CHAPTER - II

MATERIAL AND METHODS

1. MATERIAL :

Catharanthus roseus G. Don. (Vinca rosea L.) plants were raised in pot soil culture from the seeds, in the Botanical Garden of Department of Botany, Shivaji University, Kolhapur. The plants, after their establishment after a month were further grown in the soil media at various NaCl salinity levels (0.0, 25, 50, 100, 200 and 300 mM NaCl) for next two months. The salinity levels in the media were raised slowly beginning with 25 mM NaCl for all the plants except control reaching the final respective levels slowly. Plants were treated twice a week with alternate watering with equal amount of tap water to check the loss of water due to evaporation from the culture containers and to maintain the salt concentration in the medium. After 2 months, when efffect of salt treatment was visually evident, the plants were used for further studies.

To minimise the errors, random sampling was done. The plants to be studied were harvested simultaneously. For photo-synthetic studies generally third leaf was used. Number of such selected leaves were cut into small discs, mixed together and used for the study. Root, stem and leaf (other than 3rd) material was collected using the same method. This material was washed thoroughly with tap water first, then rinsed with distilled water and surface dried.

2. METHODS :

A) Growth Analysis :

The plants, after completion of NaCl salt treatments were used for growth analysis. The plants were uprooted carefully and thoroughly washed with tap water and blotted to dryness. The growth analysis includes, shoot length, root length, total length and shoot/root ratio; the number of leaves, leaf area, number of buds, number of flowers and number of pods produced per plant and the biomass production (fresh and dry matter production) of each part of plant and plant as a whole. From each treatment pot ten plants were used and mean was calculated.

B) Organic Constituents :

Organic constituents were determined from both fresh as well as oven dried plant material. From the fresh plant material, TAN and organic constituents like chlorophylls, polyphenols and proline while from the oven dried plant material carbohydrates, total nitrogen, and total alkaloids were determined.

i) Moisture Content :

The plants grown in pot soil culture were uprooted carefully and thoroughly washed under tapwater and blotted to dryness.Leaves, stem and roots of plants were separated. Well washed and blotted plant material was cut into small bits and weighed accurately. This weight served as a fresh weight of plant material. The material was kept in oven for drying at 60°C. After 4-5 days the dried plant material was weighed. This was repeated for 2-3 times for next 2-3 days till it showed a constant weight. The difference between fresh and dry weights was used for calculation of moisture percentage.

ii) Titratable Acid Number (TAN) :

The method described by Thomas and Beevers (1949) was used to determine TAN. The fresh leaf material was washed and rinsed with distilled water and blotted to dryness. It was accurately weighed (2 g) and cut into small bits. Then it was imersed in boiling water and boiled for half an hour in about 20 ml. boiling water with occasional addition of hot water to prevent drying. It was cooled and filtered through double layered cheese-cloth and volume was made with distilled water (20 ml). The filtrate was then titrated against standardized NaOH (approx. N/40) using phenolphthalein as an indicator. NaOH was standardised against N/40 oxalic acid using the same indicator. Titratable Acid Number (TAN) represents the number of ml of decinormal NaOH required to neutralise the acid contents in 100 g of fresh tissue.

iii) Carbohydrates :

Carbohydrates were estimated according to the method described by Nelson (1944). 1 g oven dried plant material was homogenised in morter with pestle and extracted with 80% alcohol. It was filtered through Buchner's funnel using Whatman No.1 filter paper. The residue on filter paper was washed with 80% alcohol repeatedly. All the washings and filtrate were mixed together. This filtrate was used for estimation of soluble sugars while the

residue was saved for estimation of starch.

The filtrate was condensed on the water bath to about 3 ml, and to it, were added lead acetate and potassium oxalate (1 g each) to decolourise it. It was mixed together with the help of glass rod with the addition of some water. It was again filtered and washed with distilled water 2-3 times, collecting the washings in the same filtrate. The final volume of filtrate was made 50 ml with distilled water. This filtrate was used for estimation of reducing sugars (A). From this 20 ml of the filtrate was taken into the conical flask and hydrolysed with 2 ml conc. HCl in an autoclave at 15 lbs pressure for half an hour. The contents were cooled, neutralised with Na_2CO_3 and filtered. This filtrate was used for the estimation of total (reducing + nonreducing) sugars (B). The volume of the filtrate was noted down.

The residue on the filter paper saved for starch estimation was transferred to a conical flask with 50 ml of distilled water and 5 ml of conc. HCl. This was hydrolysed, neutralised and filtered as stated above. This filtrate contains reducing sugars produced as a result of hydrolysis of starch. The sugars so available were estimated to determine the starch present in the tissue (C). The volume of the filtrate was also noted down.

The requisite quantity (preferably 0.1 ml) of the above filtrates A, B and C was taken separately in 10 ml marked test tubes. Different concentrations (0.1, 0.2, 0.3, 0.4 and 0.5 ml) of standard glucose solution (0.1 mg ml^{-1}) were also taken in other test tubes. 1 ml of alkaline copper tartarate reagent - (4 g $CuSO_4.5H_2O$, 24 g unhydrous Na_2CO_3, 16 g Na-K-tartarate and 180 g unhydrous Na_2SO_4 were dissolved in distilled water and volume was made to 1000 ml) was added to each test tube.

All the test tubes containing the reaction mixtures were subjected to boiling water bath for about 15 min. and then cooled to room temperature. 1 ml of arsenomolybdate reagent (25 g ammonium molybdate in 450 ml distilled water and to this were added 21 ml of conc. H_2SO_4. This was mixed with solution containing 3 g sodium arsenate dissolved in 25 ml distilled water. The mixture of the solutions was placed in an incubator at 37°C for 48 hours) was added to each test tube and shaken vigorously. The volume of the reaction mixture in each test tube was made 10 ml with distilled water. A blank was prepared by the same way but without sugar solution. After 15 minutes, the optical density of the colour developed was measured at 560 nm on double beam spectrophotometer (Shimadzu, UV-190).

iv) Polyphenols :

Polyphenols were estimated by the method of Folin and Denis (1915). Fresh leaves were washed with tap water, rinsed

with distilled water and blotted to dryness and cut into small pieces. Polyphenols were extracted from 0.5 g material in 80% acetone. The extract was filtered through Buchner's funnel under suction using Whatman No.1 filter paper. Residue on filter paper was washed repeatedly with 80% acetone, to ensure complete extraction of polyphenols. The volume of the filtrate was made to 50 ml with 80% acetone. This filtrate was used for the estimation of polyphenols.

From the filtrate 4 ml were taken in 50 ml marked Nesslor's tube. In other such tubes different concentrations (0.5, 1.0, 2.0, 4.0 ml) of standard polyphenol solution (tannic acid, 0.1 mg ml^{-1}) were taken. 10 ml of 20% Na_2CO_3 were then added to each tube to make the medium alkaline. 2 ml of Folin Denis reagent (100 g sodium tungstate and 20 g of phosphomolybdic acid dissolved in 200 ml distilled water were mixed with 200 ml of 25% orthophosphoric acid and refluxed for about 3 hours, cooled to room temperature and volume was made to 1000 ml with distilled water) were then added to each test tube and finally the volume was made to 50 ml with distilled water. A blank was prepared similarly but without standard tannic acid. The ingradients were allowed to mix thoroughly. After 15 minutes, the absorbance was read at 660 nm on the double beam spectrophotometer (Shimadzu, UV-190) using reagent blank. Polyphenols were calculated from the calibration curve of standard tannic acid.

v) Total Nitrogen :

The spectrophotometric method described by Hawk et al. (1948) was used to estimate total nitrogen. 0.5 g of oven dried material was digested in a Kjeldahl flask with 10 ml conc. sulphuric acid and a pinch of microsalt (unhydrous $CuSO_4$ and K_2SO_4 mixed in the proportion of 1:40) till a colourless liquid was obtained at the bottom of the flask. It was then cooled to room temperature and transferred quantitatively to a 100 ml capacity volumetric flask and volume was made to 100 ml with distilled water. It was kept overnight and was filtered through dry Whatman No.1 filter paper. The filtrate was used for the estimation of total nitrogen.

2 ml of filtrate was taken in Nesslor's tube (35 and 50 ml marked).In other such tubes different concentrations of standard ammonium sulphate (0.05 mg N ml^{-1}) were taken. One test tube was kept as a blank without ammonium sulphate. To these tubes was added a drop of 80% $KHSO_4$. The volume of all the tubes was adjuted to 35 ml with distilled water. 15 ml Nesslor's reagent was then added in each tube, (Nesslor's reagent : - A.7 g of KI and 10 g of HgI_2 dissolved in 40 ml distilled water. B. 10 g NaOH dissolved in 50 ml distilled water. A and B were mixed in the proportion of 4:5). The reaction between the sample and the reagent gives the product $NH_4Hg_2I_3$ which has orange-

brown colour. This colour was measured at 520 nm on double beam spectrophotometer using reagent blank. The absorbance readings for plant extracts were compared with those of standard ammonium sulphate and total nitrogen was calculated.

vi) Proline :

The method of Bates et al. (1973) was adopted for estimation of proline. Proline was extracted from 0.5 g oven dried plant material in 10 ml of 3% sulphosalicylic acid and filtered through Whatman No.1 filter paper using Buchner's funnel. 2 ml of the filtrate were taken in 10 ml test tube and to this 2 ml of freshly prepared acid ninhydrin reagent (1.25 g ninhydrin added into the mixture of 50 ml glacial acetic acid and 20 ml of 6 M orthophosphoric acid - 33 ml orthophosphoric acid mixed with 67 ml distilled water, and warmed until ninhydrin was dissolved. It was cooled to room temperature) was added and shaken well. Various concentrations of standard proline solution (0.1 mg ml^{-1}) were also taken and subjected to reaction with acid ninhydrin as above. All the test tubes containing reaction mixtures were heated in boiling water bath for one hour. The reaction was terminated very rapidly by cooling at 0°C. To each test tube, 4 ml of toluene were added and shaken vigorously for few seconds. The toluene chromophore was pipetted out and its absorbance was read at 520 nm on double beam spectrophotometer.

vii) Total Alkaloids :

Estimation of Alkaloids :

Alkaloids were estimated following the method described by Thomas and Sharma (1979) with slight modification. 2 g dried powdered plant material was moistened with 28% NH_4OH in an evaporating dish and dried on a water bath. It was then refluxed with 50 ml chloroform on a water bath for about 3 hours. It was cooled, and filtered through the Buchner's funnel using Whatman No.1 filter paper. The residue was saved.

The filtrate was concentrated on water bath to 2-3 ml volume. To remove chlorophylls this volume was introduced in a microseparating funnel. To it 1 ml 1% HCl was added and mixture was allowed to separate into two zones. The chloroform layer was removed from the microseparating funnel. Few drops (5-10) of 28% NH_4OH were added to the aqueous acid layer in the microseparating funnel to liberate the free bases, and small amount (5 ml) of chloroform was added to it, to liberate all the alkaloids. These two chloroform extracts were combined and concentrated on water bath to 2-3 ml volume to give fraction I.

The residue was similarly refluxed with 15 ml ethanol, containing 0.5% HCl. It was cooled, filtered and concentrated on a water bath as above to give fraction II. The two fractions were

combined together in a crucible of known weight and evaporated to dryness. The difference between the weights of empty and alkaloid containing crucible gave the weight of total alkaloids.

Thin Layer Chromatography of Alkaloids :

The thin layer chromatograms of uniform thickness were prepared by using the glass plates and slury of Silica gel G (Silica gel G - Slury : 20 g of Silica gel G powder added to 100 ml distilled water with constant stirring) with the help of glass rod. It was dried at room temperature and then activated at 110°C for about 1 hour.

Equal amount of alkaloid samples (prepared by adding 3 ml of chloroform to the dry powder of total alkaloids) were scored at 2 cm intervals, parallel to the direction of solvent development according to the technique of McLaughin et al. (1964) The plates were then kept in ascending separation chamber, containing freshly prepared n-butyl alcohol: glacial acetic acid: distilled water (4:1:1) as solvent, till the solvent covered 12-15 cm path over the plates. The spots were developed by spraying the chromatogram with Dragendorff's reagent (Dragendorff's reagent : A. 8 g Bismuth nitrate dissolved in 20 ml conc. HNO_3. B. 27.2 g Potassium iodide dissolved in 50 ml distilled water. A and B solutions were mixed and allowed to stand, till the KNO_3 was precipitated out. The supernatant was

decanted off and made upto 1000 ml with distilled water. This gives orange red colour with the alkaloids). Coloured spots (orange, red, yellow) were outlined and Rf values were calculated.

C) Photosynthetic Studies :

i) Photosynthetic Pigments :

Chlorophylls :

Chlorophylls were estimated following the method by Arnon (1949). Chlorophylls were extracted in 80% acetone from 0.5 g of fresh plant material. A pinch of $MgCO_3$ was added during crushing to protect and stabilize the chlorophylls. This extract was filtered through Whatman No.1 filter paper using Buchner's funnel. Residue was washed repeatedly with 80% acetone collecting the washings in the same filtrate.The volume of the filtrate was made to 100 ml with 80% acetone. The absorbance was read at 663 and 645 nm for chlorophyll 'a' and 'b' respectively, on double beam spectrophotometer (Shimadzu, Japan) using 80% acetone as blank. The chlorophylls were calculated using the formulae:

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For Chl - a, $X = (12.7 \times A\ 663) - (2.69 \times A\ 645)$

For Chl - b, $Y = (22.9 \times A\ 645) - (4.68 \times A\ 663)$

For Total Chl - (a + b), $Z = (8.02 \times A\ 663) + (20.20 \times A\ 645)$

$$\text{Chl-a/Chl-b/Total Chl (mg } 100^{-1} \text{ g fresh tissue)} = \frac{X/Y/Z \times \text{Volume of extract} \times 100}{1000 \times \text{Wt. of the plant material (g)}}$$

ii) Stomatal Index :

Stomatal index was calculated following the method developed by Cutter, (1969). Healthy leaves at different developmental stages were taken and surface cleaned with the help of dry cotton. The uniform layer of nail paint was applied on both the surfaces of leaf and was allowed to dry for some time. Peels from the upper and lower surfaces of leaf were removed. Temporary slides were prepared by using glycerine. A window of 1 mm^2 area was prepared on 1 cm^2 graph paper and placed on the coverslip, number of stomata and epidermal cells per 1 mm^2 area were counted from both the surfaces of leaf. Three such readings at different places were taken and stomatal index was calculated by using the formula;

$$\text{Stomatal index} = \frac{S}{S + E} \times 100$$

where, S = Number of stomata per unit area (mm^{-2}),

E = Number of epidermal cells per unit area (mm^{-2}).

iii) Diffusive resistance for CO_2 :

The diffusive resistance for CO_2 and transpiration rate in the intact third leaf were determined with the help of steady state autoporometer (LE-1600, Li-Cor, USA).

D) Inorganic Constituents :

i) Preparation of acid digest : For the estimation of different inorganic constituents (except chlorides) an acid digest was prepared following the method of Toth et al. (1948). 1 g of the oven dried, powdered material was transferred to 150 ml capacity beaker to which 20 ml concentrated HNO_3 were added. The beaker was covered with watch glass and was kept till the primary reactions subsided. It was then heated on hot plate till all the material was completely dissolved. It was allowed to cool to room temperature and then 15 ml of perchloric acid (60 %) were added to it and mixed thoroughly. It was then heated strongly on the hot plate until the solution became colourless

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and reduced to about 3 ml. While heating, the solution was not allowed to dry. After cooling, it was transferred quantitatively to 100 ml capacity volumetric flask, diluted to 100 ml with distilled water and kept overnight. Next day it was filtered through dry Whatman No.44 (ashless) filter paper and the filtrate was stored in well washed and distilled water rinsed PVC containers.

ii) Estimation of Inorganic Constituents : Potassium was estimated flamephotometrically (Elico, Hyderabad) while sodium, calcium, iron, manganese and zinc were estimated using atomic absorption spectrophotometer (Perkin-Elmer 3030). For flamephotometric estimation, standard solutions of known concentrations in parts per million (ppm) of K in KCl (1 to 10 ppm) were used for calibration curve. From this calibration curve the concentration of K in the acid digest was calculated. Requisite standards and dilutions of other elements were also prepared and used for estimation of these elements with atomic absorption spectrophotometer.

Phosphorus was estimated from the same acid digest using the method described by Sekine et al. (1965). 2 ml of acid digest was pipetted in a test tube to which 2 ml of 2N HNO_3 were added followed by 1 ml of Molybdate-Vanadate reagent (Reagent A : 1.25 g ammonium vanadate dissolved in 1 N HNO_3 and volume was made to 500 ml./ Reagent B : 25 g ammonium molybdate dissolved with 1N HNO_3.

in distilled water and volume was made to 500 ml. Then A and B were mixed in equal volumes). The volume was made to 10 ml with distilled water. The reaction mixture was shaken well and kept for 20 minutes. The yellow colour developed was measured at 420 nm on the double beam spectrophotometer (Shimadzu, UV-190), using reagent blank. The colour developed by standards of known concentration of phosphorus in KH_2PO_4 (0.025 mg P ml^{-1} i.e. 25 ppm) with Molybdate-Vanadate reagent was used for plotting the standard curve, with the help of which, the concentration of phosphorus in the samples was calculated.

For estimation of chlorides, method described by Imamul Huq and Larher (1983) was employed with slight modifications. The chlorides were extracted in distilled water at 100°C for 1 hour with occasional addition of hot distilled water to prevent drying. After cooling, the extract was filtered through a layer of cheese cloth. The residue on the cheese cloth was squeezed and washed 2-3 times with small amount of distilled water to collect maximum possible extract. The filtrate was collected in 100 ml capacity volumetric flask and volume was made to 100 ml with distilled water. 10 ml of the extract was titrated against standardized $AgNO_3$ using few (2-3) drops of 1% potassium chromate indicator.

Standardization :

10 ml of 0.1 N sodium chloride was taken in Erlenmeyer flask to which 50 ml of D.W.were added. This was titrated against 0.1 N $AgNO_3$, using 1% potassium chromate indicator.

1 ml of 0.1 N $AgNO_3$ = 3.54 mg Cl^-.

E) Cytology :

Meiotic Studies :

i) Fixation of buds :

Young flower buds of Catharanthus roseus G.Don.were fixed in freshly prepared Carnoy's fluid (Absolute alcohol: Glacial acetic acid, 3:1) in the morning hours between 7 to 8 a.m. Forty-eight hours after fixation, they were transferred to 70% ethanol and stored in a refrigerator.

ii) Preparation of stain :

For meiotic study, so called anther smear technique has been employed. The stain used was acetocarmine. It was prepared by adding 1 g carmine powder in boiling 100 ml 45% acetic acid. It was allowed to boil for about 1/2 hour then cooled and kept overnight. It was filtered through Whatman No.1 filter paper and kept in refrigerator.

iii) Preparation of slides for microscopic examination :

Prior to the preparation of anther smear the anthers of

appropriate size were carefully selected by dissecting out the buds. To ensure complete removal of ethanol several changes of distilled water were given. The anthers were hydrolysed in 1 N HCl on spirit lamp, it was cooled and washed repeatedly with distilled water to remove HCl. Then the anthers were taken on a clear microslide with a drop of freshly prepared acetocarmine. It was horizontally cut with a sharp razor blade and pressed with stainless steel needle. This step brought out sac of pollen mother cells from anther as well as brightened direct contact of them with stain. The clean cover glass was placed on it. It was warmed on a spirit lamp to effect proper spreading. Excess stain was removed by using blotting paper. Inverted technique was employed to blot the excess stain. The slide was sealed with wax for temporary observations. Keeping the slide overnight intensified the stain and chromosomes exhibited brilliant staining. Different stages of meiosis were studied from this preparation. Photomicrography was carried out at this stage.