Chapter-III
MATERIALS AND METHODS

1. CULTURE :

A. SOIL CULTURE :

Plantlets of Moth bean (Phaseolus aconitifolius) were grown in pot soil culture-from seeds of local variety and selected for uniformity in size, shape and colour. About 25 seeds were sown in each pot. Plants were irrigated twice a week with two litres of tap water every time. 1 month after stabilization of plants, salt treatments were started. The concentrations of NaCl used in the experiment were 0 (control), 10, 25, 50 and 100 mM. The treatments were given twice a week, alternating with watering the plants with equal amount of water to avoid excess salt accumulation and loss of water due to evaporation in the soil. After interval of every 10 days, plants were analysed for growth parameters, organic constituents and inorganic constituents.

B. SAND CULTURE :

This experiment was carried out in two sets. In the first set slow salt treatment was given while in the second set shock salt treatment was given to the growing P.aconitifolius plants. 25 seeds of local variety of moth bean (P.aconitifolius) were sown in each pot filled with acid free silica sand. After 1 week plants were nourished with Hoagland culture solution only. Nutrient solution was given twice a week alternating with equal amount of water to avoid salt accumulation and to

check the loss of water from pots due to evaporation. When the seedlings were well established (i.e. after 1 month) the pots were arranged equally in two sets. Plants from one of the sets were treated with salt solution as slow salt treatment or increasing the concentration of salt in the medium slowly shock treatments (plants were treated with salt solutions at different concentrations from the begining) were given. For the treatments NaCl and $CaCl_2$ solutions were used in the following concentrations. Set-I : Slow treatments - NaCl (0) Control, 25 and 100 (mM). Set-II : Shock treatments - NaCl 25, 100 (mM) and NaCl + $CaCl_2$ 12.5 + 12.5, 25 + 25, 50 + 50 and 100 + 100 (mM) NaCl alone or mixture of NaCl and $CaCl_2$ was mixed with Hoagland solution every time so as to give the required salt concentrations. The treatments were given twice a week alternating with watering the plants with equal amount of water. After 1 month the plants were analysed to estimate inorganic constituents.

2. METHODS :

A. GROWTH ANALYSIS :

For the growth analysis plants were obtained at the intervals of every 10 days after the commencement of treatments. For growth analysis at a time 5 plants were collected from each pot. From this fresh plant material growth parameters like average shoot length, average root length, average plant height, root to shoot ratio, number of leaves $plant^{-1}$, number of

internodes and fresh weight plant^{-1} were determined. Plants were then separated in the individual parts like leaves, stem, roots and pods and used for determination of fresh and dry matter of each individual plant part, leaf area plant^{-1}, leaf area index and number of pods plant^{-1} were analysed. From the above data Relative Growth Rate (RGR), Net Assimilation Rate (NAR) and Leaf Area Ratio (LAR) were computed following the formulae -

1. $$RGR = \frac{\log_{10} (W_2 - W_1) \times 2.303}{t_2 - t_1}$$

2. $$NAR = \frac{2.303 (W_2 - W_1) \log_{10} (A_2 - A_1)}{(A_2 - A_1) (t_2 - t_1)}$$

3. $$LAR = \frac{2.303 (A_2 - A_1) \log_{10} (W_2 - W_1)}{2.303 (W_2 - W_1) \log_{10} (A_2 - A_1)}$$

B. ORGANIC CONSTITUENTS :

Organic constituents were determined from both fresh as well as oven dried plant material. To obtain dry plant material, it was subjected to oven for a period till the dried material gave constant weight. From the fresh plant material organic constituents like TAN, Chlorophylls, Polyphenols while from oven dried plant material carbohydrates, total nitrogen, proteins and proline were determined.

i) TAN of leaf and stem was determined by the method of Thomas and Beevers (1949).

ii) Polyphenols of young and mature leaves were estimated from acetone extract according to method of Folin and Denis (1915). The polyphenols were calculated from the callibration curve of standard tannic acid.

iii) Chlorophylls were estimated from young and mature leaves following the method of Arnon (1949). Chlorophylls were extracted in 80% acetone from 0.5 g of fresh plant material. The extract was filtered through Buchner's funnel using Whatman No.1 filter paper. Residue was washed repeatedly with 80% acetone, collecting the washings in the same filterate. The volume of filterate was made to 100 ml with 80% acetone. The absorbance was read at 663 nm and 645 nm for chlorophylls 'a' and 'b' respectively on Spectrophotometer. Chlorophylls (mg/100 g fresh plant tissue) were calculated using the following formulae.

Chloro. 'a' = 12.7 x A 663 - 2.69 x A 645 = 'x'

Chloro. 'b' = 22.9 x A 645 - 4.68 x A 663 = 'y'

Chloro.(a+b) = 8.02 x A 663 + 20.20 x A 645 = 'z'

$$\text{Chl. 'a' or 'b' mg/100 g fresh tissue} = \frac{x/y \times \text{Volume of extraction} \times 100}{1000 \times \text{wt. of plant material (g)}}$$

iv) Carbohydrates : Soluble sugars and starch content of leaf and pods were determined by Somogyl Nelson's (1944) method using Arseno molybdate reagent.

v) Total nitrogen of leaves, stem, roots and pods were determined by the method of Hawk et al., (1948).

vi) Proteins of different plant parts were obtained by multiplying the total nitrogen content by a factor 5.7.

vii) Proline : Free proline content of leaf, stem and roots was determined by the method of Bates et al., (1973).

C. ENZYMES :

Enzymes hydroxylperoxidases (Peroxidase, EC 1.11.1.7 and Catalase, EC 1.11.1.6), acid phosphatase (EC 3.1.3.2) and Nitrate reductase (EC 1.6.6.1) were isolated from the fresh seedlings. Isolation and assay of enzymes were done at low temperatures (0-4°C).

i) Hydroxyperoxidases :

a) Peroxidase : Peroxidase from leaf and roots of fresh seedlings was determined following the method described by Machly (1954). Enzyme was extracted by homogenizing the plant material in cold water. It was then filtered through moist cheese cloth and the filterate so obtained was centrifuged at 10,000 rpm for 10 min at 0 to 4°C and supernatant was used as

an enzyme source. Enzyme assay mixture contained 2 ml phosphate buffer (pH 7, 0.1 M), 1 ml guiacol (20 mM) and 1 ml enzyme. The reaction was started by the addition of 0.04 ml H_2O_2 (10 mM). Change in optical density due to oxidation of guiacol was recorded per min at 470 nm on Spectrophotometer with frequent stirring of reaction mixture with glass rod. Activity is expressed as

Δ O.D. min^{-1} g^{-1} fresh tissue.

b) Catalase : The enzyme was isolated from fresh leaves and roots in cold distilled water following the usual method of extraction, filteration and centrifugation. Isolated enzyme was stored at 0-4°C temperature. Assay of the enzyme was done following the method described by Herbert (1955). Assay mixture contained 1 ml 0.45 M H_2O_2 in phosphate buffer (pH-6.8, 0.1 M), 0.5 ml enzyme. Reaction was stopped by adding 5 ml 1N H_2SO_4 after 5 minutes of the reaction. To this 1 ml 10% KI, a drop of 1% Ammonium molybdate were added and liberated Iodine was titrated against 0.01 N Sodium thiosulfate using starch as an indicator. Blue to colourless was taken as end point. Blank or 0 minutes reading was taken by mixing all the ingrediants except enzyme as given above. Activity of the enzyme is expressed as mg of H_2O_2 broken min^{-1} g^{-1} fresh tissue.

ii) Acid Phosphatase :

The enzyme was isolated from leaf and roots following the method of DeLeo and Sacher (1970). Enzyme was prepared in cold distilled water by crushing fresh leaves and roots in cold morter with pestle. Filtered through cheese cloth and filterate was centrifuged at 10,000 rpm for 10 min. supernetant was stored at 0-10°C and used as an enzyme source.

The 5 ml of assay mixture contained 3 ml of p-nitrophenyl phosphate (0.1 mg ml^{-1}), 2 ml of acetate buffer (pH 5; 0.1 M) and 1 ml enzyme. Enzymatic reaction was stopped after 1 hr by adding 1.5 ml of NaOH (1.68 N). Yellow complex produced as a result of reaction between p-nitrophenol in enzymatic break down of p-nitrophenyl phosphate and NaOH, was estimated colorometrically at 400 nm. Enzyme activity is expressed as Δ O.D. hr^{-1} g^{-1} fresh tissue.

iii) Nitrate Reductase :

This enzyme was prepared and assayed following the method by Evans (1982).

0.5 g of fresh leaves and roots were incubated separately in 5 ml mixture of 0.1 M phosphate buffer (pH 7.5), 0.02 M KNO_3, and 5% propanol taken in vials kept at 25°C in the dark. Incubation was continued for 1 hour. After this period nitrite produced in the enzymatic reaction was determined by mixing 0.5

ml reaction mixture with 0.5 ml each of 1% sulfanilamide in 3 M HCl and 0.02% N-1-naphthyl-ethylene amide hydrochloride and kept for 20 minutes. After appropriate dilution (3 ml with distilled water) the absorbance was read at 540 nm. Activity of the enzyme was expressed as μg nitrite liberated hr^{-1} g^{-1} fresh tissue.

D. INORGANIC CONSTITUENTS :

For estimation of inorganic constituents an acid digest from the oven dried plant material was used. The plant material was digested following the method of Toth et al., (1948). 0.5 g of dried powdered material was transferred to a 100 ml capacity beaker to which 10-25 ml. conc. HNO_3 were added. The beaker was covered with Watch glass and was kept till primary reaction subsided. It was then subjected to heating till the particles of plant get dissolved. Then, about 5 ml. of perchloric acid (66%) were added to it and mixed throughly after cooling to room temperature. It was then heated until the volume of extract was reduced to approximately 2-3 ml. It was cooled and transferred quantitatively to 100 ml volumetric flask and volume was made with distilled water. It was kept over night and filtered through a dry Whatman No.44 filter paper next day. The filterate was used for estimation of inorganic constituents.

i) Estimation of Na^+, K^+ and Ca^{2+} :

Na^+, K^+ and Ca^{2+} were estimated flame photometricaly. Following the procedure standardised in our laboratory. Stock solution of known concentration in parts per million (ppm) of K^+ in KCl, Na^+ in NaCl and Ca^{2+} in $CaCl_2$ were prepared and readings of these standard solutions were used for calculating the concentration of Na^+, K^+ and Ca^{2+} respectively in the acid digests of the plant material.

ii) Estimation of P^{5+} :

Phosphorus was determined following the method given by Sekine et al., (1965). Phosphorus gives yellow colour reaction with molybdate-vanadate reagent. By estimating colorimetrically the intensity of colour developed and by comparing it with the colour intensity of known standards phosphorus was estimated.

iii) Estimation of Cl^- :

Cl^- of the leaves, stem and roots were estimated following the method by Volhard (1956). In this method chlorides are extracted in hot water and dilute HNO_3. Chlorides are allowed to precipitate out by the standard silver nitrate and excess of silver nitrate (remaining) is titrated against ammonium thiocynate. From this known quantity of silver nitrate consumed by chlorides present in the plant material was calculated. Chlorides were calculated then using the formula -

1 ml 0.1 N $AgNO_3$ ≡ 3.55 mg chlorides.

Ammonium thiocynate and silver nitrate were standardized against silver nitrate and standard (0.1 N) potassium chloride solution respectively using ferric allum and 5% K-chromate indicators respectively.

Amount of Cl^- (%) present in the plant material was calculated using the formula

$$\% \text{ Chlorides} = \frac{x-y \times 0.355}{\text{wt. of material (g)}}$$

Where X = Amount of $AgNO_3$ (0.1 N) added in 250 ml volumetric flask for precipitation of chloride in extract and y = amount of 0.1 N $AgNO_3$ calculated by titration with extract, unused.

iv) Estimation of Mg^{2+} :

Mg^{2+} was estimated following the method described by Drausdoff and Nearpass (1948).

v) Estimation of Fe^{3+} and Mn^{2+} :

Fe and Mn were estimated following the methods described by Durie et al., (1965).