3. Materials and Methods:

3.1. Plant materials: Description of the species studied:

The present study was conducted with plants that were either purchased from several nurseries in Siliguri or collected from Santiniketan, where the plants were growing in natural habitats or in private collections. The plants under study have been finally identified in the Central National Herbarium Sibpur, Howrah.

Genus: *Dendrobium*

Systematic position:

- Family: Orchidaceae
- Subfamily: Epidendroideae
- Tribe: Dendrobieae
- Subtribe: Dendrobiinae

Genus: *Dendrobium* Swartz.

Species studied:

- *Dendrobium aphyllum* (Roxb.) Fischer
- *Dendrobium chrysanthum* Lindl. ex Wall.
- *Dendrobium farmeri* Paxt.
- *Dendrobium nobile* Lindl.
- *Dendrobium parishii* Rchb.f.
- *Dendrobium primulinum* Lindl.
General characters of *Dendrobium* Swartz. Species:

*Dendrobium* Swartz is a taxonomically complex genus (Carlsward *et al.*, 1997). All six species predominately epiphytic, sympodial. They are evergreen perennial. Exclusively epiphytic species having connected stems (‘pseudobulbs’) each of which can produce one or few inflorescence at a time. Flowers in spike grown from pseudobulbs of last year.

**Distribution:**

The species of *Dendrobium* have wide geographical distribution across continents (Dressler, 1993). They are found in South, East and South-East Asia, including Philippines, Borneo, Australia, Papua New Guinea and New Zealand.

**Economic Importance:**

- Most commonly encountered orchids in retail trade today
- All the species having ornamental and medicinal attributes. The extracts from various plant parts are used by the tribal people for strengthening the immune system.
- *Dendrobium nobile:* This species is widely cultivated in China for medicinal uses. Extracts of pseudobulb generally used as tonic in pulmonary tuberculosis, general debility, dyspepsia, reduced salivation, night sweates, fever and anorexia. Various important phytochemicals have been identified. Among this, alkaloids like dendobine and nobiline having neuropharmacological importance (Curtis *et al.* 1971; Kudo *et al.* 1983), gigantol and moscatilin, both with potential antimutagenic activity (Miyazawa *et al.* 1997, 1999)
and several immunomodulatory sesquiterpene glycosides (Zhao et al. 2001; Ye et al. 2002) are worthy to mention.

- **Dendrobium chrysanthum**: In Chinese Pharmacopoeia natives of North-East India (CPEC, 2000) it also has medicinal properties.

- **Dendrobium farmeri**: This species has delicious perfume and are worth growing just for the fragrance during the warmth of the day.

**Genus**: *Hygrochilus*

**Systematic position:**
- **Family**: Orchidaceae
- **Sub family**: Epidendroideae
- **Tribe**: Vandeae
- **Sub tribe**: Aeridinae
- **Genus**: *Hygrochilus*

**Species studied**: *Hygrochilus parishii* Veitch & Rchb.f.

**General characters:**
- Large epiphyte with short stem.
- Inflorescence longer than the leaves, bear 1-6 long lasting fleshy flowers.

**Distribution:**

North-east India, Myanmar, Thailand, southern China, Laos and Vietnam.

**Economic Importance:**

Ornamental value
Genus: *Thunia*

Systematic position:

- Family: Orchidaceae
- Sub family: Coelogynae
- Tribe: Coelogyninae
- Sub tribe: Thuniinae

Species studied: *Thunia alba* Reichb.f.

General characters:

- Terrestrial or semi-epiphyte

Distribution:

- Tropical and sub tropical Himalayas, Garhwal, Sikkim, Meghalaya.

Economic Importance:

- Ornamental value

3.2. Methodology:

3.2.1. *In vitro* studies:

Nutrient medium:-

The mineral salts of the culture media used were based on Knudson’s C (Knudson, 1946) with some modifications, the iron source prescribed for Knudson’s C (KC) medium was replaced by the iron-EDTA as described by Murashige and Skoog (1962). The composition of the KC basal medium is mentioned in the Table 3.2.1:
Chapter – 3 : Materials and Methods

In vitro studies of some selected orchids: histological and cytological investigations of the regenerants.

Table: 3.2.1 Composition of KC basal medium:-

<p>| | |</p>
<table>
<thead>
<tr>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Ca(NO₃)₂.4H₂O (mg l⁻¹)</td>
<td>1000.00</td>
</tr>
<tr>
<td>(NH₄)₂SO₄ (mg l⁻¹)</td>
<td>500.00</td>
</tr>
<tr>
<td>H₃PO₄ (mg l⁻¹)</td>
<td>250.00</td>
</tr>
<tr>
<td>MgSO₄.7H₂O (mg l⁻¹)</td>
<td>250.00</td>
</tr>
<tr>
<td>Na₂EDTA (mg l⁻¹)</td>
<td>37.25</td>
</tr>
<tr>
<td>FeSO₄.7H₂O (mg l⁻¹)</td>
<td>27.25</td>
</tr>
<tr>
<td>MnSO₄.4H₂O (mg l⁻¹)</td>
<td>7.50</td>
</tr>
<tr>
<td>Sucrose</td>
<td>20.00</td>
</tr>
<tr>
<td>Water</td>
<td>1.00</td>
</tr>
</tbody>
</table>

Preparations of stock solution for nutrient medium:-

**Macro salts:** For preparation of macrosalt I Ca (NO₃)₂ [5g], KH₂PO₄ [1.25g] and MnSO₄ [1.25g] were weighed and successively dissolved in 500ml of double distilled water in a volumetric flask. For preparing macrosalt II, 2.5 g (NH₄)₂ SO₄ was weighed and dissolved in 500ml double distilled water. The stock solution was maintained at 10 times the final strength of the medium and stored in refrigerator at 10±2⁰C. This stock solution was utilized for one month from the date of preparation.

**Microsalts:** H₃BO₃ (620mg), Na₂MoO₄, 2H₂O (25mg), CoCl₂, 6H₂O (2.5mg), CuSO₄,5H₂O (2.5mg), MnSO₄, 4H₂O (1690mg), ZnSO₄, 7H₂O (860mg) were weighed and successively dissolved in 100ml double distilled water. The stock was maintained at 2000 times the final strength of the medium and kept in a refrigerator at 10±2⁰C. The stock solution was utilized for 30 days from the date of preparation.

**KI Solution:** 83 mg of potassium iodide was weighed and dissolved in100 ml double distilled water. The stock solution was maintained at 2000 times the final strength of the medium and kept in a refrigerator at 10±2⁰C. The stock solution was utilized for 30 days from the date of preparation.

**Iron Source:** 745 mg of Na₂EDTA (Ethylene diamine tetra acetic acid, disodium salt) and 557mg of FeSO₄, 7H₂O were weighed and dissolved in 100ml boiled distilled water. The solution was stirred for 45 minutes placing on a hot magnetic stirrer. The final volume of the
solution after stirring was made up to 100 ml. The mixture was then poured into an amber colored bottle and kept in refrigerator at 10±2°C. The stock solution was utilized for 30 days from the date of preparation.

3.2.1. a] Asymbiotic seed culture:-

- **Pollination and capsule collection**

  With help of a needle, the anther cap of a flower was removed and the pollinium was first taken out and then transferred to the sticky stigmatic surface of another flower, whose pollinium was already removed. After this, the pollen receiving flower (female parent) was labelled with a tag containing the date of pollination and the name of the pollen-donor species. The cross-pollinated flower was then left as such for the development of capsule. This procedure was applied for both siblings (crossing two plants of the same species) and hybrids (crossing of two plants of different species). After several months the undehisced fruits were harvested while they were still green and immediately taken to the laboratory for the culture of seeds. The age of the fruits of each species and hybrid would be mentioned individually in later sections.

**Determination of seed viability:**

The viability of the seeds was tested with 1% (w/v) TTC (2, 3, 5- triphenyl tetrazolium chloride) solution, as described by Shivanna and Johri (1985) for the angiosperm pollen grains. The solution was prepared in 0.15M Tris-HCl buffer (pH 7.8) and stored in a dark bottle under refrigeration. The test was performed on cavity slides.

First, a thin film of petroleum jelly was applied around the cavity of the slide and a drop of TTC solution was added inside the cavity. The seeds were then added in the solution and a cover glass was placed above. Care was taken to prevent entrapment of air bubbles inside the cavity chamber. For each fruit sample three replicate slides were prepared. The slides were then placed inside a pair of Petri plates lined with moistened filter paper and kept under dark condition at 30±2°C for 48 hour.
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In vitro studies of some selected orchids: histological and cytological investigations of the regenerants.

In vitro studies of some selected orchids: histological and cytological investigations of the regenerants.

Fig.3.2.1A-E. Qualitative testing of viability through TTC profile for seeds of A. D. nobile (10x); B.D.chrysanthum(10x); C. D.farmeri (10x); D. D.parishii (10x); E. H. parishii(10x).

After the completion of the treatment period the slides were observed under a light microscope. The seeds showed a gradation in response, i.e. the color of seeds varied from dark red to orange and white. The seeds with white embryo could be easily recognized as nonviable ones, but in other cases (with various shades of red and orange) it was difficult to fix a cut-off point. Therefore, the test was used only for the qualitative assessment of seed conditions, i.e., whether majority of the seeds were viable (with red to orange coloration) or not.

Study of seed structure:

The mature seeds of different species were freshly collected from the naturally dehiscing capsules. The seed structure of all the above taxa was studied through light microscopy as well as by scanning electron microscopy (SEM). For each species 25 randomly selected seeds were examined under light microscope. Length and width were also measured with the help of stage micrometer at the longest and widest axis of the seed. Seeds exhibit different forms. Therefore, seed volumes were calculated using the formula $2[(W/2)^2 (1/2 L) (1.047)]$, where, $1.047=\pi/3$, $W=$ Width and $L=$ Seed length (Arditti et al. 1980). Orchid embryos are elliptical in cross section.
and therefore their volume was calculated by using the formula: \( \frac{4}{3} \pi ab^2 \) where \( a = \frac{1}{2} \) its length and \( b = \frac{1}{2} \) its width.

For SEM the seeds were directly (without any pre-treatment) attached to the aluminum stubs using double- sided adhesive tape and surrounded the specimens with silver conducting paint to form an electrical connection to the ground. The specimens were then coated with gold using the sputter coating unit and examined in the SEM Hitachi model S530.

**Fig.3.2.1.F-I.** Scanning electron micrographs of seeds studied *in vitro* germination: *D. aphyllum* (F1-F3) (1000X, 300X, 1000X); *D. chrysanthum* (G1-G3) (1000X, 1500X, 300X); *D. farmeri* (H1-H3) (1500X, 300X, 1500X); *D. parishii* (I1-I3) (1000X, 200X, 1500X).
### Chapter – 3: Materials and Methods

**In vitro studies of some selected orchids: histological and cytological investigations of the regenerants.**

#### Table 1 - Measurement Data of Seeds

<table>
<thead>
<tr>
<th>Name of the species</th>
<th>Colour</th>
<th>Length (mm)</th>
<th>Width (mm)</th>
<th>L/W</th>
<th>Seed volume (mm² x 10²)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dendrobium aphyllum</td>
<td>Yellow</td>
<td>0.2401 ± 0.00689</td>
<td>0.11487 ± 0.00499</td>
<td>2.0901</td>
<td>8.2926 x 10⁴</td>
</tr>
<tr>
<td>Dendrobium chrysanthum</td>
<td>Yellow</td>
<td>0.4659 ± 0.00893</td>
<td>0.12509 ± 0.01360</td>
<td>3.7245</td>
<td>1.9082 x 10⁵</td>
</tr>
<tr>
<td>Dendrobium farreri</td>
<td>Yellow</td>
<td>0.2468 ± 0.00518</td>
<td>0.09117 ± 0.00327</td>
<td>2.7076</td>
<td>5.3708 x 10⁴</td>
</tr>
<tr>
<td>Dendrobium nobile</td>
<td>Yellow</td>
<td>0.6464 ± 0.01432</td>
<td>0.18771 ± 0.00719</td>
<td>3.4439</td>
<td>5.9621 x 10⁵</td>
</tr>
<tr>
<td>Dendrobium parishii</td>
<td>Yellow</td>
<td>0.28061 ± 0.0047</td>
<td>0.10301 ± 0.00290</td>
<td>2.7241</td>
<td>7.7937 x 10⁴</td>
</tr>
<tr>
<td>Dendrobium primulinum</td>
<td>Pale yellow</td>
<td>0.4268 ± 0.00814</td>
<td>0.10301 ± 0.00707</td>
<td>4.1345</td>
<td>1.1854 x 10⁶</td>
</tr>
<tr>
<td>Thunia alba</td>
<td>Greenish yellow</td>
<td>0.5538 ± 0.02567</td>
<td>0.1433 ± 0.00325</td>
<td>3.8644</td>
<td>2.9772 x 10⁵</td>
</tr>
</tbody>
</table>

#### Table 2 - Measurement Data of Embryo

<table>
<thead>
<tr>
<th>Name of the species</th>
<th>Colour</th>
<th>Length (mm)</th>
<th>Width (mm)</th>
<th>L/W</th>
<th>Volume (mm³ x 10⁻⁵)</th>
<th>Seeévol/ Embryo volume</th>
<th>Air space (%)</th>
<th>Seeévol-Embryo Vol/Seeévol x100</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dendrobium aphyllum</td>
<td>Yellow</td>
<td>0.106341 ± 0.00407</td>
<td>0.06096 ± 0.00638</td>
<td>1.5508</td>
<td>1.3839 x 10⁴</td>
<td>5.9093</td>
<td>83.3534</td>
<td></td>
</tr>
<tr>
<td>Dendrobium chrysanthum</td>
<td>Yellow</td>
<td>0.20610 ± 0.00593</td>
<td>0.10182 ± 0.00334</td>
<td>1.9652</td>
<td>5.4300 x 10⁴</td>
<td>3.4990</td>
<td>71.42</td>
<td></td>
</tr>
<tr>
<td>Dendrobium farreri</td>
<td>Yellow</td>
<td>0.13653 ± 0.00633</td>
<td>0.06334 ± 0.00234</td>
<td>2.1870</td>
<td>1.4547 x 10⁴</td>
<td>3.7034</td>
<td>72.99</td>
<td></td>
</tr>
<tr>
<td>Dendrobium nobile</td>
<td>Yellow</td>
<td>0.26576 ± 0.00737</td>
<td>0.15600 ± 0.00764</td>
<td>1.7035</td>
<td>1.6828 x 10⁵</td>
<td>3.5266</td>
<td>71.64</td>
<td></td>
</tr>
<tr>
<td>Dendrobium parishii</td>
<td>Yellow</td>
<td>0.10478 ± 0.00351</td>
<td>0.05328 ± 0.00226</td>
<td>1.9665</td>
<td>7.7856 x 10⁵</td>
<td>10.0218</td>
<td>90.01</td>
<td></td>
</tr>
<tr>
<td>Dendrobium primulinum</td>
<td>Pale yellow</td>
<td>0.16576 ± 0.00610</td>
<td>0.08880 ± 0.00419</td>
<td>1.8666</td>
<td>4.213 x 10⁻⁵</td>
<td>3.4502</td>
<td>71.01</td>
<td></td>
</tr>
<tr>
<td>Thunia alba</td>
<td>Greenish yellow</td>
<td>0.20686 ± 0.00450</td>
<td>0.07909 ± 0.00288</td>
<td>2.6155</td>
<td>3.3869 x 10⁴</td>
<td>8.7869</td>
<td>88.61</td>
<td></td>
</tr>
</tbody>
</table>

- **Culture establishment and incubation:**

  Depending upon the species the pods were harvested from the plant and the capsules were rinsed with tap water for about 30 minutes. After that surface disinfestation of the capsules was performed by rinsing in 95% (v/v) ethanol for 60 seconds, followed by soaking in 0.1% (w/v) mercuric chloride solution for 15 minutes and finally washing thrice with sterile distilled water and swift passing of the capsules through the flame. Surface sterilized capsules were dissected aseptically with a scalpel and the seeds were taken out and sown on the surface of semisolid culture media. The cultures were maintained at 25 ± 2°C under 10h photoperiod provided by Philips white fluorescent lights of 3000 lux intensity.
Chapter 3: Materials and Methods

- **Nutrient medium:**
  
The germination media were based on inorganic salts of Knudson’s C with some modification. The iron source prescribed for KC media was replaced by the iron –EDTA as described by Murashige and Skoog (1962). The medium was also modified by the inclusion of 0.1% peptone and 2% (w/v) sucrose served as carbon source. The detailed composition of the culture media is given in Table 1. In all instances, the pH of the medium was adjusted to 5.2, prior to autoclaving with 0.05(N) KOH and 0.05(N) HCl. The media were solidified with 0.9% (w/v) agar (Merck Ltd. Mumbai, India). The culture vessels were plugged with non-absorbent cotton. The sterilization of culture media was performed by autoclaving at 1.05 kgcm⁻² for 20 min.

- **Media modifications:**
  
  Seeds were inoculated into culture tubes each containing 20ml of KCP basal medium. In addition to the constituents shown in the Table 1, different organic substances like peptone (.05%, 0.1%, 0.2% and 0.4%), yeast extract (.00625%, .025%, .0125% and .05%) and coconut water (5%, 10%, 20% and 40%), different plant growth regulators like auxin (Indole acetic acid, α-napthalene acetic acid, 2,4- dichlorophenoxyacetic acid) and cytokinin (6- benzylaminopurine) were also added to medium in various concentrations. For *in vitro* seed germination of *Dendrobium primulinum* and *Dendrobium nobile* two auxin modulators (p-coumaric acid and chlorogenic acid) and two polar transport inhibitors were supplemented in the basal KCP medium.

- **Germination and seedling growth:**
  
  Germination of seeds was considered to have occurred when the embryo emerged from the ruptured seed coat (De Pauw *et al.* 1995). The growth of the seedling in different treatments was expressed through quantitative assessment of the relative stage of seedling development, such as seedlings with or without visible shoot apex, seedlings with expanded leaves and seedlings with roots. In the latter case the growth was expressed as a percentage of the total live seedlings at the end of the three-month culture period.

- **Statistical analysis:**
  
  The mean values of different morphogenetic responses were shown along with their respective standard errors (SE), and were analyzed by single factor ‘analysis of variance’ (ANOVA). After obtaining a significant p-value (< 0.05), the comparison of treatment means
was done by determination of ‘least significance difference (LSD)’ values in conjunction with Duncan’s Multiple Range Test (DMRT). All statistical analysis was performed according to Little and Hills (1978).

3.2.1. B In vitro clonal propagation:

- **Plant material and inoculation:**
  
The clonal propagation of different species was carried out with seedlings growing in aseptic conditions. Therefore, there was no need for surface sterilization of the explants. The cultures were performed with intact seedlings, shoot-tips (4-6 mm in length) and leaf segments (4-8 mm in length), excised from aseptically raised 3-months old seedlings of various species. For shoot-tip derived callus culture of different species, the calli originally induced in Knudson’s C (1946) basal media supplemented with 0.1% peptone and auxin-cytokinin combination. But subcultured on PGR-free basal medium for more than 12 months. For each treatment 42 explants were cultured in five replicate flasks (250 ml Erlenmeyer), each containing 100 ml culture medium. The details of age and size of inoculum, number of observational units have given along with the results of individual experiments. The cultures were incubated in the culture room at 25 ± 2° C under a 10-h photoperiod provided by Philips white fluorescent lights of 37.5 µmol/m²/S intensity.

- **Nutrient medium:**
  
The basic culture medium was based on modified nutrient solution of Knudson’s C (1946) and the original iron source was replaced by Fe–EDTA as described by Murashige and Skoog (1962). The medium was supplemented with 2% (w/v) sucrose and 0.1% (w/v) peptone. The PGR treatments consisted of different concentrations and combinations of cytokinins (6-benzyleaminopurine, kinetin, indole propionic acid and thidiazurone) at 0, 1, 2, 4, 8 and 16 µM and auxin (α-naptheleneacetic acid) at 0, 1 and 2 µM. In case of *Dendrobium farmeri* and *Dendrobium nobile* shoot-tip culture experiments, three different polyamine (Spermine, spermidine and putrescine) treatments consisted of five different concentrations viz. 0.2, 0.4, 0.6, 0.8 and 1 µM were tested. Different carbohydrates like lactose, maltose, sucrose, galactose, mannose and glucose were also added at 3% concentration in *Dendrobium farmei* shoot-tip culture experiment in order to optimize regeneration of plantlets. The pH of the culture medium was adjusted to 5.2 prior to autoclaving. After adding 0.9% (w/v) agar (Merck Ltd. Mumbai,
India) the media were autoclaved at 1.02 kg/cm² for 20 min. The shoot-tip or leaf derived callus masses and PLBs were later maintained in PGR-free medium. The callus-derived PLBs, 2-3 mm in length with a visible shoot apex, were later transferred to fresh PGR-free medium (pH 5.2) for further development of plantlets.

- **Data collection and statistical analysis:**
  Various morphogenetic responses, derived from different explants were recorded periodically and the final data for analysis were gathered after 3 months of culture. Since the morphogenetic responses are multi-character in nature, multivariate analysis of variance (MANOVA) was conducted to evaluate the significance of the main effects of different cytokinin and auxin (NAA) and their interaction. The data is presented in result section of different experiments.

- **Acclimatization of seedling and plantlets:**
  Seedlings or plantlets with well-developed roots were selected for acclimatization. After taking out from the flasks the well rooted plantlets were rinsed thoroughly with distilled water to remove traces of nutrient medium from the plant body and then transplanted to the clay pots containing a potting mixture. The potting mixture consisted of dried coconut husk, small pieces of brick and charcoal (1:1:1 v/v). Immediately after transplantation, plantlets were treated with 0.1% (w/v) thiram solution (tetramethyl thiram disulphide) to control fungal infection. Plantlets were sprinkled with water twice daily. The transplants were kept in a well-ventilated location in the experimental garden and maintained under subdued light and 80% humidity condition for initiation of further growth. For each species about 20-30 individual seedlings/plantlets were used in the hardening process.

3.2.2. Molecular studies:

**Assessment of clonal fidelity of micropropagated plantlets with their parents:**

The clonal fidelity of micropropagated plantlets was evaluated in the present study through ISSR and RAPD fingerprinting. DNA extraction followed by PCR amplification with ISSR and RAPD analysis was done, where DNA samples were collected from the in vitro generated clones of 5 plants and their respective parent.


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**Extraction of DNA:**

**Materials:**

1. DNA extraction buffer: - 1M Tris-HCl (pH-8) [Merck, India], 5M NaCl, 0.5M EDTA (pH-8)
2. 10% SDS [SRL, India]
3. 5M Potassium acetate [SRL, India]
4. RNA ase (Ribonuclease) [Genei, Bangalore, India]
5. Isopropanol [SRL, India]

**Procedure:**

Fresh green tender leaves of 5 samples were collected from the departmental orchidarium and were immediately wrapped in aluminum foil and stored in an ice-pack till the material reached the laboratory. Tender plantlets (25 samples) were collected from 25 different culture bottles. Then they were washed thoroughly and soaked with tissue paper. DNA extraction was carried out using the method described by Bhattacharyya and Mandal (1999). To extract genomic DNA about 40 mg leaf tissue was taken. The tissue was crushed well in mortar pestle by adding 600µl pre-autoclaved DNA extraction buffer and homogenized properly in different eppendorf tubes. 75 µl of 10% SDS was then added and mixed thoroughly followed by incubating it at 65°C hot water bath for 10 minutes. 200 µl of 5M Potassium acetate was added and mixed uniformly. Then the mixture was kept in ice for 20 minutes, followed by centrifugation at 12,000 rpm for 15 minutes at 26°C in micro centrifuge. The resultant supernatants were then carefully collected in a fresh eppendorf tube. 1 µl RNA ase was added and kept for 30 minutes at 37°C dry water bath for degradation of RNA present in the sample. Following this, Isopropanol, equivalent to 60% volume of the supernatant was added to the mixture and incubated at room temperature. To get precipitation of DNA, the supernatant was discarded and the pellet was air dried till there was no smell of alcohol followed by suspension in 100 µl of sterile water.

After extraction, the quality and quantity of all samples were tested by running each extracted DNA sample in 1% agarose gel stained with ethydium bromide. After verification the samples were further diluted to a uniform concentration of 40ng/ µl. The extracted DNA samples along with the diluted samples were stored in -20°C freezer.
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In vitro studies of some selected orchids: histological and cytological investigations of the regenerants.

**PCR amplification through RAPD primers:**

**Materials:**

1. Genomic DNA (40ng)
2. 10x Taq buffer (PCR buffer) [Genei, Bangalore]
3. 2.5mM dNTPs Mix [Genei, Bangalore]
4. Primers- The primer sequence and annealing temperature of two RAPD primers were depicted below:

<table>
<thead>
<tr>
<th>Primers</th>
<th>Sequence</th>
<th>Tm</th>
</tr>
</thead>
<tbody>
<tr>
<td>S1</td>
<td>5’ CAGGCCCTTC3'</td>
<td>34°C</td>
</tr>
<tr>
<td>S2</td>
<td>5’ TGCCGAGCTG3'</td>
<td>34°C</td>
</tr>
</tbody>
</table>

5. Gel loading buffer (Dye) - 50% glycerol in TE buffer (50mM Tris, 10mM EDTA), a pinch of Bromophenol Blue.

**Procedure:**

Diluted DNA samples were subjected to PCR amplification, using the selected RAPD primers in Gene AMP PCR system 9700. The 25µl optimized PCR mixture contained 2 µl (40ng) template DNA, 2.5 µl 10x PCR buffer, 4 µl 2.5mM dNTPs, 0.5U Taq DNA polymerase and 1 µl RAPD primers and 15 µl double distilled sterile water. Reaction condition was initiated by initial denaturation at 94°C for 5 minutes followed by 35 cycles of 30 seconds at 94°C, annealing of primer at 36°C for 30 seconds and final extension at 72°C for 1:30 minute and 7 minute followed by final hold at 4°C for 5 minutes. The annealing temperature was adjusted according to the Tm of the primer being used in the reaction. Reactions were verified using gel-electrophoresis and were preserved at -20°C.

**PCR amplification through ISSR primers:**

**Materials:**

1. Genomic DNA (40ng)
2. 10x Taq buffer (PCR buffer)
3. 2.5mM dNTPs mix
4. 0.5 Taq DNA polymerase
5. Gel loading buffer- 50% Glycerol [Merck, India] in TE buffer (50mM Tris, 10mM EDTA), a pinch of Bromophenol Blue.

6. Primers- The primer sequence and annealing temperature of 6 ISSR primers were shown below:

<table>
<thead>
<tr>
<th>Primers</th>
<th>Sequence</th>
<th>Tm</th>
</tr>
</thead>
<tbody>
<tr>
<td>IS8</td>
<td>5'-AGAGAGAGAGAGAGAGC-3'</td>
<td>52°C</td>
</tr>
<tr>
<td>IS10</td>
<td>5'-CGAGAGAGAGAGAGAGA-3'</td>
<td>52°C</td>
</tr>
<tr>
<td>IS11</td>
<td>5'-CACACACACACACACACAG-3'</td>
<td>52°C</td>
</tr>
<tr>
<td>IS12</td>
<td>5'-GTGTGTGTGTGTGTGTC-3'</td>
<td>52°C</td>
</tr>
</tbody>
</table>

Procedure:-

Diluted DNA samples were subjected to PCR amplification using the selected ISSR primers in Gene Amp PCR system 9700. The 25 µl optimized PCR mixture contained 2 µl (40ng) diluted DNA, 1.5 µl primer, 2.5 µl 10x Taq polymerase assay buffer, 3.5 µl 2.5mM dNTPS, 0.5U Taq DNA polymerase and 15 µl sterile water. PCR performance consisted of an initial denaturation at 94°C for 5 minutes followed by 35 cycles of 45s at 94°C, 45s at annealing temperature 46°C, 1:30 minutes at 72°C and final extension at 72°C for 7 minutes, 4°C for 5 minutes. On completion of reaction PCR products were stored in -4°C freezer.

Gel Electrophoresis and Documentation:-

Materials:-

1x TBE buffer- 100mM Tris (pH – 8) [Merck, India], 89mM Boric acid [SRL, India], 1mM EDTA 0.5% (5mg/ml) Ethydium bromide (SRL, India)

Low melting agarose [SRL, India, Genei, Bangalore]
Chapter 3: Materials and Methods

In vitro studies of some selected orchids: histological and cytological investigations of the regenerants.

Procedure:-

Amplification products were subjected to gel electrophoresis in 1.5% agarose gel i.e. 1.5 gm of agarose was weighed and dissolved in 100ml 1x TBE buffer. Then it was allowed to melt with frequent shaking, gradually the solution becomes clear. It was kept in room temperature and 1 µl EDTA was added and mixed by gentle swirling. Finally it was poured into gel tray to solidify. Amplified products were loaded and run for half an hour at fixed voltage 80 volt. Banding patterns were photographed on gel documentation system (KODAK).

3.2.3. Cytological studies:

Study of mitotic chromosome of micropropagated plantlets:

For karyological study, fresh healthy root-tips, which showed peak mitotic activity from 10 A.M. to 11 A.M. were taken out from the flasks and thoroughly washed in distilled water. For removal of the heavy latex, root tips were longitudinally split into pieces instead of taking root tips as a whole (Sharma and Bhattacharjee, 1952) and were placed under white lamp for half an hour.

Pretreatment:- For scattering and clarification of chromosome morphology, among several pretreatment chemicals in general saturated solution of para-dichloro benzene (PDB) with trace amount of aesculine was found to be most effective. Chilling treatment of pretreatment chemical at 1-3°C for 10 minutes was essential before pretreatment. Then root tips were poured in pretreatment chemicals and incubated at 14-16 ºC for six hours.

Fixation: - Pretreated root tips were then transferred to a suitable fixative such as glacial acetic acid and ethanol (1:3) for 12 hours.

Staining: - After fixation the root tips were transferred to 45% acetic acid for 15 minutes. Then they were treated with a mixture of 1N HCl and 2% aceto-orcein in the proportion of 1:9 and gently heated for 2-3 seconds and kept for 5-6 hours for staining.

Squashing: - Finally root-tips were squashed in 45% acetic-acid applying uniform pressure over the cover glass (La Cour, 1941) for better scattering of the cells. Excess fluid from slide was blotted off and the cover glass was sealed with paraffin and cytogenetic examination and
In vitro studies of some selected orchids: histological and cytological investigations of the regenerants.

Chapter – 3: Materials and Methods

Chromosome counting were carried out and photographed with an Olympus CH 30 research microscope.

Data regarding the chromosome morphology was obtained from well spread metaphase plate and the features of chromosomes studied are follows:

1. Length of short arm of the chromosome
2. Length of whole chromosome

From these two values, the centromeric index (F %) was calculated with the help of following formula: \[ F\% = \frac{\text{Length of short arm (\(\mu m\))}}{\text{Total length of the chromosome (\(\mu m\))}} \times 100 \]

Chromosome morphology was designated according to Levan et al. 1964 on the basis of centromeric index (F %). The centromeric index (F %) or range of centromeric index (F %) indicating the nature of constriction are given in following table:

<table>
<thead>
<tr>
<th>Centromeric index (F %)</th>
<th>Nature of constriction</th>
</tr>
</thead>
<tbody>
<tr>
<td>50</td>
<td>Median</td>
</tr>
<tr>
<td>49.9-37.5</td>
<td>Nearly median</td>
</tr>
<tr>
<td>37.4-25.1</td>
<td>Nearly sub median</td>
</tr>
<tr>
<td>25</td>
<td>Sub median</td>
</tr>
<tr>
<td>24.9-18.75</td>
<td>Nearly sub median</td>
</tr>
<tr>
<td>18.74-12.6</td>
<td>Nearly sub terminal</td>
</tr>
<tr>
<td>12.5</td>
<td>Sub terminal</td>
</tr>
<tr>
<td>12.4-6.26</td>
<td>Nearly sub terminal</td>
</tr>
<tr>
<td>6.25-1.00</td>
<td>Extremely sub terminal</td>
</tr>
<tr>
<td>&lt;1</td>
<td>Terminal</td>
</tr>
</tbody>
</table>

3.2.4. Histological studies of the regenerants:

For histological studies, the tissues of appropriate stages of the regenerants were taken out from the flasks and washed thoroughly with distilled water after 30 days of inoculation. Thin free-hand sections were made sequentially. Finally the slides were mounted with glycerin and observed under microscope. Images were captured and recorded with a Olympus CH-30 research microscope fitted with dedicated photographic attachment.