MATERIALS AND METHODS

This chapter deals with the approach and procedure that have been followed to accomplish the objectives of the study. The study was carried out in four stages, i.e., germination stage, seedling stage, vegetative stage and reproductive stage. The experiments were conducted in the experimental garden of Department of Botany, University of North Bengal, India (26.42° N Latitude and 88.25° E Longitude) between February 2004 and March 2007.

3.1. Growth and maintenance of plant material

Okra [*Abelmoschus esculentus*(L.)Moench] seedlings of different cultivars (Arka Anamika, Deepti, Najuka F1, Paras Soumya, PB-57 and Parbhani Kranti) were raised in the experimental garden of Botany Department, North Bengal University. Seeds were surface sterilised in 0.01% $HgCl_2$. The seeds were then thoroughly washed with distilled water and set for germination in Petri plates lined with moistened filter paper. Germinated seeds were then sown in 10″ inches diameter earthenware pots lined with plastic sheets and filled with sandy–loam soil during the summer and kharif season. The pots were filled with garden soil and farmyard manure. Recommended package of practices were followed for growing the crop. Two weeks after germination the seedlings not showing the optimum growth and development were eliminated/thinned out. Plants of the different cultivars were maintained until fruiting stage (Plates II and III).

3.2. Heavy metal treatment

Heavy metal solutions were applied to the seeds and plants at different intervals of time to determine their effect at the different stages of the plant growth and development.

3.2.1. Selection of compounds

The heavy metal compounds selected for the study were cadmium nitrate 4-hydrate [$Cd(NO_3)_2$ $4H_20$], copper(II) sulphate-5-hydrate [$CuSO_45H_2O$], mercury(II) chloride [$HgCl_2$] and lead(II) nitrate [$Pb(NO_3)_2$].

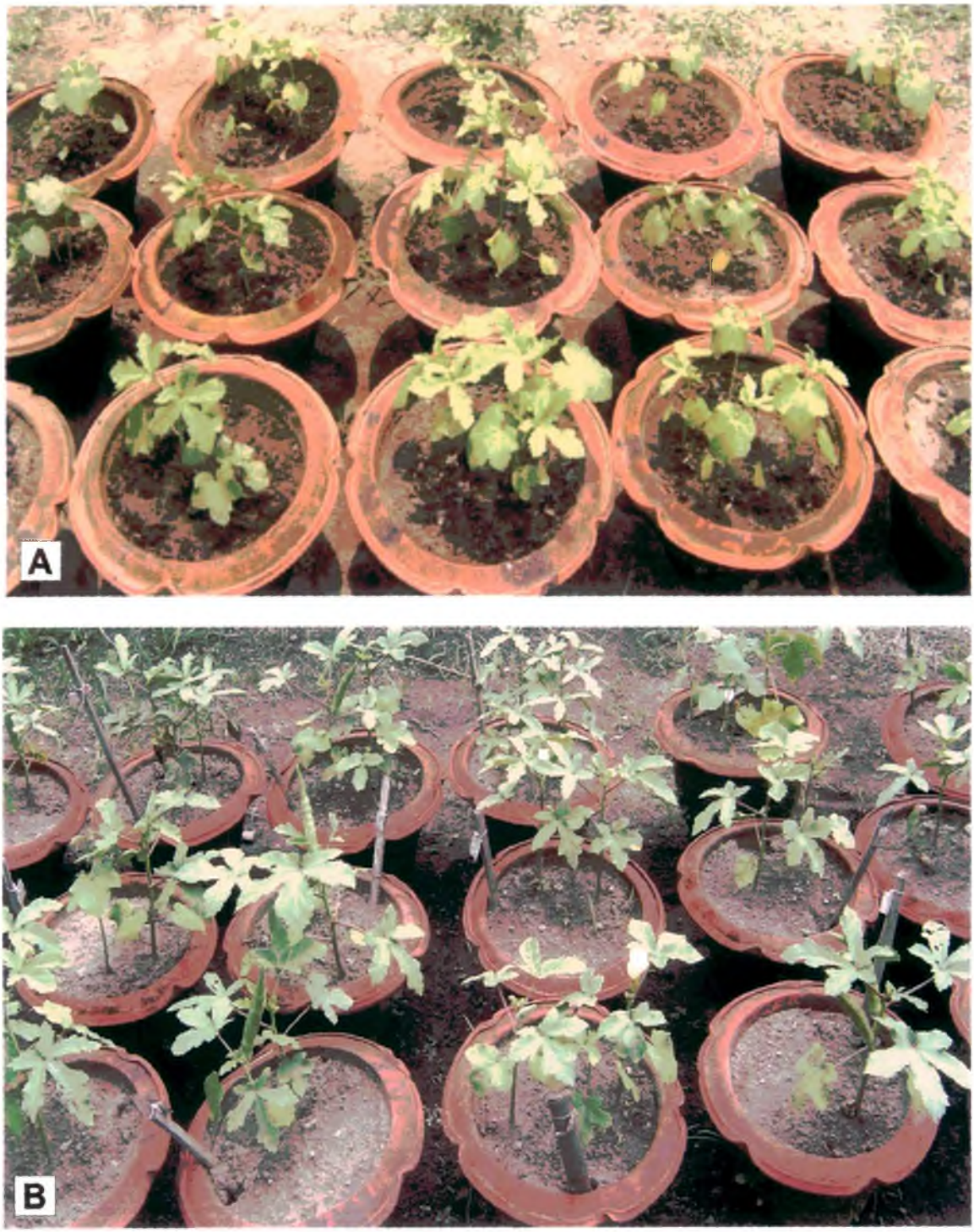

Plate II: Okra plants grown in pots. A: Seedling stage (cv. Arka Anamika) B: Fruiting stage (cv. Parbhani Kranti)

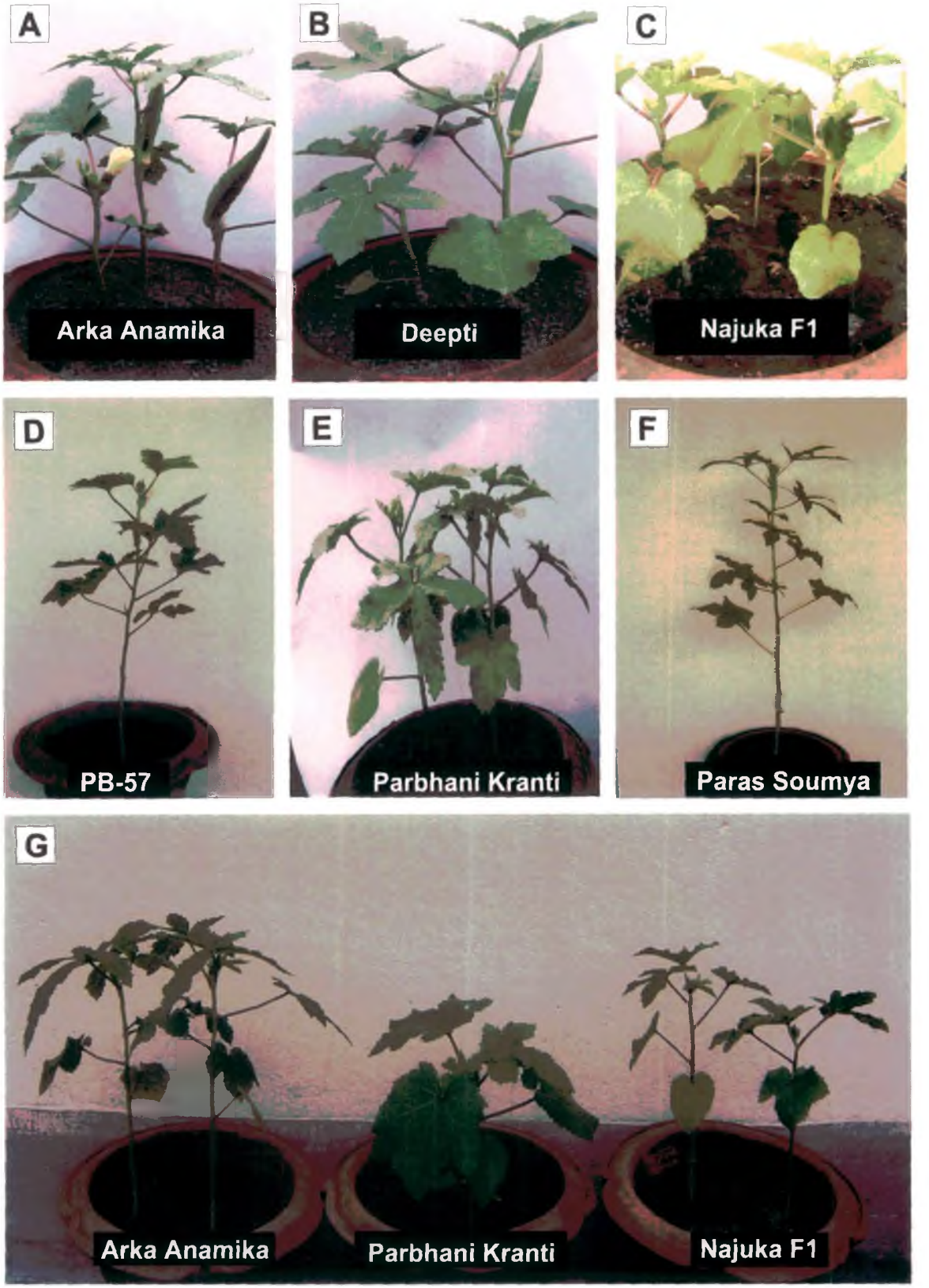

Plate III (A - G): Different cultivars of okra grown in pots.

3.2.2. Application of chemicals

Solution of $Cd(NO_3)_2$ $4H_20$, $CuSO_45H_2O$, $HgCl_2$ and $Pb(NO_3)$ were prepared at concentrations of 100 μg ml^{-1} and 1000 μg ml^{-1}. These solutions were then applied as respective treatments from time to time at different stages. For control only water was applied.

3.2.2.1 . Seed

The seeds of all the cultivars were surface sterilized in 0.01% mercury chloride ($HgCl_2$) solution for 1 min and repeatedly washed with distilled water. 10 seeds were put in each Petri plate lined with filter paper moistened with respective solutions in three replicates for germination test. The Petri plates were covered to prevent loss of moisture by evaporation. Whenever needed, the treatment solution was again applied to avoid drying. Germination percentage was recorded every 24 h for 3 days after putting the seeds in the Petri plates with respective solutions. Seeds were considered germinated when the emergent radicle reached 2 mm in length.

3.2.2.2. Soil

The seedlings were allowed to grow for 3 weeks. After 3rd week the underdeveloped/unhealthy seedlings were thinned out. 200 ml of the respective treatment solution were applied in each pot. For each treatment, 3 applications were done-once each in seedling stage, vegetative stage and reproductive stage. On control only water was applied.

3.2.2.3. Application of ameliorating compounds

Ameliorating compounds were also applied to amend the effects of heavy metals. The ameliorating compounds used were calcium chloride ($CaCl_2$) and potassium nitrate ($KNO_3.2H_2O$) at concentration of 1000 μg ml^{-1} each.

The treatments were done by soaking the seeds in a combination of the respective ameliorating compounds and heavy metals. For control the seeds were treated only with heavy metals.

3.3. Seed germination

The seeds of all the cultivars were surface sterilized in 0.01% mercury chloride ($HgCl_2$) solution for 1 min and repeatedly washed with distilled water. 10

seeds were put in each Petri plate lined with filter paper moistened with respective treatment solutions in three replicates for germination test. The Petri plates were covered to prevent loss of moisture by evaporation. Whenever needed, the treatment solution was again applied to avoid drying. Germination percentage was recorded every 24 h for 3 days after putting the seeds in the Petri plates in respective treatment solution. Seeds were considered germinated when the emergent radicle reached 2mm in length.

3.4. Determination of growth parameters and yield

The determination of growth parameters mainly depended on estimation of fresh weight, dry matter and leaf area over a period of time.

3.4.1. Fresh weight and dry weight

The plants were uprooted and washed with water. The excess water was soaked with blotting paper and the different parts were separated. The fresh weight of the different parts of representative sample on plant basis was recorded. The samples were than oven dried at 60°C to 70°C for 72-90 h till a constant dry weight was obtained.

3.4.2. Tolerance index

Heavy metal tolerance of the cultivars were calculated as the tolerance index (TI) which gives the percentage of shoot and root fresh biomass(g plant^{-1}) of heavy metal treated (FWt) over untreated control (FWc) plants according to the equation given by Metwally *et al.* 2005.

$$TI\ (\%) = FWt / FWc\ X\ 100 - 100$$

3.4.3. Relative growth index

Relative growth index (RGI) is the ratio of average dry matter of seedling in treatment to the average dry matter of seedling in control. It is expressed in per cent.

$$RGI = \frac{\text{Average dry matter of seedling in treatment}}{\text{Average dry matter of seedling in control}} X\ 100$$

3.4.4. Leaf area

Leaf area was estimated by graph paper method. Leaves were placed in the graph paper and the outline was drawn. The area covered by the outline was then calculated out.

3.4.5. Absolute growth rate

Absolute growth rate (AGR) is the increase in dry matter per unit time. The unit is mg day^{-1}.

$AGR = w_2 - w_1 / t_2 - t_1$

where, w_1 and w_2 are the total dry weights at time t_1 and t_2.

3.4.6. Relative growth rate

Relative growth rate (RGR) is the increase in dry matter over initial dry matter over a period of time. It is calculated by the formula given by Blackman (1919) and expressed as $g^{-1}g^{-1}day$.

$RGR = \log_e w_2 - \log_e w_1 / t_2 - t_1$

where, w_1 and w_2 are the total dry weights at time t_1 and t_2 respectively.

3.4.7. Specific leaf area and Specific leaf weight

Specific leaf area (SLA) is defined as leaf area per unit leaf biomass. It is expressed in $cm^2\ g^{-1}$. Specific leaf weight is defined as leaf area per unit leaf area. It also gives the measure of leaf thickness and is expressed in mg cm^{-2}.

3.4.8. Yield

Yield was calculated by recording the fresh weight of fruits per plant.

3.5. Biochemical analyses

Various biochemical analyses were performed from the treated plants at different stages of growth. Sampling at each stage was done after 48 h of application in the soil.

3.5.1. Extraction and quantification of carbohydrate

3.5.1.1.Extraction of total soluble and reducing sugar

Sugar was extracted following the method of Harbone (1973). Fresh tissues were crushed with 95% ethanol and centrifuged at 5000 rpm. Then the alcoholic

fractions of the filtrate were evaporated off on a boiling water bath. The volume was finally made up to 5 ml with distilled water and was used for estimation of both total soluble and reducing sugar.

3.5.1.2. Estimation total soluble and reducing sugar

Estimation of total soluble sugar was done following Anthrone's method as described by Plummer (1978). To 1ml of test solution 4 ml of Anthrone's reagent was added and mixed thoroughly. Mixtures were placed in a boiling water bath for 10 min. The reaction mixture was then cooled under running tap water. Absorbance was measured at 570 nm in a colorimeter. Total soluble sugar content was then calculated from the standard curve of dextrose solution.

Somogyi's method as described by Plummer (1978) was followed for the estimation of reducing sugar. 1 ml alkaline Cu-tartarate solution was added to 1 ml of test solution.Reaction mixture was then allowed to boil in a water bath for 15 min. The mixture was then cooled under running tap water and to each reaction mixture 1ml of Nelson's arseno molybdate reagent was added. Reaction mixtures were diluted to 5 ml by adding 2 ml more distilled water and absorbances were measured in a colorimeter at 540 nm. The concentration of reducing sugar was determined by plotting the values on the standard curve of dextrose solution.

3.5.1.3.Starch

Starch was extracted by homogenizing 0.5 g of the plant tissue in hot 80% ethanol to remove sugars (Thimmaiah, 1999). The extract was centrifuged and the residue was retained. The residue was repeatedly washed with hot 80% ethanol till washings did not give any colour with Anthrone reagent. The residue was then dried over a water bath. To the dried residue 5 ml of water and 6.5 ml of 52% perchloric acid was added and centrifuged at 0°C for 20 minutes. The supernatant was saved and the process was repeated using fresh perchloric acid each time. The supernatant was pooled and the volume was made up to 100 ml.

For estimation, to 1 ml of test solution 4 ml of Anthrone reagent was added and mixed thoroughly. Mixtures were placed in a boiling water bath for 8 min. The reaction mixture was then cooled under running tap water. Absorbance was

measured at 630 nm in a colorimeter. Starch content was then calculated from the standard curve of glucose solution.

3.5.2.Extraction and estimation of proline content of leaves

Proline was extracted from leaves in 3% sulfosalicylic acid. The leaf tissues were homogenized in 3% sulfosalycylic acid and filtered through Whatman No.1 filter paper. The filtrate were collected and used for the estimation of proline.

Proline was estimated following the method of Bates *et al.* (1973). To 1 ml of the test solution 1 ml of Ninhydrin solution was added [1 g Ninhydrin in 25 ml acetone:water (2:3)]. After this the solution was boiled in hot water bath for 30 min. The solution was cooled and separated with 5 ml toluene in a separating funnel. The lower layer was collected and the upper layer was discarded. The absorbance of the lower layer was measured in a colorimeter at 520 nm.

3.5.3.Extraction and quantification of pigments

3.5.3.1.Chlorophyll

Chlorophyll was extracted according to the method of Harbone (1973) by homogenizing 1 g 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 25 ml.The chlorophyll content was estimated by observing the absorbance values at 645 nm and 663 nm respectively in a UV-VIS spectrophotometer (DIGISPEC-200GL) and calculation was done following the method of 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 = $(22.9\ A_{645} - 4.68\ A_{663})$ mg g^{-1} fresh weight

3.5.3.2.Carotenoids

Carotenoids were extracted and estimated according to the method given by Lichtenthaler (1987). Extraction of carotenoids was done by homogenizing 1 g of the plant sample in methanol. The homogenate was filtered through Whatman No.1 filter paper and the volume was made up. Absorbance of filtrate was observed at 480 nm,

645 nm and 663 nm in a UV-VIS spectrophotometer (DIGISPEC-200GL) and the carotenoid content was calculated by using the formula

Carotenoids = A_{480} - (0.114 x A_{663}) - 0.638(A_{645}) μg g^{-1} fresh weight

3.6. Protein analysis

3.6.1. Extraction of soluble proteins from seeds and different plant parts

Soluble protein was extracted from seeds, leaves, roots of different cultivars following the method of Chakraborty *et al.* (1995). Plant tissues were homogenized using 0.05 M sodium phosphate buffer (pH 7.2) to which PVPP(Polyvinyl pyrollidone phosphate) was added and centrifuged at 4°C for 20 min at 10,000 rpm. The supernatant was used as crude protein extract for the estimation of protein content.

3.6.2. Estimation of protein content

Soluble proteins were estimated following the method as described by Lowry *et al.* (1951). To 1 ml of protein sample 5 ml of alkaline reagent (1 ml of 1% $CuSO_4$ and 1 ml of 2% sodium potassium tartarate, dissolved in 100 ml of 2% Na_2CO_3 in 0.1 N NaOH) was added. This was incubated for 15 min at room temperature and then 0.5 ml of 1 N Folin Ciocalteau reagent was added and again incubated for further 15 min following which absorbance was noted at 720 nm. Quantity of protein was estimated from the standard curve made with bovine serum albumin (BSA).

3.6.3. SDS-PAGE analysis of total soluble protein

Total soluble proteins extracted in 0.05 M sodium phosphate buffer were used as crude protein extract for analysis of protein pattern. Analysis was carried out on 10% SDS-PAGE gels following the method of Sambrook *et al.* (1989). Protein samples were loaded on the gel and separated for 2 h at 200 V and 15-20 mA current. Following electrophoresis the gel was fixed, stained in Coomassie Brilliant Blue (R-250) solution and finally destained in a solution of methanol: glacial acetic acid: dH_2O(4.5: 4.5: 1).

3.6.3.1. 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 warm 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. 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 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 use.

(D) Ammonium Persulphate (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. The solution was made by dissolving 3.02 g Tris base, 18.8 g Glycine and 10 ml of 10% SDS in 1 L of distilled water.

(F) SDS gel loading buffer

This buffer contains 50 mM Tris-HCl (pH 6.8), 10 mM β-mercaptoethanol, 2% SDS, 0.1% bromophenol blue, 10% glycerol. The solution was prepared by dissolving 0.5 ml of 1 M Tris buffer (pH 6.8), 0.5 ml of 14.4 M β-mercaptoethanol, 2 ml of 10% SDS, 10 mg bromophenol blue, 1 ml glycerol in 6.8 ml of distilled water.

3.6.3.2. Preparation of gel

Mini slab gel (plate size 8 cm x10 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 of 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		**5% stacking gel**	
Name of the compond	**Amount (ml)**	**Name of the Coumpound**	**Amount (ml)**
Distilled water`	2.85	Distilled water	2.10
30% acrylamide	2.55	30% acrylamide	0.50
1.5 M Tris buffer (pH 8.8)	1.95	1 M Tris buffer (pH 6.8)	0.38
10% SDS	0.075	10% SDS	0.030
10% APS	0.075	10% APS	0.030
TEMED	0.003	TEMED	0.003

After pouring the resolving gel solution, it was immediately over layered with water and kept for polymerization for 2 h. After polymerization of the resolving gel was complete, overlay of water 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 30-45 min. After polymerization of the stacking gel the comb was removed and the wells were 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.

3.6.3.3. Sample Preparation

Sample (50 µl) was prepared by mixing the sample protein (35 µl) with the SDS gel loading buffer (15 µl) in cyclomixer. All the samples were floated in boiling water bath for 3 min 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 a mixture of six proteins ranging from high to low molecular weight (Phosphorylase b- 97,4000; Bovine Serum Albumin- 68,000; Ovalbumin- 43,000; Carbolic Anhydrase- 29,000; Soyabean Trypsin inhibitor-20,000; Lysozyme- 14,300) was treated as the other sample and loaded in a separate well.

3.6.3.4. Electrophoresis

Electrophoresis was performed at a constant 18 mA current for a period of 2 h until the dye front reached the bottom of the gel.

3.6.3.5. Fixing and staining

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: d H_2O(10:20:70). The staining solution was prepared by dissolving 250 mg 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.

The gel was removed from the fixer and stained in this stain solution for 4 h at 35°C with constant shaking at low speed. After staining the gel was finally distained with distaining solution containing methanol:dH_2O:acetic acid (4.5: 4.5: 1) at 35°C with constant shaking until the background became clear.

3.7. Extraction of enzymes

3.7.1. Catalase (CAT, EC 1.11.1.6), Peroxidase (POX, EC 1.11.1.7) and Ascorbate peroxidase (APOX, EC 1.11.11)

CAT, POX and APOX were extracted by homogenizing the plant tissues in 0.05 M sodium phosphate buffer (pH 7.2) containing 1% (w/v) insoluble polyvinyl pyrrolidone phosphate (PVPP) and 2 mM β mercaptoethanol under ice cold condition. The homogenate was centrifuged immediately at 10,000 rpm for 20 min at 4°C. After centrifugation the supernatant was collected and after recording its volume was used immediately for assay.

3.7.2. Glutathione reductase (GR, EC 1.6.4.2) and Superoxide dismutase (SOD, EC 1.15.1.1)

GR and SOD were extracted by homogenizing the plant tissue in 0.1 M potassium phosphate buffer (pH 7.8) containing 1% (w/v) insoluble polyvinyl pyrrolidone phosphate (PVPP) and 2 mM β mercaptoethanol. Insoluble material was removed by centrifugation at 12,000 rpm for 15 min at 4°C.

3.8. Assay of enzyme activities

3.8.1. Catalase

CAT activity was assayed according to Chance and Machly (1955). Enzyme extract(20 µl) was added to 3 ml of H_2O_2-phosphate buffer(0.16 ml of H_2O_2 to100 ml of phosphate buffer, pH 7.2) 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 enzyme units mg protein^{-1} where one enzyme unit was defined as a change of 0.01 absorbance min^{-1} caused by the enzyme aliquot.

3.8.2. Peroxidase

Peroxidase activity was assayed by adding 100 µl of freshly prepared crude enzyme extract to the reaction mixture containing 1 ml of 0.2 M sodium phosphate buffer (pH 7.2), 100 µl of 4mM H_2O_2, 100 µl of O-dianisidine (5 mg ml^{-1} methanol) and 1.7 ml of distilled water. Peroxidase activity was assayed spectrophotometrically

at 460 nm by monitoring the oxidation of O-dianisidine in presence of H_2O_2. Specific activity was expressed as the increase in absorbance at 460 nm g^{-1}tissue min^{-1} (Chakraborty *et al.* 1993).

3.8.3. Ascorbate peroxidase

APOX activity was assayed as decrease in absorbance by monitoring the oxidation of ascorbate at 290 nm according to the method of Asada (1994) with some modification. The reaction mixture consisted of 100 µl of enzyme extract, 100 µl of 0.5 mM ascorbic acid, 100 µl of H_2O_2 and 2.7 ml of sodium phosphate buffer (pH 7.2). Enzyme activity was finally expressed as change (decrease) in absorbance (ΔA_{290}) mg protein $^{-1}$ min $^{-1}$.

3.8.4. Glutathione reductase

GR activity was assayed by the oxidation of NADPH at 340 nm with extinction coefficient of 6.2 mM cm^{-1} as described by Lee and Lee (2000). The reaction mixture consisted of 100 mM potassium phosphate buffer (pH 7.8), 2 mM EDTA, 0.2 mM NADPH, 0.5 mM glutathione (oxidized form, GSSG) with an appropriate volume of enzyme extract. The reaction was initiated by the addition of NADPH at 25°C. Enzyme activity was finally expressed as µM NADPH oxidized min^{-1} mg $protein^{-1}$.

3.8.5. Superoxide dismutase

SOD 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 3 ml of the assay mixture constituted of 0.1 ml enzyme extract, 1.5 ml phosphate buffer(0.1 M, pH 7.8), 0.1 ml Na_2CO_3 (1.5 M), 0.1 ml NBT (2.25 mM), 0.2 ml methionine (200 mM), 0.1 ml EDTA (3 mM), 0.1 ml riboflavin (60 µM) and 0.8 ml of distilled water.

The reaction tubes containing enzyme samples were illuminated with 15 W fluorescent lamp for 10 min. The other set of tubes lacking enzymes were also illuminated and served as control. A non- irradiated complete reaction mixture did not develop any colour and served as blank. The absorbance of samples was measured at 560 nm 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. Isozymes analysis of peroxidase by PAGE

Extract for isozyme analysis was prepared by crushing 1 g of leaf tissue in a mortar and pestle in 0.1 M sodium phosphate buffer (pH 7.0) on ice and insoluble material was removed by centrifugation at 12,000 rpm for 15 min at 4°C. Polyacrylamide gel electrophoresis (PAGE) was performed for isozyme analysis of peroxidase as described by Davis (1964) and immediately used for the isozyme analysis.

3.9.1. Preparation of stock solution

(A) Acrylamide stock solution (Resolving gel)

Acrylamide stock solution for resolving gel was prepared by dissolving 28 g of acrylamide and 0.74 g of N' N' methelene bisacrylamide in distilled water and the volume was made up to 100 ml. The stock solution was filtered with Whatman No. 1 filter paper and stored at 4°C in dark bottle.

(B) Acrylamide stock solution (stacking gel)

Acrylamide stock solution for stacking gel was prepared by dissolving 10 g of acrylamide and 2.5 g of bis- acrylamide in distilled water and the volume was made up to 100 ml. The stock solution was filtered and stored at 4°C in dark bottle.

(C) Tris- HCl (Resolving gel)

Tris-HCl for resolving gel was prepared by dissolving 36.6 g of Tris base in distilled water and 0.25 ml of TEMED was added. The pH was adjusted to 8.9 with concentrated HCl. The volume of the solution was made up to 100 ml with distilled water. The solution was then stored at 4°C for further use.

(D) Tris- HCl (Stacking gel)

Tris-HCl for stacking gel was prepared by dissolving 5.98 g of Tris base in distilled water and 0.46 ml of TEMED was added. The pH was adjusted to 6.7 with conc. HCl. The volume of the solution was made up to 100 ml with distilled water. The solution was stored at 4°C for further use.

(E) Ammonium persulphate solution (APS)

Fresh solution of APS was prepared by dissolving 0.15 g of APS in 10 ml of distilled water.

(F) Riboflavin solution

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

(G) Electrode buffer

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

3.9.2. Preparation of gel

Mini slab gel for the polyacrylamide gel electrophoresis of peroxidase isozymes was prepared. For slab gel preparation, two glass plates were thoroughly cleaned with dehydrated alcohol to remove any trace of grease and then dried. Spacers of 1.5 mm thickness were placed between the glass plates on three sides and these were sealed with high pressure 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: dH_2O (1: 1: 4: 1) leaving sufficient space for (comb + 1 cm) the stacking gel. This resolving gel was immediately over layered with water and kept for polymerization for 2 h. After polymerization of the resolving gel was complete, over layer was poured off and washed with water to remove any unpolymerized acrylamide.

The stacking gel solution was prepared by mixing solutions B: D: F: dH_2O (2:1:1:4). Stacking gel solution was poured over the resolving gel and comb was inserted immediately and over layered with water. Finally the gel was kept for polymerization for 30-45 min in strong sunlight. After polymerization of the stacking gel the comb was removed and washed thoroughly. The gel was now finally mounted in the electrophoresis apparatus. Tris-Glycine running buffer 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.

3.9.3. Sample preparation

Sample (32 µl) was prepared by mixing the sample enzyme (20 µl) with gel loading dye (40% sucrose and 1% bromophenol blue in distilled water) in cyclomixer in ice. All the solutions for electrophoresis were cooled. The samples were immediately loaded in a predetermined order into the bottom of the wells with a microlitre syringe.

3.9.4. Electrophoresis

Electrophoresis was performed at constant 15 mA current for a period of around 3-4 h at 4°C until the dye font reached the bottom of the gel.

3.9.5. Fixing and Staining

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. Staining of the gel was performed following the method of Reddy and Gasber (1973).

The gel was incubated in an aqueous (80 ml) solution of benzidine(2.08 g), acetic acid (18 ml), 3% H_2O_2 (100 ml) for 5 minutes. The reaction was stopped with 7% acetic acid after the appearance of clear blue coloured bands. Analysis of isozyme was done immediately.

3.10. Determination of peroxidation of cell membrane lipids

Lipid peroxidation was measured according to the method described by Heath and Packer (1968). Leaf tissue was homogenized in 2 ml of 0.1% (w/v) trichloroacetic acid (TCA) and centrifuged at 10,000 rpm for 10 min. To 0.5 ml of the supernatant 2 ml of 0.5% thiobarbituric acid (TBA) in 20% TCA was added. The reaction mixture was incubated at 95°C for 30 min. After cooling it was again centrifuged. The absorbance of the supernatant was recorded at 532 nm and corrected for 600 nm. Amount of malonedialdehyde produced was calculated by using the extinction coefficient of 155 mM cm^{-1}.

3.11. Extraction of flavonoids

Fruits samples (0.5 g dry weight) of okra were extracted in 25 ml of 1.2 M HCl in 50% aqueous methanol. For extraction hydrolysis was carried out for 2 h at 98°C reflux as per the method described by Vargas-Alvarez *et al.* (2004). After refluxing the pH was adjusted to 2.5 and 500 µl aliquots from the hydrolyzed mixture were filtered with 0.45 µm membrane filters.

3.12. Analysis of flavonoids

For analysis of flavonoids high performance liquid chromatography (HPLC) was carried out.

3.12.1. High performance liquid chromatography (HPLC)

HPLC analyses of the samples were carried out in the HPLC system (Shimadzu Advanced VP Binary Gradient) using C-18 hypersil column with linear gradient elution system as follows: mobile phase A 100% acetonitrile; mobile phase B 2% acetic acid in water. Elution: 88% B for 6 min then linear gradient to 75% B over 5 min. The elution was complete after a total of 25 min. Flow rate was fixed as 1 ml min^{-1} with sensivity of 0.5 aufs, injection volume 20 ml and monitored at 278 nm (Obanda and Owuor, 1994).

3.13. Determination of heavy metal contents in plant material

Heavy metal content in the various parts of the plants was estimated with the help of atomic absorption spectrophotometer (AAS). The plant materials were oven dried to a constant dry weight and digested in a ternary acid mixture of nitric acid: perchloric acid: sulphuric acid (10:4:1). One gram of the plant material was digested at a temperature of 180°C for 15 min. After the digestion was complete the volume was made up to 100 ml with distilled water and the AAS (Perkin Elmer model 1100) reading was recorded.

3.14. Microscopic studies

Microscopic studies were carried out to observe the accumulation of starch and alkaloids in the different parts of okra plants treated with the heavy metals at 1000 µg ml^{-1} concentration.

3.14.1. Starch

In case of starch the transverse sections of roots were cut and dipped in Melzers reagent(aqueous solution of 1% iodine and 1% potassium iodide)for 15 min. Mounting was done with glycerol and then the slides were observed under microscope in both low and high power.

3.14.2. Alkaloids

For observing the alkaloids the transverse sections of roots and fruits were cut and dipped in Wagners reagent (1.27 g iodine and 2 g potassium iodide dissolved in 100 ml of distilled water) for 10 min. The sections were then thoroughly washed in distilled water and mounting was done with lactophenol. The mounted slides were then observed under light microscope in both low and high power.

3.15. Treatment with chemicals for amendment of heavy metal stress

Ameliorating compounds were applied as treatments to amend the effects of heavy metals. The ameliorating compounds used were calcium chloride ($CaCl_2$) and potassium nitrate (KNO_3) at a concentration of 1000 $\mu g\ ml^{-1}$. For seed treatment, the seeds were sown in Petri dishes lined with filter paper moistened with the ameliorating compounds and with combination with the different heavy metals (cadmium nitrate 4-hydrate [$Cd(NO_3)_2\ 4H_20$], copper(II) sulphate-5-hydrate [$CuSO_45H_2O$], mercury(II) chloride [$HgCl_2$] and lead(II) nitrate [$Pb(NO_3)_2$] at concentrations of 1000 $\mu g\ ml^{-1}$). One set was soaked in combination with distilled water and the respective heavy metal solutions to serve as control.

3.16. Glass wares and chemicals used in the experiment

All the glass wares *viz.*, Petri plates, test tubes, conical flask, pipettes, beakers, funnels used in the experiment were either of Borosil and Riviera make. Chemicals used in the experiment were of analytical reagent grade.

3.17. Statistical Analysis

All the data were recorded in triplicate and analysed using the standard error mean and ANOVA was done with the application of randomised block design. The test of significance was calculated with the help of Students 't' test.

Results of all experiments conducted in the present study have been detailed in the following pages. All the experiments were conducted using standard procedures as given under materials and methods.

4.1. Effect of heavy metals on seed germination

Seeds of six cultivars of okra (Arka Anamika, Deepti, Najuka F-1, Paras Soumya, Parbhani Kranti and PB-57) were treated with $Cd(NO_3)_24H_20$, $CuSO_45H_2O$, $HgCl_2$ and $Pb(NO_3)_2$ at both 100 and 1000 μg ml^{-1} concentrations. Maximum inhibition of germination was observed by Hg at 1000 μgml^{-1} after 24 h of germination. In this case no germination was recorded for the cultivar PB-57. However, after 48 h 30% germination was recorded. In most of the cultivars 1000 μg ml^{-1} treatments with the other heavy metal salts also inhibited germination significantly (Table 1). Analysis of variance revealed that there was significant difference between control and all the treatments after 24 h of germination. There was also significant difference in germination per cent between 100 μg ml^{-1} concentrations of the different salts. After 48 h of germination significant difference was also obtained between control and all heavy metal salts at both concentrations except $CuSO_45H_2O$ at 100 μg ml^{-1}(Tables 1A and 1B; Plate IV). After 72 h different cultivars exhibited germination between 77% (Parbhani Kranti) and 100% (Arka Anamika) in control. Higher concentration of Pb and Hg inhibited germination in the range between 30 to 40% (Figs.1 and 2).

4.2. Analysis of the effect of heavy metals on growth and yield of okra

Effect of the different heavy metals on the growth and yield of okra cultivars was determined on the basis of different parameters.All growth parameters were determined at the vegetative stage. Treatments of heavy metal salts were at 1000 μg ml^{-1} concentrations. Morphological effects of the heavy metals on different cultivars of okra have been presented in Plates V-IX.

4.2.1. Tolerance Index

Tolerance of the different cultivars and different plant parts was determined by the tolerance index computed from the fresh weight biomass. Results revealed that among the different cultivars Deepti was most tolerant as evidenced by