2. MATERIALS AND METHODS

2.1 Experimental design

The present study was carried out in Rajkot city area (22° 17’ Lat. and 70° 49’ Lon.) (Fig., 1.1).

2.1.1 Soil selection and preparation

Experiments on seedling emergence and seedling growth were performed on a coarse loam soil found in the natural habitats where the selected plants cultivated by seed germination. Soil was collected from natural habitats, air dried and passed through a 2 mm sieve. For the study of the effect of heavy metals on plant growth, this soil was mixed with heavy metal salts and prepared for the cultivation of experimental plants. The metal salts of selected metals were mixed in eight different lots of soil (each lot of 10kg) to get 0.2, 0.4, 0.6, 0.8, 1.0, 1.2, 1.4 and 1.6 mM concentrations of metal salts. The quantity of different metal salts added into the soil is presented in Table 2.1. The soil mixed with metal salt was placed in polyethylene bags and cultivation of experimental plants was carried out in these bags. The soil without metal salt was control. The initial metal concentration of control soil was negligible and considered as zero.

Tap water was added to the soil in polyethylene bags to field capacity and then allowed to dry for 6 days.
2.1.2 Seed sowing and germination

The seeds of *Glycine max*, *Vigna unguiculata* and *Vigna aconitifolia* were collected from Sanjiv Agro Center, Rajkot.

Metal salt mixed soils were then raked with fingers and seeds were sown on 16/08/2008 and 07/07/2009 after surface sterilization with H₂O₂. Ten seeds were sown in each bag at the depth of about 8 - 10 mm in evening. Immediately after sowing soils were watered and then after watering was carried out at alternate days.

All the seedlings in each bag (Plate 1A-C) for each metal concentration were allowed to grow for five weeks after the germination of seeds and collected and brought to laboratory for biochemical analysis and other investigations. The study was started in August, 2008 and repeated from July, 2009. The results are average of the study of these two sets of germination.

2.1.3 Plant harvesting

After specific time duration plants were harvested in such a way that the tap root and root hairs were not damaged or damage was minimum. Soil particles were removed from the root by gentle washing. The roots and stem were analyzed for protein, sugar, and contents of various minerals such as potassium (K), phosphorous (P), calcium (Ca), and magnesium (Mg) and heavy metal bioaccumulation.
2.2 Seedling morphology

2.2.1 Growth rate

The plants collected for the study brought in the laboratory, washed with water and carefully blotted on the blotting sheets after washing to remove moisture on their surface. The length of entire plant was measured. The mean of 20 measurements was calculated as final reading. The growth rate of control and treated plant was studied on the basis of length of entire plant five weeks after the germination.

2.2.2 Biomass study

The method of Hunt (1978) was used to study the biomass of experimental plants. The fresh weight of root, stem and leaves was determined separately after blotting in the laboratory. They were cut into small pieces after weighing and placed in brown paper bags separately and kept in oven at 80°C for a period of 8 days for uniform drying. The dry weight of these organs was recorded.

2.3 Anatomical studies

2.3.1 Sectioning and staining

Fresh as well as fixed materials were used for anatomical studies. The small pieces of root, stem and leaves were fixed in Formalin Acetic Acid Alcohol (FAA) (Jenson, 1962). The fixed material was preserved in 70 % ethyl alcohol.
The hand sections of root, stem and lamina were taken by sharp blades. Usually the fresh organs and occasionally the preserved organs were utilized.

The transactions were stained either with safranin or with FCF fast green combination (Berlyn and Miksche, 1976) and mounted in glycerin.

For the study of epidermal structures of leaves, the epidermal peels were taken with the help of razor blade, needle and fine forceps. They were stained with Delafield’s hematoxylin, then counter stained with safranin and mounted in glycerin.

2.3.2 Cambial activity

The cambial activity was investigated in root and stem of experimental plants. The effect of two different concentrations 0.2 mM and 1.6 mM of selected heavy metals was studied. It was investigated by the number of cells of secondary xylem and phloem produced in radial rows during the growth of sixty days of plants after germination. The study was carried out with the help of transactions of roots and stems and based on the average of 20 readings.

2.3.3 Histochemical studies

The following histochemical tests in transactions of fresh materials were carried out to localize various metabolites:
2. Rhuthenium red for pecting (Johansen, 1940).
4. Sudan IV (Johansen, 1940) and sudan black B in 70 % ethanol for cutin (Von Techman, 1987).
5. Ferric chloride and sodium carbonate for tannins (Johansen, 1940).

2.4 Biochemical studies

Biochemical analyses were done by using various techniques for different parameters as per methods given in Table 2.2.

2.4.1 Total sugar estimation

2.4.1.1 Reagents

(1) Reagent - A
   12.5 gm Na₂CO₃ + 12.5 gm sodium potassium tartrate + 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 concentrated H₂SO₄ + 3 gm sodium arsenate + 25 ml distilled water.
2.4.1.2 Standard curve of total sugar

For the preparation of standard curve 100 mg glucose was dissolved in 100 ml of distilled water. This was a stock solution which was used for the preparation of glucose solutions of different concentration.

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. This was kept for 15 minutes for color development. After this, optical density was measured with the help of spectrophotometer. A standard curve was prepared by using optical density and concentration of glucose (Fig., 2.1).

2.4.1.3 Extraction and determination of total sugar

100 mg of plant material was taken; 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 was taken and 1 ml of reagent C was added in this solution. This solution was kept in water bath for 10-15 minutes. 1.0 ml of arsenic molybdate was added in this solution after cooling.

The solution was kept for 15 minutes for color development and optical density was measured with the help of spectrophotometer using 620 nm wavelengths.
Regression equation for sugar concentration and optical density was evaluated and trend line was determined from the following regression equation.

\[ Y = 0.2317 \cdot (X) - 0.2827 \]

\[ R^2 = 0.9879 \]

Where, \( Y \) = Concentration
\( X \) = Optical density

2.4.2 Protein estimation

2.4.2.1 Reagents
(1) Reagent - A
   2 gm Na\(_2\)CO\(_3\) + 100 ml 0.1 N NaOH
(2) Reagent - B
   0.5 gm CuSO\(_4\) + 100 ml distilled water + 10 gm sodium potassium tartrate + 100 ml 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 + 9 ml distilled water

2.4.2.2 Standard curve of protein

Albumin was used for the preparation of standard curve. 100 mg albumin was dissolved in 100 ml of 0.1N NaOH, this was stock
solution. 1.0 ml of stock solution contains 1mg/ml albumin. Range of albumin concentration was prepared from stock solution.

5 ml reagent C was added to 1.0 ml of different aliquots of standard protein solution containing 1 mg/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 spectrophotometer at 680 nm, optical density was noted and a standard curve of protein was prepared (Fig., 2.2).

2.4.2.3 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 reagent D was added rapidly and mixed well. The solution was allowed to stand for 30 minutes. The developed blue color was measured as optical density in spectrophotometer at 680 nm and the content of protein in terms of mg/gm was calculated by using following regression formula.
$Y = 0.0041 \times X - 0.0048$

$R^2 = 0.9625$

Where, $Y =$ Concentration

$X =$ Optical density

### 2.4.3 Mineral contents

From oven dried and acid digested samples the contents of potassium, phosphorous, calcium and magnesium minerals were determined from Roots and stems. The protocols were as per followings:

#### 2.4.3.1 Potassium

#### 2.4.3.1.1 Reagents

(1) Nitric acid 1 N

42 ml of concentrated nitric acid diluted to make final volume of 1000 ml with distilled water.

(2) Trisodium cobalt nitrite 1 N

30 gm of sodium cobalt nitrite dissolved in distilled water to make final volume 100 ml.

(3) Sulphuric acid concentrated

#### 2.4.3.1.2 Standard curve of potassium

For the preparation of stock solution of potassium (1000 mg/l K) 1.907 gm of oven dried potassium chloride was dissolved in distilled water to make final volume 1000 ml. 1.00 ml stock solution was
diluted with 99 ml distilled water (100 mg/l K). This was an intermediate potassium solution. 1.00 ml intermediate potassium solution was diluted with 99 ml distilled water to prepare standard potassium solution (10 mg/l K).

From the standard potassium solution 10 ml of 100 to 500 µg/ml potassium solutions were prepared in separate flasks. 1 ml of trisodium cobalt nitrite and 5 ml of concentrated sulphuric acid were added to each flask and then allowed to cool at room temperature. The absorbance of solution was read at 425 nm with spectrophotometer. A graph of optical density against concentration of potassium was prepared (Fig., 2.3).

**2.4.3.1.3 Procedure**

10 ml of oven dried acid digested sample (Brayton, 1992) was taken in a volumetric flask. 1 ml of trisodium cobalt nitrite and 5 ml of concentrated sulphuric acid were added to each flask. The optical density was measured at 425 nm after cooling at room temperature and the content of potassium in terms of mg/l was calculated by using following regression formula:

\[ y = 0.0711 (X) - 0.074 \]

\[ R^2 = 0.998 \]

Where, \( Y \) = Concentration

\( X \) = Optical density
2.4.3.1.4 Calculation

Potassium (mg/l) = \( \frac{m \times 1000}{V} \)

Where,
\( m \) = mg of potassium from calibration curve
\( V \) = sample volume in ml

2.4.3.2 Estimation of phosphorus

2.3.4.2.1 Reagents

(1) Vandomolybdate reagent
1 gm ammonium molybdate + 1 gm ammonium vanadate + 100 ml HNO₃.

2.4.3.2.2 Standard curve

0.2195 gm of KH₂PO₄ was dissolved in 1.0 litre distilled water. This solution contained 50 µg phosphorus/ml. From this standard solution 1.0 to 5.0 µg/ml solutions were prepared in separate volumetric flasks. 10 ml of vandomolybdate reagent was added to each flask and final volume was made 50 ml by adding distilled water. The absorbance of solution was read after 10 minutes at 470 nm with spectrophotometer. A graph of optical density against concentration of phosphorous was prepared (Fig., 2.4).
2.4.3.2.3 Procedure

10 ml of oven dried acid digested sample (Brayton, 1992) was taken in a volumetric flask. 10 ml of vandomolybdate reagent was added. Then volume was made 50 ml by adding distilled water. After 10 minutes the developed colour was measured as optical density in spectrophotometer at 470 nm and the content of phosphorous in terms of mg/l was calculated by using following regression formula:

\[ Y = 0.0529 \times (X) - 0.0533 \]
\[ R^2 = 0.9961 \]

Where, \( Y \) = Concentration
\( X \) = Optical density

2.4.3.2.4 Calculation

Phosphorus (mg/l) = \( \frac{m \times 1000}{V} \)

Where,
m = mg of phosphorous (in 50 ml final solution)
\( V \) = sample volume in ml

2.4.3.3 Calcium

2.4.3.3.1 Reagents
(1) 0.01 M EDTA solution.
(2) 1.0 N Sodium hydroxide.
(3) Murexide indicator.
2.4.3.3.2 Procedure

50 ml of sample was taken in a conical flask and 2.0 ml of sodium hydroxide solution was added. 100 to 200 mg of murexide indicator was added to form pink color. This was titrated against EDTA solution until the pink color changed to purple.

2.4.3.3.3 Calculation

Calcium (mg/l) = \( \frac{A \times B \times 1000}{V} \)

Where,

A = volume in ml of EDTA solution used for titration
B = mass in mg of calcium equivalent to 1 ml of EDTA solution
V = volume in ml of the sample taken for the test

2.4.3.4 Magnesium

2.4.3.4.1 Reagents

(1) 0.01 M EDTA solution.
(2) Buffer solution.
(3) Erichrome black T.

2.4.3.4.2 Procedure

The volume of EDTA found in the process of determination of calcium was used here. The volume of EDTA used in titration is utilized for the determination of magnesium.
2.4.3.4.3 Calculation

\[
\text{Magnesium (mg/l)} = \frac{0.02435 \times 1000 \times (V_2 - V_1)}{V}
\]

Where,
\(V\) = volume in ml of sample taken for the test.
\(V_1\) = EDTA used in calcium determination for the same volume of the sample.
\(V_2\) = volume in ml of EDTA used in titration.

2.4.4 Metal content in plant organs

Metal contents in each plant part were determined from oven dried acid digested samples (Brayton, 1992) by using following methods:

2.4.4.1 Copper estimation

Copper content was determined spectrophotometrically at 457 nm following Neocuproine method as per Bureau of Indian Standards (1992).

2.4.4.2 Chromium estimation

Chromium content was determined spectrophotometrically at 457 nm following Diphenylcarbazide method as per Bureau of Indian Standards (2003a).
2.4.4.3 Iron (Ferrous) estimation

Ferrous (iron) content was determined spectrophotometrically at 510 nm following 1, 10 Phenanthroline method as per Bureau of Indian Standards (2003b).

2.4.4.4 Nickel estimation

Nickel content was determined spectrophotometrically at 445 nm following Dimethylglyoxime method as per Bureau of Indian Standards (2003c).

2.4.4.5 Manganese estimation

Manganese content was determined spectrophotometrically at 525 nm following Periodate method as per Bureau of Indian Standards (1964).

2.4.4.6 Zinc estimation

Zinc content was determined spectrophotometrically at 620 nm following Zincon method as per Bureau of Indian Standards (1994).

2.5 In vitro heavy metal removal by *Pseudomonas* species

2.5.1 Isolation and identification of *Pseudomonas* species
Several garden soil samples were collected from different locations. About one gram of soil was added in five ml water to get liquid solution. These soil solutions were inoculated on sterile nutrient agar plates and incubated at 37°C for 24 hours. After 24 hours microscopic and physical analysis was done to identify the colonies of *Pseudomonas* spp. The main remarkable characteristic was bluish-greenish colony on nutrient agar (Plate 1D - F). After identification of the organism the colonies were isolated and re-inoculated on nutrient agar plates to get pure culture (Pleczar et al., 1993). This pure culture was used for the experiments. Pure colonies of *Pseudomonas* spp. were preserved on nutrient agar slants at 7°C till the experiments were completed. Nutrient agar was prepared as under (APHA, 1920):

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>Peptone</td>
<td>1 gm</td>
</tr>
<tr>
<td>Meat Extract</td>
<td>0.3 gm</td>
</tr>
<tr>
<td>NaCl</td>
<td>0.5 gm</td>
</tr>
<tr>
<td>Distilled water</td>
<td>100 ml</td>
</tr>
<tr>
<td>Adjust to pH 7.6</td>
<td></td>
</tr>
</tbody>
</table>

### 1.5.2 Bacterial cell growth conditions

To increase the bacterial cell number King’s B medium was used. It was prepared as follow (www):

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>Proteose peptone</td>
<td>10 gm</td>
</tr>
<tr>
<td>Anhydrous K$_2$HPO$_4$</td>
<td>1.5 gm</td>
</tr>
<tr>
<td>Glycerol</td>
<td>15 gm</td>
</tr>
<tr>
<td>MgSO$_4$ (1 M; sterile)</td>
<td>5 ml</td>
</tr>
</tbody>
</table>
Distilled water 1000 ml
Adjust to pH 7.0

First three ingredients were added in distilled water to bring volume to 1 L; pH was adjusted to 7.0 with HCl and autoclaved. Then 5 ml of sterile 1 M MgSO$_4$ was added.

Two loopful of the bacterium *Pseudomonas* species was transferred aseptically from nutrient agar to King’s B medium to increase the cell numbers. The flasks having medium with *Pseudomonas* cells were kept at 37\(^\circ\) C for 18 - 24 hours with shaking at 200 rpm called incubation.

After incubation optical density of the medium containing *Pseudomonas* cells was measured and dilution was carried out with sterile distilled water if optical density was higher than 0.2. The medium having 0.2 O.D. with *Pseudomonas* cells is called activated cell culture. *Pseudomonas* cells from this culture were used aseptically for the heavy metal removal experiments.

**2.5.3 Heavy metal uptake experiments**

Six different metals namely copper, chromium, ferrous, nickel, manganese and zinc were taken for experiments in form of their salts as shown in the Table 2.1.
2.5.4 Metal solutions

Each metal salt was taken in five different concentrations viz., 100, 200, 300, 400 and 500 ppm. The solutions were prepared in tap water to get natural conditions. Five flasks of 500 ml for each concentration were prepared. All the solutions were autoclaved at 15 lbs pressure and 121° C temperature for 20 minutes. No other growth factors were added in the solution as *Pseudomonas* species can survive/grow in the growth factors present in the tap water. All the sterile flasks were kept at refrigerated condition at below 4° C till the experiments were completed.

2.5.5 Heavy metal uptake reactions

5 ml of activated *Pseudomonas* cells having 0.2 O.D. was inoculated aseptically in the each flask having previously sterile metal solutions. At every 30 minute interval 5 ml sample was taken aseptically and further utilized for the metal estimation. The experiments were carried out up to 330 minutes for the treatment of 100, 200, 300, 400 and 500 ppm solutions of selected metals.

2.5.6 Pre treatment and harvest of microorganism

Three types of metal estimations were carried out:

1. Residual metal concentration.
2. Heavy metal adsorption on *Pseudomonas* cells.
3. Bioaccumulation of heavy metal in *Pseudomonas* cells.
2.5.6.1 Study of residual metal concentration

Each 5 ml sample was centrifuged at 5000 rpm for 10 minutes to separate biomass and supernatant solution. Biomass was used for the study of bioaccumulation and adsorption of heavy metals by *Pseudomonas* cells. Supernatant solution was utilised to estimate residual heavy metal concentration (Patel *et al.*, 2005, Nandan *et al.*, 2003).

2.5.6.2 Heavy metal adsorption on *Pseudomonas* cells

The adsorption of heavy metals on the biomass cell membrane was studied. For this, the biomass was treated with the 2 ml of 50 % ethanol. *Pseudomonas* is gram negative organism in nature and its cell membrane has lipid concentration.

Ethanol treatment dissolves the lipid cell membrane and heavy metal molecules if attached to cell membrane come in solution (Gram, 1884; Pleczar *et al.*, 1993; Prescott, 2002; Patel *et al.*, 2005; Nandan *et al.*, 2003). This solution was used for heavy metal estimation.

2.5.6.3 Bioaccumulation of heavy metal in *Pseudomonas* cells

The biomass obtained from centrifugation was treated with 2 ml concentrated H$_2$SO$_4$ to make cell lyses (Brayton, 1992). The metal molecules accumulated inside as well as on the cell surface of the *Pseudomonas* cells were released in the solution. The concentration of this metal solution was estimated to get total metal uptake. By using
following equation heavy metal bioaccumulation in *Pseudomonas* cells was calculated (Nandan *et al.*, 2003).

Cellular accumulation of heavy metal = Total uptake of heavy metal - Heavy metal adsorbed on the cell surface

### 2.5.6.4 Metal estimation for *Pseudomonas* species

The methods for the estimation of heavy metals bio-accumulated and/or adsorbed by *Pseudomonas* and residual metal concentration were similar to that used for the estimation of metals in plant organs.
Table 2.1: Quantities of metal salts used for the soil preparation in concentration of g/10 kg

<table>
<thead>
<tr>
<th>Sr. No.</th>
<th>Concentration</th>
<th>K₂Cr₂O₇ Chromium</th>
<th>CuSO₄5H₂O Copper</th>
<th>Fe SO₄ 7 H₂O Iron</th>
<th>MnSO₄ H₂O Manganese</th>
<th>Ni SO₄6 H₂O Nickel</th>
<th>ZnSO₄ 7 H₂O Zinc</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Control</td>
<td>Nil</td>
<td>Nil</td>
<td>Nil</td>
<td>Nil</td>
<td>Nil</td>
<td>Nil</td>
</tr>
<tr>
<td>2</td>
<td>0.2</td>
<td>3.3</td>
<td>1.95</td>
<td>2.80</td>
<td>1.04</td>
<td>2.4</td>
<td>2.50</td>
</tr>
<tr>
<td>3</td>
<td>0.4</td>
<td>6.6</td>
<td>3.90</td>
<td>5.50</td>
<td>2.00</td>
<td>4.7</td>
<td>5.00</td>
</tr>
<tr>
<td>4</td>
<td>0.6</td>
<td>10.0</td>
<td>6.0</td>
<td>8.30</td>
<td>3.10</td>
<td>7.1</td>
<td>7.60</td>
</tr>
<tr>
<td>5</td>
<td>0.8</td>
<td>13.3</td>
<td>7.8</td>
<td>11.10</td>
<td>4.20</td>
<td>9.4</td>
<td>10.0</td>
</tr>
<tr>
<td>6</td>
<td>1.0</td>
<td>16.6</td>
<td>9.74</td>
<td>13.84</td>
<td>5.20</td>
<td>11.8</td>
<td>12.6</td>
</tr>
<tr>
<td>7</td>
<td>1.2</td>
<td>19.9</td>
<td>11.7</td>
<td>16.60</td>
<td>6.24</td>
<td>14.20</td>
<td>15.10</td>
</tr>
<tr>
<td>8</td>
<td>1.4</td>
<td>23.2</td>
<td>13.6</td>
<td>19.40</td>
<td>7.30</td>
<td>16.50</td>
<td>17.60</td>
</tr>
<tr>
<td>9</td>
<td>1.6</td>
<td>26.6</td>
<td>15.6</td>
<td>22.10</td>
<td>8.30</td>
<td>18.90</td>
<td>20.20</td>
</tr>
</tbody>
</table>
Table 2.2: Methods applied in biochemical estimation

<table>
<thead>
<tr>
<th>Sr. No.</th>
<th>Parameters</th>
<th>Method</th>
<th>Year</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Total sugar</td>
<td>Nelson, Somogyi</td>
<td>1944</td>
</tr>
<tr>
<td>2</td>
<td>Protein</td>
<td>Lowery <em>et al.</em></td>
<td>1951</td>
</tr>
<tr>
<td>3</td>
<td>Potassium</td>
<td>Bureau of Indian Standards</td>
<td>1993</td>
</tr>
<tr>
<td>4</td>
<td>Phosphorus</td>
<td>Thimmaiah</td>
<td>1991</td>
</tr>
<tr>
<td>5</td>
<td>Calcium</td>
<td>Thimmaiah</td>
<td>1991</td>
</tr>
<tr>
<td>6</td>
<td>Magnesium</td>
<td>Thimmaiah</td>
<td>1991</td>
</tr>
</tbody>
</table>