Materials and Methods
MATERIALS AND METHODS

Explant source

The undehisced capsules of Coelogyne stricta (D.Don) Schltr. and Eria bambusifolia Lindl. were collected from the National Orchidarium, Botanical Survey of India, Yercaud (1500 MSL) in the Servarayan Hills (Tamil Nadu), Southern India.

Sterilization

The freshly collected capsules were thoroughly washed with running tap water and then with the detergent teepol (0.1%). They were surface sterilized with mercuric chloride solution (0.1%) for 3 minutes and were subsequently rinsed in sterilized double distilled water. The capsules were dipped in 80% ethyl alcohol for a minute and flamed. The sterilized capsules were cut longitudinally with the help of a sharp sterilized surgical blade for extracting seeds and were inoculated on two different basal media under aseptic conditions.

Preparation of the media

For the preparation of the media only analytical reagents of “Hi-media” grade chemicals and Borosil glasswares were used. Double distilled water was used for preparing the media. The nutrient media basically consists of inorganic nutrients, carbon source, vitamins and organic supplements (optional). Stock solutions were prepared separately for macronutrients, micronutrients, iron, potassium iodide and vitamins. All the chemicals were weighed accurately in electronic weighing machine (A & D company Ltd). All stock solutions were poured in well-stoppered sterilized bottles and preserved in a refrigerator at 4°C. Specific quantity of the stock solutions of the chemicals and growth regulators were pipetted onto a 1 litre beaker. Required sucrose,
other organic supplements and complex additives (optional) were added. The final volume was made up with distilled water and the pH was adjusted to 5-5.8 (Table 1) with either 0.1 N NaOH or 0.1 N HCl using a pH meter (ELICO).

To the above said media, 0.8 to 0.9% agar (extra pure gelling point 32-35°C Hi-media-Bombay) was added, melted in a water bath and the medium was dispensed into 250 ml conical flasks (50 ml of medium) or onto 50 ml test tubes (10-20 ml of medium). The tubes or the flasks after covering with aluminium foil were autoclaved at 1.06 kg pressure/sq. cm for about 20 minutes at 121°C. The autoclaved medium in the culture tubes were cooled and allowed to solidify as slants and it was stored at 25°C for future use. The inoculations were done after 4 days to ensure that the flasks were free from contamination.

Preparation of growth regulators, growth adjuvants

Three important groups of growth regulators such as auxins, cytokinins and gibberellic acid (GA₃), growth adjuvants like YE, CH, other compounds such as CW and AC were used in the experiments.

Auxins

Two auxins namely α-naphthalene acetic acid and indole-3-acetic acid were used in these experiments. The stock solution was prepared by dissolving 10 mg of auxin individually in 1 ml of ethanol. The volume was made upto 100 ml with sterile distilled water. The required volumes of auxins were added to the nutrient media, before autoclaving. Three concentrations (0.5, 1.0 and 2.0 mg/l) were used.
Cytokinins

The stock solution was prepared by dissolving 10 mg of 6-benzyl adenine and kinetin 6-furfurylamino purine in 1 ml of 0.1 N Hydrochloric acid (HCl) and the volume was made up to 100 ml by adding sterile distilled water. The concentration of 0.5, 1.0 and 2.0 mg/l were used in these experiments.

Gibberellic acid

The stock solution was prepared by dissolving 10 mg of GA3 in small amount of distilled water and the volume was made up to 100 ml with sterile distilled water. The concentrations used in these experiments were 0.5, 1.0 and 2.0 mg/l.

Coconut water

Coconut water was collected from tender coconuts and filtered through cheesecloth. The filtered water was boiled for approximately 10 minutes in order to precipitate the proteins, then cooled to room temperature, decanted and the supernatant was again filtered through a Whatman No. 1 filter paper. The required amount of coconut water was added to the medium before autoclaving.

Inoculation and Incubation

For raising aseptic culture, laminar airflow chamber (Thermadyne) was used. Before use, the interior of the laminar flow chamber was cleaned with alcohol. The nutrient media and instruments were exposed to UV light for 10-15 minutes to assist asepsis. The various instruments used in aseptic operations were sterilized by flaming them with absolute alcohol inside the cabinet. For each experiment a set of 5 cultures...
were raised, and were maintained at 25±2°C under the diffused white fluorescent light (3000 lux) with a photoperiod of 12 hours daily and a relative humidity of 55 to 60%. The cultures were photographed whenever found necessary.

Asymbiotic seed germination

Knudson C (1946) and Murashige and Skoog (1962) (Table 1) media were tested initially to find out the suitable medium for seed germination. Bursting of the seed coat and emergence of the enlarged embryos, i.e., the protocorm was considered as germination. The germination of seeds was recorded and percentage of seed germination was calculated.

Five samples of the seeds were taken out after 8 and 13 weeks at random and the slides were prepared for observations by placing a drop of glycerin and covering it with a cover slip. The seeds were scooped out and scrutinized randomly in a petriplate and observed under the microscope. The seeds were classified as germinated / ungerminated for calculating germination percentage. The percentage of germination is calculated by counting the total number of seeds germinated with that of total number of seeds observed.

\[
\text{Percentage of germination} = \frac{\text{Total no. of seeds germinated}}{\text{Total no. of seeds observed}} \times 100
\]

Seedling development

Among the two media tried, the best medium for germination was selected and was supplemented with auxins (IAA and NAA), cytokinins (BA and KN), gibberellin (GA₃) individually and in combinations at various concentrations 0.5, 1.0 and 2.0 mg/l and the medium was enriched with growth adjuvants like AC, CH, YE (100, 200 and 400 mg/l) and CW (10, 20 and 40%) for further proliferation of protocorms and development of plantlets.
Table 1. Chemical composition of Murashige and Skoog (1962) and Knudson C (1946) media (mg/l)

<table>
<thead>
<tr>
<th>S. No.</th>
<th>Component</th>
<th>MS</th>
<th>KC</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>NH$_4$NO$_3$</td>
<td>1650</td>
<td>-</td>
</tr>
<tr>
<td>2.</td>
<td>(NH$_4$)$_2$SO$_4$</td>
<td>-</td>
<td>500</td>
</tr>
<tr>
<td>3.</td>
<td>KNO$_3$</td>
<td>1900</td>
<td>-</td>
</tr>
<tr>
<td>4.</td>
<td>CaCl$_2$.2H$_2$O</td>
<td>440</td>
<td>-</td>
</tr>
<tr>
<td>5.</td>
<td>MgSO$_4$.7H$_2$O</td>
<td>370</td>
<td>250</td>
</tr>
<tr>
<td>6.</td>
<td>KH$_2$PO$_4$</td>
<td>170</td>
<td>250</td>
</tr>
<tr>
<td>7.</td>
<td>Ca(NO$_3$)$_2$.4$H_2$O</td>
<td>-</td>
<td>1000</td>
</tr>
<tr>
<td>8.</td>
<td>KI</td>
<td>0.83</td>
<td>-</td>
</tr>
<tr>
<td>9.</td>
<td>FeSO$_4$.7H$_2$O</td>
<td>-</td>
<td>25</td>
</tr>
<tr>
<td>10.</td>
<td>H$_3$BO$_3$</td>
<td>6.2</td>
<td>-</td>
</tr>
<tr>
<td>11.</td>
<td>MnSO$_4$.4H$_2$O</td>
<td>22.3</td>
<td>7.5</td>
</tr>
<tr>
<td>12.</td>
<td>ZnSO$_4$.7H$_2$O</td>
<td>8.6</td>
<td>-</td>
</tr>
<tr>
<td>13.</td>
<td>Na$_2$MoO$_4$.2H$_2$O</td>
<td>0.25</td>
<td>-</td>
</tr>
<tr>
<td>14.</td>
<td>CuSO$_4$.5H$_2$O</td>
<td>0.025</td>
<td>-</td>
</tr>
<tr>
<td>15.</td>
<td>CoCl$_2$.6H$_2$O</td>
<td>0.025</td>
<td>-</td>
</tr>
<tr>
<td>16.</td>
<td>C$<em>{10}$H$</em>{12}$N$_2$O$_3$FeNa</td>
<td>0.73</td>
<td>-</td>
</tr>
<tr>
<td>17.</td>
<td>C$<em>6$H$</em>{12}$O$_6$</td>
<td>100</td>
<td>-</td>
</tr>
<tr>
<td>18.</td>
<td>C$_2$H$_3$NO$_2$</td>
<td>0.5</td>
<td>-</td>
</tr>
<tr>
<td>19.</td>
<td>C$_6$H$_3$NO$_2$</td>
<td>0.5</td>
<td>-</td>
</tr>
<tr>
<td>20.</td>
<td>C$_4$H$_7$CINO$_3$</td>
<td>0.1</td>
<td>-</td>
</tr>
<tr>
<td>21.</td>
<td>C$<em>{12}$H$</em>{17}$CIN$_4$O$_3$.HCl</td>
<td>2.0</td>
<td>-</td>
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<tr>
<td>22.</td>
<td>Sucrose</td>
<td>30 g/l</td>
<td>20g/l</td>
</tr>
<tr>
<td>23.</td>
<td>Agar</td>
<td>8 g/l</td>
<td>9g/l</td>
</tr>
<tr>
<td>24.</td>
<td>pH</td>
<td>5.8</td>
<td>5.0</td>
</tr>
</tbody>
</table>
**Pseudobulb segment culture**

Pseudobulb segments (0.5-1.0 cm) of *C. stricta* obtained from the *in vitro* grown one year old seedlings were cut longitudinally into 2 pieces and cultured in half strength MS medium supplemented with various plant growth regulators like IAA, NAA, BA and KN alone and in combinations.

**Encapsulation of protocorms**

For encapsulation 90 days old protocorms obtained *via* seed culture were selected randomly and 3% of sodium alginate (Loba) were prepared by mixing it with distilled water, MS basal medium and MS medium along with mannitol (3%).

For complexation, 1.036/150 ml calcium chloride (CaCl₂) solutions were prepared with distilled water. Both the gel matrix and complexing agents were autoclaved at 121°C for 20 minutes. Protocorms were mixed with sodium alginate solution and were subsequently singly dropped into calcium chloride solution using a wide mouthed glass dropper (inside diameter 4 mm). Calcium alginate beads were formed within 30-45 minutes on a gyratory shaker moving at 80 rpm under light (1000 lux). Sodium alginate at 3% formed firm, clear, isodiametric beads. The beads were recovered by decanting CaCl₂ solution and washed thrice with sterilized double distilled water. The beads each containing a single protocorm were cultured on MS basal medium, MS medium supplemented with NAA, BA, GA₃ (1.0 and 2.0 mg/l) and growth adjuvants like AC, CH, YE (200 and 400 mg/l) and CW (20 and 40%).

**Storage**

A set of 50 beads (prepared in MS and MS medium along with 3% mannitol) was stored in dark at 2 different temperatures *viz,* 4°C and 25±2°C in sterile petridishes, sealed.
with parafilm. They were taken out at regular intervals of 30 days inoculated in MS basal medium and the conversion percent of the stored beads was assessed periodically up to 210 days.

**Rooting**

The well-developed plantlets were transferred to the half strength MS medium supplemented with NAA (*C. stricta*) and IAA (*E. bambusifolia*) individually in different concentrations ranging from 0.5-2.0 mg/l.

**Hardening**

The fully developed healthy plantlets were removed from the culture flasks and the plantlets were thoroughly washed in running tap water to remove the adhering nutrient medium completely without causing damage to the roots. Then the plantlets were treated with (0.5%) fungicide (Roko) and transferred to flasks filled with various types of sterilized potting media as detailed below.

1. Perlite + sterile soil.
2. Sand + charcoal + brick pieces.

A tray with sterilized distilled water was kept under the pots. The pots were initially kept in culture room 25±2°C temperature and 3000 lux light at a photoperiod of 12 hours covered with polythene bag and after 3 weeks the potted plantlets were transferred to the greenhouse condition.
Estimation of mycorrhizal fungi colonization

The in vitro raised seedlings of *C. stricta* were taken out and thoroughly washed in running tap water to remove the adhering nutrient medium completely without causing damage to the roots. To the best potting medium the plantlets are transferred directly and another set of plantlets were immersed in water containing spores of *Rhizoctonia solani* and planted in the potting media inoculated with the *R. solani*. The percentage of seedling survival was calculated after three months, mycorrhizal colonization was estimated.

The procedure described by Phillips and Hayman (1970) was adopted for clearing and staining the root segments for rapid assay of mycorrhizal colonization. The root segments preserved in FAA were cut into pieces of 1cm length and washed thoroughly in distilled water. They were softened by boiling in 10% KOH at 90°C for few minutes to an hour. Then the root bits were washed 3-4 times in distilled water and cleared in 3% H₂O₂ for 5-10 minutes. The cleared specimens were again washed in distilled water, neutralized with 5N HCl for 10 minutes and stained with neutral 0.05% trypan blue (in lactophenol) for 15-30 minutes. The excess stain, if any, was removed with clear lactophenol. Stained root bits were mounted on glass slides in lactophenol and examined in compound microscope under low power (100X) for the observation.

\[
\text{Percentage of root colonization} = \frac{\text{No. of intersections showing fungal structures}}{\text{Total no. of intersections}} \times 100
\]

Antimicrobial activity

To study the antimicrobial activity, the in vitro and in vivo grown plantlets were used.
Preparation of crude extract

The whole plants were homogenized with methanol. The extract was filtered (Whatman No. 1 filter paper) and the filtrate was dried at room temperature. The dried materials were taken and redissolved in the same solvent made into known volume. From the known volume of the four samples 0.25, 0.50 and 1.0% solution were prepared. Antimicrobial activity of the above mentioned extracts were assayed separately using modified disc diffusion method (Bauer et al., 1966).

The petriplates were sterilized using autoclave at 121°C for 15-20 minutes. Freshly prepared nutrient agar and PDA medium were poured into the petriplates. The composition of the media is represented in Table 2 and 3. Different bacterial and fungal species were inoculated onto the medium by streak plate method. The strains were enriched before culturing.

Table 2. Composition of Nutrient agar Medium

<table>
<thead>
<tr>
<th>Components</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>Peptone</td>
<td>5.0 g</td>
</tr>
<tr>
<td>Beef extract</td>
<td>3.0 g</td>
</tr>
<tr>
<td>Agar</td>
<td>15.0 g</td>
</tr>
<tr>
<td>Distilled water</td>
<td>1000 ml</td>
</tr>
<tr>
<td>pH</td>
<td>7.0</td>
</tr>
</tbody>
</table>
Table 3. Composition of PDA medium

<table>
<thead>
<tr>
<th>Components</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>Potato</td>
<td>200.0 g</td>
</tr>
<tr>
<td>Dextrose</td>
<td>20.0 g</td>
</tr>
<tr>
<td>Agar</td>
<td>15.0 g</td>
</tr>
<tr>
<td>Distilled Water</td>
<td>1000 ml</td>
</tr>
<tr>
<td>PH</td>
<td>6.2</td>
</tr>
</tbody>
</table>

The bacterial and fungal isolates were obtained from the Microbiology lab. Department of Botany, Bharathiar University, Coimbatore, Tamil Nadu, India. The following isolates were used to test the antimicrobial activity.

**Bacteria**

*Aeromonas hydrophila, Bacillus subtilis, Pseudomonas aeruginosa, Staphylococcus aureus, Salmonella typhi* and *Vibrio cholerae*

**Fungi**

*Alternaria alternata, Aspergillus niger* and *Pyricularia oryzae*.

Sterile filter paper disc (6 mm diameter) containing methanol (control) and plant extracts (0.25, 0.50 and 1.0%) were placed on the medium. Bacterial cultures were incubated at 30±2°C for 12-16 hours and the fungal cultures at the same temperature upto 48-52 hours. The assessment of the antimicrobial activity was based on the measurement of the inhibition zone formed around the disc. The inhibition zone was measured and expressed in mm.
Phytochemical analysis

The method of Harborne (1960, 1973a, 1973b) was adopted for the analysis of alkaloids, phenols and sugars. Bate-Smith’s (1962) was followed for the analysis of flavonoids using TLC.

ALKALOIDS

Preparation of sample (Harborne, 1973a)

The alkaloids were extracted from in vitro and in vivo plant and plant tissue by 1N HCl and the crude extract was left for 12 hours. The crude extract was filtered and concentrated to one quarter of the original volume. The above extract was precipitated with concentrated ammonia solution and centrifuged with 1N ammonia solution and then spotted on TLC plates.

FLAVONOIDS

Preparation of sample (Bate-Smith, 1962)

10 g of plant tissue was immersed in 2N HCl and heated for 30-40 minutes at 100°C. The extract was then cooled and the cooled extract was mixed with ethyl acetate and separated with separating funnel. The ethyl acetate extract was concentrated to dryness, taken up in 1-2 drops of ethanol and aliquots chromatographed one dimensionally.

PHENOLS

Preparation of sample (Harborne, 1973b)

Fresh plant tissue of in vitro and in vivo each 10 g was hydrolysed with 2N HCl at 40°C for half an hour. It was cooled and filtered and the filtrate was mixed with equal volume of petroleum ether and separated using separating funnel. The ether extracts so obtained was spotted directly on the chromatogram.
SUGARS

Preparation of sample (Harborne, 1960)

The fresh plant tissues of *in vitro* and *in vivo* were extracted with 95% methanol. The extract was concentrated to remove methanol and filtered through Whatman No.1 filter paper. The clear extract was spotted directly on the chromatogram.

Identification of the above compounds was confirmed by spectral measurements using UV spectrophotometer (Shimadzu 1601).

PREPARATION OF TLC PLATES

The glass plates to be coated were cleaned thoroughly, wiped with alcohol and dried in special racks. They were lined on the applicator pad with a few drops of water used as a means for dicing these plates immobile on the pad.

COATING MATERIAL

The weighed absorbent silica gel-G (50 g/100 ml) was mixed with water either by grinding in a mortar or by vigorous shaking for 10 minutes in a stoppered conical flask. Later it is transferred quickly to the spreader to have a uniform layer.

COATING THE PLATES

The aperture opening in the applicator was set for desired thickness of the coating (250µ). The slurry was poured into the applicator placed on the last plate on the left of the pad. The lever was turned 180°C. When the slurry could be seen coming out, the applicator is drawn smoothly on the plates.
DRYING AND STORING

The coated plates were left in position overnight free from dust. When necessary, these plates were activated after drying in a hot air oven at 105°C for 30-60 minutes. The plates were placed in special racks provided, to prevent the layers from chipping off. Since these active plates are easily deactivated in moisture, they were stored in a desiccator until use.

APPLICATION OF THE SAMPLES

A small spot was applied to the silica gel plates and the chromatograms were developed in various solvent systems.

Statistical Method

The data obtained from the experiments were analyzed by using new Duncan’s Multiple Range Test (DMRT) wherever necessary for mean comparison of shoot length, number of multiple protocorms, root number and root length (Duncan, 1955). Five replicates were maintained for each experiment.