way from adult or young explants can be developed normally under *ex vitro* conditions. Considerably increasing its multiplication rate using the *in vitro* protocol described in this work makes it potentially useful for large-scale production.

8.2 Isoenzyme analysis of *in vitro* propagated and mother plants of *Naringi crenulata* and *Aegle marmelos* for genetic confirmation

The term isozyme is used to refer multiple form of an enzyme with similar or identical catalytic activities occurring within the same plant. Isozymes may differ in primary structure, because they are encoded in different genes. The genetic of isozyme is well understood and thus can be used as an effective marker in developmental genetics and differentiation. They are valuable tools to study the genetic variability within the population of plants.

The present experiment was carried out to study the genetic stability between the *in vitro* raised plantlet and mother plant of *Naringi crenulata* and *Aegle marmelos* using five isoenzyme systems namely, peroxidase, esterase, acid phosphatase, alkaline phosphatase and polyphenol oxidase. The anionic system of Davis (1964) and Anbalagan (1999) was adopted for separation of isozymes using native Poly Acrylamide Gel Electrophoresis (PAGE).

The young leaves of both *Naringi crenulata* and *Aegle marmelos* were collected from the mother plant and *in vitro* raised plantlet and the internode derived callus from both the plant species were mashed in a pre-chilled pestle and mortar by using respective extracting buffer (0.1M sodium phosphate...
buffer, pH 7.0 for peroxidase and esterase; 0.01M potassium phosphate buffer, pH 7.0, for polyphenol oxidase; 50mM citrate buffer, pH 5.3, for acid phosphatase; 50mM citrate buffer, pH 9.0, for alkaline phosphatase). The slurry was centrifuged at 10,000 rpm for 20 minutes at 4°C. The supernatant was collected and used as the enzyme source for the study.

The stacking and separating gels were prepared according to Davis, (1964) for peroxidase and acid phosphatase and according to Anbalagan, (1999) for esterase, alkaline phosphatase and polyphenol oxidase isoenzyme analysis. The gels were incubated in specific staining solutions for characterizing isoenzyme pattern of the above enzymes using Reddy and Gasber, (1971); Abbott et al., (1984); Sadasivam and Manickam, (1991). Each isoenzyme band was characterized by its Rf value and their similarity index was calculated.

8.2.1 Peroxidase

The peroxidase isozyme pattern showed no variation between the regenerated plantlets and the mother plant with the Rf value 0.081 for Naringi crenulata and 0.093 for Aegle marmelos whereas variation was observed in the callus, which showed an Rf value of 0.093 for Naringi crenulata. But in Aegle marmelos, the callus showed 100% similarity.

8.2.2 Esterase

Comparison of the esterase isoenzyme system between the mother plant and in vitro raised plantlet showed 92.3% similarity for Naringi crenulata and 88.9% for Aegle marmelos. Callus showed more variations 42.9% and 40% respectively in Naringi crenulata and Aegle marmelos.

8.2.3 Acid phosphatase

The acid phosphatase isoenzyme banding pattern of Naringi crenulata had revealed that in vitro raised plantlet and callus showed 100% pairing
affinity towards the mother plant. In the case of *Aegle marmelos*, pairing affinity of the mother plant, *in vitro* raised plantlet and callus was 66%.

### 8.2.4 Alkaline phosphatase

The Alkaline phosphatase isoenzyme banding pattern of *Naringi crenulata* showed that 66.7% of similarity in the mother plant, *in vitro* raised plantlet and callus; whereas, in *Aegle marmelos* the *in vitro* raised plantlet and callus showed 100% similarity towards the mother plant.

### 8.2.5 Polyphenol oxidase

The polyphenol oxidase isoenzyme banding pattern revealed that, there was 100% pairing affinity between the mother plant and *in vitro* raised plantlet of *Naringi crenulata*, but the callus exhibited variation. In *Aegle marmelos*, the *in vitro* raised plantlets showed 66.7% similarity, and the callus had 100% similarity with the mother plants.

### 8.2.6 Total soluble proteins

The tissue homogenate of mother plant leaves, *in vitro* raised plantlet leaves and callus of both *Naringi crenulata* and *Aegle marmelos* was used to determine the position of the protein bands and their relative molecular weight by SDS-PAGE. It was found that the callus and *in vitro* raised plantlet of *Naringi crenulata* produced maximum number of bands. The protein bands of *Naringi crenulata* had the molecular weight ranging from 66 KD to 18.4 KD and 66 KD to 6.5 KD in *Aegle marmelos*.

The genetic confirmity experiments proved that *in vitro* raised plantlets had more similarity to the mother plant. The callus of *Naringi crenulata* and *Aegle marmelos* illustrated variations from their mother plant. This might be either due to the dedifferentiated nature of the callus, in which the gene expression may vary from that of the differentiated tissues or by the somaclonal variations. This type of variations can be used for the improvement of plant breeding in future.
8.3 Studies on antibacterial activity of different extracts of *Naringi crenulata* and *Aegle marmelos* and identification of the active principle

In the present investigation, antimicrobial activity of both the plant extracts against six microbial species namely, *Bacillus subtilis*, *Escherichia coli*, *Klebsiella pneumoniae*, *Proteus vulgaris*, *Salmonella typhi* and *Staphylococcus aureus* were recorded. Considerable antimicrobial activity was detected in isopropanol, methanol and ethanol extracts of *Naringi crenulata* and benzene, methanol and ethanol extract of *Aegle marmelos*.

8.3.1 Antibacterial activity of *Naringi crenulata*

The isopropanol, methanol and ethanol extracts of *Naringi crenulata* showed better inhibition for all the six pathogens. The highest degree of inhibition was exerted on *Bacillus subtilis*, *Klebsiella pneumoniae*, *Escherichia coli* and *Salmonella typhi*. All the six pathogens exhibited resistance to the benzene and aqueous extracts of *Naringi crenulata*.

8.3.2 Antibacterial activity of *Aegle marmelos*

The present study reported that *Bacillus subtilis* showed more susceptibility to the benzene extract of *Aegle marmelos* leaves. The methanol extract was found to be superior in its activity and it was succeeded by ethanol and aqueous extracts. The acetone extract exhibited resistance to all the other organisms except *Bacillus subtilis* and *Staphylococcus aureus*. Isopropanol extract had no antibacterial activity.

8.3.3 Purification, Characterization and Structure Elucidation of Isolated Compound

In the present study, it was observed that the isopropanol extract of *Naringi crenulata* showed antibacterial activity against all the six pathogens. The growth of *Bacillus subtilis*, *Proteus vulgaris* and *Staphylococcus aureus* were inhibited more by isopropanol extract when compared to the others. Hence the isopropanol extract of *Naringi crenulata* was selected for further
purification by chromatographic techniques. Among the six fractions obtained, fraction number 6 showed strong antibacterial activity against four pathogens (*Bacillus subtilis*, *Salmonella typhi*, *Klebsiella pneumoniae* and *Proteus vulgaris*) tested and it was confirmed that the fraction number 6 contains the active principle. So it was further purified by rechromatography and was characterized by mass and NMR spectrophotometric methods. From the spectral data the molecular formula of the compound was predicted as $C_{14}H_{14}O_4$. This indicates that the active compound might be Marmesin, a linear dihydrofuranocoumarin. The literature sited confirmed that furanocoumarins have medicinal properties and antifungal activity.

As Marmelosin is the active compound of *Aegle marmelos* and it has been already explored more in literature, our study concentrates mainly on the Marmesin produced by *Naringi crenulata*.

In conclusion, our results revealed that Marmesin from the leaf extract of *Naringi crenulata* possessed a broad-spectrum of antibacterial activity against a panel of bacteria responsible for the most common bacterial diseases. Hence this compound can be used for the treatment of various diseases.

8.4 Histological studies of the mother plant and *in vitro* propagated plantlets of *Naringi crenulata* and *Aegle marmelos*

The present experiment was conducted to study the histological variations that occurred between the mother and micropropagated plants of *Naringi crenulata* and *Aegle marmelos*. The thin sections of leaf and stem were used for the study.

8.4.1 *Naringi crenulata*

Changes in the morphological and histological features of leaves were observed between mother and *in vitro* propagated plant of *Naringi crenulata*. The abaxial epidermis was stomatiferous in the mother plant leaves. Both the abaxial and adaxial epidermis was stomatiferous in the micropropagated plantlet leaves. In both mother and *in vitro* raised plantlet leaves the stomata were
cyclocytic with a whorl of subsidiary cells around a stoma. The in vitro leaf has developed a new potential histogenous development of the stoma on the adaxial side; in contrast to absence of stomata on the adaxial side of the mother leaf.

The stomatal frequency was 170/ mm$^2$ in the mother plant but it was just 20/ mm$^2$ in the in vitro plantlet leaf. Reduction in the stomatal frequency of in vitro plantlet leaf might be due to total reduction in the size of lamina.

The veins were thin and dense in the case of in vitro plantlet leaf because there was a general reduction in the differentiation of vascular bundle. The venation was dense because the lamina expands to limited extent. Absence of secretory cavities in the in vitro plantlet leaves might be due to absence of some specific auxins responsible for differentiation of secretory cavities.

Calcium oxalate crystals were abundant in the stem of the mother plant when compared to the in vitro plant.

8.4.2 Aegle marmelos

The morphological and histological features of leaf and stem of Aegle marmelos in vitro plantlets showed variation with their mother plant. The thickness of the midrib was lesser in the in vitro raised plantlet leaf (550µm) than the mother paint leaf (90µm). This reduction in thickness is due to the lack of some growth hormones naturally available in the in vitro condition.

The vascular system consisted of a wide boat shaped main bundle and two top shaped adaxial accessory bundles in the in vivo leaf. In the in vitro plantlet leaf, the vascular system consisted of a broad thick band of main strand and a small less prominent adaxial strand.

The abaxial epidermises of both in vitro and mother leaves were stomatiferous. Apart from the normal stomata, the in vitro leaf had “giant stomata”. It may be due to abnormal accumulation of growth factors in the in vitro plantlet leaf. There was a variation in the stomatal frequency of mother plant (85/mm$^2$) and in the in vitro (50/ mm$^2$) leaf. This reduction of stomatal number in the in vitro leaf was due to reduction in the size of the lamina.
Calcium oxalate crystals were localized with veins in the *in vivo* plant and were prismatic type.

The stem of the mother plant was slightly angular in outline and roughly circular in the *in vitro* plant. A thin epidermal layer of small squarish cells with thick cuticle was observed in both stem sections. The secretory canal cells were seen in the cortex. The vascular cylinder was hollow enclosing a wide pith.

The histological studies of the *in vitro* and mother plant leaves; stem of *Naringi crenulata* and *Aegle marmelos*, revealed no much variations. Therefore, *in vitro* propagation via nodal explants is the best method in producing plants identical to the mother plant in their vegetative specifications, than propagation via somatic embryogenesis, because the method employed in the present study does not pass the callus stage. It is known that passing the callus stage enhances the occurrence of chromosomal mutation related to its number and structure.