CHAPTER II

MATERIALS AND METHODS

A) Soil Culture :

The plantlets of local cultivar of moth bean (Phaseolus aconitifolius) were raised in pot soil culture. The seeds of local strain were collected through market and selected for uniformity in size, shape and colour. About 15-20 seeds were sown in each pot. The plants were irrigated thrice a week with two litres of tap water every time. Two months after the growth of the plants water stress treatment was started.

Water stress was given by withholding water from the pot by irrigating the plants in different pots for different periods such as one of the pots was irrigated daily. The other pots were irrigated for every 2,3,7 and 15 days. At the final stage five different types of plants with water stress treatment such as control (no stress), 2, 3, 7 and 15 days stress were obtained. After fifteen days the plants were analysed for growth, organic and inorganic constituents and some enzyme systems. The culture was raised in triplicate.

B) Methods of Analysis :

i) Growth Analysis :

Plants were analysed for their growth with respect to biomass production. For this, 20 plants from each treatment pot were uprooted carefully and their root system was thoroughly and as quickly as possible, washed with tap water to remove adhered soil and quickly blotted to dryness. Each plant was

quickly weighed intact. The plant parts such as root, stem and leaves were then separated and accurately weighed separately. The plant material was then subjected to oven at about 60°C for 4-7 days till the dried material gave constant dry weights. Thus the biomass production (fresh and dry matter production) was analysed statistically.

ii) Organic constituents :

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

a) Carbohydrates :

Carbohydrates were determined following Nelson's (1944) method. 2 g fresh plant material was homogenised in morter with pestle and extracted with 80% ethanol. It was filtered through Buchner's funnel using Whatman No.1 filter paper. The filtrate was used for estimation of soluble sugars while the residue was used for starch determination. The filtrate thus obtained was condensed on a boiling water bath till the volume was reduced to about 3-5 ml and treated with lead acetate and potassium oxalate (1:1) to decolourise it. To this 20 ml distilled water was added and filtered. It was

again washed with distilled water 2-3 times, collecting the washings in the same filtrate. This filtrate was used for estimation of reducing sugars, A.

20 ml of this extract was hydrolysed with HCl (2 ml) in a pressure cooker at 15 lbs pressure for half an hour. The contents were cooled, neutralised with Na_2CO_3 and filtered through filter paper. The filtrate was used for the estimation of total sugars, B.

The residue obtained in the first filtration (ethanol extract) was transferred to a conical flask with 50 ml of distilled water and 5 ml of concentrated 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 requisite quantity (preferably 0.1 ml) of the above filtrates A, B and C was taken separately in 10 ml marked test tubes. In other such test tubes different concentrations (0.1, 0.2, 0.3, 0.4 and 0.5 ml) of standard glucose solution (0.1 mg/ml) were taken. 1 ml of Somogyi's alkaline copper tartarate solution (4 g $CuSO_4$, $5H_2O$; 24 g anhydrous Na_2CO_3; 16 g Na - K tartarate (Rochelle salt); and 180 g anhydrous Na_2SO_4 dissolved in 1000 ml distilled water) was added to each tube. All the reaction mixtures were then subjected to boiling water bath

for about 10 min. After cooling to room temperature 1 ml of Arsenomolybdate reagent (25 g ammonium molybdate in 450 ml distilled water, to which were added 21 ml concentrated H_2SO_4. To this was then added 3 g sodium arsenate, Na_2HASO_4, 7 H_2O; dissolved in 25 ml distilled water. All ingradients were mixed well and the solution was placed in an incubator at 37°C for 48 h before use) was added to each reaction mixture. The contents of each test tube were then diluted with distilled water to a volume (50 ml). A blank was prepared by the same way but without sugar solution. After 10 min the absorbance of each reaction mixture was read at 560 nm on UV - VS double beam spectrophotometer (Shimadzoo).

From the glucose standard curve, the sugar percentage in the above three fractions was determined.

b) Polyphenols :

Polyphenols were estimated from the leaves following Folin-Denis method (1915).

Polyphenols from fresh material were extracted in 80% acetone (30 ml). Extract was filtered through Whatman No.1 filter paper using Buchner's funnel under suction. Polyphenols were extracted repeatedly from the residue. The volume of the filtrate was made to 50 ml. This filtrate was used for the estimation of polyphenols.

2 ml of this filtrate was taken in 50 ml marked Nessler's

tube. In other such tubes different concentrations (0.5, 1, 2 and 4 ml) of standard polyphenol solution (tonnic 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 of sodium tungstate and 20 g of phosphomolybdic acid, dissolved in 200 ml distilled water were mixed with phosphoric acid 25%. It was then refluxed for $2\frac{1}{2}$ hours, cooled to room temperature and diluted to one litre with distilled water) were then added to each tube and finally the volume was made to 50 ml with distilled water. A blank was prepared without polyphenolics. The ingradients were allowed to mix thoroughly. After 10 min the optical density of each mixture was read at 660 nm on spectrophotometer.

c) Total nitrogen :

Total nitrogen was estimated from root, stem and leaves following the method by Hawk et al. (1948).

0.5 g of oven dried plant material was digested in a Kjeldahl flask with sulphuric acid (1:1) and a pinch of micro-salt (mixture of anhydrous copper sulphate and potassium sulphate in the proportion of 1:40) till a colourless liquid is obtained at the bottom of the flask. It was then cooled to room temperature and transferred quantitatively to the volumetric flask and the volume was made to 100 ml with distilled water. It was kept overnight and was filtered through the filter paper. The filtrate was used for the estimation of nitrogen.

2 ml of this filtrate was taken in a Nesselor's tube (35 and 50 ml marked). In other such tubes different concentrations (0.5, 1, 2 and 4 ml) of standard ammonium sulphate (0.05 mg nitrogen ml^{-1}) were taken . One tube was kept as a blank without ammonium sulphate. To these tubes was added a drop of 8% potassium bisulphate ($KHSO_4$) and 1 ml H_2SO_4 (1:1, wherever needed). The volume of all these tubes was adjusted to 35 ml with distilled water, 15 ml of Nesselor's reagent was then added in each tube. Nesselor's reagent is a mixture of reagent A (7 g of KI and 10 g of HgI_2 dissolved in 40 ml of distilled water) and B (10 g of NaOH, dissolved in 50 ml of distilled water) in the proportion of 4 : 5. The colour intensity of the orange brown product produced by the reaction between NH_3 liberated from the sample and the reagent was measured at 520 nm on spectrophotometer. The amount of nitrogen in the sample was calculated from the standard curve of ammonium sulphate.

d) Proline :

Proline content of root, stem and leaves has been determined following the method by Bates et al. (1973).

The known quantity (0.5 g) of fresh material was homogenized in 10 ml of 3% salfosalicylic acid and filtered through Whatman No.1 filter paper. The volume of the filtrate was recorded. 2 ml of this filtrate was taken in a test tube to

which were added 2 ml of acid ninhydrin reagent (prepared freshly by adding 2.5 g ninhydrin in 60 ml of glacial acetic acid, warmed to dissolved after adding 40 ml of 6 M ortho-phosphoric acid. It was then cooled and stored at 20°C) and 2 ml of glacial acetic acid and the whole reaction mixture was boiled in water bath for an hour.

The reaction was terminated in ice bath and 4 ml of toluene were added in each reaction mixture. The reaction mixture was shaken vigorously for 15-20 seconds. The reaction mixture was then brought to room temperature and the absorbance of toluene chromophore was measured at 520 nm on spectrophotometer using reaction blank. To take toluene chromophore vaccu-pipette was used. At the same time reaction mixture for standard curve of proline (0.25 $\mu g\ ml^{-1}$) were prepared in the same way by taking different concentrations of proline solution. The amount of free proline in root, stem and leaves has been calculated from the standard curve and expressed in terms of $\mu g\ 100g^{-1}$ fresh tissue.

e) Chlorophylls :

Chlorophylls were estimated from the leaves, following the method by Arnon (1949).

Chlorophylls were extracted in 80% acetone from 0.5 g of fresh plant material. The extract was filtered through Whatman No.1 filter paper using Buchner's funnel. Residue on filter

paper was washed thoroughly with 5-10 ml aliquotes of 80% acetone. All the washings were collected in the same filtrate. The volume of filtrate was made to 100 ml with 80% acetone. The preparation of plant extract for chlorophylls was done in dull light conditions and at 0-4°C. The absorbance was read at 663 and 645 nm for chlorophylls 'a' and 'b' respectively on UV - Vis spectrophotometer (Shimadzoo). Chlorophylls (mg 100^{-1} g fresh plant tissue) were calculated using the following formulae,

$$\text{Chl. 'a'} = (12.7 \times A_{663}) - (2.69 \times A_{645}) = X$$

$$\text{Chl. 'b'} = (22.9 \times A_{645}) - (4.68 \times A_{663}) = Y$$

$$\text{Chl. '(a + b)'} = (8.02 \times A_{663}) + (20.2 \times A_{645}) = Z$$

$$\text{Chl.'a' or 'b' or 'total'} = \frac{X/Y/Z \times \text{Vol. of extract} \times 100}{1000 \times \text{wt. of plant material (g)}}$$

$$= \text{mg } 100^{-1} \text{ g fresh tissue.}$$

iii) Enzymes :

Enzymes peroxidase (EC 1.11.1.7) and acid phosphatase (EC 3.1.3.2) were isolated from the fresh roots and leaves. Isolation and assay of enzymes were done at low temperature (0-4°C).

a) Peroxidase :

Peroxidase from fresh plant material i.e. roots and leaves was determined following the method describe by Maehly (1954).

Enzyme was extracted by homogenizing the plant material (0.5 g) in ice cold water (10 ml). It was then filtered through two layers of cheese cloth and the filtrate was centrifuged for 15 minutes at 500 rpm. at 0 to 4°C and the 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 0.5 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 the reaction mixture with glass rod. Enzyme activity is expressed as Δ O.D. min^{-1} g^{-1} fresh tissue.

b) Acid Phosphatase :

The enzyme was isolated from roots and leaves following the method of Leo and Sacher (1970).

The enzyme was prepared by homogenizing 0.5 g of plant material in 10 ml of 0.1 M acetate buffer (pH 5) in a mortar with pestle. The extract was filtered through muslin cloth already moistened with acetate buffer and the filtrate was centrifuged at full speed for 10 minutes at 500 rpm. The supernatant was stored at 0-4°C and used as an enzyme source.

Enzyme 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 initiated by the addition of enzyme and was stopped after 1 hour by adding 1.5 ml

of NaOH (1.68 N). Yellow coloured complex produced as a result of reaction between p-nitrophenol produced in enzymatic breakdown of p-nitrophenyl phosphate and NaOH, was estimated spectrophotometrically at 400 nm. Enzyme activity is expressed as Δ O.D. hr^{-1} g^{-1} fresh tissue.

iv) Inorganic constituents :

a) Preparation of plant extract :

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 the oven dried powdered plant material was taken in 150 ml beaker and 20 ml concentrated HNO_3 were added to it and allowed to stand for sometime till the initial reactions were subsided. It was then subjected to heating till the particles of plant material were completely dissolved. Then, 10 ml of concentrated perchloric acid (60%) were added to it. It was then heated strongly until the volume of the plant extract was reduced to approximately 3-5 ml. It was cooled to room temperature, transferred quantitatively to 100 ml capacity volumetric flask and diluted to 100 ml with distilled water. It was kept over night and next day filtered through a dry Whatman No.44 filter paper. The filtrate was used for estimation of inorganic constituents.

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

Na^+, K^+ and Ca^{2+} were estimated flame photometrically following the procedure standardised in our laboratory. Stock solutions of known concentration in parts per million (ppm) of K^+ in KCl, Na^+ in NaCl and Ca^{2+} in $CaCl_2$ were prepared. For the calibration curve of these elements various concentrations of these elements were prepared as follows.

Various concentrations of Na^+ in NaCl ranging from 1 to 10 ppm were prepared by diluting the stock solution of NaCl (10 ppm) accordingly. Similar concentrations were prepared for K^+ in KCl also. However, various concentrations of Ca^{2+} in $CaCl_2$ ranging from 20 to 200 ppm were prepared by diluting the stock solution of $CaCl_2$ (200 ppm) accordingly. The deflections in galvanometer for these different concentrations of Na^+, K^+ and Ca^{2+} using Na^+, K^+ and Ca^{2+} specific filters respectively were noted and calibration curves for these three elements were prepared. From these calibration curves the concentrations of Na^+, K^+ and Ca^{2+} in the acid digest were calculated.

c) Estimation of P^{5+} :

Phosphorus was estimated following the method given by Sekine et al. (1965).

Phosphorus gives yellow colour reaction with molybdate vanadate reagent. By estimating colorimetrically the intensity of the developed colour and by comparing it with the colour

intensity of known standards, P^{5+} can be estimated.

2 ml of acid digest was pipetted in a test tube to which 2 ml of 2 N HNO_3 were added followed by 1 ml of molybdate vanadate reagent (A - 1.25 g of ammonium vanadate dissolved in 500 ml of 1 N HNO_3. B - 25 g of ammonium molybdate in 500 ml distilled water. Then A and B were mixed in equal volumes). Volume was made to 10 ml with distilled water. The reaction mixture was shaken well and kept for 20 minutes to allow the colour to develop. Absorbance was measured at 420 nm using blank reaction mixture containing no phosphorus.

A curve of standard P^{5+} was prepared for different concentrations of phosphorus (standards containing 0.5, 1, 2 and 4 ml) using standard KH_2PO_4 solution of the strength of 0.025 mg^P ml^{-1}. By comparing the absorbance of the extract with that for standard curve amount of phosphorus in the plant material is calculated.

d) Estimation of Mg^{2+} :

Magnesium was estimated following the method of Drosdoff and Nearpass (1948).

To 5 ml acid digest in a 50 ml marked test tube the following reagents were added in the order and mixed thoroughly, 1 ml hydroxylamine hydrochloride (5% w/v), 5 ml of starch compensating solution (equal volumes of freshly prepared starch solution 2% and compensating solution, 3.7 g calcium chloride, 0.74 g aluminium sulphate, 0.36 g magnesium chloride, 0.6 g trisodium

phosphate, all dissolved in distilled water containing 10 ml of concentrated hydrochloric acid and then volume was made to 1 litre), 1 ml thiazole yellow (0.1%) and 5 ml 2.5 N sodium hydroxide. The volume was made to 50 ml with distilled water and allowed to stand for 30 minutes. Colour intensity was measured at 525 nm on spectrophotometer.

Reagent blank was prepared in the same manner as above except the Mg^{2+} solution. The Mg^{2+} content of the extract was calculated with the help of Mg^{2+} standard curve for which different concentrations of Mg in Mg SO_4 solution (1 mg $MgSO_4$ ml^{-1}) were prepared.

e) Estimation of Mn^{2+} :

Manganese was estimated following the method described by Durie et al. (1965). In this method manganese is oxidized to per-mangnate with potassium periodate. The optical density of this per mangnate can be measured at 525 nm on spectrophotometer.

25 ml acid digest, 5 ml acid mixture (10 ml orthophosphoric acid and 25 ml H_2SO_4 diluted to 100 ml with distilled water) and 25 ml blank solution (water) were taken in 100 ml capacity beakers separately. 0.3 g of potassium periodate was added to each beaker. Beakers were covered properly, subjected to boiling water bath and boiled for half an hour. Cooled to room temperature and then reaction mixtures were transferred

quantitatively to 50 ml volumetric flask separately and volume was made with distilled water. Optical density of the pink violet colour that was developed due to formation of potassium permanganate was measured at 525 nm against distilled water blank.

Standard curve was prepared from different concentrations (0.5, 1.0, 1.5 and 2.0 ml) of Mn in K MnO_4 solution (10 mg 100^{-1} ml distilled water) and with the help of this total manganese content in the stem and the leaves were calculated.

f) Estimation of Fe^{3+} :

Iron was determined by the method described by Durie et al. (1965). In this method ferric ion is reduced to ferrous ion. The colour developed between the latter and O-phenenthroline is read at 510 nm spectrophotometer.

For estimation of Fe^{3+}, 5 ml of acid digest and 5 ml of standard iron solution (3 μg Fe_2O_3 ml^{-1}) were taken separately into 50 ml marked test tubes. One more 50 ml marked test tube was reserved for reagent blank containing no iron solution. To this 10 ml of hydroxylamine hydrochloride (10% w/v) were added followed by small piece of congo red paper (cm^2). It was mixed well. The acetate buffer solution (140 g sodium acetate in distilled water + 60 ml acetic acid → diluted to one litre with distilled water) was added dropwise until the indicator

paper changed just from blue to red. 8 ml of O-phenenthroline (0.25% w/v) were then added, volume was adjusted to 35 ml with distilled water and optical density was read at 510 nm against reagent blank. By comparing the optical density of the test solution with that of standard Fe_2O_3 solution, total amount of Fe in the plant material was calculated.

g) Estimation of Cl^- :

Chloride was estimated according to the method given by Chapman and Pratt (1961).

0.5 g of oven dried, powdered plant material was mixed in a beaker with 50 ml distilled water. 50 ml distilled water was again added after 15 minutes. This process was done in three times. It was then boiled until the volume of extract was reduced to approximately 5-10 ml. It was then filtered through Whatman No.44 paper and diluted to 25 ml with distilled water. 10 ml of this plant extract was titrated against 0.05 N $AgNO_3$. Potassium cromate (5%) was used as an indicator and end point was reddish brown ppt. The values of chlorides are expressed in g 100^{-1} g dry tissue.

v) Rate of Transpiration and Diffusive Resistance :

The effect of water stress on the rate of transpiration, diffusive resistance, flow rate, relative humidity, quantum and leaf temperature has been studied using steady state autoporometer (Model LI-1600, Li-Cor, U.S.A.).