Materials & Methods

3.1. Plant material

3.1.1. Collection

The seeds of different varieties of soybean (*Glycine max* (L.) Merr.) JS 335; JS 71-05; NRC; Rossio were obtained from the National Centre for Soybean Research, Indore, M.P. and ICAR Gangtok. Two varieties of lentil seeds (*Lens culinaris* Medik.) (Asha and Subrata) were collected from Pulses and Oil Research Centre, Berhampore, two (IPL 81; IPL 406) from Indian Institute Of Pulses Research , Kanpur, Uttar Pradesh India, and Lv from Bagdogra market , Siliguri. Viability was checked in laboratory and seedlings of different varieties were then raised from these stock of seeds in Department of Botany, University of North Bengal. Four varieties of soybean and six varieties of lentil seeds were selected for experimental purposes (Plates II, III and IV).

3.1.2. Propagation

The propagation of both soybean and lentil were done from the seeds obtained from the National Centre for Soybean Research, Indore, M.P. ,ICAR Gangtok and Pulses and Oil Research Centre, Berhampore respectively.

3.1.3. Plantation

Both soybean and lentil seeds of different verities were soaked overnight in distilled water after surface sterilization with 0.1% $HgCl_2$ w/v for 3minutes and washed thoroughly three to four times with sterilized distilled water and grown in Petri dishes at room temperatures (20°C for lentil and 30 °C for soybean)and humidity 50-60 % in lentil and 60-70% in soybean, during last week of April – first week of May and middle of October – first week of November in case of soybean and lentil respectively. For experimental purposes one week old seedlings were transferred to 5.5" height and 5" diameter size plastic pots containing sandy loam soil mixed with farmyard manure in the proportion of 2:1 by weight.

3.1.4. Maintenance

Plants were regularly watered twice a day early morning and evening and maintained properly by weeding once a week at normal atmospheric temperature.

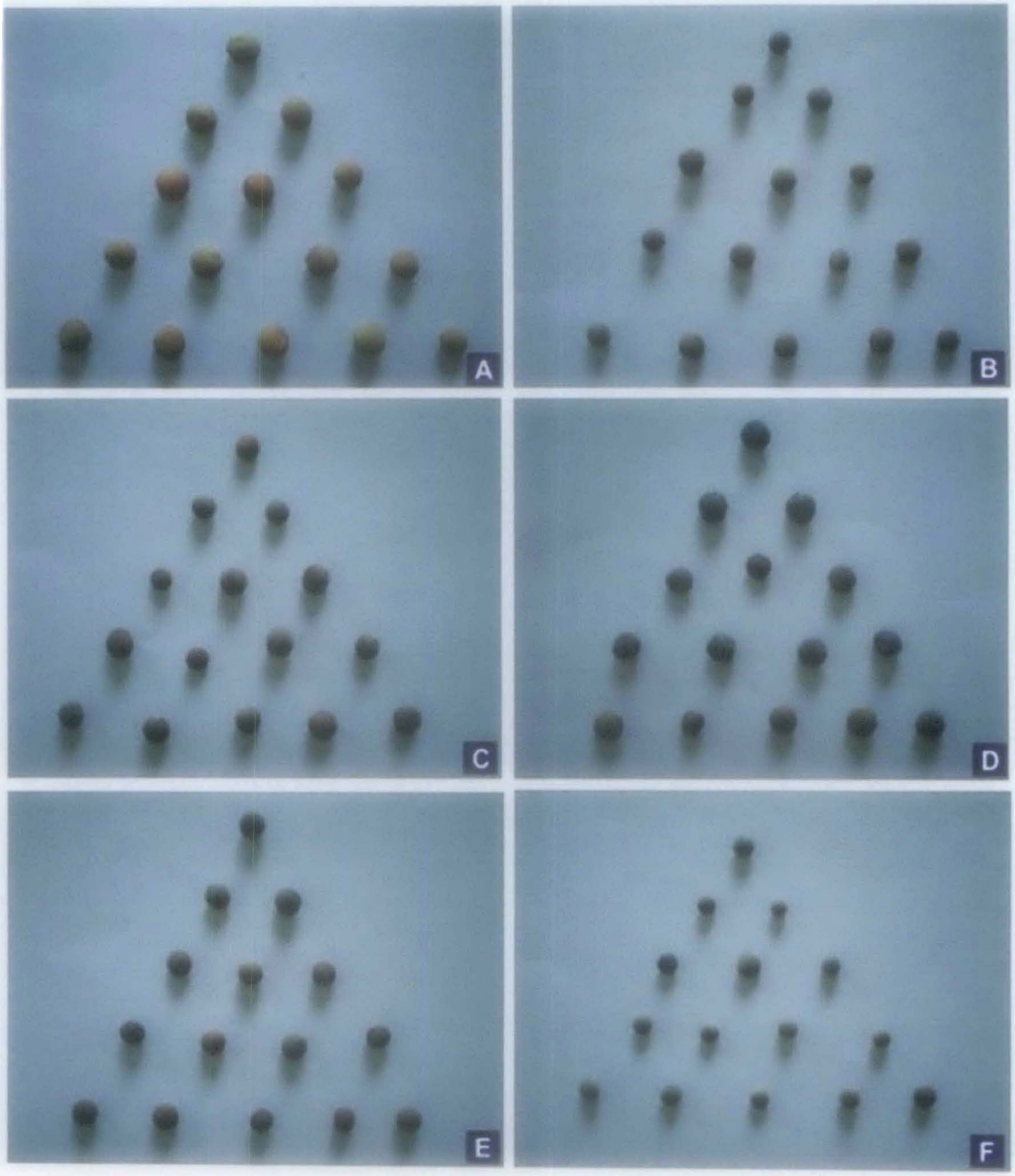

Plate II: Seeds of different varieties of lentil A: IPL 406 B: IPL 81 C: Sehore D: Asha E:Subrata F: Lv.

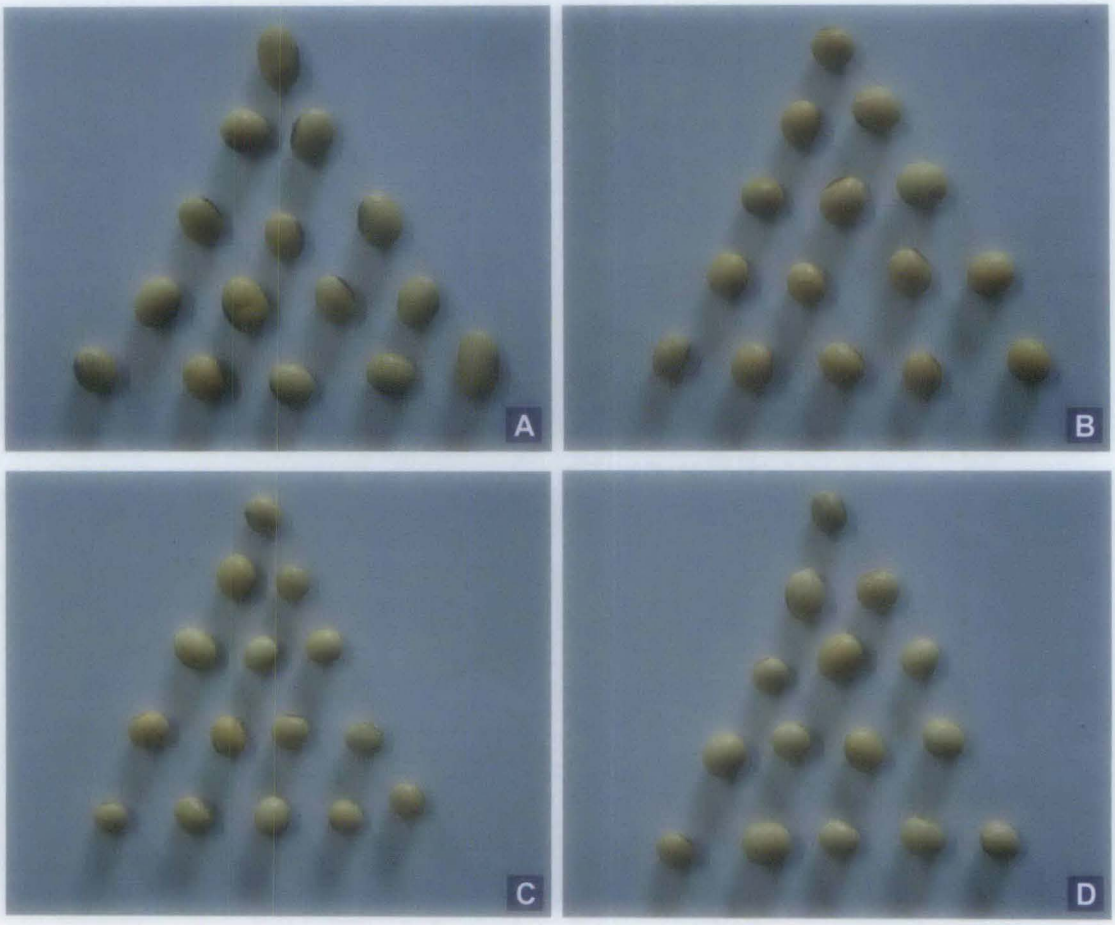

Plate III: Seeds of different varieties of Soybean A:Rossio B: JS 335 C: JS 71-05 D: NRC 37.

Plate IV : A and B: Different varieties of soybean in experimental field.

3.2. Temperature treatment

3.2.1. Seed treatment

Seeds were soaked overnight in sterile distilled water after surface sterilization with 0.1 % $HgCl_2$ (w/v) and transferred to moist Petri dishes. Then these Petri dishes were kept at elevated temperatures of 30°C, 35°C, 40°C, 45°C and 50°C in case of lentil and in case of soybean seeds were treated at low temperatures of 20°C, 15°C, 10°C and 5°C for 4 hrs duration following which they were allowed to germinate at room temperature and percentage of germination was observed after one week.

3.2.2. Seedling treatment

For seedling treatment , seeds of both soybean and lentil were soaked overnight in sterile distilled water after surface sterilized with 0.1 % $HgCl_2$(w/v) and transferred to moist Petri dishes following which they were allowed to germinated at room temperature. After one week seedlings were transferred to 5.5" height and 5" diameter size plastic pots containing sandy loam soil mixed with farmyard manure in the proportion of 2:1 by weight.

One month old seedlings of soybean were exposed to low temperature of 20°C, 15°C, 10°C and 5°C for 4 hrs further seedlings were also exposed to different hours at 5°C. Similarly one month old seedlings of lentil were exposed to high elevated temperature of 30°C, 35°C, 40°C, 45°C and 50°C. Since seedlings showed maximum effects at 50°C, hence this temperature was considered as lethal temperature for lentil (Plates V and VI).

3.3. Foliar application of chemicals

3.3.1. Salicylic acid (SA)

For chemical pre-treatments one month old seedlings of the different verities of lentil were sprayed with solutions of 100μM salicylic acid, twice a day (early in the morning and also evening) for a week and finally just prior to exposure to lethal temperature. The pre – treated seedlings and distilled water treated (control) seedlings were dipped in respective solutions in conical flasks after which seedlings were exposed to 50°C as described.

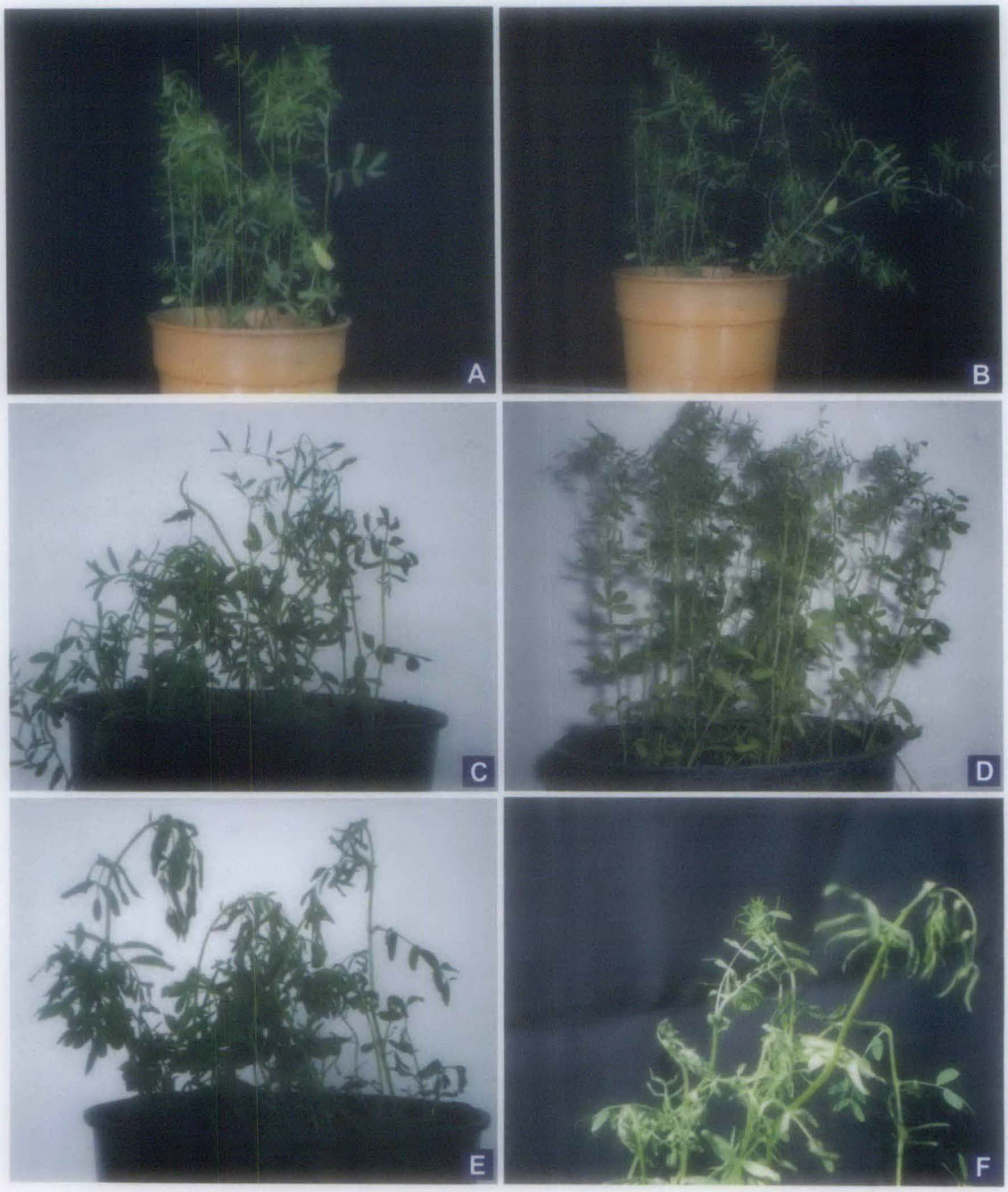

Plate V: One month old lentil seedlings (var. Sehore) subjected to different high temperatures A : control , B: 30°C, C: 35°C, D: 40°C, E: 45°C, F: 50°C.

Plate VI: One month old lentil seedlings (var. Lv and IPL 406) subjected to lethal temperature A: Lv control , B: treated, C: IPL 406 control, D: treated.

3.3.2. Abscisic acid (ABA)

One month old seedlings of lentil were sprayed with 50μM Abscissic acid (ABA) solution twice a day (early in the morning and evening) in the form of foliar spray, for a week and finally just prior to exposure to lethal temperature. The same volume (50ml) of distilled water was sprayed on control seedlings. The temperature treatment was carried out exactly in the same manner as mentioned in case of SA.

3.3.3. Calcium chloride ($CaCl_2$)

Calcium chloride solution of 10mM strength was sprayed on one month old seedlings of lentil same as SA and ABA. Controls consisted of plants on which the same volume of distilled water was sprayed and treated in the same manner as test plants. The seedlings were immersed in respective solutions in conical flasks for heat treatments.

3.4. Tolerance index determination

Variation in heat tolerance of the seedlings was calculated as the tolerance index (TI) which gives the percentage of shoot and /or root fresh biomass (g/plant) of treated (FW_t) over untreated control (FW_c) plants according to the following equation as suggested by Metwally et al. (2005) $TI\ (\%)=(FW_t / FW_c\ X100) - 100$

3.5. Determination of cell membrane stability

Membrane thermostability was tested by cell membrane stability (CMS) test with the pinnules obtained from seedlings following the method of Martineau *et at.* (1979). 1g leaves were washed with 3-4 changes of distilled water and placed in test tubes (150x25mm) containing 2ml of pre heated (to the treatment temperature) water. Tubes were covered with plastic wrap and placed in a water bath at the desired temperature for 15min, while the control tubes were kept at 25°C. After cooling to room temperature, distilled water was added to make the volume up to 10 ml. Sample were incubated at 10°C for 16 hrs and conductivity measured with a conductance meter (Labindia pH and conductivity meter Pico+). The tubes were covered with aluminium foil and autoclaved at 120°C for 15min to released all electrolytes. After cooling tubes to 25°C the contents were mixed and final conductance measured. The injury was determined as follows:

Relative injury [R1] (%)= $[1-\{1-(T_1/T_2)\}/\{1-(C_1/C_2)\}] \times 100$

Where T and C refer to the conductance in treatment and control tubes and subscripts 1 and 2 refer to readings before and after autoclaving respectively.

3.6. Determination of lipid peroxidation

Lipid peroxidation was measured in terms of malondialdehyde (MDA) content as described by Dhindsa *et al* (1981). Mature intermediate leaf tissue were homogenized in 2ml of 0.1% (w/v) trichloroacetic acid (TCA) and centrifuged. 0.5 ml of supernatant of leaf extracts was mixed with 2ml of 20%(v/v) trichloroacetic acid containing 0.5% (v/v)thiobarbituric acid. The mixture was heated at 95°C for 30min, quickly cooled and centrifuged at 10000rpmfor 10min. The absorbance of the supernatant was read at 532nm and 600nm. The concentration of MDA was calculated by means of an extinction coefficient of $155mM^{-1}cm^{-1}$(Heath and Packer 1968).

3.7. Extraction of antioxidative enzymes

3.7.1. Catalase (CAT: EC.1.11.1.6)

The mature leaf samples were ground to powder in liquid nitrogen and extracted with 3 ml of 50mM sodium phosphate buffer (pH 6.8) using polyvinylpyrrolidone under ice cold conditions, following the method of Chance and Machly (1955). The homogenate was centrifuged at -4°C for 15 min at 10,000 rpm. The supernatant was used as crude enzyme extracts.

3.7.2. Peroxidase (POX: EC. 1.11.1.7)

For the extraction of POX, 0.5 g of plant tissues was ground to powder in liquid nitrogen and extracted in 3ml of 100mM Sodium phosphate buffer (pH 6.8) using polyvinylpyrrolidone under ice cold conditions. The homogenate was centrifuged immediately at 10,000rpm for 15 min at -4°C. After centrifugation the supernatant was collected and after recording its volume was used immediately for assay or stored at -20^0C (Chakraborty *et al.* 1993).

3.7.3. Ascorbate peroxidase (APOX: EC.1.11.1.11)

APOX enzyme extract was prepared by powdering 0.5 g of tissue in liquid nitrogen and extracting in 3ml of 50mM Na_2HPO_4/NaH_2PO_4 buffer (pH7.2)

containing 2mM polyvinylpyrrolidone (PVP) following the method of Asada (1984). The homogenate was centrifuged at - 4°C for 15minutes at 10,000 rpm. The supernatant obtained was used for enzyme assay.

3.7.4. Glutathione reductase (GR: EC. 1.6.4.2)

The enzyme extract for GR was prepared by crushing the plant tissue in liquid nitrogen and extracting with 100mM Potassium phosphate buffer (pH 7.6) containing 20mM polyvinylpyrrolidone (PVP) The homogenate was centrifuged at - 4°C for 15min at 10,000 rpm. The supernatant obtained was used for enzyme assay and estimation of total soluble protein content. The activity was determined following the method of Lee and Lee (2000).

3.7.5. Superoxide dismutase (SOD: EC. 1.15.1.1)

The SOD enzyme extract was prepared by grinding the plant tissue in liquid nitrogen to powder form and extracting in 100mM Potassium phosphate buffer (pH7.6) containing 20mM polyvinylpyrrolidone (PVP), following the method of Dhindsa et.al(1981) . Insoluble material was removed by centrifugation at 10,000 rpm for 10 minutes at -4°C and the supernatant was used as enzyme extract.

3.8. Assay of enzymes activities: Prior to assay of enzyme activities , protein content of the extract was determined in each case following the procedure described elsewhere .

3.8. Assay of enzymes activities.

3.8.1. Catalase (CAT: EC.1.11.1.6)

Catalase activity was measured according to Chance and Machly (1955). Enzyme extract (40 µl) was added to 3ml of H_2O_2 phosphate buffer (0.16ml of H_2O_2 to 100 ml of phosphate buffer, pH 7.0) and the breakdown of H_2O_2 was measured at 240 nm in a spectrophotometer. An equivalent amount of buffer containing H_2O_2 was used as reference. The enzyme activity was expressed as µM H_2O_2 oxidised mg^{-1} protein min^{-1}.

3.8.2. Peroxidase (POX: EC. 1.11.17)

For determination of peroxidase activity , 100µl of freshly prepared crude enzyme extract was added to the reaction mixture containing 1ml of 200mM sodium

phosphate buffer (pH 5.4). 45µl of 30% H_2O_2 , 100 µl of O-dianisidine (5 mg/ml methanol) and 1.7ml of distilled water. Peroxidase activity was assayed spectrophotometrically in UV VIS spectrophotometer (UV-VIS Spectrophotometer 118 systronics) at 460 nm by monitoring the oxidation of O-dianisidine in presence of H_2O_2 (Chakrabotry *et al.* 1993). Specific activity was expressed as mM O- dianisidine oxidized mg^{-1} protein min^{-1}.

3.8.3. Ascorbate peroxidase (APOX: EC.1.11.1.11)

Ascorbate peroxidise activity was assayed as decrease in absorbance by monitoring the oxidation of ascorbate at 290nm according to the method of Asada (1984) with some modification . The reaction mixture consisted of 0.1 ml of enzyme extract ,0.1 ml of 0.5mM ascorbic acid ,0.1 ml of 30% H_2O_2 (v/v) and 2.7ml of 50mM sodium phosphate buffer (pH 7.2). Enzyme activity was finally expressed as mM Ascorbate (reduced/ oxidised) mg^{-1} protein min^{-1}.

3.8.4. Glutathione reductase (GR: EC 1.6.4.2)

Glutathione reductase activity was determined by the oxidation of NADPH at 340nm with extinction coefficient of 6.2 $mMcm^{-1}$as described by Lee and Lee(2000). The reaction mixture consisted of 100mM potassium phosphate buffer (pH7.6), 2mM EDTA , 0.1mM NADPH, 0.6mM glutathione (oxidised form, GSSG) with 0.1ml of enzyme extract. The reaction was initiated by addition of NADPH at 25°C.Enzyme activity was finally expressed as µM NADPH oxidized mg $protein^{-1}$ min^{-1}.

3.8.5. Superoxide dismutase (SOD: EC 1.15.1.1)

Superoxide dismutase activity was assayed by monitoring the inhibition of the photochemical reduction of nitroblue tetrazolium (NBT) according to the method of Dhindsa *et al.*(1981)with some modification. Each 3ml of the assay mixture constituted of 0.1ml enzyme extract, 1.5ml of 100mM phosphate buffer (pH 7.8), 0.1ml Na_2CO_3 (1500mM). 0.1ml NBT (2.25mM), 0.2ml ml methionine (200mM), 0.1mlEDTA (3 mM), 0.1 ml riboflavin (60 µM) and 0.8 ml of distilled water. The reaction tubes containing enzyme samples were illuminated with 15W fluorescent lamp for 10min. Another set of tubes lacking enzymes were also illuminated and served as control. A non-irradiated complete reaction mixture served as blank. The absorbance of samples were measured at 560nm and 1 unit of activity was defined as

the amount of enzyme required to inhibit 50% of the NBT reduction rate in the controls containing no enzymes.

3.9. Isozyme analyses by polyacrylamide gel electrophoresis (PAGE)

Polyacrylamide gel electrophoresis (PAGE) using 8% resolving gel and 5% stacking gel in Tris-glycine buffer (pH 8.3) was performed for isozyme analyses of different enzymes following the method of Davis (1964). The various solutions required for the analysis were prepared as follows:

3.9.1. Preparation of the stock solution

Solution A: Acrylamide stock solution (Resolving gel)

For the preparation of Acrylamide stock solution for resolving gel 28g of acrylamide and 0.76g of N'N' methelene bis-acrylamide was dissolved in 100ml of distilled water and filtered through No. 1 filter paper and stored at 4°C in dark bottle.

Solution B: Acrylamide stock solution (stacking gel)

For the preparation of acrylamide stock solution for stacking gel 10g of acrylamide and 2.5g of bis-acrylamide was dissolved in 100 ml of warm distilled water. The stock solution was filtered and stored at 4°C in dark bottle.

Solution C: Tris –HCl (Resolving gel)

36.6g of Tris –base was mixed with distilled water and 0.25ml of TEMED was added. The pH was adjusted to 8.9 with conc. HCl. The volume of the solution was made up to 100ml with distilled water. The solution was then stored at 4°C for further use.

Solution D : Tris-HCl (Stacking gel)

5.98g of Tris base was mixed with distilled water and 0.46ml of TEMED and the pH was adjusted to 6.7 with conc. HCl. The volume was made up to100ml with distilled water. The solution was stored at 4°C for further use.

Solution E: Ammonium persulphate solution (APS)

Fresh solution of APS was prepared by dissolving 0.15g of APS in 100ml of distilled water.

Solution F: Riboflavin solution

Fresh solution of Riboflavin was prepared by dissolving 0.4mg of riboflavin in 10ml of distilled water. The solution was kept in dark bottle to protect from light.

Solution G: Electrode buffer

Electrode buffer was prepared freshly by dissolving 0.6g of the Tris base and 2.9g of Glycine in 1L of distilled water.

3.9.2. Preparation of gel and electrophoresis

For the polyacrylamide gel electrophoresis of isozymes mini slab gel was prepared. For slab gel preparation, two glass plates were taken and were thoroughly cleaned with dehydrated alcohol to remove any trace of grease and then dried. 1.5mm thick spacers were placed between the glass plates on three sides and these were sealed with high vacuum grease and clipped thoroughly to prevent any leakage of the gel solution during pouring. 7.5%resolving gel was prepared by mixing solution A:C:E : distilled water in the ratio of 1:1:4:1 by pasture pipette leaving sufficient space for (Comb +1cm) the stacking gel. This resolving gel immediately over layered with water and kept for polymerization for 2h. After polymerization of the resolving gel was completed over layer water was poured off and washed with distilled water to remove any unpolymerized acrylamide. The staking gel solution was prepared by mixing solutions B: D: F: distilled water in the ratio of 2:1:1: 4 and poured over the resolving gel and comb was inserted immediately and over layered with distilled water. The gel was kept for polymerization for 30-45min in strong sunlight. After polymerization of the stacking gel the comb was removed carefully and wells were washed thoroughly. Lower spacer was also removed and the space was cleaned with tissue paper to remove extra grease.

The gel was now finally mounted in the electrophoresis apparatus. 300ml Tris –Glycine running buffer (for mini gel) was added sufficiently in both upper and lower reservoir. Any bubble, trapped at the bottom of the gel was removed very carefully with a bent syringe.

Sample preparation

Sample was prepared by mixing the sample enzyme with gel loading dye (40% sucrose and 1% bromophenol blue in distilled water). All the solutions for

electrophoresis were cooled. The samples were immediately loaded in a pre-determined order into the bottom of the wells with a micro litre syringe.

Electrophoresis

Electrophoresis was performed at constant 10mA current for a period of 3-4h at 4°C until the dyefront reached the bottom of the gel.

3.9.3. Staining procedure

After electrophoresis the gel was removed carefully from the glass plates and then the stacking gel was cut off from the resolving gel and finally stained using suitable staining dye.

3.9.3.1. Catalase

Extract for isozymes analysis was prepared by grinding 0.5g of tissue in liquid nitrogen in pre-chilled mortar and pestle and finally extracted in 0.1M sodium phosphate buffer (pH7.0) as described by Davis (1964) and used for the isozyme analysis.

Catalase isozymes are visualized by following the method of Woodbury *et al.* (1971) After electrophoresis the gel was soaked in 3.3mM H_2O_2 for about 20min. The gel was then rinsed with distilled water and incubated in a freshly mixed solution of equal volume of 1% potassium ferrocyanide and 1% of ferric chloride for about 20minutes. Analysis was done immediately after the appearance of yellow bands in a green background and R_m values for different isozymes were calculated.

3.9.3.2. Peroxidase

Sample was prepared by grinding 0.5g of tissue in liquid nitrogen in pre-chilled mortar and pestle and finally extracted in 0.1M sodium phosphate buffer (pH7.0) as described by Davis (1964). Peroxidase isozyme pattern was estimated by staining the gel in Benzidine dye in acetic acid water mixture consisting of Benzidine (2.08 g), Acetic acid (18 ml), 3% H_2O_2 with (100ml) for 5 min by following the method of Reddy and Garber (1971). The reaction was finally stopped with 7% acetic acid after the appearance of blue coloured bands. Analysis of isozymes was done immediately.

3.9.3.3. Superoxide dismutase

Extract for isozymes analysis was prepared by grinding 0.5g of tissue in liquid nitrogen in pre-chilled mortar and pestle and finally extracted in 0.1M potassium phosphate buffer(pH7.6) and used for the isozymes analysis as described by Burk and Oliver (1992) with slight modification.

After electrophoresis, gel was soaked in 50mM potassium phosphate buffer (pH7.6) , 0.1mM EDTA, 0.02% TEMED , 3mM Riboflavin, 0.25mM NBT and incubated in dark for 30min at room temperature with constant shaking. After 30min gel was rinsed with distilled water and placed in distilled water. The gel was then exposed to light under 400 WHP Sodium lamp for 5-10min, at 25°C. Analysis was done immediately and R_m values for different isozymes were calculated.

3.10. Determination of hydrogen peroxide accumulation

3.10.1. Quantification

The hydrogen peroxide was extracted following the method of Jena and Choudhuri(1981) by homogenizing 50mg leaf tissue with 3ml of phosphate buffer (50mM , pH 6.5). the homogenate was centrifuged at 6000rpmfor 25min . The supernatant was used for quantification of hydrogen peroxide.

To determine H_2O_2 level 3ml of extracted solution was mixed with 1ml of 0.1% Titanium sulphate in 20% H_2SO_4(v/v), and the mixture was then centrifuged at 6000rpm for 15min . The intensity of the yellow colour of the supernatant was measured at 410nm. H_2O_2 level was calculated using the extinction coefficient ($0.28\mu mol^{-1}cm^{-1}$)

3.10.2. Microscopic detection

Detection of hydrogen peroxide in leaf tissue was performed according to Thordal- Christensen *et al.* (1997). The detached leaf was washed thoroughly three to four times and incubated for 14h in dark with 1mg/ml 3,3'-diaminobenzidine (DAB) pH 3.8 and subsequently , the chlorophyll was removed by rinsing the leaf several times in 96% ethanol and observed under microscope.

3.11. Extraction and estimation of non enzymatic antioxidants

3.11.1. Ascorbate

Ascorbate was estimated by following the method of Mukherjee and Choudhuri (1983). The seedlings were homogenised in a cold mortar and pestle on ice using 10ml of 6% Tricholoacetic acid and filtered. To 4ml of the extract, 2ml of 2%dinitrophenylhydrazine (in acidic medium) and 1 drop of 10% Thiourea (in 70% ethanol) were added. The mixture was kept in boiling water bath for 15 min and cooled at room temperature. 5ml of 80% (v/v) Sulphuric acid (H_2SO_4) was added to the mixture at 0°C. The absorbance at 530nm was recorded. The concentration of ascorbate was calculated from a standard curve plotted with known concentration of ascorbic acid.

3.11.2. Carotenoids

Carotenoids were extracted and estimated according to the method given by Lichtenthaler (1987). 0.5g of plant sample was homogenized in methanol for the extraction of carotenoid. After extraction the absorbance value was noted at 480nm in a VIS spectrophotometer and the carotenoid content was calculated using the following standard formula.

A_{480}-(0.114xA_{663})-0.638(A_{645}) μg g^{-1}fresh weight.

3.12. Estimation of total antioxidant activity

Mature intermediate leaves were powdered and 1 g of the powder was combined with 4ml of methanol, centrifuged at 4000 rpm for 10 min, supernatant was collected and placed in a 60°C water bath for twenty five minutes (Miller *et al.* 2000). The semi-dried extract was gathered and for estimation, 0.2g of the extract was dissolved in 10ml of methanol. Two ml of 2, 2-Diphenyl-1-picrylhydrazyl (DPPH) solution with a concentration of 0.025g of DPPH in 1000ml of methanol was mixed with 40 μl of extract solution, the initial reading of purple colour was taken. After 30 min incubation at room temperature, the reaction solution colour turns from purple to yellow was examined at 517 nm by using a spectrophotometer (UV-VIS Spectrophotometer, Model 118 Systronics). The inhibition percentage of the absorbance of DPPH solution was calculated using the following equation

$$\text{Inhibition \%} = \frac{A\,T_0 - A\,T_{30}}{A\,T_0} \times 100$$

(A T_0 was the absorbance of DPPH at time zero. A T_{30} was the absorbance of DPPH after 30 min of incubation). Total antioxidant activity was thus measured as free radical scavenging ability in terms of inhibition of absorbance by DPPH.

3.13. Protein analysis

3.13.1. Extraction of soluble proteins

Soluble proteins were extracted from seeds and leaves following the method of Chakraborty et. al. (1995). For this, the tissue was ground in a mortar using 50mM sodium phosphate buffer (PH 7.2) and centrifuged at - 4°C for 15 minutes at 10,000 rpm. The supernatant obtained will be used as crude protein extract for the estimation of protein by the method of Lowry (1951).

3.13.2. Quantification

Soluble proteins were quantified by following the method as described by Lowry et.al (1951). To 1ml of protein sample 5ml of alkaline reagent (1ml of 1%$CuSO_4$ and 1ml of 2% sodium potassium tartarate, dissolved in 100ml of 2%Na_2CO_2 in 0.1 N NaOH) was added. This was incubated for 15mins at room temperature and then 0.5 ml of 1 N Folin Ciocalteau reagent was added and again incubated for further 20 minutes following which absorbance was measured at 720nm. Quantity of protein was estimated from the standard curve made with bovine serum albumin (BSA).

3.13.3. SDS-PAGE analysis

Total soluble protein extracted in 0.05M sodium phosphate buffer were used as crude protein extract for analysis of protein pattern. Analysis was carried out on 10% SDS-PAGE gel following the method of Sambrook et al. (1989).

3.13.3.1. Preparation of stock solution

For the preparation of gel the following stock solutions were prepared:

(A) Acrylamide and N'N'-methylene bis acrylamide

A stock solution containing 29% acrylamide and 1% bisacrylamide was prepared in water. As both of these amides are slowly deaminated to acrylic and bis acrylic acid by alkali and light the pH of the solution was kept below 7.0. the stock solution was

then filtered through Whatman No. 1 filter paper , kept in brown bottle and stored at 4°C and used within one month.

(B) Sodium Dodecyl Sulphate (SDS)

A 10% stock solution of SDS was prepared in warm water and stored at room temperature.

(C) Tris Buffer

i) 1.5 M Tris buffer was prepared for resolving gel. The pH of the buffer was adjusted to 8.8 with concentrated HCl and stored at 4°C for further use.

ii) 1.0 M Tris buffer was prepared for use in the stacking and loading buffer. The pH of this buffer was adjusted to 6.8 with conc. HCl and stored at 4°C for further use.

(D) Ammonium Per sulphate sulution (APS)

Fresh 10% APS solution was prepared with distilled water each time before use.

(E) Tris – Glycine electrophoresis buffer

Tris running buffer consists of 25 mM Tris base, 250 mM Glycine (pH 8.3)and 0.1% SDS. A solution was made by dissolving 3.02g Tris base, 18.8 Glycine and 10ml of 10% SDS in 1liter of distilled water.

(F) SDS gel loading buffer

This buffer consists 50m M Tris –HCl (pH 6.8), 10 mM β-mercaptoethanol, 2% SDS,0.1% bromophenol blue, 10% glycerol. A 1x solution was prepared by dissolving 0.5 ml of 1M.

Tris buffer (pH 6-8), 0.5 ml of 14.4 M β-mercaptoethanol , 2ml of 10% SDS, 10mg bromophenol blue, 1ml glycerol in 6.8ml of distilled water.

3.13.3.2. Preparation of gel and electrophoresis

Mini slab gel (Plate size 8 cm x 10 cm) was prepared for the analysis of protein patterns by SDS-PAGE. For gel preparation, two glass plates were thoroughly cleaned with dehydrated alcohol to remove any traces of grease and then dried. Then 1.5 mm thick spacers were placed between the glass plates at three sides and sealed

with high vacuum grease and clipped tightly to prevent any leakage and the gel solution during pouring. Resolving and stacking gels were prepared by mixing compounds in the following order and poured by Pasture pipette leaving sufficient space for comb in the stacking gel (comb +1cm)

Composition of solutions

10% resolving gel

Name of the compound	Amount (ml)
Distilled water	2.85
30% acrylamide	2.55
1.5 M Tris (pH 8.8)	1.95
10% SDS	0.075
10% APS	0.075
TEMED	0.003

5% stacking gel

Name of the compound	Amount (ml)
Distilled water	2.10
30% acrylamide	0.50
1.5M Tris (pH 8.8)	0.38
10% SDS	0.030
10% APS	0.030
TEMED	0.003

After pouring the resolving gel solution, it was immediately over layered with iso-butanol and kept for polymerization for 2 hours. After polymerization of the resolving gel was complete, overlayer was poured off and washed with water to remove any unpolymerized acrylamide, Stacking gel solution was poured over the resolving gel and the comb was inserted immediately and over layered with water. Finally the gel was kept for polymerization for 30-40 minutes. After polymerization of the stacking gel the comb was removed and washed thoroughly. The gel was then finally mounted in the electrophoresis apparatus. Tris-Glycine buffer was added sufficiently in both upper and lower reservoir. Any bubble trapped at the bottom of the gel , was removed carefully with a bent syringe.

Sample preparation

Sample (50µl) was prepared by mixing the sample protein (35µl) with 1 x SDS gel loading buffer (15 µl). All the samples were floated in boiling water bath for 4minutes to denature the protein sample. The samples were immediately loaded in a pre-determined order into the bottom of the wells with a micro litre syringe. Along with the samples, protein markers consisting of mixture of five proteins ranging from

high to low molecular masses (Phosphorylase b-98; Bovine Serum Albumins- 67; Ovalbumin -44; GST-29; and Lysozyme 16)was treated as the other sample and loaded in a separate well.

Electrophoresis

Electrophoresis was performed at a constant 18 mA current for a period of three hours until the dye front reached the bottom of the gel.

3.13.3.3. Fixing and staining and destaining

After electrophoresis the gel was removed carefully from the glass plates and then the stacking gel was cut off from the resolving gel and finally fixed in glacial acetic acid : methanol : water(10:20:70) for overnight. The gel was removed from the fixer and stained in Coomassie blue stain for 4 h at 37^0C with constant shaking at low speed. The staining solution was prepared by dissolving 250mg of Coomassie brilliant blue (Sigma R 250) in 45 ml of methanol. After the stain was completely dissolved, 45 ml of water and 10 ml of glacial acetic acid were added. The prepared stain was filtered through Whatman No. 1 filter paper.

After staining the gel was finally destained with destaining solution containing methanol, water, acetic acid (4.5 : 4.5 :1) at 40^0C with constant shaking until the background became clear.

3.14. Extraction and Estimation of Phenol

Phenol was extracted by following the method of Mahadevan and Sridhar (1982). 1g of leaf tissue was immersed in boiling absolute alcohol at the rate of 5ml alcohol per g of tissue for 5-10min cooled and then crushed in mortar and pestle using 80% alcohol in dark and filtered in dark chamber. The residues were re-extracted with 80% alcohol, and then the final volume was made up with 80% alcohol to 10ml.

3.14.1. Total Phenol

Total phenol was estimated by following the method of Bray and Thorpe (1954). In one ml of extract, 1ml of 50% diluted Folin ciocaltean and 2ml of 20% Na_2CO_3 solution were added. Then the mixture was boiled in water bath for 1min.

After cooling under running tap water the mixture was diluted with distilled water and final volume was made 25ml. The absorbance was measured at 650nm in colorimeter.

3.14.2. Ortho- dihydroxy Phenol

Arnow's method (1933) was followed to estimate ortho-hydroxy phenol in which 2ml of 0.5(N) HCl, 1ml of Arnow's reagent (10g $NaNO_2$ + 10g Na_2MOO_4 in 100ml of distilled water) and 2ml of 1(N) NaOH were added in 1ml of crude extract. After reaction pink colour was developed and the volume was raised up to 10ml with distilled water, after shaking the absorbance was measured in colorimeter at 515nm.

3.15. HPLC analysis of Phenolics

Phenolics compounds was extracted from leaves of both soybean and lentil tissues following the method of Obanda and Owuor(1994) with slight modification. Leaf sample (5g) were extracted with 50ml of acetone at 45°C in water bath for 30 min. Extracts were decanted and filtered thoroughly Whatman No. 1 filter paper. Acetone extract was concentrated to dryness and finally the residue was dissolved in 10ml distilled water. Water solution was extracted with equal volume of chloroform for four times. The pH of the water layer was adjusted to 2 by adding 2 drops of 2 N HCl and extracted with methyl isobutyl ketone. Methyl isobutyl ketone extract was concentrated to dryness and dissolved in 1.5 ml of 2% acetic acid. The extract was finally filtered through milipore filter (milipore 0.4μm HA filter paper).

High performance liquid chromatography (HPLC) of the samples were performed according to Sarma et al. (2002). The HPLC system (Shimadzu Advanced VP Binary Gradietn) equipped with C-18 hypersil column with linear gradient elution system as follows – mobile phase A 100% acetonitrile ; mobile phase B 2% acetic acid in water was used for sample analysis.Elution : 88% B for 6 min then linear gradient to 75 % B over 5 min. The elution was complete after 25 min. Flow rate was fixed as 1 ml min^{-1} with sensitivity of 0.5 aufs. Injection volume was 20 μl and monitored at 278nm.

3.16. Extraction and Estimation of Proline

Proline was extracted by following the method of Bates *et al.*(1973). 0.5g of plant tissue was crushed with 5ml of 3%Sulfosalicylic acid in mortar and pestle then was filtered through whatman No.1 filter paper at room temperature. The supernatant was collected for estimation.

Proline content of the extract was estimated as described by Bates *et al.* (1973) with some modification. To 1ml of extract, 3ml of distilled water and 1ml of Ninhydrin solution (1g ninhydrin + 10ml acetone+ 15ml distilled water) was added. The mixture was kept on a boiling water bath for 30 min. After cooling, the reaction mixture was transferred in a separating funnel and 5ml of Toluene was added and mixed vigorously. Lower coloured solution was taken and absorbance values were measured at 520 nm and quantified from a standard curve of proline.

3.17. Extraction and Estimation of Chlorophyll

Chlorophyll was extracted according to the method of Harborne (1973) by homogenizing 1g of leaf sample in 80% acetone and filtering through whatman No. 1 filter paper. 80% acetone was repeatedly added from the top till the residue became colourless. The filtrate was collected and the total volume was made up to 10ml. The chlorophyll content was estimated by observing the O.D. values at 645nm and 663nm respectively in a UV-VIS spectrophotometer (UV-VIS Spectrophotometer 118 systronics) and calculated by using the following formulae (Arnon, 1949)

Total chlorophyll: $(20.2\ A_{645} + 8.02\ A_{663})$ mg g^{-1}fresh weight

Chlorophyll a: $(12.7\ A_{663} - 2.69\ A_{645})$ mg g^{-1}fresh weight

Chlorophyll b: (22.9 A645 – 4.68 A663) mg g-1fresh weight