Chapter - II

Materials and Methods

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A) Material :

In order to study the effect of salts on germination, the seeds of Dolichos biflorus Roxb., Lens esculenta Moench., Phaseolus aconitifolius Jacq., Phaseolus aureus Roxb. and Trigonella foenumgraecum Linn. were obtained from the local market. The healthy seeds were selected and surface sterilized with 0.1% $HgCl_2$ solution for 2-3 minutes. The seeds were then washed thoroughly with distilled water and germinated in sterilized pettridishes on filter papers (Watman No.1). For salt tolerance studies, the seeds were subjected to various concentrations of NaCl from 2.5 to 300 mM and distilled water was used as a control. The seeds and filter papers were moistened with respective salt solutions and also with distilled water (as control). The Pettridishes were kept in dark at room temperature and as per requirement, the respective salt solutions were added into Pettridishes so that the filter paper was kept always moist with the respective salt solutions. The treatments were continued upto 120 h (5 days). The germination counts in each treatment were recorded after every 24 h. The radicle emergence was taken as the criterion for germination.

The effect of salt at the above concentrations on seedling growth with respect to root and shoot length and fresh as well as dry matter of the seedlings was studied at every 24 h, till the end of 120 h of growth.

At the above stages, the changes in the moisture content, carbohydrate and soluble protein contents and the activity of

certain enzymes such as peroxidase, catalase and acid phosphatase in the seedlings were also studied.

B) Methods :

1) Carbohydrates :

Carbohydrates (soluble sugars and starch) were estimated from the oven dried whole seedling material following the method by Nelson (1944).

a) Soluble sugars :

The soluble sugars were extracted from 0.5 g plant material with 80% alcohol. The extract was filtered through Whatman No.1 filter paper using Buchner's funnel under suction. The filtrate thus obtained was condensed on water bath to about 1 ml. To this were added lead acetate and potassium oxalate (about 1 g each) and about 40 ml distilled water for decolourization. After 5 min, the extract was filtered through Whatman No.1 filter paper using Buchner's funnel under suction. This extract was hydrolysed with about 3-4 ml concentrated HCl by autoclaving at 15 lbs pressure for half an hour. The hydrolysate was cooled, neutralized with anhydrous sodium carbonate and filtered as before. The volume of the filtrate was measured and this filtrate was used for the estimation of total (reducing and non-reducing together) sugars.

The sugars from the filtrate were estimated by determining the reducing power by employing arsenomolybdate reagent introduced

by Nelson (1944) for the calorimetric determination of the cuprous oxide formed in the oxidation of sugars by alkaline copper reagent. For this 0.5 ml aliquots were taken in test tubes along with different concentrations of standard glucose solution (0.1 mg/ml) in other test tubes. To this, required amount of distilled water was added to make the final volume of each reaction mixture 1 ml. In case of blank, instead of filtrate or standard glucose, distilled water was used to begin with the reaction. One ml of alkaline copper tartarate reagent (4 g $CuSO_4,5H_2O$, 24 g anhydrous Na_2CO , 16 g Na-K-tartarate and 180 g anhydrous Na_2SO_4 dissolved in 1 litre distilled water) was added in each test tube and all these reaction mixtures were transferred to boiling water bath and boiled for 10 minutes. After cooling to room temperature, 1 ml Arsenomolybdate reagent (25 g ammonium molybdate in 420 ml water to which 21 ml concentrated H_2SO_4 were added, followed by 3 g sodium arsenate, $Na_2AsO_4,7H_2O$, dissolved in 25 ml water. These ingradients were mixed well and digested for 48 h at 37 C in incubator befor use) was added to each reaction mixture which were further diluted to 10 ml with distilled water. After 10 minutes, absorbance was read at 560 nm on Spectrophotometer (UV-VIS, Shimadzu, Japan). The values are expressed as 100 g^{-1} dry matter.

b) Starch :

For the estimation of starch, the insoluble residue obtained at the beginning during filteration of the alcoholic

extract, was transferred to a 100 ml capacity conical flask with about 40 ml distilled water and 4 ml concentrated HCl. The same was hydrolysed at 15 lbs pressure for half an hour and cooled to room temperature, neutralized by addition of anhydrous Na_2CO_3 and filtered. The volume of the filtrate was measured. This extract contained reducing sugars formed as a result of hydrolysis of starch. The reducing sugars in the extract equivalent to starch were determined according to the procedure described earlier for estimation of soluble sugars. The values are expressed as g 100 g^{-1} dry tissues.

2) Soluble proteins :

The soluble proteins were estimated following the method by Lowry et al.(1951). From the oven dried plant material the proteins were extracted with 80% alcohol. The extract was filtered through Buchner's funnel using Whatman No.1 filter paper. The filtrate thus obtained was condensed on water bath to about 2-3 ml. To this, 10 ml of distilled water was added and again filtered through Whatman No.1 filter paper. The filtrate thus obtained served as a source of soluble proteins.

For the assay, 0.1 ml extract was taken to which 0.9 ml distilled water and 5 ml reagent 'C' (50 ml 'A' + 1 ml 'B' where A - 2% Na_2CO_3 in 0.1 N aqueous NaOH, B - 0.05% $CuSO_4, 5H_2O$ in 1% Na tartarate) were added and after 15 min 0.5 ml folin phenol reagent (100 g sodium tungstate, Na_2WO_4, $2H_2O$ and 20 g sodium molybdate, $Na_2MoO_4, 2H_2O$ were dissolved in 700 ml distilled water,

50 ml 85% phosphoric acid and 10 ml Conc. HCl were then added. This mixture was refluxed gently for 10 h using water condenser. 50 g of lithium sulphate, 50 ml distilled water and a few drops of brome water were added to the mixture. This was boiled for about 15 min without water condenser to remove excess amount of bromine. It was cooled to room temperature and diluted to 1 N acidity by titrating it against 1 N NaOH) was added. After 30 minutes absorbance was read at 660 nm on Spectrophotometer.

3) Enzymes :

Enzyme hydroperoxidases (Peroxidase and Catalase) and acid phosphatase were isolated from the fresh seedlings. Isolation and assay of enzymes were done at low temperatures (0 to 4°C).

a) Hydroxyperoxidases :

i) Peroxidase :

Peroxidase from fresh seedlings was determined by the method of Maehly (1954). Enzyme was extracted by homogenizing the plant material in cold water. It was then filtered through double layered moist cheese cloth and the filtrate so obtained was centrifuged at 10,000 x g for 20 min at 0 to 4C and the supernatent was used as an enzyme source.

The enzyme assay mixture contained 1 ml phosphate buffer (pH 7, 0.1M), 1 ml guicol (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 guicol was recorded per minute at 470 nm on Spectrophotometer (UV-VIS, Shimadzu, Japan) with frequent stirring of reaction mixture with glass rod. Activity is expressed as Δ.O.D. $min^{-1}g^{-1}$ fresh tissue as well as Δ.O.D. min^{-1} mg^{-1} protein.

ii) Catalase :

The enzyme was isolated from the fresh seedlings in cold distilled water following the usual method of extraction, filtration and centrifugation as described above. Isolated enzyme was stored at 0-4C.

The assay of the enzyme was done following the method of Herbert (1955). Assay mixture contained 2 ml 0.45 M H_2O_2 in phosphate buffer (pH 7, 0.1M) and 1 ml enzyme. Reaction was stopped by adding 5 ml 1 N H_2SO_4 after 5 minutes of the reaction. To this 1 ml 10% KI and a drop of 1% ammonium molybdate were added and liberated iodine was titrated against 0.01 N Sodium thiosulphate using starch as an indicator. Blue to colourless was taken as the end point of titration. Blank or 0 min reading was taken by mixing all the ingradients except enzyme as given above. Activity of the enzyme is expressed as mg of H_2O_2 broken min^{-1} g^{-1} fresh tissue as well as mg H_2O_2 broken $min^{-1}mg^{-1}$protein

b) Acid phosphatase :

The enzyme activity was determined according to the procedure described by D.Leo and Sacher (1970) and McLachlan

(1980). Enzyme was prepared in cold distilled water by homogenizing the seedlings in an ice cold morter with pestle. The resultant homogenate was filtered through four layered cheese cloth and the filtrate was centrifuged at 10,000 X g for 20 min supernatent was stored at 0.4C and served as an enzyme source.

The assay mixture contained 3 ml of p-nitrophenyle phosphate (0.1 mg p-nitrophenyl phosphate ml^{-1} of acetate buffer, 0.1M, pH 5), 2 ml acetate buffer (pH5, 0.1M) and 1 ml enzyme. The reaction was allowed to proceed for 30 min and then was terminated by the addition of 1.5 ml of 1.68 N NaOH. The reaction was terminated immediately in 0 min reaction which served as control. The optical density of the developed yellow coloured complex was read at 420 nm on UV-VIS Spectrophotometer (Shimadz , Japan). The enzyme activity is expressed as ΔO.D. h^{-1} g^{-1} fresh tissue as well as ΔO.D. h^{-1} mg^{-1} protein.

c) Enzyme proteins :

Enzyme proteins were determined following the method by Lowry et al.(1951). The method has been described earlier. For assay, 0.05 ml enzyme preparation was taken.