Chapter II

MATERIALS AND METHODS

1. MATERIAL :

To study the effects of salts on germination, two leguminous seeds viz. Crotalaria juncea (L.) and Phaseolus aconitifolius (Jacq.) have been selected. The seeds of these varieties were procurred from the local market. The healthy seeds were surface sterilized with 0.1% $HgCl_2$ solution for 5 minutes. The seeds were then washed throughly with distilled water and germinated in sterilised Petridishes on filter papers (Whatman No.1). For salt tolerance studies at germination level; the seeds were subjected to various concentrations of NaCl and $CaCl_2$. The concentrations used were ranging from 2.5 mM to 300 mM. Control was maintained under non-saline conditions (distilled water). The seeds were then moistened with respective solutions and control with distilled water. The Petridishes were kept at room temperature. Further doses of respective solutions were added as per the requirements, so that the filter paper was always moist with the respective solution. The treatments were continued upto 120 h (5 days). The germination counts in each treatment were recorded after every 24 h till 96 hours. The emergence of radicle is taken to be the criterion of germination.

In order to see the performance in general, the seeds of both species namely Crotalaria juncea and Phaseolus aconitifolius were primarily subjected to wide range of NaCl and

$CaCl_2$ concentrations from 10 mM to 300 mM. The concentrations used were 10, 20, 40, 60, 80, 100, 150, 200 and 300 mM.

The seeds were also screened within lower concentrations along with some higher concentrations of both the salts. The concentrations used were 2.5, 5.0, 7.5, 10, 50, 100 and 200 mM.

In the preliminary studies, the effect of salt at the above concentrations on the germination was observed after every 24 h till 96 h and the pattern of seedling growth was studied at the end (after 120 h) of growth. Here the effect of salts on root and shoot length and fresh weight of the seedlings was studied. From the above preliminary observations the higher toxic doses were omitted and then the concentrations like 2.5, 5, 10, 50, and 100 mM of salts were used with only one or two toxic doses in the further studies.

2. **METHODS** :

Germination percentage, ~~dry matter~~, proline, some enzymes like peroxidase, catalase, amylase, acid phosphatase and nitrate reductase have been studied after every 24 hours of growth till 120 hours. However, the carbohydrates were estimated from the seedlings after 6, 48 and 96 hrs of germination at the levels of 5, 10 and 100 mM NaCl concentration. In all the studies the control was maintained in distilled water.

A) Moisture content :

The moisture percentage was determined by subjecting a known amount (wt) of fresh seedlings to high temperature (60°C) in oven until a constant dry weight was obtained.

B) Carbohydrates :

Carbohydrates (total sugars and starch) were estimated spectrophotometrically by the method of Nelson (1944). Some known quantity of fresh material (seedlings) was honogenized in 80% ethanol. The extract was filtered through Buchner's funnel using Whatman No.1 filter paper. The filtrate was used to estimate sugars while the residue was used for starch estimation.

The filtrate thus obtained was condensed on a water bath to about 3-5 ml. This extract was then treated with lead acetate and potassium oxalate (1 g : 1 g) with 30-50 ml distilled water to decolorize it. After decolorization the qliquotes were filtered and the volume was measured. A known volume was hydrolysed with 2 ml con. HCl for 30 min under 15 lbs pressure in a pressure cooker. The contents were cooled, neutralized with Na_2CO_3 and filtered. The filtrate was used for the estimation of total sugars.

The residue of alcoholic extract obtained at the beginning was transferred in a conical flask. To it 50 ml of D.W. and 5 ml of concentrated HCl were added and hydrolysed at 15 lbs pressure for half an hour and cooled to room temperature. It was then neutralised by Na_2CO_3 and filtered through filter paper Whatman No.1. The volume of the filtrate was noted. This filtrate containing sugars which was produced as a result of hydrolysis served as a sample for starch estimation. The sugars so obtained were estimated to determine the starch present in the material.

The requisite quantity of the above filtrates (extracts) was taken separately in 10 ml marked test tubes. In such other 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 Somagyi's alkaline copper tartarate reagent (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 test 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 water to which were added 21 ml concentrated H_2SO_4. To this was then added 3 g sodium arsenate, Na_2HASO_4, $7H_2O$; dissolved in 25 ml water. All ingredients 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 tube were then diluted to a volume (10 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 spectrophotometer.

By the interpolation from the glucose standard curve, the sugar percentage in the above fractions was determined.

C) Proline :

Proline was estimated by the method of Bates et al., (1973). The known quantity (0.5 g) of fresh seedlings was homogenized in 10 ml of 3% sulfosalicylic acid. After homogenization, it was filtered. 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 by adding 2.5 g ninhydrin in 60 ml of glacial acetic acid warmed to dissolve after adding 40 ml of 6 M arthophosphoric acid, (cooled and used fresh) and 2 ml of glacial acetic acid. The reaction was allowed to continue for 1 h at 100°C on water bath.

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 vaccupipette was used.

At the same time, reaction mixture for standard curve of proline (0.1 mg/ml) were prepared in the same way by taking different concentrations of proline solution. The calibration curve for standard proline was drawn and the amount of free proline in seedlings was determined and expressed in terms of µg/g fresh tissue.

D) Enzymes :

i) Peroxidase :

For the study of peroxidase (EC 1.1.17) the method followed was of Maehley (1954). The enzyme was extracted in cold distilled water by homogenizing 0.5 g of germinating seeds in cold mortar with pestle, followed by filteration through 4 layers of moist cheese cloth. The filtrate was centrifuged at 10,000 g for 20 min and the supernatent was used as an enzyme source. The enzyme assay mixture was prepared by mixing 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 sterring the reaction mixture with glass rod. Activity of the enzyme is expressed as Δ O.D. min^{-1} g^{-1} fresh weight.

ii) Catalase :

Catalase (EC 1.11.1.6) was studied by the method of Herbert (1955). For this 0.5 g of germinating seeds were homogenised in chilled mortar with pestle in 10 ml cold distilled water followed by filtration through four layers of moist cheese cloth. The filtrate was centrifuged at 10,000 g for 20 min in refrigerated centrifuge and the supernatent was stored at 0°C and used as an enzyme source. The assay mixture was prepared by mixing 1 ml H_2O_2 (0.045 M) and 1 ml enzyme. Assay mixture was incubated at room temperature for 1 min and the reaction was terminated by the addition of 5 ml H_2SO_4 (5 N). To this 1 ml KI (10%) solution and a drop of ammonium molybdate were added and liberated iodine was titrated against 0.01 N sodium thiosulphate using starch as an indicator. The difference between '0' and '1' min reaction was used as enzyme activity. For '0' min reaction and blank the reaction mixture was the same as in case of enzyme assay mixture but for blank without enzyme and for '0' min the reaction was terminated immediately

after or before the addition of enzyme. Activity of enzyme is expressed as mg H_2O_2 broken min^{-1} g^{-1} fresh weight.

iii) <u>oc- Amylase</u> :

For the study of enzyme oc- amylase (EC.3.2.1.1) a blue value method of Katsumi and Fukuhara (1969) was employed. For this 0.5 g of germinating seeds were homogenized in a chilled mortar with pestle in 10 ml of cold acetate buffer (pH 5,0.1 M) and filtered through four layers of cheese cloth already moistened with acetate buffer. The filtrate was centrifuged at 10,000 g for 20 min and the supernatent was used as an enzyme source. Two sets of reaction mixtures were prepared, one serving as '0' min (control) and the other as the reaction mixture. 1 ml of acetate buffer (pH 5, 0.1 M) and 1 ml of enzyme preparation were mixed in a test tube. After 10 minutes of equilibrium, 1 ml of 0.2% amylase solution (200 mg of amylose was dissolved in 4 ml of 1 N NaOH and kept in a refregerator overnight to dissolve completely. Then it was diluted to 800 ml with distilled water and adjusted to pH 7.5 with 1 N acetic acid and brought to a final volume of 100 ml with the addition of distilled water) was added. The reaction in control ('0' min) set was terminated immediately following the addition of substrate by adding 10 ml 0.5 N acetic acid. The reaction mixture in other set was shaken exactly for 1 h and was terminated by

adding 10 ml 0.5 N acetic acid. 1 ml of reaction mixture was sampled and mixed with 10 ml I_2 KI solution. The O.D. of the solution was read at 700 nm on spectrophotometer (ELICO CL-24) against I_2KI solution used as blank. The enzyme activity was expressed as change in O.D. h^{-1} g^{-1} fresh tissue.

iv) Acid Phosphatase :

Activity of enzyme acid phosphatase (EC 3.1.3.2) was determined according to the procedure described by De Leo and Sacher (1970) and McLachlan (1980). The extraction of the enzyme was done by macerating 0.5 g of seedling tissue in ice cold acetate buffer (pH 5, 0.1 M) in a ice cooled mortar with pestle. The resulting suspension was filtered through four layers of cheese cloth already moistened with acetate buffer and the filtrate was centrifuged at 10,000 g for 10 min and supernatent was used as an enzyme source. The assay mixture contained 3 ml of P-nitrophenyl phosphate (0.1 mg P-nitrophenyl phosphate/ml of acetate buffer, pH 5, 0.1 M), 2 ml of acetate buffer (pH 5, 0.1 M) and 1 ml of enzyme. The reaction was stopped by the addition of 1.5 ml of 1.68 N NaOH after one hour. The reaction terminated immediately ('0' min) served as control. The optical density of the developed colour was determined at 420 nm on spectrophotometer. The enzyme activity is expressed as Δ O.D. h^{-1} g^{-1} fresh weight.

v) Nitrate reductase :

Nitrate reductase (EC 1.6.6.1) was determined (in vivo) by employing the leaf-disc method (Evans, 1982). The known quantity of seedlings were suspended in a 5 ml reaction mixture containing 0.1 M KNO_3, 1% (v/v) n-propanol, 0.1% (v/v) Triton X-100 and 0.1 M phosphate buffer at pH 7.5 after cutting them once or twice into pieces. The test tubes containing reaction mixture and pieces of seedling were then sealed and kept in darkness for incubation for 30 min. The enzyme activity was measured by treating 0.4 ml of incubation mixture with 0.3 ml of 1% sulfanylamide and 0.3 ml of 0.02% NEEDA (N-(1-napthyl) ethylene diamine dihydrochloride). To this 2 ml distilled water added and after one hour of incubation the absorbance of colour developed was read at 540 nm on spectrophotometer. The activity of enzyme is expressed in terms of $\mu g\ NO_2^-\ h^{-1}\ g^{-1}$ fresh tissue.