Materials and Methods
MATERIALS AND METHODS

3.0 MATERIALS

3.1 Plant species

Pimpinella tirupatiensis Bal and Sutr (1960).

3.2 Chemicals and Glassware

<table>
<thead>
<tr>
<th>Chemical</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>Taq Polymerase</td>
<td>Strategens</td>
</tr>
<tr>
<td>Primers</td>
<td>Operon tech, USA</td>
</tr>
<tr>
<td>DNA Marker</td>
<td>Bangalore Genei, India.</td>
</tr>
<tr>
<td>Ethidium bromide</td>
<td>Bangalore Genei, India.</td>
</tr>
<tr>
<td>Tween-20</td>
<td>Glaxo India (P) Ltd., Mumbai, India.</td>
</tr>
<tr>
<td>Mercuric Chloride (Hg Cl₂)</td>
<td>E.Merck (P) Ltd., Mumbai, India.</td>
</tr>
<tr>
<td>Plant Growth Regulators</td>
<td>Sigma Chem.Co., U.S.A.</td>
</tr>
<tr>
<td>Macro-Micro salts</td>
<td>Hi-media, Mumbai, India.</td>
</tr>
<tr>
<td>Sucrose</td>
<td>S.D.fine chem.Ltd., India.</td>
</tr>
<tr>
<td>Agar</td>
<td>CDH (P) Ltd., Mumbai, India.</td>
</tr>
<tr>
<td>Phytagel</td>
<td>Sigma Chem. Co., USA</td>
</tr>
</tbody>
</table>
3.2.b Equipment

- Electrophoresis system: Broviga, Balaji Scientific Services, Madras, India.
- pH meter: Elico, India.
- Weighing Balance: Dona Instruments (P) Ltd., Calcutta, India.
- Fluorescent Tubes: Philips India (P) Ltd., Mumbai, India.
- Stereo microscope: Getner (P) Ltd., India.
- Phase contrast microscope: NIKON.
- PCR thermocycler: Perkin Elmer, (480 series) U.K.
- Gel documentation system: Amersham Pharmacia - Biotech. Ltd., USA.
- Atomic Absorption Spectrophotometer: Perkin Elmer 2280

3.3 MICROPROPAGATION

3.3.a Source of explants

A limited number of seedlings of *P.tirupatiensis* brought from Tirumala hills to the botanical garden of S.V.University were maintained for collecting shoot tip and nodal explants. For certain experiments explants were also directly collected from the hills and immediately used for raising cultures. Adequate care was taken throughout the study to reduce damage to the populations and its viability during explant collection. Essentially, explant collection was made less frequently and as far as possible with necessary conservation precautions.
3.3.b Surface sterilization

(i) Seed explants: Seeds collected during March - September, were washed in running tap water for 10 min and surface sterilized using 0.1% HgCl₂ (5 min) followed by several rinses with sterile double distilled water.

(ii) Axillary buds: Axillary buds explants were surface sterilized by initially washing in tap water followed by a rinse in 1% Tween 20 for 5 min. Explants were further disinfected with 0.05% HgCl₂ solution for 5 min. After a quick rinse in 70% ethanol (30 sec) they were washed 5-6 times in sterile double distilled water prior to placing them vertically on culture initiation medium in 25x150 mm culture tubes.

(iii) Tuber explants: Due to subterranean nature, tubers required more care to reduce both the endogenous as well as exogenous sources of contamination. A limited number of tubers collected and used in the present study were disinfected using 0.02% HgCl₂ solution for 10 mins followed by several rinses in sterile double distilled water.

3.3.c Preparation of Media

Analytical grade (AR) chemicals and sterile, double distilled water were used to prepare stock solutions and media. Stock solutions of macronutrients (40x), micronutrients (100x), vitamins and amino acids (100x), were prepared by dissolving the constituents individually in a 500 ml flask over a magnetic stirrer. The stock solutions of Iron was prepared by dissolving FeSO₄ 7H₂O and Na₂ EDTA separately and then both the solutions were mixed in an amber coloured bottle and stored until use. All the stock solutions were stored at 4°C in dark. Stocks of growth regulators and other growth adjuvants were prepared afresh.
Table 1: Nutritional components of various media used in the present study

<table>
<thead>
<tr>
<th>Components</th>
<th>Murashige and skoog's medium (1962) (mg/l)</th>
<th>White's medium (1954) (mg/l)</th>
<th>Blaydes medium (1966) (mg/l)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Macronutrients</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>KNO₃</td>
<td>1900</td>
<td>80</td>
<td>1000</td>
</tr>
<tr>
<td>NH₄NO₃</td>
<td>1650</td>
<td>-</td>
<td>1000</td>
</tr>
<tr>
<td>Ca(NO₃)₂ 4H₂O</td>
<td>-</td>
<td>300</td>
<td>499</td>
</tr>
<tr>
<td>CaCl₂ 2H₂O</td>
<td>440</td>
<td></td>
<td></td>
</tr>
<tr>
<td>KH₂PO₄</td>
<td>170</td>
<td>-</td>
<td>300</td>
</tr>
<tr>
<td>NaH₂PO₄ 2H₂O</td>
<td>19</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(NH₄)₂SO₄</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>MgSO₄ 7H₂O</td>
<td>370</td>
<td>750</td>
<td></td>
</tr>
<tr>
<td><strong>Micronutrients</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MnSO₄ 4H₂O</td>
<td>22.3</td>
<td>5</td>
<td>4.4</td>
</tr>
<tr>
<td>ZnSO₄ 7H₂O</td>
<td>8.6</td>
<td>3.0</td>
<td>1.5</td>
</tr>
<tr>
<td>CuSO₄ 5H₂O</td>
<td>0.025</td>
<td>0.01</td>
<td>-</td>
</tr>
<tr>
<td>H₃BO₃</td>
<td>6.2</td>
<td>1.5</td>
<td>1.5</td>
</tr>
<tr>
<td>KI</td>
<td>0.83</td>
<td>0.75</td>
<td>0.8</td>
</tr>
<tr>
<td>Na₂SO₄</td>
<td></td>
<td></td>
<td>200</td>
</tr>
<tr>
<td>Na₂MoO₄ 2H₂O</td>
<td>0.25</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CoCl₂ 6H₂O</td>
<td>0.025</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Iron Source</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>FeSO₄ 7H₂O</td>
<td>27.8</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Na₂EDTA H₂O</td>
<td>37.3</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Vitamins</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Thiamine HCl</td>
<td>0.5</td>
<td>0.01</td>
<td>0.1</td>
</tr>
<tr>
<td>Pyridoxine HCl</td>
<td>0.5</td>
<td>0.01</td>
<td>0.1</td>
</tr>
<tr>
<td>Niacin acid</td>
<td>0.5</td>
<td>0.05</td>
<td>0.5</td>
</tr>
<tr>
<td>Glycine</td>
<td>2.0</td>
<td>3</td>
<td>2.0</td>
</tr>
<tr>
<td>αy-inositol</td>
<td>100.0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Casein hydrolysate</td>
<td></td>
<td>-</td>
<td>15.0</td>
</tr>
<tr>
<td>Mannitol</td>
<td>1</td>
<td></td>
<td>10.0g</td>
</tr>
<tr>
<td><strong>Carbohydrate Source</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sucrose</td>
<td>30 gml⁻¹</td>
<td>20 gml⁻¹</td>
<td>30 gml⁻¹</td>
</tr>
<tr>
<td><strong>Solidifying Agent</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Agar</td>
<td>8 gml⁻¹</td>
<td>8 gml⁻¹</td>
<td>8 gml⁻¹</td>
</tr>
</tbody>
</table>
For the preparation of media, the stock solutions were added to sterile double distilled water or molten agar and the final volume was made up with double distilled water (Fig.2). Unless mentioned otherwise, the pH of the medium was adjusted to 5.8 with 0.1 N HCl or 0.1 N NaOH, prior to adding agar and autoclaving at 121°C under 1.06 Kg cm² pressure for 15 min. However, heat liable substances such as ABA, GA₃ and antibiotics etc. were filter sterilized and added to the autoclaved medium after cooling to 50°C - 55°C.

3.4. NUTRITIONAL ANALYSIS OF THE MATURE TUBER

In the present investigation the nutritive value of the tubers were analysed including some important minerals and proximate principle. The tubers were washed and dried at room temperature (25°C ± 1°C) and then ground to a fine powder. The flour was chemically analysed and ash was determined according to the methods recommended by AACC (1962).

3.4.b Mineral analysis

The mineral constituents in the *P. tirupatiensis* tuber were analysed using an Atomic Absorption Spectrophotometer (Perkin-Elmer Model 2280). About 5g of flour was taken in a porcelain container, ignited and ashed on the muffle furnace (Gallenkamp Hot Spot) at about 700-750°C for 15 hrs. The percentage of total ash was calculated after the ignition.

3.4.c Fat analysis

Dietary crude fibre was determined gravimetrically after ethyl ether extraction in a soxhlet apparatus as described in the Association of Official Analytical Chemists (1980).

3.5 MATURE EXPLANTS

3.5a. Induction of callus

Various explants like leaves, petioles, tubers and internodes collected from mature plantlets were surface sterilized and inoculated on MS media containing different concentrations of the following auxins (2,4-D, IAA, NAA and IBA). Data on frequency of callusing, nature and colour of the callus derived from different mature explants were determined at the end of 6 weeks of culture.
3.5.b Morphogenetic studies on callus derived from mature explants

For morphogenetic studies from various callus cultures the response of different calli derived from mature leaves, nodal segments, petiole and tuber segments were evaluated. MS medium fortified with various concentrations of BA and NAA were used for subculturing 200 mg FW of callus derived from each explant. At the end of 8 weeks of culture, data on frequency of shoot organogenesis and mean shoot number were monitored.

3.5.c Culture initiation from axillary bud and shoot tip explants

Experiments initially done with various combinations of BA+NAA along with MS medium were used to screen the best explant and optimal growth regulator concentration for shoot multiplication from the field grown plants. Further shoot multiplication was done on MS medium fortified with various concentrations and combinations of auxins, (NAA, IAA and IBA) and cytokinins (BA, Kn and TDZ). Explants were implanted vertically on medium solidified with 0.8% agar or 0.23% phytagel.

To study the effect of culture vessels on shoot multiplication 250ml culture bottles with polypropylene caps were used at shoot multiplication stage. Regeneration potential of various in vitro explants like shoot tips (0.3 - 0.5 cm), axillary buds (all the subtending nodes below the shoot tips) (0.5 –1.0 cm) and flower buds (1.0 - 2.0 cm) were evaluated. Unless, otherwise mentioned all the explants were implanted vertically and a minimum of 20 replicates were maintained and all the experiments were repeated at least thrice. For all experiments, control was maintained as MS medium devoid of growth regulators.

3.5.d Subculture of multiple shoots

Subculturing was done at the log phase of growth for callus and shoot proliferation. Individual shoot rosettes were either dissected or separated and transferred on to fresh medium.

3.5.e Effect of position of axillary bud on shoot organogenesis

The axillary bud explants excised from various nodes starting from the crown of the nature plants proceeding towards the flower bud (numbered as node 1 - node 6) were used to
Fig-2. Preparation of MS stock solutions and volumes taken from the stocks to prepare the medium (Dodds and Roberts, 1982)

**STOCK SOLUTIONS**

<table>
<thead>
<tr>
<th>Micronutrients (× 100)</th>
<th>Weights for 100 ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>MnSO₄.4H₂O</td>
<td>2230 mg</td>
</tr>
<tr>
<td>ZnSO₄.7H₂O</td>
<td>860 mg</td>
</tr>
<tr>
<td>H₃BO₃</td>
<td>620 mg</td>
</tr>
<tr>
<td>KI</td>
<td>83 mg</td>
</tr>
<tr>
<td>Na₂MoO₄.2H₂O</td>
<td>25 mg</td>
</tr>
<tr>
<td>CuSO₄.5H₂O</td>
<td>2.5 mg</td>
</tr>
<tr>
<td>CoCl₂.6H₂O</td>
<td>2.5 mg</td>
</tr>
</tbody>
</table>

**Iron-EDTA (× 100)**

<table>
<thead>
<tr>
<th>Weights for 100 ml stock solution</th>
</tr>
</thead>
<tbody>
<tr>
<td>FeSO₄.7H₂O</td>
</tr>
<tr>
<td>Na₂EDTA.2H₂O</td>
</tr>
</tbody>
</table>

**Cytokinin Stock Solution** *(Authors suggest × 100)*

**Vitamin Stock (× 100)**

<table>
<thead>
<tr>
<th>Weights for 100 ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glycine</td>
</tr>
<tr>
<td>Nicotinic acid</td>
</tr>
<tr>
<td>Pyridoxine-HCl</td>
</tr>
<tr>
<td>Thiamine-HCl</td>
</tr>
</tbody>
</table>

**DIRECT OPERATIONS**

To make one litre
Weigh out directly:

- NH₄NO₃ 1650 mg/l
- KNO₃ 1900
- MgSO₄.7H₂O 370
- KH₂PO₄ 170
- CaCl₂.2H₂O 440

To make one litre, dissolve the quantities above in ca. 400 ml DDW

Add 10 ml microelement stock solution

Add 5 ml of iron stock solution

Weigh and dissolve 100 mg myo-inositol

Add aliquot of cytokinin stock

Weigh out auxin*, dissolve and add to medium directly

Make up to 800 ml with DDW

Adjust pH to 5.7-5.8

Make up to 1 litre with DDW

Store in refrigerator if necessary

Divide into 2 lots of 100 ml and place each in 250 ml flask

Add 3 g sucrose and 0.8 g agar** to each 100 ml flask

AUTOCLAVE

Cool to 50°C and add 1 ml filter-sterilised vitamins to each 100 ml flask

Dispense to pre-sterilized vessels

* = Included as required at various levels
DDW = Double distilled water
3.5.b Morphogenetic studies on callus derived from mature explants

For morphogenetic studies from various callus cultures the response of different calli derived from mature leaves, nodal segments, petiole and tuber segments were evaluated. MS medium fortified with various concentrations of BA and NAA were used for subculturing 200 mg FW of callus derived from each explant. At the end of 8 weeks of culture data on frequency of shoot organogenesis and mean shoot number were monitored.

3.5.c Culture initiation from axillary bud and shoot tip explants

Experiments initially done with various combinations of BA+NAA along with MS medium were used to screen the best explant and optimal growth regulator concentration for shoot multiplication by using explants derived from the field grown plants. Further shoot multiplication was done on MS medium fortified with various concentrations and combinations of auxins, (NAA, IAA and IBA) and cytokinins (BA, Kn and TDZ). Explants were implanted vertically on medium solidified with 0.8% agar or 0.23% phytgel.

To study the effect of culture vessels on shoot multiplication 250ml culture bottles with polypropylene caps were used at shoot multiplication stage. Regeneration potential of various in vitro explants like shoot tips (0.3 -0.5 cm), axillary buds (all the subtending nodes below the shoot tips) (0.5 -1.0cm) and flower buds (1.0 - 2.0 cm) were evaluated. Unless, otherwise mentioned all the explants were implanted vertically and a minimum of 20 replicates were maintained involved in each experiment and all the experiments were repeated at least thrice. For all experiments, control was maintained as MS medium devoid of growth regulators.

3.5.d Subculture of multiple shoots

Subculturing was done at the log phase of growth curve for callus and shoot proliferation. Individual shoot rosettes were either dissected or separated and transferred on to fresh medium.

3.5.e Effect of position of axillary bud on shoot organogenesis

The axillary explants excised from various nodes starting from the crown of the mature plants proceeding to the flower bud (numbered as node 1 - node 6) were used to
study the effect of their position in the plant shoot regeneration capacity. Explants were surface sterilized and inoculated onto MS medium containing BA 1.0 mg/l and NAA 0.1 mg/l. For each treatment 8 explants were used and the experiment was repeated twice. Data on frequency of shoot organogenesis, mean shoot number and mean shoot length were calculated at the end of 4 weeks of culture period.

3.6 IN VITRO FLOWERING

Multiple shoots derived from hypocotyl callus through indirect regeneration were transferred onto MS medium supplemented with various concentrations of BA, NAA and GA3, 30.0 g/l sucrose and 8.0 g/l agar. After 4 weeks of culture, plantlets were transferred to fresh medium containing the same composition of growth regulators. Following two consecutive passages, data on number of plantlets with initiation and opening of flowers were collected. Number of flowers forming seeds in the culture were collected after 35 days of 2nd passage on the same medium. Each treatment comprised of 20 plantlets and the experiment was repeated thrice.

3.7 ROOTING OF IN VITRO MULTIPLIED SHOOTS

Rooting of the shoots was attempted, both ex vitro and in vitro conditions. For ex vitro rooting, the entire clusters of multiple shoots obtained at the end of four week cycle of shoot multiplication were used. Shoots measuring 3.0 - 3.5 cm were planted in 1:1 (w/w) peat moss and soilrite mixture moistened with inorganic nutrients of MS basal medium. In vitro, shoots were either individually separated or excised from the clumps and transferred to medium containing various auxins (IAA, IBA and NAA) with White's media. Data on frequency of rooting, number of roots, mean root length and degree of callusing were collected at the end of 6 weeks of culture.

3.8 JUVENILE EXPLANTS

3.8.a In vitro seed germination

For seed germination in vitro, fully mature seeds were collected and surface sterilized by immersing in 0.1% HgCl2 for 10 min and rinsing with sterile distilled water for 5-6 times. Disinfected seeds were aseptically placed on to MS media supplemented with BA (0.1-2.0 mg/l) or GA3 (0.5-2.0 mg/l).
3.8.b In vitro Tuberization

Aseptic seedlings germinated on MS medium containing GA$_3$ were transferred to MS medium containing BA 1.0 mg l$^{-1}$ and NAA 0.5 mg l$^{-1}$ with different concentrations of sucrose (10, 30, 50 and 100 g l$^{-1}$). All cultures were incubated under 16/8 hrs light and dark photo period. The number of tubers formed and the mean diameter of the tubers were noted after 9 weeks of culture.

3.8.c Induction of callus from aseptic seedlings

Leaf, internode and hypocotyl segments excised from 3 weeks old aseptic seedlings were cultured on MS medium supplemented with different concentrations of BA and NAA. Frequency (%) of callusing, degree of callusing and the nature of the callus obtained from various explants were monitored at the end of 6 weeks of culture.

3.8.d Morphogenetic studies on callus derived from various aseptic seedling explants

To induce morphogenesis from callus derived from various aseptic seedling explants (leaf and hypocotyl segments), MS medium containing various cytokinins (BA, 2-iP and TDZ) and auxins (NAA, IAA and IBA) in different combinations were used. Data on frequency of shoot organogenesis, mean number of shoots and mean shoot length were noted after 5 weeks of culture.

3.9 SOMATIC EMBRYOGENESIS

3.9.a Induction of Somatic Embryogenesis

Friable, dark pink regions (200 mg FW) of the proliferating hypocotyl callus obtained on MS medium containing TDZ (0.1 - 2.0 mg l$^{-1}$) and NAA (0.5 mg l$^{-1}$) were transferred on to MS medium with different combinations of TDZ, BA and 2,4-D. Data on frequency of embryogenic callus formation, mean number of mature somatic embryos per callus segment and the nature of the callus were determined at the end of 8 weeks of culture.
3.9.b Maturation of Somatic Embryos

All maturation experiments were carried out with one embryogenic line. Ten days after subculture on the embryo induction medium, pieces of actively growing PEM (Pro Embryogenic Mass) were transferred onto MS medium with 0.8% w/v agar containing BA with GA₃, TDZ with GA₃ and ABA in various concentrations.

The proembryogenic mass cultures were placed on 30 ml of agar solidified MS medium in 90 mm petri dishes. Cultures were initiated in a growth chamber at 25°C with a continuous photoperiod at a light intensity of 25 μmol m⁻² S⁻¹ from cool-white fluorescent lamps. ABA was filter sterilized and added to the medium after autoclaving. Number of somatic embryos were counted after 6 weeks of culture. The somatic embryo is a rudimentary plant and considered as matured embryo only when it had an embryonic root and shoot and contained the first leaves and primordia.

3.10 Encapsulation and low temperature storage of shoot buds

3.10.a Encapsulation of shoots

For encapsulation of shoots, in vitro grown multiple shoots measuring (0.5 - 3.0 mm) comprising the meristematic dome with one or two pairs of foliar primordia were isolated under a stereo microscope from multiple shoot cultures 6 weeks after their last transfer. The shoots were blotted for 1 min on sterile filter paper and incubated for 2 min in 2% (w/v) sodium alginate solution prepared in MS medium. Each shoot with approx. 0.09 ml sodium alginate was then dropped into a solution of calcium chloride (50 mM) and kept on a gyratory shaker (75 rpm) for 3 mins to accomplish encapsulation. Calcium alginate capsules containing axillary buds were collected after 30 min and rinsed 2-3 times in autoclaved water to remove traces of calcium chloride.

3.10.b Low temperature storage of encapsulated shoot buds

A set of encapsulated buds were stored in a laboratory refrigerator at 4°C for 30, 60 and 90 days and data on the percentage shoot development was collected at 30 days intervals upto 90 days in initiation medium. A set of un encapsulated buds were stored under above conditions to serve as a control.
3.10.3 Regeneration of encapsulated shoot buds

Encapsulated cold stored (at 4°C) buds were dehydrated in front of laminar air flow chamber for 5 hrs and transferred aseptically on semisolid MS medium containing BA 1.0 mg/l and NAA 0.1 mg/l for recovery of plantlets. The initiation medium was also supplemented with 500 mg/l activated charcoal. Shoot regrowth was expressed as the percentage of the number of shoot tips that resumed growth and formed new shoots and roots after cold storage of different duration.

3.11 Acclimatization

Rooted plantlets were removed from the medium and washed with sterile water prior to transferring them on to potting mix containing soil and vermiculite (1:1) and covered with Polythene sheets which was gradually removed during acclimatization and field transfer. The survival of the plants at each condition was assessed periodically. Established plantlets were maintained in the same potting medium for a period of 3-4 months and then replanted in fresh potting mixture of the same composition in 15 cm pots and kept under 50% shade for further growth.

3.12 Culture Conditions

All cultures aseptic seedlings, callus cultures, somatic embryos and plantlets were maintained at 25 ± 2°C under 16 hrs photoperiod (1900-2000 Lux) provided by cool white fluorescent lamps (Phillips, India). Seed cultures were terminated at the end of 3 weeks. In all other instances, a culture was maintained in the same vial for a maximum of 4 weeks. Depending on the experiment the parent culture was either transferred or subcultured every four weeks. Through periodic transfer/subcultures a culture was maintained for a total period of 8,12, or 16 weeks.
The following corrections were included in the thesis:

1. Fig. 1  The table was modified removing the % symbol.
2. Page 5  The number of red listed species was corrected as 814 from 620.
3. Page 11  Line 12 from bottom was modified as documented ‘by’
4. Page 13  line 4 from the top and 8 from bottom. The first letters of the species Chrysanthemum X Marifolium were corrected into capitals.
5. Page 21: Spellings of Pyridoxine HCL and casein hydrolysate have been corrected. mg/l was put forth to the 2nd, 3rd and 4th column.
6. Page 27: Line 11 from the bottom reported was corrected as replanted.
7. Page 28: Table 2 was deleted.
8. Page 31: Line 4 from bottom sentence corrected as 20 replicates ‘were’ used.
9. Page 32: 6th line from the bottom Nei and Li(1979) the author name was correct.
10. Page 34: Table 4.2 the standard error was correctly entered.
11. Plate V to XX Magnification and bars have been mentioned in all photographs.
12. Page XX  The Journal name was corrected as Plant Cell Rep.
13. Titles have been included for the following references, Sharma and Chandel, Sharma et al., Valobra and James, Tandon and Rathore, Sulaiman and babu, Cordell et al.,
14. Page 3 & xii  Martin and Rosanne Teukaberry was corrected as Kellman & Teukaberry.
15. Page 9: Heywood et al was corrected as Heywood and Wyse Jackson.
16. Page 12: Bira was corrected as Birah.
17. Page 13: Alstoemeria was corrected as Alstoemeria.
18. Page 13 & iii: Michael E Compton was corrected as Compton, M.E.,
19. Page 16  William Collings was corrected as William and Collins.
   P. brachycarpa was corrected as Pimpinella brachycarpa.
   Earnst et al 1984 was changed Ernst and Oesterhelt.
   Stephen and Jayabalain 1997 was corrected to 1998.
20. Page 55: Ivana is corrected as Ivano
21. Page 56: Arrilaga et al., was corrected as Arrilaga and Merkle.
26. The following missing references have been included in the respective pages.
   Page 11: Constable, 1990
   Page 16: Jha 1980, Moon and Lee 1993 was corrected as Moon and Lee 1994
   Steward et al 1958, Kudielka and Theimer, 1983
   Page 55: Philip et al., 1992
27. Page xvii  Rajasthan was corrected as Rajasthan.
28. Page 5  Tumbaggia was corrected as Tumbaggaia
29. Page xix  Vedavathi was corrected as Vedavathy
30. Page 8  Taxa was corrected as taxon
31. Page 12 & xiv  Phyllanthus was corrected as Phyllanthus
32. Page x  Roufia was corrected as Rouvofia
33. Fig. 4 & 5  X-axis was changed as Concentration of Growth regulators “as represented in Fig.4 page 35”.
34. Fig 6.  X-axis was mentioned as ‘Position of node from the Crown’. 
3.12 Genetic Diversity Study

3.12.a Sample Collection

Young leaves from eleven individuals widely separated in each location were used in this study. Fully expanded leaves were harvested, packed in polybags and transferred immediately to an ice box containing dry ice and transported to M S Swaminathan Research Foundation, Chennai for DNA isolation. Samples were washed in ice cold distilled water to remove traces of all external particles on the leaf surface. After blotting with tissue paper samples were packed in polybags and stored in deep freezer (-80°C) until DNA extraction.

3.12.b DNA isolation

Genomic DNA of individual plants was isolated from leaf tissue following Doyle and Doyle (1987) with little modifications. Five grams of leaf tissue was weighed and ground to a fine powder in liquid nitrogen using mortar and pestle. Before allowing the tissue to thaw, the powder was gently dispersed in 20 ml of extraction buffer (containing 50 mM EDTA, 100 mM Tris HCl, 0.8 M NaCl, 0.5M Sucrose, 2% Triton X-100, (2%), 2-Mercaptoethanol in a 100 ml beaker. The contents were stirred till the powder got dispersed in the solution. After filtering the homogenate through a cheese cloth in to a 50 ml centrifuge tube it was incubated at 60°C for 30 min. The homogenate was centrifuged at 10,000 rpm for 10 min at 20°C and this step was repeated 3-4 times by suspending the pellet in fresh buffer (with incubation at 65°C for 15 min) so as to remove the cytoplasmic contents indicated by the pellet turning almost pale yellow/white in colour.

The pellet containing nuclei was resuspended in extraction buffer (250 mM EDTA, 100mM Tris HCl, 1.4 M NaCl, 2% Cetyl Trimethyl Ammonium Bromide (CTAB) and incubated at 60°C for 30 min. The nuclear homogenate was extracted with 20ml Chloroform : Isoamyl alcohol (24 : 1 V/V), and after centrifugation at 10,000 rpm and 25°C (for 10 min) the aqueous phase containing the DNA was removed with a large bore pipette to a clean 50 ml centrifuge tube. About 3/4th volume of isopropanol was added to aqueous phase and stored at freezer overnight. The contents were later centrifuged at 10,000 rpm for
10 min and supernatant was decanted. The pellet was dissolved in 400μl TE (Tris 10mM pH 8.0 + EDTA 1mM-250mM) and 200μl of Phenol and 200 μl chloroform were added and centrifuged at 10,000 rpm for 10 min. The aqueous phase was mixed with equal volumes of Chloroform and again centrifuged at 10,000 rpm for 10 min. With the aqueous phase 50μl of sodium acetate (3 M, pH 5.2) and 1 ml of freezer stored ethanol were added and again centrifuged at 10,000 rpm for 10 min. The pellet was dissolved in 1 ml of 70% ethanol and centrifuged at 10,000 rpm for 5 min and the final pellet was dissolved in 100μl TE after decanting ethanol and air drying the pellet.

3.12.c Quantification of DNA

DNA obtained after RNAase treatment and phenol chloroform extraction were quantified by agarose gel electrophoresis using 100 ng of lambda Hind III digest DNA. Standard 8μl of the DNA solution was loaded in an agarose gel (1.4%) and electrophoresis was carried out in TBE buffer at 50 mA current for 90 min and observed on a UV transilluminator. The DNA concentration was determined by taking OD (Optical Density) values at 260/280 nm.

3.12.d Primers

Random oligonucleotide primers (10 mers) were obtained from Operon Tech Inc. (USA) and the sequence of the primers used were given in Table- 15. Totally 20 primers were used in the present study which gave reproducible amplification products.

3.12.e Random Amplified Polymorphic DNA (RAPD) Analysis

The protocol was essentially the same (Williams et al., 1999) except for the composition of the extraction buffer (100 mM Tris-HCl, 1.4M NaCl, 20mM Na2 EDTA, 2% CTAB and 2% 2-Mercaptoethanol) and 1:3 ratio of tissue to extraction buffer was used for DNA isolation. To study RAPD, genomic DNA was amplified by Polymerase Chain Reaction (PCR) with 10 mer random Oligonucleotide primers (Operon Tech, CA, USA). Totally
twenty primers were used to amplify the DNA from 22 genotypes. A typical 25 μl reaction mixture consisted of 2.5 μl of 10 x assay buffer (100 mM Tris - HCl [pH 8.3], 500 mM KCl and 0.01% Gelatin) 2mM MgCl₂. 100μM of each dNTPs (ATP, GTP, CTP and TTP,) 15 ng of primer and 1.0 , μl of Taq DNA polymerase enzyme and DNA. The concentration of DNA (1 μg) was maintained same in all experiments.

The reaction mixture was overlaid with equal volumes of mineral oil, and DNA amplification was performed in a Perkin Elmer DNA Thermal Cycler 480 series. The amplification condition included a total of 45 cycles with 1 min (3 min for the first cycle) at 94°C for template denaturation, 1 min at 37°C for primer annealing and 2 min (10 min for the final cycle) at 72°C for primer extension. The amplification products were analyzed on 1.4 % agarose gels. RAPD analyses of the same genotypes were repeated twice to confirm the observed banding pattern. As a negative control, a tube with out target DNA but with all other contents were included in each set of experiments.

3.12.f Agarose Gel Electrophoresis

DNA amplification products were separated in 1.4 % agarose gel. Electrophoresis was carried out in 1 X TBE at 50 mA until the Bromophenol blue dye front reached the end of the gel. Ethedium bromide (0.5 μg ml⁻¹ was added to the gel. Standard markers were loaded in one lane (100 ng Lambda Hind III digest (NIL). The gels were visualized through UV-transilluminator and photographed with either Polaroid film (type 667) or documented in the gel documentation system (Amersham Pharmacia, USA).

3.12.g Data Observation and statistical analysis

Despite scarcity and limitations encountered with the plant material, for most of the treatments a minimum of 20 replicates were used. Nevertheless, experiments were performed with larger replicates, wherever possible. All the experiments were repeated at least twice and the cultures were observed at regular intervals. Unless otherwise mentioned, the values for various growth parameters were recorded only at the end of incubation period. After
probing the data from all the cultures in the treatment, the values for different characters were calculated which have been presented in the form of tables and graphs. The quantitative data were subjected to statistical analysis. Mean and Standard error of means (SE) were calculated for Shoot length, rate of shoot multiplication and the number of roots per shoot. The standard error of the mean was presented as \( \pm \), while the intensity of few responses such as degree of callusing was indicated by the number of (+) and absence thereof by (-) signs.

R4PD data

The polymorphic loci were determined using the electrophoretic migration of the fragments in R4PD. The DNA profiles were scored manually, directly from photographs of the gels, by assigning a value of 1 for band presence and 0 for band absence. The scores of the presence or absence of a band rather than difference in intensity among bands was calculated by a pair wise genetic distance matrix using the formula of Nei and Li (1979). Similarity index in all pair wise combinations was calculated as 

\[ 2m_{xy} / (m_x + m_y) \]

where \( m_{xy} \) was the member of fragments shared by two species and \( m_x \) and \( m_y \) were the member of fragments in each species. Finally, a dendrogram based on Un Weighted Pair Group with arithmetic Mean Average (UPGMA) method (Sneath and Shokal, 1973) was prepared to provide an overview of genetic relationships between the plants analyzed.