Chapter 3

Materials and Methods
CHAPTER 3

MATERIALS AND METHODS

To achieve the aims and objectives framed in chapter 1, a factorial randomized design pot experiment was conducted during the ‘Rabi’ (winter) season of 2006-07, to investigate the effect of various plant growth regulators (PGRs) i.e., IAA, IBA, NAA, BAP, KIN, TDZ, GA₃, SA, HBR and TRIA, respectively on the performance of two common cultivars of periwinkle (*Catharanthus roseus* L.), namely ‘rosea’ and ‘alba’. The pot experiment was conducted in the net house of the Department of Botany, Aligarh Muslim University, Aligarh. The details of agro-climatic conditions, soil analyses of the experimental pots and the techniques and procedures employed in this regard are given below.

3.1 Agro-climatic conditions

Aligarh, is a town of western Uttar Pradesh (North India) and is located 130 km east of Delhi at 27°52’ N latitude, 78°51’E longitude and is 187.45 m altitude above sea level. It has semi-arid and subtropical climate, with severest hot dry summers and intense cold winters. The winter extends from the middle of October to the end of March. The mean temperatures for December and January, the coldest months, are about 15°C and 13°C, respectively. The extreme minimum recorded for any single day is 2°C and 0.5°C respectively. The summer season extends from April to June and the average temperature for May is 34.5°C and 45.5°C, respectively.

The monsoon extends from the end of June to the middle of October and temperature ranges between 26°C to 30°C. The mean annual rainfall is about 847.3 mm. More than 85% of the total down pour is delivered during a short span of four months from June to September. The remaining showers are received during winter. Winter season ranges between 56% and 77% with an average of 66.5%. In the summer, it ranges between 37% to 49% with an average of 43%. Whereas, in the monsoon season, the R.H. ranges between
63% and 73% with an average of 68%. The metrological data were recorded during the investigation period at the Meteorological Observatory, Department of Physics, Aligarh Muslim University, Aligarh. Aligarh district has the same soil composition and appearances as those found generally in the plains of Uttar Pradesh. Different types of soils, such as sandy, loamy, sandy-loam and clay-loam are found in the district.

3.2 Soil analysis

Prior to transplanting, soil samples were collected randomly from different pots for the analysis of the soil characteristics. The samples were analyzed in the soil testing Laboratory, Government Agriculture Farm, Quarsi, Aligarh. The physico-chemical properties of soil are given in Table 1.

3.3 Preparation of pots

Prior to transplanting, a 5.0 kg homogenous mixture of soil and cow dung manure in the ratio of 5:1 was filled in the earthen pots of 10" size. These filled pots were arranged according to the simple randomized complete block design of the experiment in the net house, Department of Botany, AMU, Aligarh.

3.4 Seedling

The healthy seedlings of two cultivars of periwinkle were collected from nursery, Aligarh. Healthy seedlings of uniform size were selected for transplanting and one seedling was maintained in each pot.

3.5 Experiment 1

Experiment 1 was conducted during the winter season of 2006-07, on Catharanthus roseus L. The aim of this experiment was to find out the best PGR, among the various plant growth regulators and the cultivar also in terms of growth, physiological and biochemical, yield and quality parameters under the agro climatic conditions of Aligarh, western Uttar Pradesh. The experiment was conducted according to a factorial randomized design. Healthy seedlings of uniform size were selected and transplanted in each pot. Transplanting was
Table 1. Physico-chemical characteristics of soil sued for Experiment 1.

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>Experiment I</th>
</tr>
</thead>
<tbody>
<tr>
<td>Texture</td>
<td>Sandy loam</td>
</tr>
<tr>
<td>pH (1:2)</td>
<td>7.3</td>
</tr>
<tr>
<td>Conductivity (E.C. (1:2) m mhos/cm)</td>
<td>0.48</td>
</tr>
<tr>
<td>Available nitrogen (mg N per kg soil)</td>
<td>98.46</td>
</tr>
<tr>
<td>Available phosphorus (mg P per kg soil)</td>
<td>6.98</td>
</tr>
<tr>
<td>Available potassium (mg K per kg soil)</td>
<td>142.9</td>
</tr>
</tbody>
</table>
done in the pots on 2\textsuperscript{nd} March, 2007. Before transplanting, a uniform basal doses of nitrogen, phosphorus and potassium were given at the rate of 15, 25 and 25 kg per ha, respectively. The plants were kept free from weeds and irrigated as and when required. The plants were sprayed with deionized water (control) or $10^{-7} \text{ M}$ each of IAA, IBA, NAA, BAP, KIN, TDZ, GA$_3$, SA, HBR and TRIA at 60 days after planting (DAP). The PGRs were sprayed regularly at intervals of 20 days. In this experiment, three plants were sampled at fruiting stage (150 DAP). Yield parameters were recorded at the time of harvest (210 DAP).

3.6 Sampling technique

Three plants from each treatment were sampled randomly to assess the growth, physiological and biochemical and quality parameters at fruiting stage and yield parameters were analyzed at the time of harvest. The parameters recorded are listed below.

3.6.1 Growth parameters

1. Shoot and root length
2. Shoot and root fresh weight
3. Shoot and root dry weight
4. Leaf-area index

3.6.2 Physiological and biochemical parameters

5. Total chlorophyll content
6. Total carotenoids content
7. Net photosynthetic rate
8. Carbonic anhydrase (CA) activity in fresh leaves
9. Nitrate reductase (NR) activity in fresh leaves
10. Leaf-N, P and K contents
3.6.3 Yield and quality parameters

11. Number of pods per plant
12. Number of seeds per pod
13. 100-seed weight
14. Seed-yield per plant
15. Alkaloid content in dried leaves and roots

3.6.1.1 Shoot and root length

The length of shoot and root of each plant from each treatment was measured separately with the help of a meter scale.

3.6.1.2 Shoot and root fresh weight

At 150 DAP, three plants from each treatment were uprooted carefully and washed with running tap water to wipe off all adhering foreign particles. They were soaked thereafter using blotting sheets. The fresh weight of shoot and root of each plant from each treatment was determined separately using an electronic balance.

3.6.1.3 Shoot and root dry weight

The shoot and root of each plant from each treatment were dried in a hot air oven at 80°C for 24 hours to record the dry weight.

3.6.1.4 Leaf area index

Leaf area index was determined by using following formula suggested by Watson (1947).

\[
\text{LAI} = \frac{\text{Leaf area per plant}}{\text{Area occupied by plant}}
\]

Physiological and biochemical parameters were determined, using the following methods.
3.6.2.1 Total chlorophyll content

Total chlorophyll content was estimated in fresh leaves collected from each treatment at 150 DAP by the method of Arnon (1949). One hundred mg fresh tissue from interveinal areas of leaves was ground using a mortar and pestle in 10 mL of 80% acetone (Appendix). The suspension was filtered through Whatman filter paper No. 1 and the filtrate was collected in a volumetric flak. Optical Density (OD) was recorded at 645 and 663 nm using a spectrophotometer (Spectronic 20D*, Milton Roy, USA). The total chlorophyll content was expressed as mg g⁻¹ FW and calculated using the following formula:

Total chlorophyll (mg g⁻¹ FW) = 20.2 × (OD 645) + 8.02 × (OD 663)

3.6.2.2 Total carotenoids content

The procedure for the preparation of solution was same as that of chlorophyll content estimation. The filtrate sample was recorded at 480 and 510 nm using a spectrophotometer for carotenoids estimation. The total carotenoids content were calculated using the following formula.

\[ \text{Carotenoids content (mg g}^{-1} \text{FW)} = 7.6 \times (\text{OD 480}) - 1.49 \times (\text{OD 510}) \times \frac{V}{d \times \text{FW} \times 1000} \]

Where,

\[ \text{OD} = \text{Optical density of the extract at given wavelength (viz. 645, 663, 480 and 510 nm)} \]

\[ V = \text{Final volume of chlorophyll extract in 80% acetone} \]

\[ \text{FW} = \text{Fresh weight of leaves (g)} \]

\[ d = \text{Length of the path of light (1 cm)} \]

3.6.2.3 Net photosynthetic rate

Net photosynthetic rate was observed on clear days at 11.00 A.M. on fully expanded leaves of *Catharanthus roseus* L. using an IRGA (Infra Red Gas Analyzer, LICOR 6200 Portable Photosynthesis System, Nebraska, USA). The

*This equipment was used for all spectrophotometric analysis presented in this Dissertation.*
IRGA was calibrated and zero was adjusted approximately every half hour during the measurement period. The fully expanded leaves were enclosed in a one-liter gas exchange chamber for 60 seconds. These measurements were recorded three times in each treatment. Net photosynthetic rate is expressed as mol CO$_2$ kg$^{-1}$ leaf FWs$^{-1}$.

### 3.6.2.4 Carbonic anhydrase (CA) activity

The carbonic anhydrase activity in fresh leaves was analyzed using the method described by Dwivedi and Randhawa (1974).

The fresh leaf samples were cut into small pieces at a temperature below 25°C. Two hundred mg of these pieces were weighed and transferred to petri plates. The leaf pieces were taken in 10 mL of 0.2M cystein hydrochloride (Appendix) and left for 20 minutes at 4°C. The leaf pieces were blotted and transferred to a test tube containing 4 mL of phosphate of pH 6.8 (Appendix). To the test tube, 4 mL of 0.2M sodium bicarbonate solution (Appendix) and 0.2 mL of 0.002% bromothymol blue (Appendix) was added. The test tubes were shaken gently and left for 20 minutes at 4°C. CO$_2$ liberated by the catalytic action of CA on NaHCO$_3$ was estimated by titrating the reaction mixture against 0.05N HCl (Appendix) using methyl red (Appendix) as indicator. In each sample, the quantity of HCl used to neutralize reaction mixture was noted and difference was calculated. A blank consisting of all the above components of reaction mixture, except the leaf sample, was run simultaneously with each set of the samples. The activity of CA was calculated by putting the values in the following formula:

\[
\frac{V \times 22 \times N}{W} \quad [\text{mol (CO}_2\text{) kg}^{-1} \text{ (leaf FW) S}^{-1}]\]

Where,

\[V = \text{Difference in volume (mL of HCl used in control and test sample titration)}\]

\[22 = \text{Equivalent weight of CO}_2\]
The activity of nitrate reductase in fresh leaves collected from each treatment was estimated by the method of Jaworski (1971).

The leaves were cut into small pieces (1 cm²). Two hundred mg of these chopped leaves were weighed and transferred to plastic vials. To each vial, 2.5 mL of phosphate buffer pH 7.5 (Appendix) and 0.5 mL of potassium nitrate solution (Appendix) were added followed by the addition of 2.5 mL of 5% isopropanol (Appendix). These vials were incubated in BOD incubator for 2 hours at 30±2°C in the dark. 0.4 mL of incubated mixture was taken in at test tube to which 0.3 mL each of sulphanilamide solution (Appendix) and NED-HCl (appendix) were added. The test tubes were left for 20 minutes for maximum colour development. The mixture was diluted to 5 mL by adding double distilled water (DDW). The OD was recorded at 540 nm using a spectrophotometer.

A blank was run simultaneously with each set of determination. Standard curve was plotted by using known graded concentration of NaNO₂ (sodium nitrite) solution. The OD of each sample was compared with that of calibration curve of sodium nitrite and nitrate reductase activity expressed as nM NO₂⁻ g⁻¹FW h⁻¹.

3.6.2.6 Leaf-N, P and K contents

The leaf samples from all the plants were collected at their fruiting stage (150 DAP) for assessing the N, P and K contents. The leaves were dried in hot-air oven at 80°C for twenty four hours. Dried leaves were powdered and the powder was meshed using a 72 mesh. The powder was labeled and stored in small polythene bags for the chemical analysis. The same leaf-powder was used for the estimation of nitrogen, phosphorus and potassium contents.
3.6.2.6.1 Digestion of leaf powder

One hundred mg of oven-dried leaf powder was carefully transferred to a digestion tube and 2 mL of AR Grade concentrated sulphuric acid was added to it. It was heated on a temperature controlled assembly for about two hours to allow complete reduction of nitrates present in the plant material. After heating, the contents of the tube turned black. It was cooled for about 15 minutes at room temperature and then 0.5 mL 30% hydrogen peroxide (H₂O₂) was added drop by drop. The solution was heated again till the colour of the solution changed from black to light yellow. Again after cooling for about 30 minutes, additional 3 to 4 drops of 30% H₂O₂ were added, followed by reheating for another 15 minutes. The addition of H₂O₂ followed by heating was repeated until the contents of the tube turned colorless. The peroxide digested material was transferred from the tube to a 100 mL volumetric flask with three washings of DDW. The volume of the volumetric flask was then made up to the mark (100 mL) with DDW. This aliquot was used to estimate N, P and K contents. The details of methods used for the analysis of these elements are given below separately.

3.6.2.6.2 Nitrogen

Nitrogen was estimated according to the method of Lindner (1944). A 10 mL aliquot of the digested material was taken in a 50 mL volumetric flask. To this, 2 mL of 2.5 sodium hydroxide (Appendix) and 1 mL of 10% sodium silicate (Appendix) solutions were added to neutralize the excess of acid and to prevent turbidity, respectively. The volume was made up to the mark with DDW. In a 10 mL graduated test tube, 5 mL aliquot of this solution was taken and 0.5 mL Nessler's reagent (Appendix) was added. The contents of the tubes were allowed to stand for 5 minutes for maximum colour development. The OD of the solution was read at 525 nm, using the spectrophotometer. The reading of each sample was compared with the standard calibration curve to estimate the per cent nitrogen content on dry weight basis. The standard curve
was plotted using different concentrations of ammonium sulphate solution versus O.D. of the solution.

3.6.2.6.3 Phosphorus

Phosphorus was estimated according to the method of Fiske and Subba Row (1925). A 5 mL aliquot was taken in a 10 mL graduated test tube. To it, 1 mL molybdic acid (2.5%) was added carefully, followed by addition of 0.4 mL 1-amino-2-naphthol-4-sulphonic acid (Appendix). When the colour turned blue, the volume was made upto 10 mL with the addition of DDW. The solution was shaken for five minutes and then transferred to a spectrophotometric tube. The OD of the solution was read at 620 nm, using a blank. The standard curve was plotted using different concentrations of potassium dihydrogen orthophosphate versus OD. The percent phosphorus content was determined on the dry weight basis.

3.6.2.6.4 Potassium

Potassium content in leaf was analyzed flame photometrically (Hald, 1946). In the flame photometer, the solution (peroxide digested material) is discharged through an atomizer in the form of a fine mist into a chamber, where it is drawn in to a flame. Combustion of the elements produces light of a particular wavelength [\( \lambda_{\text{max}} \) for K = 767 nm (violet)]. The light produced was conducted through the appropriate filters to impinge upon a photoelectric cell that activates a galvanometer.

The air was supplied through an air pump and LPG (Liquid Petroleum Gas) was used for combustion. The chimney of the equipment was removed and the gas was ignited using an electric lighter. The final pressure of the two gases was adjusted to 15 pounds per inch. When the flame formed sharp blue cones, the correct filter was set and DDW was introduced (using a beaker) and the galvanometer was set to zero. Then the standard solution of the element was sucked through a capillary tube and the galvanometer was adjusted to the 100 position by using the amplifier. Unless the 0 and 100 points are maintained on successive readings, the gas pressure, air-pressure or both were adjusted to
bring about a stable position. Thereafter, intermediate standards (i.e. diluted solutions of known concentrations between 0 and 100 per cent) were checked and a graph was prepared. The relationship between the galvanometer reading and the concentration do not appear in a straight line. Rather it appears in a curvilinear fashion. Lastly, the samples were run and exact concentration of the element was calculated using the graph. A standard curve was prepared, using different dilutions of potassium chloride solution versus the reading on the scale of the galvanometer. The per cent potassium content was estimated on dry weight basis.

3.6.3.1 Total alkaloid content in leaves/roots

Total alkaloid content was estimated in leaves and roots of each treatment as described by Afaq et al. (1994). The leaves/roots were dried in a hot-air oven at 80°C for twenty four hours. The sample was powdered and filtered through a 72 mesh screen. Five hundred mg powder of leaves/roots was taken in a 100 mL round bottom reflux flask, to this a known volume of ethyl alcohol was added. Then the mixture was refluxed for 6 hours. Thereafter, it was filtered and a 50 mL of dilute HCl was added and the later on it was transferred to a separating funnel to which 50 mL of diethyl ether was added. This mixture was shaken for 15-20 minutes. The upper diethyl ether layer was discarded and the lower water layer was decanted in a beaker and was made slightly basic by adding ammonia solution and by checking it with litmus paper. The decant was again transferred into a separating funnel with 50 mL of diethyl ether layer and was decanted. Again to this decant, anhydrous sodium carbonate was added. The mixture was again decanted in a preweighed dry porcelain dish and evaporated till dryness and weighed again.

Total alkaloid content (%) was calculated using following formula:

\[
\frac{W_E - W_A}{W_R} \times 100
\]
Where,

\[ W_E = \text{Weight of empty porcelain dishing (g)} \]
\[ W_A = \text{Weight of porcelain dish after evaporation (g)} \]
\[ W_R = \text{Weight of the powder (g)} \]

3.6.4. Statistical analysis

The data of all experiments were analyzed statistically by adopting the analysis of variance technique, according to Gomez and Gomez (1984). For the F test, the error due to replicates was also determined. When F value was found to be significant at 5% level of probability, critical difference (CD) was calculated. The models of analysis of variance for the designs employed are given in Table 2.
Table 2. Model of the ‘analysis of variance’ (ANOVA) for Experiment 1.

<table>
<thead>
<tr>
<th>Source of variation</th>
<th>Experiment 1 (Factorial randomized design)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>DF</td>
</tr>
<tr>
<td>Replicates (R)</td>
<td>2</td>
</tr>
<tr>
<td>PGR treatment (T)</td>
<td>10</td>
</tr>
<tr>
<td>Cultivars (Cv)</td>
<td>1</td>
</tr>
<tr>
<td>T x Cv</td>
<td>10</td>
</tr>
<tr>
<td>Error</td>
<td>42</td>
</tr>
<tr>
<td>Total</td>
<td>65</td>
</tr>
</tbody>
</table>