II

MATERIAL AND METHODS

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A) PRESOWING SOAKING TREATMENT

Seeds of soybean variety were obtained from authentic source in the local market. The viability of a seed sample was tested first and latter on the seedlot was employed for the investigation. Healthy uniform seeds were sorted out. In initial phase of investigation for the presowing soaking treatment, the seeds were soaked in various plant growth regulator solutions prepared in distilled water. But it was noticed that during drying following soaking treatment the seed coats cracked. This aspect has been discussed in detail in the first part of chapter 'Results and Discussion'. Hence, organic solvent acetone was used for preparing solutions 100 ppm solutions of following plant growth regulators were prepared in acetone.

1) Ascorbic acid
2) Salicylic acid
3) Chlorocholine chloride
4) Ethephon

The method of Salim and Tood (1968) was followed for seed pretreatment. 10 g of healthy seeds were kept in 500 ml beakers containing 400 ml of respective solutions under natural day light for 8 hours. After this soaking treatment the seeds were removed and air dried in tray under

natural light till they attained their original weight. The untreated healthy seeds served as control.

B) GERMINATION STUDIES

For study of influence of presowing soaking treatments in various physiological parameters in the germinating soybean seeds. Seeds from each treatment were taken and they were surface sterilized with 0.1% $HgCl_2$ solution for 2 minutes. The seeds were washed, thoroughly and blotted dry. Twenty seeds from each treatment were kept in sterilized petridishes for germination over whatman No.1 filter paper at room temperature in germination chamber. The filter paper was moistened with 20 ml glass distilled water. Water uptake was studied after 12 and 24 h intervals, while respiration rate and activities of various enzymes and fate of various organic constituents were studied after 24 h of seed germination.

a) <u>Water Uptake</u>

After 12 and 24 h germination 10 seeds from each treatment were carefully removed blotted dry and their fresh weight was recorded. Then the seeds were kept in oven at 60°C till a constant weight (dry weight) was obtained. The difference in these two weights was considered as an amount of water taken by seeds during germination.

b) **Respiration Rate**

Two g of seeds from each treatment were transferred to a small vial which was hold carefully in a jar containing 2 ml 0.025 N Barium hydroxide solution. The jar was properly sealed and made air tight. simultaneously another jar (control) containing 2 ml 0.025 N. Barium hydroxide solution but without seed material was also sealed and made air tight. The experimental set up was placed in dark for 60 minutes the vial containing seeds was carefully removed and the Barium hydroxide solution was titrated against 0.01 N HCl using phenolphthalein indicator. The Barium hydroxide solution in the control jar was also titrated in similar manner. The difference in titer values give a measure of respiratory CO_2 evolution and the rate of respiration was calculated according to formula given by Malean and Ivimey-Cook (1968) mg CO_2 liberated $h^{-1}g^{-1}$ seed material.

$$= \frac{(\text{control titration reading} - \text{Experimental titration reading}) \times 0.22 \times 50 \times 60}{\text{Experimental time in minutes} \times \text{Weight of material in g.}}$$

c) **Enzyme α-Amylase** (EC 3.2.1.1)

A blue valve method Katsumi and Fukuhara (1969) was followed for study of enzyme α amylase. The seed coat was removed 0.5 g of germinating seeds were crushed in a chilled mortar and pestle in 10 ml of cold acetate buffer

(pH 5) and filtered through 4 layeres of cheese cloth. The filterate was centrifuged at 10,000 rpm for 20 min and supernatent was used as enzyme source. Two sets of reaction mixture were prepared one serving as 'O' minute contorl and other serving as a reaction mixture. One ml of acetate buffer (pH 5) and one ml enzyme solution were mixted in a test tube. After 10 minutes of equiliberation period one ml of 0.2% amylose solution (200 mg of amylose were dissolved in 4 ml of 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 1N acetic acid and brought to final volume of 100 ml with the addition of distilled water). The reaction in control (0 min.) set was terminated immediately following the addition of substrate by additing 10 ml of 0.5 N acetic acid. The reaction mixture in another set was shaken for exactly 30 min. and then the reaction was terminated by adding 10 ml 0.5 N acetic acid. One ml of each reaction mixture was taken in a another set of test tubes and mixed with 10 ml of dilute I_2KI solution. The O.D. of the solution was measured at 700 nm against I_2KI solution used as blank. The energy activity was expressed as change in OD $min^{-1}g^{-1}$protein.

The soluble proteins in the enzyme extract were estimated following the method of Lowry et al. (1951) which is described in latter part of this chapter.

d) <u>Enzyme Lipase</u> (EC 3.1.1.3)

For extraction and study of lipase activity titrimetric methods of Wolf (1968) and Chinoy <u>et al</u>. (1969) were followed. Five g of germinating seeds from each treatment were taken and seed coat was removed. The seed material was repeatedly homogenized in 50 ml of chilled acetone. This was filtered through two Layers of cheese cloth. The oil free residue left on the cheese cloth served as the source of enzyme lipase. For study of this enzyme Triacetin was employed as substrate, 1 ml of triacetin was allowed to react with 500 mg of enzyme in 10 ml of 0.1 M phosphate buffer pH 7.5 in a conical flask. The conical flask was continuously shaken for 60 minutes and the reaction was terminated by addition of mixture of 30 ml ethyl alcohol and 10 ml solvent ether. The contents of conical flask containing free fatty acids liberated due to lipase action were titrated against 0.1 N NaOH using phenolphthalene indicator. simultaneously in another conical flask a similar reaction mixture was taken but the reaction was terminated immediately following addition of enzyme (0 min). The difference in 60 min and 0 min titration readings was considered as a measure of enzyme activity and expressed as ml alkali consumed $h^{-1}g^{-1}$ fresh tissue.

e) <u>Enzyme Acid Phosphatase</u> (EC 3.1.3.2)

Enzyme acid phosphatase was assayed according to the method of McLachlan (1980). 0.5 g of germinating seeds material from each treatment was homogenized separately in 10 ml of cold acetate buffer (pH 5) in ice cold mortar with pestle. The resultant homogenate was filtered through four layers of cheese cloth and filterate was centrifuged at 10,000 rpm for 20 minutes. The supernatant served as enzyme source.

The assay mixture contained 3 ml of p-nitrophenyl phosphate (0.1 mg P-nitrophenyl phosphate ml^{-1} of acetate buffer pH 5), 2 ml acetate buffer and 1 ml enzyme. The reaction was allowed to proceed for 30 minutes and then was terminated by addition of 1.5 ml of 1.68 N NaOH. The reaction terminated immediately (0 min) served as control. The optical densities of the developed pale yellow colour complex were read at 420 nm. The soluble proteins in the enzyme extract were estimated accordingly to method of Lowry <u>et al</u>. (1951) described in latter part of the chapter. The enzyme activity is expressed as change in OD $h^{-1}mg^{-1}$ protein.

f) <u>Enzyme Phytase</u> (EC 3.1.3.8)

The enzyme phytase from cotyledons were extracted and assayed according to the method of Kuvaera and Kritovich

(1978) and liberated phosphorus was estimated by using method of Fiske and Subba Row (1925). The seed coat was removed and 10 cotyledons were homogenized in cold 20 ml 0.1 M acetate buffer pH 5.6. Homogenized solution was filtered through 2 layers of muslin cloth. Filterate was centrifuged at 15000 rpm for 20 minutes. Supernatent served as enzyme source. Reaction mixture contained 4 ml acetate buffer pH 5.6, 1 ml phytin, 1 ml enzyme. The reaction was stopped at 0 and 60 minutes by adding 5 ml TCA (Trichloroacetic acid). This was followed by centrifugation at 10,000 rpm for 10 minutes. The inorganic phosphorus (P) in supernatent was determined by Fiske and Subba Row method. To 1 ml of supernatent, 4ml distilled water, 1ml 3% ammonium molybdate, 0.4 ml ANSA (1 amino 2-Naphthol 4-sulphonic acid) reagent was added. After 5 minutes the absorbance of developed blue colour complex was measured at 660 nm. The change in O.D. in 0 min and 60 min was determined and amount of Pi liberated was calculated with the help of standard curve. The enzyme activity is expressed as change in OD $h^{-1}mg^{-1}$ protein.

g) <u>Enzyme Nitrate Reductase</u> (EC.1.6.6.1)

Activity of nitrate reductase was determined following the in vivo method described by Jaworski (1971). Germinating seeds from different treatments were taken and the seed coat was carefully removed. The seeds were cut into small pieces. A weighed amount of seed pieces were

incubated in the medium containing 1 ml 1M KNO_3, 2 ml 5% n-propanol, 5 ml 0.2 M phosphate buffer pH 7.5 and 2 ml 0.5% Triton-X-100 for 1 h in dark under anaerobic conditions. After 1 hour 1 ml reaction mixture transfered to another testtube and mixed with 1 ml 1% sulfanylamide in 2N HCl and 1 ml 0.2% NEEDA [N-(1-Napthyl) ethylene Diamide Dihydrochloride]. While mixture of 1 ml incubation medium, 1 ml sulfanylamide one ml NEEDA served as blank. The absorbance was read at 540 nm on Spectronic-20 spectrophotometer. Standard curve was prepared with the help of different concentrations of KNO_2, and enzyme activity is expressed as µ moles of NO_2 liberated $h^{-}g^{-}$ fresh tissue.

h) <u>Enzyme Dehydrogenase</u>

For the study of dehydrogenase from cotyledon and embryo axis the tetrazolium method of Kittoch and Law (1957) was followed. The seed coat was removed from germinating seeds and 1 g of seeds are broken into pieces and incubated for 4 hours in 4 ml 0.2% 2-3-5 Triphenyl tetrazolium chloride (TTC) solution in dark. After this seedlings are washed 2-3 times with distilled water, surface was dried and were treated with 10 ml of 2-methoxyethanol for extraction of red coloured formazan, which is formed due to activity of dehydrogenase. The optical density of coloured formazan was measured at 470 nm. The enzyme activity is expressed as change in OD $h^{-1}g^{-1}$ fresh weight.

i) **Enzyme Catalase** (EC.1.11.1.6)

Activity of enzyme catalase was studied following method described by Herbert (1955). The seed coat was removed and one g of germinating seeds were homogenised in 10 ml of distilled water. It was filtered through moist double layered cheese cloth. The filterate was centrifuged at 10,000 rpm for 10 minutes at 0-4°C and the supernatent was used as an enzyme source. Assay mixture contained 1 ml 0.1 N H_2O_2 in 0.02 M phosphate buffer pH 6.8 and 0.5 ml enzyme. Reaction was terminated by adding 5 ml 1N H_2SO_4, after one minute of reaction, to this one ml of 10% KI and 3 drops of 1% ammonium molybdate were added and liberated Iodine was titrated against 0.01 N sodium thiosulphate using starch as an indicator. The decolourization of blue colour was taken as an end point. Blank or zero minute reading was taken by mixing all ingradients except enzyme. The proteins in enzyme extract were determined following method of Lowry et al. (1951). Activity of the enzyme is expressed as mg of H_2O_2 broken down $min^{-1}mg^{-1}$ protein.

j) **Enzyme Peroxidase** (EC.1.11.1.7)

Peroxidase activity was assayed according to method of Maehly (1954). The seed coat was removed and one g of germinating seeds were homogenised in 15 mls of (1/15 M) phosphate buffer (pH 6.8) and filtered through 4 layered

muslin cloth. The filterate was centrifuged at 10,000 rpm for 20 minutes and supernatent was used as source of enzyme.

The reaction mixture contained 5 ml of 1/15 M Acetate buffer, 0.5 ml of 0.1 % Guicol, 1 ml enzyme extract, 5 ml distilled water, 0.5 ml of 0.08%. H_2O_2 were mixed and incubated at 30°C. After 15 minutes of incubation, 1 ml of 1N H_2SO_4 was added to stop the reaction followed by measurement of absorbance at 470 nm. The soluble proteins in the enzyme extract were assayed according to method of Lowry's et al. (1951). The enzyme activity is expressed as change in OD per mg of protein per hour.

Study of isozyme pattern of peroxidase was done by polyacrylamide gel electrophoresis.

k) **Study of Peroxidase Isozyme Pattern**

The isozyme pattern of enzyme peroxidase obtained from germinating seeds subjected to vaious treatments was studied employing polyacrylamide gel electrophoresis (Davis, 1964, Dange and Reddy, 1983). For this purpose electrophoresis apparatus (Tube gel electropheresis, Hoffer Madras) was used,

I. Preparation of stock solutions

Solution A : 1 N HCl = 48 ml

Tris buffer = 36.6 gms

TEMED = 0.23 ml

Distilled water add to make = 100 ml

Solution B : Acrylamide = 28 gms

N.N methylene Bisacrylamide = 0.735 gms

Distilled water added to make 100 ml

Solution C : Ammonium per sulphate = 140 mg/100 ml

Running buffer Tris = 3 gm

Glycine = 14.4 gm

Distilled water added to make 500 ml (pH 8.4)

dilute 10 X 1 ml before use

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

III) Preparation of gel columns : The tubes (8½ X 0.5 cm) were fixed to a gel tube holder to seal one end of the tube. The gel solution prepared as mentioned above was layered slowly by means of fine pipette to fill the tube (about 8 cm). To avoid meniscus formation, few drops of water were layered over the gel solution. The tubes were left for gelling for about 40 mts strong light was avoided.

IV) Extraction of Peroxidase Isozyme : Two gm germinating seed tissue sample were extracted with 0.1 M tris-HCl

buffer (pH 7.5) containing 0.02 M EDTA, 0.05 M NaCl, 0.03 M ß-mercaptoethanol with morter and pestle. The sample was centrifuged at 12000 rpm and the spernantant was collected. The filter paper disc with the sample observed was placed over the gel layered with about 20µ of 40% sucrose containing bromophenol blue. The gel tubes were kept at 4°C for the protein separation.

The gel tubes were fixed to the upper electrode vessel. The lower and the upper electrode vessel were filled with the electrode buffere until the electrodes were completely immersed. The two terminals were connected to the power source. The current was kept constant at 3 milliamperes pertube, with initial voltage adjusted to 100V. The run was continued until bromophenol blue front reaches the end of the tubes which required approximately 90 minutes. The gel were removed with the help of long needles inserted between the wall of the gel tube and the gel, with rotatory motion, along with flow of water.

Staining for the Detection of Enzymes

After removing the gels they were stained simultaneously in trays containing the proper substrate to detect peroxidase activity. Isoperoxidase were stained with a solution consisting of benzidine, ammonium chloride (30%), 0.5% hydrogen peroxide (50:15:35). The gels were incubated

for 10 mts at root temperature, destained in a solution of 7 acetic acid and fixed in 2% acetic acid. The gel tubes were photographed and the position of isozymes was marked. On destaining, the Rm value of each band was calculated using the following formula.

$$Rm = \frac{\text{Distance of the band from origin}}{\text{Distance of dye marker front from origin}}$$

1) **Soluble Proteins**

The soluble proteins in enzyme extracts for various enzymes mentioned earlier were estimated by the method of Lowry et al. (1951). 0.5 ml crude enzyme extract was taken intest tube and diluted to 1 ml with distilled water. To this 5 ml of reagent C (50 ml of 'A' containing 2% sodium carbonate in 0.1 N aqueous NaOH is mixed with 1 ml of 'B' containing 0.5% copper sulphate in 1% Na-K tartarate. Solution was mixed well allowed to stand for 15 minutes at room temperature. After 15 minutes 0.5 ml of Folin and Ciocalteau's phenol reagent was added raidly with immediate mixing. This was allowed to stand for 30 minutes and intensity of developed blue colour was measured at 600 ppm on spectronic 20. Protein concentration was calculated by comparing with standard curve of different concentration of Bovine serum albumin (0.1 mg/ml) prepared in a similar manner.

m) **Soluble Sugars**

The soluble sugars were estimated following the method of Nelson (1944). The soluble sugar's were extracted from 0.5 g oven dried seedling tissue with 80% neutral ethanol. The extract was filtered through Whatman No.1 filter paper in Buchner funnel. The filterate thus obtained was condensed on water bath to about 5 to 3 ml. To this 1 g lead acetate and potassium oxalate (1:1) were added for decolourization. Fifty ml of distilled water were added and aliquot was filtered. The volume of filterate was recorded. A known volume of this extract (20 ml) was hydrolyzed with 2ml conc. HCl by autocalving at 15 lbs atm pressure for half an hour. The content was cooled neutralized with sodium carbonate and filtered. The volume of filterate was recorded and this filterate was used for the estimation of total soluble sugars.

The soluble sugars (now all converted into reducing sugar form) were estimated by determining the reducing power by employing Arsenomylybdate reagent introduced by Nelson (1944) for the colorimetric determination of the cuprous oxide formed in the oxidation of sugars by alkaline copper tartarate reagent. For this 0.4 ml aliquotes were taken in the test tube, to this requisite amount of distilled water was added to make final volume 1 ml. In case

of blank instead of filterate a standard glucose distilled water was taken to begin with the reaction one ml of somogy's alkaline copper tartarate reagent (4 g $CuSO_4$, $5H_2O$; 24 g anhydrous Na_2SO_4 dissolved in one litre distilled water) was added in each test tube and all these reaction mixtures were transferred to boiling water bath for 10 minutes. After cooling to room temperature, 1 ml Aresenomolybdate reagent (25 g NH_4MOO_4 in 450 ml water to which 21 ml concentration H_2SO_4 were added followed by 3 g $Na_2HASO_4 \cdot 7H_2O$ dissolved in 25 ml water. These ingradients were mixed well and digested for 48 h. at 37°C incubator before use) was added to each reaction mixture which were further diluted to 10 ml with distilled water. After 10 minutes absorbance was noted at 560 nm on the spectronic 20. In another set of test tubes different concentrations of standard glucose solution (0.1 mg/ml distilled water) were taken and calibration curve was prepared following the procedure similar to that employed for test solution. With the help of this calibration curve the soluble sugar levels in the seed material were estimated.

n) <u>Ascorbic Acid</u>

Ascorbic acid content in germinating seeds was estimated as the method described by Dhopte and Phadnawis (1989). Germinating seeds was taken and seed coat was removed and immediately blotted to dry. It was weight

accurately (3 g) and cut into 2-3 mm pieces. Then pieces were placed in mortar and oxalic acid (0.4 %W/V) was added at the rate of 4 ml/g seedlings (tissues). Seedling were thoroughly crushed for about 5 minutes and extract was filtered through double layers of cheese cloth. Then it was centrifuged at 1000 g for 20 minutes. The volume of supernatant was made to 15 ml (1 g tissue in 5 ml oxalic acid) with oxalic acid reagent (0.4 %W/V). Ascorbic acid in extract was estimated by visual titration method, based on reduction of 2-6, dichlorophenol indophenol dye. Standard ascorbic acid solution was prepared by adding 50 mg of ascorbic acid to 50 ml of 0.4% oxalic acid solution in 250 ml volumetric flask and finally volume was made with to 250 ml oxalic acid. One ml of this solution contains 0.2 mg of ascorbic acid.

Indophenol reagent was prepared as follows. 150 ml of glass distilled water was added to 200 ml volumetric flask. Then 50 mg of sodium 2,6 dichlorophenol indophenol was added to it. The flask was gently heated in a hot water bath to dissolve the dye. Then 42 mg of $NaHCO_3$ were added. The flask was allowed to cool. After cooling the volume of solution was made to 200 ml with glass distilled water and the reagent was stored in dark glass bottle at 2°C. For standardization of indophenol reagent 5 ml of ascorbic acid solution was taken in a white porcelain dish and then it

was titrated against the indophenol dye until the solution become pink (The pink colour persisted at least 15 sec.).

After standardization of indophenol reagent 5 ml of seedling extract (prepared as described earlier) was titrated against standardized Indophenol reagent as above.

The ascorbic acid content of the extract was calculated by using formula.

$$I \times S \times D/A \times 100/W = \text{mg of ascorbic acid } 100\ g^{-1} \text{ fresh tissue}$$

where,

I = ml of indophenol reagent used in the titration

S = mg of ascorbic acid reacting with 1 ml of the reagent

D = Volume of the extract in ml

A = The aliquot titrated in ml

W = The weight of sample in g.

The value of ascorbic acid so obtained was further corrected on the basis of moisture content in the fresh tissue and expressed on dry weight basis.

o) <u>Sulphydryl Group</u>

The content of sulphydryl (SH) group was determined following the method of Ellman (1959). Germinating seeds were washed with distilled water and blotted to dryness. Then they were cut into small bits 0.2 g

seedling material was accurately weighed and homogenized in cold mortar in 10 ml phosphate buffer (pH 7). Extract was filtered through Buchner's funnel using Whatman No.1 filter paper. The reaction mixtures were prepared for 0 minute and 1 minute. 0 minute reaction mixture contained 7.3 ml phosphate buffer (pH =7), 0.2 ml 1N NaOH and 0.5 ml of extract. To this reaction mixture 0.02 ml of 5'-5' dithiobis (2-nitrobenzoic acid) (DTNB) was added. It was prepared by dissolving 39.69 of 5'-5' dithiobis (2-nitrobenzoic acid in 10 ml of phosphate buffer (μ = 0.1 buffer pH 7). The absorbance was read for 0 min and 1 min reaction mixture at 410 nm on spectronic 20, spectrophotometer. The sulphydryl content was calculated by sung formula.

$$Co = \frac{A}{t} \times D$$

where Co = original concentration

A = Absorbance at 410 nm (difference between 1 ml and 0 min reading)

t = extinction coefficient - 136/m/cm

D = dilution factor

$$D = \frac{\text{Volume at 1 min (after addition of DTNB)}}{\text{Volume at 1 min (without addition of DTNB)}}$$

The results are expressed on fresh weight basis taking into

consideration, the moisture content in the seedling material.

p) **Free Proline**

Free proline content in the germinating seeds of soybean was determined according to method of Bates et al. (1973). For this 1 g germinating seeds were homogenized in mortar with 10 ml 3% sulfosalicylic acid thoroughly and filtered through Buchner funnel using Whatman No.1 filter paper. The volume of the filterate was made to 20 ml with 3% sulfosalicylic acid. This filterate served as source of proline. Then 2 ml filterate was allowed to with 2 ml glacial acetic acid and 2 ml acid ninhydrin reagent (prepared by warming 1.25 g ninhydrin in 30 ml glacial acetic acid and 20 ml 6 M phosphoric acid with agitation cooled and stored at 4°C) in a test tube for 1 hour at 100°C in boiling water bath. Similar procedure was also followed for another set of test tubes containing various concentrations of standard proline solution (0.1 mg proline/ml). After one hour the reaction was germinated by transferring the test tubes immediately to ice bath. To this 4 ml of Toluene were added and mixed vigourously for 15-20 seconds. Reaction mixtures were then brought to room temperature and the absorbance of toluene chromophore was measured at 520 nm on spectrometer against toluene using blank. Proline concentration was calculated from calibration

curve and final values were expressed as mg 100^{-1} fresh tissue.

The values of various enzyme activities and organic constituents depicted in the chapter 'Results and Discussion' represent average of three independent determinations.