2. MATERIALS AND METHODS

2.1 Seeds and germination

Experiments were carried out in laboratory of Biosciences department of Veer Narmad South Gujarat University, Surat. The seeds of *Cajanus cajan* and *Trigonella foenum-graecum* were obtained from the Gandhi Agro Pvt. Ltd., Surat. The germination was carried out in Petri dishes.

Seeds were surface sterilized with H_2O_2 for the prevention of surface fungal/bacterial contamination. Different ppm solutions were prepared in pure distilled water in laboratory by using $ZnSO_4$ and Cd $(NO_3)_2$ and pure distilled water was used as control for the study. Ten seeds were placed on cotton in each petri dish and 40 ml solution of each concentration was supplied once for seed germination. Tap water applied every alternate day after this treatment. The Petri dishes were monitored daily for fungal and other inspections.

For each metal sub lethal (LC_{20}), lethal (LC_{50}) and super lethal (LC_{80}) values were determined for both plants. Experiments were carried out up to 20 days after the sowing of seeds.

The growth parameters viz., germination, fresh weight, dry weight, root length, shoot length studied. Biochemical estimation of protein, sugar, leaf pigments, ascorbic acid, proline, free amino acid, tannin and DNA was carried out. In cytological studies mitotic index, percentage of aberrant cells and different kind of abnormalities were measured. Change in the activity of enzyme catalase, peroxidase and amylase were also studied. The anatomical change occurred due to zinc and cadmium in root and stem of young and old seedling has been studied. Finally accumulation of zinc and cadmium in

different organs of plant were also investigated. An experiment for the removal of zinc and cadmium from aqueous solution was also carried out.

The fresh weight, dry weight, root length and shoot length of seedling were studied on 10^{th} and 20^{th} day after sowing of seeds. The biochemical estimation of protein, sugar, ascorbic acid, proline, free amino acid, tannin and enzyme activity of root, stem and leaf of seedlings was carried out on 10^{th} and 20^{th} day after sowing of seeds. DNA content was studied in whole plant on 10^{th} and 20^{th} day of growth. In *Trigonella foenum-graecum* the leaf development was not completed during study period. Therefore in this plant the biochemical parameters were studied in cotyledons.

The leaf pigments content was also investigated. It was studied in the leaves of *Cajanus cajan* on 10^{th} and 20^{th} day after sowing of seeds. In *Trigonella foenum-graecum* the leaf pigments content of cotyledons were studied on 10^{th} and 20^{th} day.

All the parameters were studied in the seedlings cultivated by the treatment of sub lethal, lethal and super lethal concentrations of cadmium and zinc and compared with the control. All the results are the mean values of the experiments, which were repeated three times.

2.2 Preparation of ppm solution

ppm solutions were prepared as under (www.delloyd. Com[1]).

2.2.1 ZnSO4

FW (Formula Weight) of salt = 161 gm

Atomic weight of zinc = 65

So that 1 gm of zinc in relation to FW of salt = 161/65 = 2.476 gm.

Therefore 2.476 gm $ZnSO_4$ was dissolved in 1 liter distilled water to make 1000 ppm zinc standard.

2.2.2 Cd $(NO_3)_2$

FW of Salt = 236 gm

Atomic weight of cadmium = 112

So that 1 gm of cadmium in relation to FW of salt = 236/112 = 2.107 gm

Therefore 2.107gm Cd $(NO_3)_2$ was dissolved in 1 liter distilled water to make 1000 ppm cadmium standard.

2.2.3 Dilution Formula

The solution of different concentrations of $ZnSO_4$ and $Cd(NO_3)_2$ were prepared by following formula from standard solution (www.delloyd.com[1]).

Number of ml for required volume = (required ppm x required volume) / stock

e.g. = 40 ml of 20 ppm from 1000 ppm = (20 x 40) / 1000

= 0.8 ml of 1000 ppm in 40 ml volume will give 20 ppm solution.

2.3 LC_{50} value

For the determination of LC_{50} value, three sets of germination and growth of seedlings treated with different concentration of $ZnSO_4$ and Cd $(NO_3)_2$ of both plants, *Cajanus cajan* and *Trigonella foenum-graecum*, were prepared. The percentage of mortality was recorded up to the growth of 20 days after the sowing of seeds. Mean value of three sets was taken. The graphs of percentage mortality against the concentration of $ZnSO_4$ and Cd $(NO_3)_2$ were prepared and a trend line was obtained which was used for the determination of sub lethal (20% mortality), lethal (50% mortality) and super lethal (80% mortality) value.

In these studies LC_{20}, LC_{50} and LC_{80} value for *Cajanus cajan* was 200 ppm, 400 ppm and 600 ppm zinc solution respectively; and 10 ppm, 20 ppm and 30 ppm cadmium solution respectively (Table1; Figs. I,II). Similarly LC_{20}, LC_{50} and LC_{80} value for *Trigonella foenum-graecum* was 100 ppm, 200 ppm and 300 ppm zinc solution respectively; and 1 ppm, 3 ppm and 5 ppm cadmium solution respectively (Table 1; Figs. III,IV). For experiments these solutions of zinc and cadmium were selected for the treatment. Plants grown in distilled water were control.

2.4 Morphological Studies

The fresh and dry weight of seedlings and length of root and shoot were studied.

2.4.1 Root and Shoot length

The seedlings of 10 and 20 days were collected and root and shoot length was measured in cm using the scale. Mean value was taken and standard deviation was calculated.

2.4.2 Fresh and Dry weight

The fresh weight (gm) of seedlings was taken by using Sartonius balance. For dry weight the seedlings were dried in the oven at 80 ^{0}C for two days or till the constant dry weight was attained. Average value was taken and standard deviation was calculated.

2.5 Biochemical

Methods were used for the estimation of protein, sugar, ascorbic acid, leaf pigments, proline, free amino acid, tannin and DNA are as under.

2.5.1 Estimation of ascorbic acid

For the estimation of ascorbic acid the method of Chinoy (1969) was followed:

2.5.1.1 Reagents

(1) Meta phosphoric acid 3%

3 gm meta phosphoric acid + 100 ml distilled water.

(2) Citric acid

12.5 gm citric acid + 100 ml 0.1 N NaOH.

(3) Buffer solution

1:1 (meta phosphoric acid and citric acid).

2.5.1.2 Standard curve of ascorbic acid

10 mg of dye 2,6-dichlorophenol indophenols was dissolved in hot glass distilled water and was cooled. 2 ml of glass distilled water and 5 ml of dye solution were taken and dye was adjusted for the value of 0 on the scale of the photochem colorimeter using green filter (540 nm). The standardization of the dye solution was done every time just before starting estimation.

0.01 mg/ml to 0.1 mg/ml of ascorbic acid solutions were prepared from a stock solution by dilution with distilled water to the requisite concentration. 1.0 ml aliquot of each ascorbic acid solution was mixed with 1.0 ml of buffered HPO_3 solution and 5.0 ml of standardized dye solution and the color was read immediately in the photochem colorimeter using green filter and optical density was noted. The readings were used to prepare standard curve (Fig. V).

2.5.1.3 Extraction and determination of ascorbic acid

100 mg weighed sample of plant material was placed in a mortar and covered 10 ml of cold distilled water. The homogenate was centrifuged and the filtrate was used for the estimation. 1.0 ml cooled buffered HPO_3 was added to 1.0 ml of the original homogenate after thorough shaking. 2.0 ml aliquot of the mixture was diluted with 5.0 ml of distilled water and the pointer on the colorimetric scale was adjusted at zero for the turbidity factor. 5.0 ml of standard dye solution was added to 2.0 ml aliquot of the same mixture and the optical density was noted in colorimeter. Ascorbic acid content was calculated by using following formula.

$$\text{Ascorbic acid content (mg/gm fresh weight)} = \frac{C \times V}{W \times 1000 \times v}$$

Where, C = Concentration of ascorbic acid from standard graph (µg/ml)

W = Weight of sample (gm)

V = Volume of extract (ml)

v = Volume of supernatant taken for analysis (ml)

2.5.2 Estimation of protein

Protein were determined by the method of Lowery *et al.* (1951)

2.5.2.1 Reagents

(1) Reagent- A

2 gm Na_2CO_3 + 100ml 0.1 N NaOH

(2) Reagent - B

0.5 gm CuSO4 + 100ml distilled water + 10 gm Sodium potassium tartrate + 100ml distilled water.

(3) Reagent- C

Reagent A (50 ml) + Reagent B (2 ml)

(4) Reagent – D

1 N Folin phenol

(5) 10% perchloric acid

1 ml perchloric acid + 6ml distilled water.

2.5.2.2 Standard curve of protein

100 mg albumin dissolved in 100ml of 0.1N NaOH. 1.0 ml of this solution contains 1mg/ml albumin range of concentration were prepared from stock solution.

5 ml reagent C was added to 1.0 ml of different aliquots of standard protein solution containing 1mg/ml; both were mixed and allowed to stand for 10 minutes at room temperature. 0.5 ml of folin phenol reagent was added rapidly and mixed well. The solution was allowed to stand for 10 minutes. The blue color developed was read in photochem colorimeter using red filter (680 nm) and optical density was noted and a standard curve of protein was prepared (Fig. VI).

2.5.2.3 Extraction and determination of protein

100 mg of plant material was crushed in 10.0 ml of 80% ethanol. The crushed material was kept for 15 minutes in boiling water bath. It was then centrifuged. The residue was suspended in water and 10 ml 10% perchloric acid was added to remove sugars and soluble nitrogen fractions. The residue was washed with distilled water and digested in water bath with 1.0 ml 0.1 N NaOH for 10 minutes. The mixture was cooled and volume was made up to 10.0 ml with distilled water. It was then centrifuged and residue was discarded. From the supernatant, 1.0 ml was taken and to this solution 5.0 ml of reagent C was added, both were mixed well and allowed to stand for 10 minutes at room temperature. 0.5 ml of folin reagent D was added rapidly and mixed well. The

solution was allowed to stand for 30 minutes. The developed blue colour was read in colorimeter and optical density was noted and the content of protein in terms of mg/gm fresh weight was calculated by using following formula.

$$\text{Protein content (mg/gm fresh weight)} = \frac{C \times V}{W \times 1000 \times v}$$

Where, C = Concentration of protein from standard graph (µg/ml)

W = Weight of sample (gm)

V = Volume of extract (ml)

v = Volume of supernatant taken for analysis (ml)

2.5.3 Estimation of total sugar

A sugar was determined by the methods of Nelson (1944) and Somagyi (1945).

2.5.3.1 Reagents

(1) Reagent - A

12.5 gm Na_2CO_3 + 12.5 gm sodium potassium tartret + 500 ml distilled water

(2) Reagent - B

1 gm CuSO4 + 100 ml distilled water

(3) Reagent - C

50 ml reagent A + 2 ml reagent B

(4) Arsenic molybdate reagent

2.5 gm ammonium molybdate + 95 ml distilled water + 21 ml cons. H_2SO_4 + 3 gm sodium arsenate +25 ml distilled water.

2.5.3.2 Standard curve of sugar

100 mg glucose was dissolved in 100 ml of distilled water. From the stock solution different concentration of solution were prepared.

1.0 ml reagent C was added to 1.0 ml of different aliquots of standard sugar solution. Then 1.0 ml of arsenic molybdate was added in this solution. Then keep it for 15 minutes for colour development. And optical density was measured with the help of colorimeter using red filter after colour development. A standard curve was prepared by using optical density and concentration (Fig. VII).

2.5.3.3 Extraction and determination of sugar

100 mg of plant material was homogenized and the volume was made up to 10.0 ml with 80% ethanol. It was then kept in boiling water bath for 20 minutes. The sample was centrifuged and the supernatant consisted of ethanol soluble free sugars. From this solution 1.0 ml of solution is taken and 1 ml of reagent C was added in this solution. Again this solution was kept in water bath for 10-15 minutes. And then it allowed cooling down. 1.0 ml of arsenic molybdate was added in this solution. The solution was kept for 15 minutes for colour development and optical density was measured with help of colorimeter using red filter.

A standard curve was prepared with known amount sugar. Total sugar where calculated by following formula.

$$\text{Sugar content (mg/gm fresh weight)} = \frac{C \times V}{W \times 1000 \times v}$$

Where, C = Concentration of sugar from standard graph (µg/ml)

W = Weight of sample (gm)

V = Volume of extract (ml)

v = Volume of supernatant taken for analysis (ml)

2.5.4 Proline

Proline content was determined by the methods of Bates *et al.* (1973).

2.5.4.1 Reagent

(1) Acid ninhydrin

Warm 1.25 gm ninhydrin in 30 ml glacial acetic acid and 20 ml 6 M phosphoric acid, with agitation until dissolved. Store at 4 ^{0}C and use within 24 hour.

(2) 3% Aqueous sulphosalicyclic acid

(3) Glacial Acetic acid

(4) Toluene

(5) Proline

2.5.4.2 Procedure

(1) Extract 0.5 gm of plant material by homogenizing in 10 ml of 3% aqueous sulphosalicyclic acid.

(2) Filter the homogenate through Whatman No. 2 filter paper.

(3) Take 2 ml of filtrate in a test tube and add 2 ml of glacial acetic acid and 2 ml acid ninhydrin.

(4) Heat it in the boiling water bath for 1 hour.

(5) Terminate the reaction by placing the tube in ice bath.

(6) Add 4 ml toluene to the reaction mixture and stir well for 20 –30 sec.

(7) Separate the toluene layer and warm to room temperature.

(8) Measure the red colour intensity at 520 nm.

(9) Run a series of standard with pure proline in a similar way and prepare a standard curve (Fig. VIII).

(10) Find out the amount of proline in the test sample from the standard curve.

2.5.4.3 Calculation

Express the proline content on fresh-weight basis as follows

$$\text{mg/gm fresh weight} = \frac{\mu g \text{ proline /ml} \times \text{ml toluene} \times 5}{115.5 \times \text{gm sample}}$$

Where, 115.5 = Molecular weight of toluene

2.5.5 Free amino acid

Free amino acid content was determined by the methods of Moore and Stein (1948).

2.5.5.1 Reagent

(1) Ninhydrin : Dissolve 0.8 gm stannous chloride in 500 ml of 0.2 M citrate buffer (pH 5.0). Add this solution to 20 gm of ninhydrin in 500 ml of methyl cellosolve (2- methoxyethanol)

(2) 0.2 M citrate buffer pH 5.0

(3) Diluent solvent: Mix equal volumes of water and n- propanol.

2.5.5.2 Extraction

Weigh 500 mg of the plant sample and grind it in a pestle and mortar with a small quantity of acid wash send. To this homogenate, add 5 to 10 ml of 80% ethanol. Filter or centrifuge. Save the filtrate or the supernatant. Repeat the extraction twice with the residue and pool all the supernatants. Reduce the volume if needed by evaporation and use the extract for the quantitative estimation of total free amino acid.

2.5.5.3 Estimation

(1) To 0.1 ml of extract, add 1 ml of ninhydrin solution.

(2) Make up the volume to 2 ml with distilled water.

(3) Heat the tube in a boiling water bath for 20 min.

(4) Add 5 ml of the diluent and mix the contents.

(5) After 15 min read the intensity of the purple colour against a reagent blank in a colorimeter at 570 nm. The colour is stable for 1 hour.

(6) Prepare the reagent blank as above by taking 0.1 ml of 80% ethanol instead of the extract.

2.5.5.4 Standard

Dissolve 50 mg leucine in 50 ml of distilled water in a volumetric flask. Take 10 ml of this stock standard and dilute to 100 ml in another volumetric flask for working standard solution. A series of volume from 0.1-1 ml of this standard solution gives a concentration range 10µg- 100µg. Proceed as that of the sample and read the colour.

A standard curve draw, using absorbance versus concentration and find out the concentration of the total free amino acids in the sample (Fig. IX). Free amino acid (mg/gm fresh weight) calculated using following formula.

$$\text{Free amino acid content (mg/gm fresh weight)} = \frac{C \times V}{W \times 1000 \times v}$$

Where, C = Concentration of free amino acid from standard graph (µg/ml)

W = Weight of sample (gm)

V = Volume of extract (ml)

v = Volume of supernatant taken for analysis (ml)

2.5.6 Tannin

Tannin content was determined by the methods of Schanderl (1970).

2.5.6.1 Reagent

(1) Folin –Denis Reagent

(2) Sodium Carbonate Solution

(3) Standard Tannic acid Solution

Dissolve 100 mg tannic in 100 ml of distilled water.

(4) Working standard solution

Dilute 5 ml of the stock solution to 100 ml with distilled water. One ml contain 50 μg tannic acid.

2.5.6.2 Extraction of Tannin

Weigh 0.5 g of the powdered material and transfer to a 250 ml conical flask. Add 75 ml water. Heat the flask gently and boil for 30 min. Centrifuge at 2,000 rpm for 20 min and collect the supernatant in 100 ml volumetric flask and make up the volume.

2.5.6.3 Procedure

(1)Transfer 1 ml of the sample extract to a 100 ml volumetric flask containing 75 ml water.

(2) Add 5 ml of folin-denis reagent, 10 ml of sodium carbonate solution and dilute to 100 ml with water. Shake it well. Read the absorbance at 700 nm after 30 min.

(3) Prepare a blank with water instead of the sample. Prepare standard graph by using 0-100 μg tannic acid.

2.5.6.4 Calculation

Calculate the tannin content of the sample as tannic acid equivalents from the standard graph (Fig. X).

2.5.7 DNA

DNA content was determined by the methods of Sambrook *et al.* (1989).

2.5.7.1 Reagent

(1) Extraction buffer

200 mM Tris-HCL (pH 8.0) + 50 mM EDTA + SDS 1% (Sodium dodisyl sulphate)

(2) 70 % Ethanol

(3) Isopropanol

(4) Chloroform : Isoamyl alcohol (24:1)

(5) RNase

(6) Sodium acetate 3M

(7) TE buffer

10 mM Tris-HCL (pH 8.0) + 1 mM EDTA

2.5.7.2 Procedure

(1) Wash the plant material in running tap water followed by sterile distilled water. Remove the water on the material by blotting with a filter paper and cut into small bits.

(2) Weigh out 0.5 gm of the above material and transfer to a suitable clean dry porcelain pestle and mortar and crushed in 7 ml extraction buffer.

(3) Incubate crushed material for 30 minute at 37^{o}C temperature.

(4) Centrifuge this material at 10,000 rpm for 15 minute at 4^{o}C, collect supernatant and add 25µl of RNase to it.

(5) Incubate for 30 minute at 37^{o}C, again incubate in ice for 30 minute.

(6) Centrifuge at 10,000 rpm for 15 minute at 4^{o}C

(7) Collect supernatant and add chloroform : isoamyl alcohol (24:1), volume should be equal of the volume of supernatant.

(8) Centrifuge at 10,000 rpm for 15 minute at 4^{o}C and take upper aqueous phase.

(9) Repeat the chloroform : isoamyl alcohol steps three times.

(10) Add isopropyl alcohol (volume equal to the volume of aqueous phase)

(11) Incubate for overnight at -20^{o}C and centrifuge at 10,000 rpm for 15 minute at 4^{o}C, take pellet and add ethanol.

(12) Centrifuge at 10,000 rpm for 15 minute at 4^{o}C and repeat ethanol steps three times.

(13) Evaporate ethanol from pellet and add TE buffer and dissolve pellet.

(14) Take DNA sample in Quartz cuvette and measure the OD at 260 nm

2.5.7.3 Calculation

OD 260 nm = 1.000 then concentration of DNA in sample is 50µg/ml.

2.6 Leaf pigments

2.6.1 Extraction and determination of pigments content

For pigment determination, 100 mg fresh weight sample were homogenized in 20 ml of 80% acetone using mortar and pestle and centrifuged at 6000 rpm for 15 minute. Finally the supernatant was made up to 20 ml and optical densities were measured at 480 and 510 nm wavelengths for carotenoids and 645 and 663 nm for chlorophyll on a spectrometer. The amount of chlorophyll-a, chlorophyll-b and carotenoids were calculated by using the formulae given by Machlachlan and Zalik (1963) and Duxbury and Yentsch (1956).

$$\text{Chlorophyll -a (mg/gm fresh weight)} = \frac{[12.3 \times D_{663} - 0.86 \times D_{645}] \times V}{d \times 1000 \times W}$$

$$\text{Chlorophyll -b (mg/gm fresh weight)} = \frac{[19.3 \times D_{645} - 3.6 \times D_{663}] \times V}{d \times 1000 \times W}$$

$$\text{Carotenoids (mg/gm fresh weight)} = \frac{[7.6 \times D_{480} - 1.49 \times D_{510}] \times V}{d \times 1000 \times W}$$

Where, V= Volume of extract (ml)

d = length of light path

W= Fresh weight of sample (gm)

Total chlorophyll was calculated by adding the amounts of chlorophyll-a and chlorophyll-b.

2.7 Enzymes activity

2.7.1 Catalase activity

Catalase activity was estimated by the methods of Luck (1974).

2.7.1.1 Reagents

(1) Phosphate buffer (0.067 M, pH 7.0)

(2) H_2O_2- phosphate buffer:

Dilute 0.16 ml of H_2O_2 to 100 ml with phosphate buffer. After its preparation it should be confirmed that its absorbance at 240 nm is not more than 0.5 O.D. units.

(3) Acid wash sand

2.7.1.2 Procedure

(1) Weigh 1 gm of plants materials and grind them in chilled pestle and mortar in presence of 20 ml of cold 0.0067 M phosphate buffer (dilute 10 times the Reagent 1 for this step).

(2) Transfer the homogenate to centrifuge tube and centrifuge at 10,000 X g for 20 min at 4^0 C. Repeat extraction of the pellet twice with cold buffer and combine the supernatants obtained after each centrifugation.

(3) Take 3 ml of phosphate buffer and 0.04 ml of the plant extract into a quartz cuvette. Mix the content gently by inversion. Use this mixture as blank for adjusting the spectrophotometer to zero absorbance or 100% transmission at 240 nm.

(4) For assaying the enzyme activity, pipette 3 ml of H_2O_2- phosphate buffer solution and add the 0.04 ml of plant extract. Mix the contents and immediately record the absorbance against blank and note the time taken for decrease in O.D. by 0.05 units. This time should not exceed 60 sec otherwise repeat the assay with less diluted enzyme preparation.

2.7.1.3 Calculations

1 gm tissue was homogenized in total volume of 20 ml and 0.04 ml was taken for assay. The absorbance at 240 nm decreased from 0.5 to 0.45 in 20 sec

17/20= 0.85

0.85/0.04= 21.25

21.25 × 20 = 425 units/gm fresh weight

2.7.2 Peroxidase activity

Peroxidase activity was estimated by the methods of Malik and Singh (1980).

2.7.2.1 Reagent

(1) Phosphate buffer 0.1 M (pH 7.0)

(2) Guaiacol solution 20 mM

(3) Hydrogen peroxide solution. Dilute 0.14 ml of 30% H_2O_2 to 100 ml with water. The extinction of this solution should be 0.485 at 240 nm.

(4) Enzyme Extract

Extract 1 gm of fresh plant tissue in 3 ml of 0.1 M phosphate buffer pH 7.0 by grinding in a pre-cooled mortar and pestle. Centrifuge the homogenate at 18,000 g at 5^0 C for 15 min. Use the supernatant as enzyme source within 2- 4 hour. Store in ice till the assay is carried out.

2.7.2.2 Procedure

(1) Pipette cut 3 ml buffer solution, 0.5 ml guaiacol solution, 0.1 ml enzyme extract and 0.03 ml hydrogen peroxide solution in a cuvette.

(2) Mix well and place the cuvette in the spectrophotometer.

(3) Wait until the absorbance has increased by 0.05. Start a stop-watch and note time required in minutes (Δt) to increase the absorbance by 0.1.

2.7.2.3 Calculation

Since the extinction of guaiacol dehydrogenation sproduct at 436 nm under the conditions specified is 6.39 per micromole, the enzyme activity per liter of extract is calculated as below:

$$\text{Enzyme activity units/liter} = \frac{3.18 \times 0.1 \times 1000}{6.39 \times 1 \times \Delta t \times 0.1}$$

2.7.3 Amylase activity

Amylase activity was estimated by the methods of Brenfield (1995).

2.7.3.1 Reagent

(1) Sodium acetate buffer, 0.1 M pH 4.7

(2) Starch, 1% solution

(3) Dinitrosalicylic acid Reagent

Dissolve 1 gm Dinitrosalicylic acid, 200 mg crystalline phenol and 50 mg sodium sulphite in 100 ml of 1% NaOH

(4) 40% potassium sodium tartrate

2.7.3.2 Extraction of Amylase

Extract 1 gm of sample material with 5-10 volumes of ice-cold 10 mM calcium chloride put for 3 hour at room temperature. Centrifuge the extract at 54,000 g at 4 ^{0}C for 20 min. The supernatant is used as enzyme source.

2.7.3.3 Procedure

(1) Pipette out 1 ml of starch solution and 1 ml of properly diluted enzyme in a test tube.

(2) Incubate it at 27 ^{0}C for 15 min

(3) Stop the reaction by the addition of 2 ml of dinitrosalicylic acid reagent

(4) Heat the solution in a boiling water-bath for 5 min.

(5) While the tubes are warm, add 1 ml potassium sodium tartrate solution.

(6) Then cool it in running tap water.

(7) Make up the volume to 10 ml by addition of 6 ml water.

(8) Read the absorbance at 560 nm and terminate the reaction at zero time in the control tubes.

(9) Prepare a standard graph with 0-100 μg maltose (Fig. XI).

2.7.3.4 Calculation

Unit of amylase activity is expressed as maltose produced mg/gm fresh weight of tissue during 5 minutes of incubation with 1% starch.

2.8 Cytogenetic study

2.8.1 Root tip excision and slide preparation

The control as well as $ZnSO_4$ and $Cd(NO_3)_2$ treated root tips were fixed in 3:1 methanol-acetic acid. Fixation period of root tip was 9:30 am to 10:30 am as the rate of mitosis was found higher during this period. The staining procedure followed was that of Conger and Fairchid (1954) and Darlington and La Canr (1976). The staining with aceto orcine as well as 1:1 aceto orcine – aceto carmine was found suitable for present study.

Temporary squash preparation of the material was used for preliminary observations and the preparations were made permanent with DPX. Mitotic index was scored after screening hundreds of cells from each group. Percentage of aberrant cells was scored per hundreds cell.

2.8.2 Calculation of mitotic index (in percentage) and percentage of aberrant cells

Mitotic index was calculated by observing the slides of root tip of control and treated plants at 3^{rd}, 5^{th} and 7^{th} days of growth. Total 100 cells were observed on slide.

The cell in the stage of prophase, metaphase, anaphase and telophase were counted. The mitotic index and percentage of aberrant cells were calculated using following formulae;

$$\text{Mitotic index (\%)} = \frac{\text{No. of dividing cells}}{\text{Total no. of cells studies}} \times 100$$

$$\text{Percentage of aberrant cells} = \frac{\text{No. of aberrant cells}}{\text{No. of dividing cells}} \times 100$$

2.9 Anatomy and stomata studies

The plant parts i.e. roots and stem harvested and cut into 10~15 cm pieces and preserved in formalin-acetic acid-alcohol (FAA), a lethal chemical preservative. Manual sectioning was done to study the plant material in cross sections.

Epidermal peels scraped from adaxial and abaxial surfaces of leaves were obtained and studied. After sectioning, the material was stained in Safranine and Fast Green stains, and then mounted in a drop of glycerin jelly on glass slides. A cover slip was placed over them and observations were made.

The stomatal index was calculated by using following formula

Stomatal index = [S / S + E] ×100

Where, S = number of stomata and E = number of epidermal cells.

2.10 Accumulation

The roots, stems and leaves were harvested, and they were washed with deionized water and the samples were dried at 80^{o} C in an oven for 48 hours for heavy metal analysis. Heavy metal analysis was done by Atomic Absorption Spectrophotometer at Dhanvantary pharmaceutical, Kim, Gujarat, India.

2.11 Phytoremediation

2.11.1 Husk of *Cajanus cajan* (Pigeon pea) and *Oryza sativa* (Rice)

Pigeon pea (*Cajanus cajan*) and Rice (*Oryza sativa)* husk (seed coat) was collected from a legume seed-splitting mill Dharampur, Valsad, Guajrat. The husk was thoroughly washed in running tap water to remove dirt and other particulate matter. This was later subjected to colour removal through washing and boiling in distilled water repeatedly. Subsequently the husk was oven dried at 80^{o}C for 24 hr, and then husk was grinding and converted in to the powder form. Batch mode adsorption studies using processed Husks were carried out to determine the adsorption of cadmium and zinc. The synthetic stock solution (1000 ppm) of cadmium nitrate and zinc sulphate prepared in the laboratory was diluted to different concentrations and to each 100 ml; 1 g of the husk was added and agitated in a rotary shaker at 150 rpm for 30 minutes. The adsorbate and the adsorbent were separated using whatman no. 1 filter paper. The metal concentration in filtrate was determined by Inductively Coupled Plasma Atomic Emission Spectroscopy at SAIF, IIT Bombay, India and percentage of adsorption was calculated.

2.11.2 Water Hyacinth (*Eichhornia crassipes*) and Water lettuce (*Pistia stratiotes)*

Aquatic plants of Water hyactih (*Eichhornia crassipes*) and Water lettuce (*Pistia stratiotes)* collected from the ponds of the Dharampur. Plants were selected for uniformity in size and shape. Before experiments, their surface was sterilized with 1% sodium hypochlorite for 1 min, and immediately washed in running tap water and then rinsed and kept in deionized water for 24 hour. Pre-clean plastic containers (volume 2 L and height 20 cm) were filling with 1.5 liter of different concentrations and pH of zinc and cadmium solution. Plants were placed into plastic containers for 48 hours. After that

the metal concentration in solution was determined by Inductively Coupled Plasma Atomic Emission Spectroscopy at SAIF, IIT Bombay, India and percentage of adsorption was calculated.

2.12 Photography

The photographs of morphology of seedlings were taken by using Sony T-10 digital camera with X5 zoom lens. The photographs were transferred to IBM Lenovo laptop computer and prints were taken by using HP-4288 Printer. Photomicrographs were taken on Carl-Zesis photomicroscope with planophotochromatic objectives using Kodak 100 ASA-35mm colour film. Daylight, yellow or green filter were used. Photomicrographs were also taken on Carl-Zesis Axio scope.