CHAPTER II

MATERALS AND METHODS

GERMINATION STUDIES

A) **Seed germination studies**:-

Seeds are obtained from the pod of *Prosopis juliflora* plants growing in the agriculture field in Sangli district in the month of April-May. The seeds were isolated from such pods and healthy seeds were sorted out. Since the seeds have hard seed coat which restricted seed germination, seeds were mechanically scarified with polish paper. The seeds were treated with 0.1 % Mercuric chloride for 5 minutes for surface sterilization. The seeds were rinsed with distilled water for 4-5 times. 10 seeds were placed in Petri plates containing filter paper moistened with 10 ml of NaCl and $Na_2 SO_4$ solution of different concentrations as (100 mM, 200 mM and 300 mM). The filter papers moistened with 10 ml of distilled water served as control. Germination percentage was recorded after 24 hours and seedling growth (with respect to root and shoot length) was studied after 24 hours.

B) Studies of influence of sodium chloride salinity on metabolism.

1) Sand culture:-

For this study, a sand culture technique was adopted. Silica sand was thoroughly washed with running water and dried in air. Equal amount of silica sand was filled in plastic container of uniform size with porous at the bottom. Mechanically scarified seeds of *Prosopis juliflora* were sown in the sand at about 10mm depth (Mutha and Burman, 1998). The treatment of various salt concentrations (100 mM, 200 mM, and 300 mM NaCl) were given to each pot

with half strength Hoagland solution. The composition of Hoagland nutrient medium (control) was as follows…

1M KNO_3 - 6 ml

1M $Ca(NO_3)_2$- 4 ml

1 M $MgSO_4$ - 2 ml

1M $(NH_4)_2 HPO_4$- 1 ml

Micronutrient - 1 ml

H_3BO_3 - 2.86 g

$MnCl_2$ 4 H_2O- 1.81 g

$ZnCl_2$ - 0.11 g

Na_2 MoO_4 2 H_2O- 0.05 g

5 % Iron tartarate- 1 ml

After one month the growth analysis with respect to total biomass, dry and fresh weight was carried out.

2) Mineral Nutrition

a) **Preparation of Acid digest**

Acid digestion method of Toth *et al.,* (1948) has been followed for the analysis of inorganic constituents. Plant material (i.e. root and leaves) was carefully washed in water and blotted to dryness leaves and roots were separated and subjected to drying at 60 ^{0}C for 10 days till dried plant had constant weight. The oven dried plant material was powdered. Five hundred mg of oven dried powder of root and leaves was transferred to 150 ml capacity beaker to which 20

ml concentrated HNO_3 were added. The beakers were covered with watch glass and kept till the primary reactions subsided. Then these beakers were heated slowly to dissolve solid particles. After cooling to room temperature, 10ml of perchloric acid (60%) were added to it and mixed thoroughly. Then these beakers were heated strongly until a clear and colorless solution (about 2-3 ml) was obtained. It was then cooled and transferred quantitatively to 100 ml capacity volumetric flask, diluted to 100 ml with distilled water and kept overnight. Next day these extracts were filtered through dry Whatman No.44 (Ash less) filter paper. Filtrates so obtained were used for estimation of different in organic constituents.

i) **Sodium, Potassium and Calcium**:-

Sodium, Potassium and Calcium were estimated using atomic absorption Spectrophotometer. For standardization various concentrations of sodium (20-100 ppm) and potassium (20-100 ppm) and calcium (20-100 ppm) from NaCl, KCl and $CaCl_2$ respectively were prepare using standard solutions, standard curve for these elements were prepared. The plant extract (acid digest) was analyzed in the similar manner. In case needed, appropriate dilution of plant extract were made with distilled water.

ii) **Phosphorus**:-

The method of Sekine *et al.,* (1965) was employed for estimation of Phosphorus. Phosphorus react with 'molybdate vanadate' reagent to give yellow coloured complex. By estimating calorimetrically the intensity of colour develop

and by comparing it with the color intensity of known standards, Phosphorus content was estimated.

4 ml of acid digest were taken in test tube and to two ml of 2 N HNO_3 and 1 ml of 'molybdate vanadate' reagent is added (A-25 g ammonium molybdate in 500 ml of distilled water, B- 1.25 g ammonium vanadate in 500 ml 1 N HNO_3, A and B were mixed at the time of experiment) were added. Then final volume of each test tube was adjusted to 10 ml with distilled water. After 20 minutes, color intensity was measured at 420 nm using a reaction blank containing no phosphorus.

Calibration curve of standard phosphorus was prepared from standard phosphorus solution containing mg per ml (0.110 g KH_2PO_4 per liter = 0.025 mg P^{5+} ml $^{-1}$) with the help of standard curve the amount of phosphorus in the plant material was calculated and it was expressed on dry weight basis.

iii) **Chloride**:-

Extraction of Chloride was done according to the method described by Imamul Huq and Larher (1983) with slight modification, and estimation was performed according to the method of Chapman and Pratt (1961). The chlorides were extracted by putting powder of oven dried plant material in distilled water at 45 ^{0}C for 1 hour with addition of hot distilled water to prevent drying. After cooling, the extract was filtered through a layer of muslin cloth. The filtrate was collected in 50 ml volumetric flask and volume was made with distilled water. From this, 10 ml extract was taken for titration against standardized $AgNO_3$. Few

drops of Acetic acid solution (dilute 200 ml concentration Acetic acid with 800 ml of distilled water) were added to the filtrate until the pH of the solution was 6-7. Then five drops of 1% potassium chromate solution were added and titrated with standardized 0.05 N Silver nitrate (8.5 g $AgNO_3$ were dissolved in 1 liter distilled water) upto appearance of permanent reddish brown colour. The chlorides (mg 100 g^{-1} dry weight) were calculated with the help of equation

1 ml 0.05 N $AgNO_3$ = 1.77 mg Cl.

3) Qualitative changes of lipid composition:-

Thin layer Chromatographic technique was employed for qualitative study of lipid composition of root and leaf tissue.

Preparation of Extract:-

Three grams of fresh leaf and root material of control and 300mM NaCl treated plant were homogenized in 100 ml of mixture of chloroform in methanol (2:1) by volume. Equal volume of water was added to lead a phase of separation between chloroform layer containing lipid and water layer. The chloroform layer was separated and condensed to 2ml

Preparation of TLC plates:

The method described by Kurt Randerath (1966) was followed for preparation of TLC plates. The clean and dry glass plates were coated with a thin uniform layer of slurry of silica gel G (40 gm silica gel G with binder 13% $CaSO_4$ with 80 ml distilled water). The plates were air dried and activated in oven at 60^0C for 12 hours.

Loading and development of TLC plates:-

Seventy five µl of extracts of leaf and root of control and 300 mM NaCl treated plant were carefully loaded on TLC plate with the help of micropipette. For separation of lipids solvent system containing Chloroform: Methanol: Acetic acid: Water (85:15:10:4 by volume) was employed. After the plates were sufficiently developed the plate were removed from glass jar and air dried. The plates were exposed to iodine vapor in close chamber until the spots were clearly visible. The phospholipids were identified by the comparing the position of bands on the TLC plates with the standard picture of plant lipid separation ( ) and by determining the Rf value and comparing them with these of standards.

The values presented in chapter Result and Discussion represents arrange of these determinations.

4) Carbohydrate Metabolism :-

a) Enzyme Invertase (E.C. 3.2.1.26)

A slightly modified method of Uppal and Kanwar (1992) was followed for the study of activity of Invertase enzyme. One gram leaf as well as root tissue from each treatment and control were homogenized in 10 ml of cold 0.1 M Phosphate buffer (pH 7.5). Homogenate was filtered through 4 layers of muslin cloth. The filtrate was centrifuged at 10,000 rpm for 20 minutes. Supernatant served as enzyme source. Assay mixture contained 4 ml 1% sucrose solution, 1 ml phosphate buffer (pH-7.5), 1 ml enzyme source. The reaction was terminated by boiling the mixture immediately 0 minute and after 60 minutes. One ml reaction

mixture was mixed with 2 ml Dinitrosalicylic acid reagent and boiled in waterbath for 5 minutes and cooled in ice bath. The volume was adjusted to 10 ml with distilled water and absorbance was measured at 530 nm. The soluble proteins in the enzyme extract were determined according to the method of Lowry *et al.*, (1951). The enzyme activity was expressed as ΔOD h^{-1} mg^{-1} protein. With the help of standard curve of glucose, the amount of glucose released corresponding to change in ΔOD h^{-1} mg^{-1} protein was estimated.

b)Soluble Protein:-

The soluble proteins in the enzyme extract were determined according to the method of Lowry *et al.*, (1951). 0.1 ml enzyme extract was taken in test tube and diluted to 1 ml with distilled water. To this 5 ml of reagent - C solution (50 ml of A containing 2% sodium carbonate in 0.1 N aqueous NaOH was mixed with 1 ml of B containing 0.5% copper sulphate in 1%, Na-K tartarate), was added, mixed well and allowed to stand for 15 minutes at room temperature. After 15 minutes 0.5 ml Folin Ciocalteau phenol reagent was added readily with immediate mixing. This was allowed to stand for 30 minutes in dark and intensity of developed blue colour was measured at 660 nm on Spectronic -20. Protein content was calculated by comparing with standard curve of different concentrations of Bovine Serum Albumin (0.1 mg/ml) prepare in a similar manner. The values were expressed as mg g^{-1} fresh tissue.

c) Enzyme α-amylase (E.C. 3.2.1.1)

A method described by Katsumi and Fukuhara, (1969) was adopted for the study of enzyme α-amylase. Five hundred mgs of leaf as well as root tissue from each treatment and control were homogenized in 10 ml ice cold acetate buffer (pH-5) and filtered through 4 layered muslin cloth. The filtrate was centrifuged at 10,000 rpm for 20 minutes and supernatant was used as enzyme source. The reaction mixture contained 1 ml acetate buffer (pH-5), 1 ml enzyme and 1 ml 0.20% amylose solution (200 mg amylose dissolved in 4 ml 1 N NaOH and kept in refrigerator overnight to dissolve completely, then it was diluted to 80 ml with distilled water and adjusted to pH-7.5 with 1 N acetic acid and brought to final volume of 100 ml with addition of distilled water). The reaction was terminated by addition of 10 ml of 0.5 N Acetic acid at 0 minutes and 30 minutes. One ml of reaction mixture was mixed with 10 ml dilute I_2KI solution. The absorbance was measured at 700 nm against I_2KI solution used as blank. The soluble proteins in the enzyme extract were determined by the method of Lowry *et al.*, (1951) described earlier. The enzyme activity was expressed as $\Delta OD\ h^{-1}\ mg^{-1}$ protein.

5. PHOSPHORUS METABOLISM

a) Enzyme Acid Phosphatase (E.C. 3.1.3.2)

The method of Mclachlan (1980) was followed for the study of activity of Acid phosphatase. Five hundred mgs of fresh leaves as well as root tissue from each treatment and control were crushed in 10 ml of ice-cold 0.1 M Acetate buffer (pH-5). The resultant homogenate was filtered through 4 layers of musline cloth and the filtrate was centrifuged at 10,000 rpm for 20 minutes. The supernatant served as enzyme source. The assay mixture contained 3 ml of synthetic substrate p-nitrophenyl phosphate (0.1 mg p-nitrophenyl phosphate per ml of acetate buffer (pH-5)), two ml acetate buffer (pH-5) and 1 ml enzyme. The reaction was allowed to proceed for 30 minutes and then was terminated by addition of 1.5 ml, 1.68 N NaOH. The optical densities of the developed yellow colour complex were recorded at 420 nm. The proteins in this enzyme extracts were estimated by the method of Lowry *et al.* (1951) which is already described. The enzyme activity was expressed as μ moles p-nitrophenol liberated h^{-1} mg^{-1} protein.

b) Enzyme Alkaline Phosphatase (E.C. 3.1.3.1)

The method described by Weimberg (1970) was employed for the study of activity of enzyme alkaline phosphatase. Five hundred mgs leaf and root tissue from each treatment and control were crushed in 10 ml ice-cold 0.1 M Tris-HCl buffer (pH-8.0) containing 1 M KCl, 0.01 M EDTA and 0.4 ml 0.2 M β-mercaptoethanol. The resultant suspension was filtered through 4 layered musline cloth and filtrate was centrifuged at 10,000 rpm for 20 minutes. The supernatant

served as enzyme source and it was saved in ice bath. During assay conditions, 0.5 ml crude enzyme were incubated with 1 ml 0.1 M Tris-HCl buffer (pH-7.5), 0.1 ml, 0.05 M $MgCl_2$, 0.1 ml 0.02 M p-nitrophenyl phosphate and 1.7 ml distilled water at 30°C. Absorbance was measured immediately after addition of enzyme at 410 nm after 1 hour of incubation. The soluble proteins in this enzyme extracts were estimated by the method of Lowry *et al.* (1951) which has been already described. Enzyme activity was expressed as μ moles p-nitrophenol liberated h^{-1} mg^{-1} protein.

c) Enzyme ATPase (E.C. 3.6.1.3)

The activity of enzyme was determined following the method described by Todd and Yoo (1964) extraction of enzyme was carried out according to the method of Weimberg (1970). Five hundred mgs leaf and root tissues from each treatment and control were homogenized in 10 ml ice-cold 0.1 M Tris-HCl buffer (pH-8.0) containing 1 M KCl, 0.01 M EDTA and 0.4 ml 0.2 M β-mercaptoethanol. The homogenate was filtered through 4 layers of musline cloth and filtrate was centrifuged at 10,000 rpm for 20 minutes. The supernatant served as enzyme source. One ml of supernatant was added to 0.5 ml of 0.01 M $CaCl_2$ and 0.5 ml 0.003 M ATP. The reaction was carried out at 38°C for 1 hour and was stopped by adding 1 ml of 0.1 M NaOH. The liberated phosphorous was estimated by the method of Fiske and Subba Rao (1925). To 1 ml of reaction mixture 4 ml distilled water, 1 ml 5 N H_2SO_4, 1 ml 2.5% Ammonium molybdate, 0.4 ml ANSA reagent was added. After 10 minutes the absorbance of developed blue colour

complex was measured at 660 nm. The change in OD at 0 min and 60 min was determined and amount of liberated P was calculated with the help of standard curve. The proteins in this enzyme extracts were estimated by the method of Lowry *et al.* (1951) which is described earlier. The enzyme activity is expressed as μ moles inorganic phosphorous liberated h^{-1} mg^{-1} protein.

d) Enzyme Alkaline inorganic Pyrophosphatase : (E.C. 3.6.1.1)

A method of Kar and Mishra (1976) was employed for the determination of the activity of enzyme alkaline inorganic pyrophosphatase. One gm leaf as well as root tissue from each treatment and control were crushed in 10 ml of 150 μ moles of Tris HCl buffer (pH 8.6). This was filtered through 4 layered muslin cloth and centrifuged at 4,000 rpm for 20 min. Supernatant serves as enzyme source. The assay mixture contained 2 ml 150 μ moles Tris HCl buffer (pH 8.6), 2 ml of 5 μ moles of tetrasodium pyrophosphate and 1 ml 100 μ moles of magnesium chloride and 1 ml of enzyme extract. The assay mixture was incubated for 10 minutes at 37°C and the reaction was stopped with the addition of 1 ml of 20 % perchloric acid. A zero time control was run at the same time with the enzyme extract added after adding perchloric acid to assay mixture. After 15 minutes reaction mixture was centrifuged at 5000 rpm for 5 minutes. The inorganic phosphate in supernatant was determined by Jaffe and Galston, (1966) method. To 1 ml supernatant and 1 ml chromogenic reagent (chromogenic reagent consist of 5 gm $FeSO_4$ and 1 gm $(NH_4)_2MoO_4$ dissolved in 100 ml of 1 N H_2SO_4). The mixture

was incubated at 30°C for 15 minutes. The resulting blue colour was measured at 650 nm. The change in OD during 60 minute was determined and amount of phosphorus liberated was calculated with the help of standard curve. The proteins in the enzyme extracts were estimated according to the method of Lowry *et al.*, (1951) which is already described. The enzyme activity was expressed at μ moles inorganic phosphorus liberated h^{-1} mg^{-1} protein.