CHAPTER - II

MATERIALS AND METHODS

1. Plant material

Sesbania grandiflora (L.); *Portulaca oleracea* (L.) and *Kalanchoe pinnata* (Lamk) pers. were grown under natural conditions in the garden of the department. The mature and healthy leaves were selected for the experiment. They were washed first with tap water followed by distilled water, blotted with blotting paper, and used for the studies in enzyme kinetics. The influence of substrate concentration, temperature and pH of the assay medium on the activities of enzymes peroxidase (E.C. 1.11.1.7) and catalase (E.C. 1.11.1.6) has been investigated. Polyphenol oxidase or phenolase (E.C. 1.10.3.1) and IAA oxidase (E.C. 1.13.16) have also studied following the various methods as given below :

1. Enzyme peroxidase : Maehly (1954)
2. Enzyme catalase : Sadasivam and Manickam (1991).
3. Polyphenol oxidase : Sato and Hasegawa (1976)
4. IAA oxidase : Tang and Bonner (1947)

2. Enzyme studies

A. Enzyme peroxidase (E.C. 1.11.1.7)

Peroxidase activity was determined in the healthy and mature leaves of *S. grandiflora*, *P. oleracea* and *K. pinnata* by the method described by Maehly (1954).

1.0 g fresh leaf material in the form of discs was homogenised in 20 ml of 0.1 M phosphate buffer (pH 7.0). Then it was filtered through 4-

layered cheeze cloth (moistened with buffer). The filtrate so obtained was centrifuged at 10,000 rpm for 10 minutes at 0 to 4°C. The supernatant was used as an enzyme source, or Crude Peroxidase Preparatin (CPP).

An enzyme assay mixture contained, 2 ml phosphate buffer (0.1 M, pH 7), 1 ml guaiacol (20 mM) and 1 ml of enzyme. The reaction was initiated by the addition of 0.05 ml H_2O_2 (1 mM). Changes in optical density due to oxidation of guaiacol was recorded per minute at 470 nm on spectrophotometer with frequent stirring of reaction mixture with glass rod. The readings were taken at different concentrations of substrate, H_2O_2 phosphate buffer, 0.05, 0.10, 0.2, 0.4, 0.6, 0.8, 1.0, 1.2, 1.4, 1.6, 1.8, 2.0, 2.5, 3.0, 3.5 and 4.0 ml. The activity of enzyme peroxidase is expressed as,

Δ O.D. $min^{-1}g^{-1}$ fresh tissue;

Δ O.D. $min^{-1}mg^{-1}$ protein

B. Enzyme catalase (E.C. 1.11.1.6)

Catalase activity was determined in the healthy and mature leaves of *S. grandiflora* (L.); *P. oleracea* (L.) and *K. pinnata* (Lumk) by the method described by Sadasivam and Manickam (1991) with slight modifications.

1.0 g fresh and mature leaf material in the form of small discs was homogenized in 20 ml of 0.1 M phosphate buffer (pH 7). Then it was filtered through 4 layered cheeze cloth (moistered with buffer) and the filtrate so obtained was centrifuged at 10,000 rpm for 10 minutes at 0 to 4°C. The supernatant was used as an enzyme source.

An assay mixture contained 3 ml H_2O_2 phosphate buffer (0.64 ml of H_2O_2 diluted to 100 ml with 0.1 M phosphate buffer, pH 7), 0.5 ml of enzyme, mixed immediately and change in optical density was recorded per minute at 240 nm on double beam UV-spectrophotometer (Shimadzu). The readings were taken at different concentrations of substrate, H_2O_2 phosphate buffer 0.2, 0.4, 0.6, 0.8, 1.0, 1.2, 1.4, 1.6, 2.0, 2.2, 2.4, 2.6, 2.8 and 3.0 ml. Activity of enzyme catalase is expressed as,

Δ O.D. $min^{-1}g^{-1}$ fresh tissue,

Δ O.D. $min^{-1}mg^{-1}$ protein

Effect of pH on the activities of enzymes peroxidase and catalase

For the study of effect of pH on the activities of the enzymes, the buffer at different pH was prepared and used in the assay medium. Thus phosphate buffer, 0.1 M at pH from 5.8 to 8.0 was used in the preparation of assay media for peroxidase while phosphate buffer 0.1 M at pH from 5.8 to 8.4 was used in the preparation of assay media for enzyme catalase. During assay of the enzyme at these different pH, pH of the assay medium containing buffer, substrate and other inclusions wherever added was recorded for each assay medium at each pH. Similarly for the assay media at every pH, it was recorded at the end of the enzymatic reaction. Mean of these two pH values, one at the beginning of the enzymatic reaction and the other at the end of enzymatic reaction, was obtained and was taken as pH value at which activity of the enzyme has been reported.

Effect of temperature on the activities of enzymes peroxidase and catalase

To study the effect of temperature on the activities of these enzymes, the enzyme assay mixtures were incubated in the water bath at different temperatures for certain time, till the desired temperature was obtained. The temperatures studied are 10°, 15°, 20°, 25°, 30°, 35° and 40°C. The enzymatic reactions were initiated when the reaction mixture had attained the desired temperature.

C. Enzyme Polyphenol oxidase (E.C. 1.10.3.1)

Polyphenol oxidase or phenolase (E.C. 1.10.3.1) from fresh and mature leaves of *S. grandiflora* (L.), *P. oleracea* (L.) and *K. pinnata* (Lamk) was determined by the method of Sato and Hasegawa (1976).

1.0 g leaf material was homogenized in morter with pestle and enzyme was extracted in 20 ml of 0.1 M phosphate buffer (pH 7). It was filtered through 4-layered cheeze cloth (moistened with buffer) and the filtrate so obtained was centrifuged at 10,000 rpm for 10 minute at 0° to 4°C and the supernatant was used as an enzyme source.

An assay mixture contained 0.5 ml of catechol (10 mM, in 0.1 M K-phosphate buffer, pH 6.8); 1 ml sulphanilic acid (10 mM); 1.0 ml of phosphate buffer (0.2 M, pH 6.8) and 0.5 ml enzyme. The enzymatic reaction was initiated by adding 0.5 ml enzyme preparation. The change in optical density was recorded at 0 min and 30 min. on spectrophotometer at 500 nm.

D. Enzyme IAA oxidase (E.C. 1.13.16)

IAA oxidase activity was determined in healthy and mature leaves of *S. grandiflora* and *P. oleracea* by the method described by Tang and Bonner (1947).

0.5 g of fresh leaf material was washed with distilled water and blotted to surface dry and homogenized and extracted in 10 ml of cold phosphate buffer (0.1 M, pH 7) in mortor with pestle. It was filtered through 4 layered cheese cloth and centrifuged at full speed (10,000 rpm) for 20 minutes. The supernatant was used as an enzyme source in every case.

An assay mixture for the enzyme contained 4 ml IAA (0.05 $mgml^{-1}$), 2 ml of enzyme, 0.5 ml of 2-4 Dichlorophenol (0.0001 M), 0.5 ml of $MnCl_2$ (0.002 M) and 3 ml of phosphate buffer (0.1 M, pH 6.2).

During the enzymatic reaction, 2 ml of assay mixture was pipetted out at different time intervals such as 0, 30 and 60 min. after the initiation of enzymatic reaction, mixed with 8 ml of Tang and Bonners reagent, mixed well and allowed to keep for 30 min. The optical density of each reaction mixture was then recorded on double beam spectrophotometer (Shimadzu) at 520 nm.

Enzyme protein

The proteins from enzyme preparations were determined by the method described by Lowry *et al.* (1951).

In the assay mixture, 0.05 ml of enzyme preparation was diluted to 1 ml with distilled water and mixed with 5 ml of freshly prepared reagent 'C' (50 ml 2% Na_2CO_3 in 0.1 N aqueous NaOH mixed with 1 ml 0.5% $CuSO_4$ $5H_2O$ in 1% Na-K-tartarate). After 15 min., 0.5 ml Folin-phenol reagent (100 g sodium tungstate mixed with 25 g. sodium molybdate, 700 ml distilled water, 50 ml 85% phosphoric acid and 100 ml concentrated HCl were refluxed gently for 10 hr using water condensor. To this, 150 g Lithium sulphate, 50 ml distilled water and a few drops of bromine water were added. The mixture was boiled for 15 min without water condenser to remove excess bromine. The mixture was cooled and then adjusted to 1 N by titrating it against 1 N NaOH) was added. The colour was allowed to develop for 30 min. and then absorbance was recorded at 660 nm.

Albumin solutin (0.1 $mgml^{-1}$) was used for preparation of standard curve of protein. Blank was prepared by mixing distilled water, reagent C and Folin-phenol reagent.

E. Qualitative determination of isozymes of peroxidase

Isozymes of enzyme peroxidase were determined employing polyacrylamide gel electrophoresis (Davis, 1964).

a) Extraction of peroxidase Isozymes

2 g of fresh and healthy plant material (leaves) (of *S. grandiflora*, *P. oleracea* and *K. pinnata*) was collected and washed thoroughly with distilled water and blotted to surface dry. It was homogenized in morter with pestle and extracted in chilled acetone (in case of *P. oleracea* and *K. pinnata*). The process was repeated thrice and the mucilage free tissue

samples were used for final extraction of enzyme. It was homogenized in morter with pestle and extracted in 20 ml ice cold Tris-HCl buffer, 0.1 M, pH 7.5 containing 0.02 M EDTA, 0.005 M NaCl and 0.03 M ß-mercaptoethanol. The extract was centrifuged at 12,000 Xg at 4°C for 30 minutes and the supernatant was collected and stored at 0-4°C. It was used as an enzyme source.

b) Preparation of gel solutions

Following stock solutions were prepared for the preparation of working solution:

Solution (A) : 1 N HCl = 48 ml
Tris = 36.6 g
TEMED = 0.23 ml

Distilled water to make 100 ml.

(1 N HCl : 87.3 ml conc. HCl per liter of distilled water).

Solution (B) : Acrylamide = 28.00 g
Bis-Acrylamide = 0.735 g
Distilled water to make 100 ml.

Solution (C) : Ammonium persulphate = 140 mg 100^{-1} ml.

c) Preparation of working solution from stock solution

The working solutions were prepared fresh. Gel solution was prepared by mixing the stock solutions A,B,C and distilled water in the proportion of 1:2:4:1 respectively.

d) Running Buffer

Tris = 3.0 g

Glycine = 14.4 g

D. water to make 500 ml (pH 8.4).

(Diluted 10 times before use), stored under freeze conditions and used chilled while loading and running the gel.

e) Preparation of gel columns

The tubes (8.5 x 0.5 cm) were fixed to gel tube holder to seal one end of the tube. The gel solution prepared as mentioned above was layered slowly by means of a fine pipette to fill the tube (about 8.0 cm). To avoid the miniscus formation, few drops of water were layered over the gel solution. The tubes were left for gelling for about 40 min. avoiding strong light.

f) Loading and Running the Gel

After the gel columns were ready, the gel of each column was layered with about 20 λ solution of 40% solution in 50% glycerol + 0.02% of bromophenol blue. Then in each cuvette 50 λ enzyme preparation (0.8 ml enzyme extract + 0.2 ml of Bromophenol blue) was layered.

The gel tubes were fixed to the upper electrode vessel. The lower and upper electrode vessels were filled with the buffer until the electrodes were completely immersed. The two terminals were connected to the power source. The current was kept constant at 3 mA per tube, with initial voltage adjusted to 100 volts. The run was continued until the marker front reached the end of the tubes at 4°C.

The gels were removed with the help of syringe provided with long needle inserted between the wall of gel tube and the gel, with a rotatory motion, along with flow of water.

g) Staining for detection of enzymes

After removing the gel columns, they were stained with solution containing, mixture of Benzidine, Ammonium chloride (30%) and 0.5 % Hydrogen peroxide in 50:15:35 proportions. The gels were incubated for 30 min. at room temperature in the dark destained in solution of 7% acetic acid and fixed in 2% acetic acid.

After completion of staining the gel tubes showing the different bands of isozymes were photographed, the length of marker front and the distance travelled by different bands (isozymes) were recorded. The 'Rm' was calculated by using formula.

$$\text{'Rm'} = \frac{\text{Distance migrated by particular band}}{\text{Distance migrated by marker front}}$$