Plant cell, tissue and organ culture studies involve the science of growing and manipulating plant cells, tissues and organs \textit{in vitro} on artificial medium which supplies the nutrients necessary for plant growth under controlled and aseptic conditions. The success of plant tissue culture as a means of plant propagation is greatly influenced by the nature of the culture medium used and conditions maintained during culture period. It includes materials and methods appropriate to research for several practical objectives.

**MATERIALS**

(i) PLANT SPECIES

\textit{Ceropegia elegans} Wall.

\textit{Ceropegia juncea} Roxb.

(ii) CHEMICALS

The chemicals used in the study were of analytical grade. Inorganic salts were supplied from Hi Media, Merck, S.D. fine and Qualigens chemicals, India. All vitamins, plant growth regulators were supplied from Sigma-Aldrich Chemical Co., USA. Casein hydrolysate, yeast extract, citric acid, PVP, activated charcoal was purchased from HiMedia Laboratories, India.

(iii) SOLUTIONS

Hydrochloric acid (HCl), Hydrogen peroxide (H\textsubscript{2}O\textsubscript{2}) etc.

(iv) GLASSWARE

Test tubes (2.5×15 cm), Petri plates (55mm and 85mm diameter), Conical flasks (100, 250, 500, 1000 ml capacity), Erlenmeyer Beakers (100, 250, 500, 1000ml capacity), DURAN Bottle (100, 250, 500, 1000 ml capacity), pipettes (1, 2, 5, 10 ml capacity) and measuring cylinders of all capacities (10ml-1000ml) were purchased from “Borosil” India. In addition glass screw capped culture bottles (6×15 cm) were used for some experiments.
Micropipettes (2-200\mu l, 100-1000\mu l and 500-5000\mu l) were obtained from Eppendorf, Microp tips (2-200\mu l, 100-1000\mu l and 500-5000\mu l) were obtained from “Sigma Chemical Co” (USA) and plastic beakers of various capacities (50ml, 100ml, 250ml, 500ml and 1000ml) were obtained from “Borosil” India.

METHODS

(I) CLEANING AND STORAGE OF GLASSWARE:

Cleaning of glassware is the first and important step in in vitro studies because glassware is also one of the sources for contamination. The glassware used in tissue culture experiments were measuring jars, volumetric flasks, beakers, test tubes (25 x 150 mm), petri plates, pipettes, Erlenmeyer flasks, glass rods etc. Required glassware was soaked overnight in 40% chromic acid solution and then scrubbed with brush followed by rinsing under running tap water. Then they were washed with 5% Labolene solution and scrubbed with a brush in a hot detergent bath then rinsed with hot tap water until soap traces were removed. Finally all the glassware was rinsed with Millipore water and sterilized in hot air oven at 150° C for 3 hours. Discarded cultures, as well as contaminated vials without opening the closers were autoclaved at 121°C at 15psi for 20 minutes to dissolve the agar and to kill any contaminants that may be present. The culture glassware is easier to wash after the spent medium has been liquefied and removed before washing.

After cooling glassware was capped and kept in polypropylene bags then tightened with rubber bands and stored in closed racks until use to minimize the spread of bacteria and fungi in the laboratory.

(II) COLLECTION OF PLANT MATERIAL AND MAINTENANCE:

The mature fruits and plantlets with tubers of Ceropedia juncea Roxb. were collected from Kalasamudram forest in Anantapur District Andhra Pradesh during June, 2007 and Ceropedia elegans Wall. plantlets along with fasciculate tuberous roots from Sirumalai hills (Tamil Nadu), Eastern Ghats during October, 2007. The collected plants were planted in pots and grown in Sri Krishnadevaraya University Botanical Garden under greenhouse condition for Ceropedia elegans and garden conditions for Ceropedia juncea. The plants were grown under natural daylight and irrigated with water as
required. After 4 weeks of plant established in pots, young shoots started growing. Actively growing shoots were used as the explant sources for in vitro studies.

(III) MEDIA PREPARATION

The chief components of plant tissue culture medium include inorganic nutrients (macronutrients and micronutrients), organic nutrients (vitamins and amino acids), carbon source, plant growth regulators, charcoal, and gelling agent. Other organic supplements such as CH, CM, and Ascorbic acid were also added to the medium when required. All the chemicals used in media preparation were analytical grade. The hormones were Sigma make. Bacteriological grade agar was used as gelling agent. In the present investigation universal media, MS (Murashige and Skoog, 1962), B₅ (Gamborg et al., 1968) and WPM (Lloyd and Mc Cown, 1981) were used. The composition of all the above three media were represented in Table-2 and 3. Stocks were prepared for convenience.

Macronutrients

Macronutrients listed in the Table-2 were dissolved one by one. Otherwise stocks solutions were prepared in concentration of 5× or 10× depending upon the frequency of usage. A separate stock solution of calcium salts (calcium chloride or calcium nitrate) was prepared to avoid precipitation problem.

Micronutrients

For convenience inorganic and organic micronutrients were prepared at 40× concentrations separately (Table 3). Iron was supplied in chelated form. It is prepared by dissolving FeSO₄·7H₂O and Na₂EDTA·2H₂O separately in two separate clean, sterile glass beakers containing double distilled water by heating and constant stirring; finally these two solutions were mixed and made to 100 ml with sterilized Millipore water. Iron stock was stored at room temperature in amber colored bottle due to its photosensitivity and others stock solutions were stored in Erlenmeyer bottle containers at 4° C. All the stocks were used within two months. After two months old stocks were discarded and prepared freshly.

Stock Solution for Plant Growth Regulators

Auxins, cytokinins, Gibberillins were prepared separately at 0.01× concentration. 10 mg of each plant growth regulator was taken in separate clean sterile beaker and
Table – 2

Chemical composition of different nutrient media -
Macro nutrients

<table>
<thead>
<tr>
<th>Component</th>
<th>Amount (mg/l)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>MS</td>
</tr>
<tr>
<td>MgSO₄·7H₂O</td>
<td>370</td>
</tr>
<tr>
<td>KH₂PO₄, H₂O</td>
<td>170</td>
</tr>
<tr>
<td>NaH₂PO₄, H₂O</td>
<td>-</td>
</tr>
<tr>
<td>KNO₃</td>
<td>1900</td>
</tr>
<tr>
<td>NH₄NO₃</td>
<td>1650</td>
</tr>
<tr>
<td>CaCl₂·2H₂O</td>
<td>440</td>
</tr>
<tr>
<td>(NH₄)₂SO₄</td>
<td>-</td>
</tr>
<tr>
<td>Ca(NO₃)₂·4H₂O</td>
<td>-</td>
</tr>
<tr>
<td>K₂SO₄</td>
<td>-</td>
</tr>
<tr>
<td>Myo-Inositol</td>
<td>100</td>
</tr>
<tr>
<td>Sucrose</td>
<td>20g</td>
</tr>
<tr>
<td>Agar 0.9%</td>
<td>9g</td>
</tr>
<tr>
<td>Activated Charcoal</td>
<td>0.5g</td>
</tr>
</tbody>
</table>
Table - 3

Chemical composition of different media - Micronutrients - 40x concentration

<table>
<thead>
<tr>
<th>Micronutrients</th>
<th>MS mg/100ml</th>
<th>Bs mg/100ml</th>
<th>WPM mg/100ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>STOCK - I</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>H$_3$BO$_3$</td>
<td>248</td>
<td>120</td>
<td>6.2</td>
</tr>
<tr>
<td>KI</td>
<td>33.2</td>
<td>30</td>
<td>-</td>
</tr>
<tr>
<td>Na$_2$MO$_4$.H$_2$O</td>
<td>10</td>
<td>10</td>
<td>0.25</td>
</tr>
<tr>
<td>CoCl$_2$.2H$_2$O</td>
<td>1</td>
<td>400</td>
<td>-</td>
</tr>
<tr>
<td>MnSO$_4$.4H$_2$O</td>
<td>-</td>
<td>80</td>
<td>22.3</td>
</tr>
<tr>
<td>ZnSO$_4$.H$_2$O</td>
<td>-</td>
<td>1</td>
<td>8.6</td>
</tr>
<tr>
<td>CuSO$_4$.5H$_2$O</td>
<td>-</td>
<td>1</td>
<td>0.25</td>
</tr>
<tr>
<td>CoCl$_2$.6H$_2$O</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>STOCK - II</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MnSO$_4$.4H$_2$O</td>
<td>892</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>ZnSO$_4$.H$_2$O</td>
<td>344</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>CuSO$_4$.5H$_2$O</td>
<td>1</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>STOCK - III</td>
<td>STOCK - II</td>
<td>STOCK - II</td>
<td></td>
</tr>
<tr>
<td>FeSO$_4$.7H$_2$O</td>
<td>1114</td>
<td>1112</td>
<td>1112</td>
</tr>
<tr>
<td>Na$_2$ EDTA, 2H$_2$O</td>
<td>1494</td>
<td>1492</td>
<td>1492</td>
</tr>
<tr>
<td>STOCK - IV</td>
<td>STOCK - III</td>
<td>STOCK - III</td>
<td></td>
</tr>
<tr>
<td>Thiamine HCl</td>
<td>40</td>
<td>400</td>
<td>1</td>
</tr>
<tr>
<td>Pyridoxine HCl</td>
<td>40</td>
<td>40</td>
<td>0.5</td>
</tr>
<tr>
<td>Nicotinic acid</td>
<td>40</td>
<td>40</td>
<td>0.5</td>
</tr>
<tr>
<td>Glycine</td>
<td>160</td>
<td>-</td>
<td>2</td>
</tr>
</tbody>
</table>
Table - 4

Solvents used for dissolving different plant growth regulators

<table>
<thead>
<tr>
<th>Growth regulator category</th>
<th>Name of the plant growth regulator</th>
<th>Solvent</th>
</tr>
</thead>
<tbody>
<tr>
<td>Auxins</td>
<td>2,4-D</td>
<td>IN NaOH</td>
</tr>
<tr>
<td></td>
<td>2,4,5-T</td>
<td>IN NaOH</td>
</tr>
<tr>
<td></td>
<td>2,4,5-TP</td>
<td>IN NaOH</td>
</tr>
<tr>
<td></td>
<td>IAA</td>
<td>EtOH / IN NaOH</td>
</tr>
<tr>
<td></td>
<td>IBA</td>
<td>IN NaOH</td>
</tr>
<tr>
<td></td>
<td>NAA</td>
<td>IN NaOH</td>
</tr>
<tr>
<td></td>
<td>Picloram</td>
<td>IN NaOH</td>
</tr>
<tr>
<td></td>
<td>Dicamba</td>
<td>IN NaOH</td>
</tr>
<tr>
<td>Cytokinins</td>
<td>BAP</td>
<td>IN NaOH</td>
</tr>
<tr>
<td></td>
<td>Kn</td>
<td>IN NaOH</td>
</tr>
<tr>
<td></td>
<td>TDZ</td>
<td>DMSO or IN NaOH</td>
</tr>
<tr>
<td></td>
<td>2iP</td>
<td>IN NaOH</td>
</tr>
<tr>
<td></td>
<td>Zeatin</td>
<td>IN NaOH</td>
</tr>
<tr>
<td>Gibberellin</td>
<td>GA$_3$</td>
<td>IN NaOH</td>
</tr>
</tbody>
</table>
Table - 5
Table showing equivalent values in molarity for different concentrations (mg/l) of PGR generally used in plant tissue culture

<table>
<thead>
<tr>
<th>Concentration (mg/l)</th>
<th>Cytokinins (μM)</th>
<th>Auxins (μM)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>BA</td>
<td>Kinetin</td>
</tr>
<tr>
<td>0.1</td>
<td>0.44</td>
<td>0.46</td>
</tr>
<tr>
<td>0.2</td>
<td>0.89</td>
<td>0.93</td>
</tr>
<tr>
<td>0.3</td>
<td>1.33</td>
<td>1.39</td>
</tr>
<tr>
<td>0.4</td>
<td>1.78</td>
<td>1.86</td>
</tr>
<tr>
<td>0.5</td>
<td>2.22</td>
<td>2.32</td>
</tr>
<tr>
<td>0.6</td>
<td>2.66</td>
<td>2.79</td>
</tr>
<tr>
<td>0.7</td>
<td>3.11</td>
<td>3.25</td>
</tr>
<tr>
<td>0.8</td>
<td>3.55</td>
<td>3.72</td>
</tr>
<tr>
<td>0.9</td>
<td>3.99</td>
<td>4.18</td>
</tr>
<tr>
<td>1.0</td>
<td>4.40</td>
<td>4.62</td>
</tr>
<tr>
<td>2.0</td>
<td>8.90</td>
<td>9.30</td>
</tr>
<tr>
<td>5.0</td>
<td>22.20</td>
<td>23.20</td>
</tr>
<tr>
<td>10.0</td>
<td>44.40</td>
<td>46.00</td>
</tr>
<tr>
<td>MW</td>
<td>225.3</td>
<td>215.2</td>
</tr>
</tbody>
</table>
dissolved in suitable solvent (Table 4) and made to 100 ml with sterile Millipore water and they were preserved at 4°C. Hormonal stocks were added depending on the requirement in appropriate amounts to the basal medium.

**Carbon Sources**

All plant tissue culture media require the presence of carbon and energy source. Glucose, fructose, sucrose, galactose and maltose were the chief carbon sources tested in the present investigation. Out of these, sucrose was preferred for all the experiments in different concentrations depending upon the type of the *in vitro* culture.

**Growth Adjuvants**

Different organic supplements such as Casein Hydrolysate (CH), Coconut Milk (CM), Yeast Extract (YE) and antioxidants like Ascorbic acid (AA), Citric Acid (CA) and Poly Vinyl Pyrrolysate (PVP) singly or in combination added to the medium as and when required. A simple procedure for storing the coconut milk was developed. Water collected from tender coconuts was deproteinized by heating up to 80 – 100°C for about 10 minutes and filtered through Whatman filter paper. The filtered liquid extract was stored in plastic vials at -10°C in deep freezer.

**Medium Matrix / Gelling agent**

Bacteriological grade agar agar 0.8% was used in almost all experiments except germination and rooting elements where 0.6% was used.

**Activated Charcoal**

Activated Charcoal (AC) removes contaminants from agar and secondary products secreted by the cultured tissues. The type of AC used is important because the absorptive characteristics and pH are dependent on the manufacturing process. Analytical grade AC at 500 mg to 2000 mg was tested. However, in both *Ceropegia elegans* and *Ceropegia juncea* addition of AC to the medium found to be less effective in getting response form all the explants than on the medium with out charcoal. Hence all the experiments including rooting were carried without AC.

**(IV) PREPARATION OF ONE LITER OF MEDIUM**

To prepare one liter of medium, 500ml of Millipore water was taken in clean Erlenmeyer flask. Required quantities of macronutrients were added sequentially followed by addition of freshly weighed sucrose (30g) and micronutrient stock solutions
### Preparation of 1 Liter Murashige and Skoog Medium

Take 500 ml of double distilled water in 1 Liter Erlenmeyer flask

Weigh, add and dissolve the following macro salts one by one in sequence

<table>
<thead>
<tr>
<th>Micro Stock – I (40X)</th>
<th>mg/100 ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>MgSO₄.7H₂O</td>
<td>370 mg</td>
</tr>
<tr>
<td>KH₂PO₄</td>
<td>170 mg</td>
</tr>
<tr>
<td>NH₄NO₃</td>
<td>1650 mg</td>
</tr>
<tr>
<td>KNO₃</td>
<td>1900 mg</td>
</tr>
<tr>
<td>CaCl₂.2H₂O</td>
<td>440 mg</td>
</tr>
<tr>
<td>Myo inositol</td>
<td>100 mg</td>
</tr>
</tbody>
</table>

Adjust pH to 5.7

Pour medium into graduated cylinder and make up to 1 liter

Add 0.8% agar agar

Melt agar agar with intermittent shaking

Dispense the medium of 12 ml each into culture tubes or 40 ml each into culture bottle

Autoclave at 15 psi at 121°C for 15 min

Weigh, add and dissolve 3% Sucrose

Add 2.5 ml of each micro stock I, II, III & IV

Add aliquots of PGR stock of desired concentration

Add MW up to 900 ml in Erlenmeyer flask

Adjust pH to 5.7

Pour medium into graduated cylinder and make up to 1 liter

Add 0.8% agar agar

Melt agar agar with intermittent shaking

Dispense the medium of 12 ml each into culture tubes or 40 ml each into culture bottle

Autoclave at 15 psi at 121°C for 15 min

---

<table>
<thead>
<tr>
<th>Micro Stock – II (40X)</th>
<th>mg/100 ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>MnSO₄.4H₂O</td>
<td>892 mg</td>
</tr>
<tr>
<td>ZnSO₄.4H₂O</td>
<td>344 mg</td>
</tr>
<tr>
<td>CuSO₄.5H₂O</td>
<td>1 mg</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Micro Stock – III (40X)</th>
<th>mg/100 ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>FeSO₄·7H₂O</td>
<td>1114 mg</td>
</tr>
<tr>
<td>Na₂EDTA</td>
<td>1494 mg</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Micro Stock – IV (40X)</th>
<th>mg/100 ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nicotinic acid</td>
<td>20 mg</td>
</tr>
<tr>
<td>Pyridoxine</td>
<td>20 mg</td>
</tr>
<tr>
<td>Thiamine HCl</td>
<td>20 mg</td>
</tr>
<tr>
<td>Glycine</td>
<td>80 mg</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>PGR Stock (0.01%)</th>
<th>10 mg</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cytokinins or auxins or gibberellins was dissolved in suitable solvent and made up to 100 ml</td>
<td></td>
</tr>
</tbody>
</table>
of each 2.5ml were added. Required quantity of plant growth regulators were added at this stage and dissolved. After addition of all constituents of media, the pH of the medium was adjusted to 5.7 with 1N NaOH or 1N HCl, then make up to 1 litre prior to addition of gelling agent agar (6-8g) as per requirement and media was kept in microwave oven to melt the gelling agent. Then it was dispensed in to culture tubes (Borosil, India) 25 x 150mm (12ml/tube) and air tied with aluminum foil and culture bottles each 40ml then screwed with screw cap. All the culture tubes and bottles containing media were sterilized at 121°C at 15 psi for 15 minutes in autoclave. After sterilization the tubes were kept in slants or in vertical position so as to solidify the media in required manner.

(V) STERILIZATION TECHNIQUES

Preparation of sterile media, containers and small instruments:

The nutrient media used in tissue culture were sterilized by autoclaving. In present investigation culture media in glass containers sealed with aluminum foil or plastic closures were autoclaved at 15 psi and 121°C for 15 minutes.

Glass culture vials were mostly sterilized along with medium whereas the glassware used for presterilized nutrient medium preparation was sterilized by dry heating in hot air oven at 160-180°C for 3 hours.

For aseptic manipulation, the instruments such as forceps, scalpels, and scissors were sterilized by dipping in ethanol, followed by flaming and cooling, which is referred as flame sterilization.

GROWTH OF THE CULTURES UNDER CONTROLLED ENVIRONMENTAL CONDITIONS

Maintainence of Aseptic Conditions

Contamination is one of the main impediments in tissue culture studies. To over come this problem all the articles used in the experiments were sterilized with autoclave. The laminar air flow chamber is used as a transfer area and was cleaned and swabbed with alcohol and all equipment used in inoculation such as medium, forceps, scalpel, Petri plates, sterilized double distilled water, spirit lamp, cotton, rubber bands, autoclaved aluminum foil etc. (expect plant material) were transferred to it and arranged conveniently and exposed to UV radiation for 30 minutes. The laminar flow cabinet was
switched on with flow velocity of $27 \pm 3$ m/sec for 30 minutes before inoculations, to remove the ozone formed by the UV radiation. An extremely important point about aseptic procedure and one of the leading causes of contamination is unclean hands. To overcome this problem prior to inoculation the hands were washed with antiseptic soap followed by swabbing with 95% alcohol. Laminar flow cabinet was frequently swabbed with alcohol while doing operations and to minimize the contamination during inoculation hands swabbed with alcohol frequently, followed by flame sterilization of forceps and scalpel.

Explants isolation and the re-flasking of cultures were conducted on a sterile surface and within an environment, which will ensure that there is very low risk of explants becoming re-infected and contaminants entering the culture vessel. Inoculations were done near spirit lamp. Sterilized plant material was transferred on to sterilized Petri plates containing filter paper with the help of forceps and scalpel. The explants were dissected and damaged ends were removed then blotted on a sterile filter paper. Then explants were inoculated on to culture medium vertically to maintain polarity. After placing the explants on nutrient medium the mouth of culture tubes were covered with sterilized aluminum foil and tied with rubber bands. Culture tubes were labeled giving the details of the experiment, name of the explant, medium and date of inoculation etc.

Plant cultures are greatly influenced by physical factors such as temperature, relative humidity and light. Test tubes containing explants for culture were incubated under culture room conditions. The culture room was provided with constant temperature of $25 \pm 2^\circ$C in culture room by air conditioner and photoperiod of 16 hours light was regulated using cool white fluorescent tubes provided with a light intensity of 10 w / cm² (Ca. 2000 lux) and 8 hours dark is maintained with an automatic timer. Frequent observations were carried out and contaminated tubes were removed carefully. Established cultures were sub cultured to fresh medium to speed up the regeneration process. After trenchant monitoring observations were noted simultaneously before each subculture. For each experiment a minimum of 15 tubes were maintained and all experiments were repeated thrice.
Chapter III Materials and Methods

(VI) SURFACE STERILIZATION OF PLANT MATERIAL

*Ceropegia juncea*

Matured healthy seeds of *Ceropegia juncea* were selected and taken in 100ml clean Erlenmeyer flask then washed in an agitated solution of liquid detergent (1% Tween-20) for 20 minutes followed by running tap water for half-an-hour. Further operations were carried under aseptic conditions inside laminar chamber. Seeds were first rinsed with Millipore water for 5 minutes and then subjected to 70% ethyl alcohol treatment for 30 seconds and again washed with sterilized Millipore water. Later surface sterilization was done with sterilants like HgCl₂ (0.1% to 0.5%), NaOCl (5% to 20%) and H₂O₂ (15% and 30%) maintaining different time intervals (3 and 8 minutes) followed by three rinses with sterile Millipore water.

Sterilized seeds were once again washed with sterilized Millipore water before they were placed on different basal media like MS, B₅ liquid / solid, MPW + Agar etc., either supplemented with plant growth regulator (BAP, NAA, GA₃) or directly (Basal). 1% sucrose and 0.8% agar-agar was used for all the experiments. For the purpose of confirmation of results, every experiment was repeated thrice.

**Initiation of Aseptic Cultures of Ceropegia elegans**

Excised internode, node and leaf explants of *Ceropegia elegans* were collected and used for the morphogenic studies. The explants were washed first under running tap water then with liquid of detergent (1%Tween 20) for 10 minutes and then rinsed with Millipore water. Further operations were carried under aseptic conditions inside laminar air flow chamber. Explants were rinsed with sterile Millipore water, thereafter immersed in 80% ethanol for 30 seconds and again washed with sterilized Millipore water. Later surface sterilization was done with sterilants like HgCl₂ (0.05% to 1.0%), NaOCl (5% to 20%) and H₂O₂ (20% and 30%) maintaining various time intervals (2, 4, 6, 8 minutes), followed by three rinses with autoclaved Millipore water.

**In vitro Seed Germination of Ceropegia juncea**

Surface sterilized seeds were inoculated on different nutrient media (one seed / each test tube) in aseptic conditions. 40-50 days old aseptic seedlings were used as explant source. Various seedling explants such as roots, cotyledons, leaves, internode,
cotyledonary node, node, shoot tips were excised aseptically and cultured on nutrient media for different experiments.

Establishment of cultures from the explants of Ceropegia elegans

Surface sterilized explants were cut into suitable sizes and cultured on appropriate sterile media fortified with various plant growth regulators and growth adjuvants. For the purpose of confirmation of results every experiment was repeated thrice and medium was supplemented with 3% sucrose and 0.8% agar-agar.

(VII) IN VITRO SHOOT MULTIPLICATION:

Explant Evaluation

Explants of 45-50 days old aseptic seedlings of Ceropegia juncea such as node, cotyledonary node and shoot tips, mature explants of Ceropegia elegans such as node and shoot tips were cultured on MS medium supplemented with 3% sucrose and BAP (1, 2, 3, 5, 7 mg/l), Kn (1, 2, 3, 5, 7 mg/l) for evaluation. The morphogenic responses of the explant were recorded and best explant for shoot regeneration was identified. Criteria used for selection are percentage of response, number of shoots and shoot length.

Medium Evaluation

MS, B5 and WPM media supplemented with 2 mg/l BAP and 3% sucrose were used for screening the influence on organogenic potentiality from various seedling explants of Ceropegia juncea such as cotyledonary node, node and shoot tip and mature explants of Ceropegia elegans such as node and shoot tips. Criteria of medium selection are percentage of explants responded, average number of shoots and shoot length per explant.

Assessment of Plant Growth Regulators and other Organic Supplements for Shoot Induction

After selecting the best medium for shoot multiplication further experimental manipulation were carried to assess the morphogenic influence of different plant growth regulators such as auxins (IAA, IBA, NAA, 2,4-D, Picloram), cytokinins (BAP, Kn, TDZ, 2-iP, Zeatin) and Gibberellin (GA_{3}) and other organic supplements such as CH, CM,YE, Ascorbic acid, Citric acid, PVP and different sugars (Glucose, Fructose, Sucrose, Galactose, Maltose).
(VIII) IN VITRO CALLUS CULTURE STUDIES

Medium Evaluation

Different seedling explants of \textit{Ceropegia juncea} such as cotyledons, internode and leaf, mature explats of \textit{Ceropegia elegans} such as petiole, leaf lamina and internode were excised aseptically and cultured on MS, B5, WP Media fortified with 2 mg/l 2,4-D for callus initiation and regeneration of plantlets. Best medium was selected based on percentage of response, fresh and dry weight of callus.

Effect of Plant Growth Regulators:

Various seedling explants of \textit{Ceropegia juncea} such as cotyledons, internodes and leaflets, mature explants of \textit{Ceropegia elegans} such as petiole, leaf lamina and internodes were used for screening the potentially of different plant growth regulators such as 2,4-D, 2,4,5-T, Picloram, NAA, IAA, IBA, etc.

(IX) IN VITRO ROOTING

Regenerated shoots of \textit{Ceropegia juncea} process were isolated and used for rooting. Full strength, half strength and quarter strength MS, B5 and WP medium supplemented with 2% sucrose, 0.7% agar and auxins such as IAA, IBA and NAA (0.1 to 3mg/l) individually and in combination were used.

(X) ACCLIMATIZATION

\textit{In vitro} raised plantlets with well-developed roots of \textit{Ceropegia juncea} and \textit{Ceropegia elegans} were taken out of the test tube and cleaned with sterilized Millipore water to remove all the traces of agar. Then these plantlets were planted in earthenware pots containing sterilized soil rite mix covered with big polythene bag and incubated at 25 ± 2°C for 20 days. During this period liquid quarter strength MS basal nutrient medium devoid of sucrose was provided instead of water. Later small perforations were made on the polythene bag to reduce the relative humidity. Slowly the width of the holes was increased until the relative humidity inside the polythene bag and outside the chamber come to equal. After this conformity polythene bag was removed and the pots were directly exposed to the controlled temperature (25 ± 2°C). Slowly the pots were transferred to room temperature having diffuse light. And finally plants were shifted to the sunlight in Botanical garden.
(XI) STATISTICAL ANALYSIS

Each culture tube with one shoot explant was considered as one replicate. Each treatment in each set of experiments consists of 15 replicates and each experiment was repeated three times. Standard error of means was calculated in each experiment. The data was statistically analyzed using one-way analysis of variance (ANOVA), means were compared using the DMR test at the 0.05% level of significance.

(XII) ANTIMICROBIAL STUDIES

1. Plant material

*Ceropegia juncea* stem segments were collected from Kalasamudram forest of Kadiri, Anantapur, Andhrapradesh. Leaves and stem segments were collected from Sirumalai hills of Palani, Tamilnadu.

2. Sterilization of Plant Material:

All the materials were surface sterilized as follows; washed the plant material under running tap water for 5 times followed by a treatment with solution containing 2-3 drops of Tween 20 for 15 minutes. Discarded the solution and treated the plant material with tap water, followed by a treatment with 70% alcohol for 1 minute. Then rinsed thrice with autoclaved Millipore water. Later the plant material was treated with 0.05% HgCl₂ for 10 minutes. The plant material was rinsed with autoclaved Millipore water 4-5 times to decant mercuric chloride.

The surface sterilized plant material was shade dried for 30 days and finally kept in hot air oven for 12 hours at 45°C. After complete drying, the dried pieces were then crushed into a coarse powder with the help of domestic mixer.

3. Preparation of Plant Extracts:

The coarsely powdered plant materials (50 gm of each) were extracted successively with Petroleum ether, methanol and millipore water by using Soxhlet apparatus and the extraction process was performed corresponding fractions. The extract was then filtered through Whatman No.1 filter paper. The filtrate was concentrated with a rotary evaporator under reduced pressure at 60°C to afford crude Petroleum ether extracts (40 gm). Each extract was transferred into clean and dried airtight vials and stored at 4°C until for use. The crude petroleum ether extract was fractionated in to methanol (250 ml) finally with aqueous fractions by solvent- solvent partitioning.
The crude water extract of *Ceropegia juncea* stem was obtained by extracting 50 grams of stem material, leaf and stem materials of *Ceropegia elegans* were dissolved in 50 ml of water than grinded it and the extract was filtered with muslin cloth and extract was desiccated under rotoevaporator.


The test microorganisms employed include bacteria and fungi. The microorganisms listed below were availed from the microbial type culture collection center, Institute of Microbial Technology (IMTECH), Sector 39-A, Chandigarh-160036, India and organisms were maintained on their respective media (as per guidelines of IMTECH) in slants at 4°C and sub cultured before use. The bacteria studied are clinically important ones, cause several infections and it is essential to overcome them through some active therapeutic agents.

These organisms include....

**GRAM POSITIVE BACTERIA**

*Proteus vulgaris* (MTCC1771)

*Bacillus subtilis* (MTCC121)

*Staphylococcus aureus* (MTCC 737)

**GRAM NEGATIVE BACTERIA**

*Pseudomonas aeruginosa* (MTCC 1688)

*Klebsiella pneumoniae* (MTCC 109)

*Escherichia coli* (MTCC 443)

**FUNGI**

*Candida albicans* (MTCC 3017)

*Aspergillus fumigatus* (MTCC1811)

All the selected organisms are pathogenic and the disease caused by them are candidiasis, amycotic disease, by *Candida albicans*, moderate to severe gastroenteritis by *Escherichia coli*, conjunctivitis iridocheroiditis by *Bacillus subtilis*, suppurative and inflammatory lesions by *Pseudomonas aeruginosa* (acts as secondary invader), post harvesting diseases of grains and legumes by *Aspergillus fumigatus*. All the microbes
were grown on aseptic media. Stock cultures were made freshly every seven days on agar slants during this scheme of work.

5. Preparations of Media and sterilization.

Media used for growth of microorganisms according to information from MTCC. Growth medium or Nutrient agar medium for bacteria and Sabouraud agar medium for fungi were used for culturing of microorganisms.

A. Nutrient agar medium or growth medium

Following chemicals are weighed and dissolved in 800 ml of sterile distilled water and made up to 1000 ml.

<table>
<thead>
<tr>
<th>S. No.</th>
<th>Composition</th>
<th>gm/L</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Beef extract</td>
<td>1</td>
</tr>
<tr>
<td>2</td>
<td>Yeast extract</td>
<td>2</td>
</tr>
<tr>
<td>3</td>
<td>Peptone</td>
<td>5</td>
</tr>
<tr>
<td>4</td>
<td>NaCl</td>
<td>5</td>
</tr>
<tr>
<td>5</td>
<td>Agar</td>
<td>15</td>
</tr>
<tr>
<td>6</td>
<td>Distilled water</td>
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</tr>
<tr>
<td>7</td>
<td>pH 7.0</td>
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</tr>
</tbody>
</table>

B. Sabouraud agar medium

Following chemicals are weighed and dissolved in 800 ml of sterile distilled water and made up to 1000 ml.

<table>
<thead>
<tr>
<th>S. No.</th>
<th>Composition</th>
<th>gm/L</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Peptone</td>
<td>10</td>
</tr>
<tr>
<td>2</td>
<td>Dextrose</td>
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</tr>
<tr>
<td>3</td>
<td>Agar</td>
<td>5</td>
</tr>
<tr>
<td>4</td>
<td>Distilled water</td>
<td>1000 ml</td>
</tr>
<tr>
<td>5</td>
<td>pH 5.6</td>
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</tr>
</tbody>
</table>
Chapter III
C. PEPTONE WATER

Peptone water was prepared by dissolving 15gm of peptone in 1000 ml of distilled water, mixed well and sterilized at 15 lbs pressure (121° C) for 15 min. in autoclave.

D. Sterilized Distilled Water

Distilled water was poured to 15 ml test tube and sterilized in an autoclave.

1. Preparation of Inoculum

Loop full of culture from 24 hours culture tubes was taken, then dissolved it in test tube containing 15 ml peptone water, and then spread on nutrient agar medium for bacteria and Sabouraud agar medium for fungi in petriplates. The petriplates were then incubated in incubator at 37°C for 16 to 18 hours. Then the plates were used for the experiment.

2. Inoculation Methodology

Previously prepared petridishes containing media (NAM for bacterial broth culture) were kept inside the incubator for drying. Placed all the things required (crude plant extracts, Petri plates having media, bacterial culture and peptone water) in the laminar air flow chamber. Sterilized the loop by heating and allowed it to cool. Taken a loop full of bacterial culture and dissolve it in peptone water. Sterilized the mouth of test tube with the help of flame. After incubation, spreaded the inoculum with help of sterile glass spreader on an applicator into the adjusted suspension. Inoculated the dried surface of a nutrient agar plate by streaking over the entire sterile agar surface. Repeated this procedure two more times, and rotated the plate 60° each time to ensure an even distribution of inoculum.

Determination of MIC

Susceptibility of test organism to all the extracts was determined by employing the standard disc diffusion technique. Whatman No.1 filter paper discs of 8 mm diameter, placed in dry petri dish were autoclaved. The sterile filter paper discs (10-20) were added to each of the test extracts and standards (antibiotics) were shaken thoroughly and kept for overnight. Later, the saturated filter paper discs were taken out and dried on the laminar airflow bench and carefully placed over the spread cultures and incubated at 37°C for 24 hours for bacteria and 48 hours for fungi. Paper discs treated with anti-
microbial standards alone served as control. The zone of inhibition if any, induced by the extracts on growth of microorganisms were examined in each plate and measured the diameters of the zones of complete inhibition, including the diameter of the disk. Measured the zones of nearest millimeter using a ruler.