CHAPTER - 3
MATERIAL
AND
METHODS
Materials and Methods

CHAPTER-3

3.1 Study area

The study was conducted in Rohtak city which is located between 76° 25' and 76° 94' East longitudes and 28° 35' and 28° 80' North latitude, lying at 219.84 meters above sea level. The climate in this area is classified as continental with extremes of heat in summer and markedly cold in winters. The annual rainfall of Rohtak is 455mm, most of which is received during the last week of June to September contributed by South-west monsoon.

3.2 Description of site and sampling

Four different sites were taken from Rohtak city, where electroplating industries are situated. Laxmi precisions screw industries ltd. is the largest electroplating industry in the city and has two different plants (branches). Plant 1 is located nearby residential areas of Rohtak city and Plant 2 of this electroplating industry is surrounded by agricultural land. Vee automatics ltd. And Nav Bharat industries are located outside the residential boundary, still their industrial discharge is contaminating the soil as well as ground water of the city. Plant 1 of Laxmi precisions screw industries ltd. was taken as site 1, plant 2 of Laxmi precisions screw industries ltd. industry as site 2, Vee automatics ltd. as site 3 and Nav Bharat industries as site 4. Soil samples (0–20 cm in depth) were collected from above mentioned different electroplating industries of Rohtak city during March 2009 to March 2010. For each site, 10–20 sub-samples were randomly collected and combined into a composite sample, and 1 kg sub-sample of composite sample was taken back to laboratory for sample preparation and analysis. Before chemical analysis, soil samples were air dried, powdered and sieved through a 2mm sieve. Stones and plant materials were removed manually.

3.3 Physico-chemical characteristics of electroplating contaminated soil

3.3.1 Hydrogen ion concentration

Hydrogen ion concentration was measured by pH meter as per the procedure outlined in Subramanian et al., (1994). This parameter was analysed from 1:2 soil water extract. Warmed up the instrument for 15 minutes. Calibrated the instrument with the known buffer solutions. Immersed the electrode in unknown sample and stirred for three minutes and noted the pH.
3.3.2 Electrical conductivity

Electrical conductivity was measured with the help of EC meter following the procedure of Gupta (2000). Warmed up the instrument for 20 minutes. Rinsed the conductivity cell with distilled water and then with the sample. Temperature and cell constant corrections were adjusted on the conductivity meter. Connected the conductivity cell to meter and dipped in the sample. Passed the current and adjusted the current by rotating the dial in such a way that maximum sensitivity was obtained. Read the conductivity value in ds/m.

3.3.3 Cation Exchange capacity (CEC)

CEC was determined by leaching with NH₄OAc (Ammonium acetate) at pH 7. 25.0 g of soil was added to a 500 mL Erlenmeyer flask. Then added 125 ml of the 1 M NH₄OAc, shook thoroughly and allowed to stand 16 hours. A 5.5 cm Buchner funnel was fitted with retentive filter paper, moistened the paper, applied light suction, and transferred the soil. Soil was gently washed, four times with 25 ml additions of the NH₄OAc, allowing each addition to filter through but not allowing the soil to crack or dry leachate was discarded. Soil was washed with eight separate additions of 95% ethanol to remove excess saturating solution and again leachate was discarded. Adsorbed NH₄ was extracted by leaching the soil with eight separate 25 ml additions of 1 M KCl, leaching slowly and completely as above. Soil was discarded and transferred leachate to a 250 ml volumetric and diluted to volume with additional KCl. Concentration of NH₄-N was determined in the KCl extract by distillation. NH₄-N (Ammonium nitrogen) was also determined in the original KCl extracting solution (blank).

CEC (cmolc/kg) = (NH₄-N in extract - NH₄-N in blank) / 18

3.3.4 Organic Matter

The soil organic matter was estimated by rapid titration method of Walkley and Black (1934) as outlined in Gupta (2000). 1.0g oven dried, ground and sieved sewage irrigated soil was taken in 500 ml titration flask, followed by addition of 20 ml of 1.0N K₂Cr₂O₇ and 20 ml concentrated H₂SO₄. After half an hour the contents of the flask were diluted with 200 ml distilled water. Before titrating it against 0.5N ferrous ammonium sulphate, 10 ml of 85% phosphoric acid and 1.0 ml of diphenylamine indicator were added. At the end point dark blue colour changed to grassy green. A blank was also run simultaneously in order to
standardize the normality of the ferrous ammonium sulphate solution used.

\[
\text{Organic matter (\%) = 10 } (\text{Blank reading} - \text{titration reading}) \times 0.003 \times 100 \\
= \text{Blank reading} \times \text{weight of plant material}
\]

3.3.5 Extraction of available nutrients from soil

For sodium and potassium estimation, 5.0 g of the soil samples were extracted with 100 ml ammonium acetate buffer (pH = 7.0±0.1) for half an hour and then filtered. After extraction, the nutrient elements were estimated by taking aliquots from the extracted samples.

3.3.6 Sodium and Potassium

An aliquot drawn from the extracted material in 25 ml beaker was fed into a flame photometer through capillary. Reading was recorded on the indicator scale after the stabilization of the flame. The total concentration of sodium and potassium was calculated from the calibration curve prepared by taking a range of standard solutions of sodium chloride and potassium chloride.

\[
\text{Sodium or potassium (\%) = mg/l of Na or K from standard curve} \times \text{solution volume} \times \frac{10^4}{\text{sample weight (g)}}
\]

3.3.7 Phosphorus

Phosphorus was determined by Olsen’s method (Olsen et al., 1954). 2.5 g of air dried soil sample was placed in a 125 ml Erlenmeyer flask. To it added a little charcoal and 50 ml of NaHCO₃ solution at 25°C. Shook for 30 minutes on a reciprocating shaker at 120 strokes per minute. Simultaneously a blank was run without the soil sample. The extract was filtered using Whatman No.40 filter paper. 10 ml aliquot of the extract was pipetted in a 50 ml volumetric flask, to it added 10 ml of distilled water and one drop of p-nitrophenol indicator. Contents were acidified to pH 5.0 by adding 2.5M H₂SO₄ dropwise till colour disappears. Added 8 ml of Murphy-Riley solution (Added 250 ml of 2.5M H₂SO₄, followed by 75 ml of ammonium molybdate solution, 50 ml of ascorbic acid solution and 25 ml of antimony tartarate solution. Then added 100 ml of distilled water and mixed on a magnetic stirrer) and brought the volume upto 50 ml with distilled water. After 15 minutes the intensity of blue
Materials and Methods

colour was read at 730nm on spectrophotometer. Measured the amount of phosphorus in 
μg/ml from the standard curve drawn with potassium dihydrogen orthophosphate.

Available Phosphorus = \( P \) (from standard curve) \( \times \) volume of extractant \( \times \) 2.24

Volume of aliquot wt. of soil

3.3.8 Total Nitrogen

The total nitrogen was estimated by Kjeldhal method (Black, 1965) as outlined in 
Gupta (2000). 2 g of sample was placed in the digestion flask. 10 ml sulfuric acid was added 
and swirled until the acid was thoroughly mixed with the sample. Mixture was allowed to 
stand for cooling. Then 2.5 g of the catalyst mixture was added and heated until the digestion 
mixture became clear. Mixture was boiled, gently for up to 5 h so that the sulfuric acid 
condenses about 1/3 of the way up to the neck of the flask or the end of the tube. After 
completion of the digestion step, flask or tube was allowed to cool and 20 ml of water was 
added slowly while shaking. Contents were transferred to the distillation apparatus. 5 ml of 
boric acid was added to a 200 ml conical flask and placed the flask under the condenser of 
the distillation apparatus in such a way that the end of the condenser dips into the solution. 
Then 20 ml of sodium hydroxide was added to the funnel of the apparatus and the alkali was 
run slowly into the distillation chamber. About 100 ml of condensate was distilled and rinsed 
the end of the condenser, and added a few drops of mixed indicator to the distillate and 
titrated with sulfuric acid to a violet endpoint.

Nitrogen (%) = \( \frac{\text{ml standard acid} - \text{ml blank}}{\text{Weight of sample in grams}} \times \text{N of acid} \times 1.4007 \)

3.4 Heavy metals

Total metal concentrations in soil were analysed by the method as outlined in Gupta 
(2000). Added 10 ml of 1:1 HNO₃ to 2 g air-dried soil in a 150 ml beaker. Placed the soil on 
a hot plate, covered with a watch glass and heated at 95°C for 15 minutes. Cooled the digest 
and added 5.0ml of conc. HNO₃. Refluxed for an additional 30 minutes at 95°C. Repeated 
the last step and reduced the solution to about 5.0 ml without boiling. Cooled the sample 
again and added 2.0 ml of deionized water and 3.0 ml of 30% H₂O₂. With the beaker 
covered, heated the sample gently to start reaction. Continued to add 30% H₂O₂ in 1.0 ml
Materials and Methods

increments, followed by gentle heating until the effervescence subsided. Added 5.0 ml of conc. HCl and 10 ml of deionized water and refluxed the sample for an additional 15 minutes without boiling. Cooled and filtered the sample through a Whatman No. 42 filter paper. Diluted to 50 ml with deionized water and analyzed for Cr, Zn, Fe, Cu and Ni by atomic absorption spectrophotometer (AAS Hitachi Z-6100).

3.5 Community analysis

An ecological survey was done from March 2009 to March 2010. Community analysis was carried out during rainy season when majority of the plants were at the peak of their growth. In every study sites, 30 quadrats of 10 m X 10 m (100 sq m) size were randomly laid to study plant species. The herbaceous species was studied by laying 50 quadrats of 1 m X 1 m (1 sq m) size randomly in each study site.

3.5.1 Quantitative analysis

The important quantitative analysis such as density and frequency of tree species, shrubs and herbs species were determined as per Curtis and McIntosh (1950).

(a) Density

Density is an expression of the numerical strength of a species where the total number of individuals of each species in all the quadrats is divided by the total number of quadrats studied.

Density is calculated by the equation:

\[
\text{Density} = \frac{\text{Total number of individuals of a species in all quadrats}}{\text{Total number of quadrats studied}}
\]

(b) Frequency (%)

This term refers to the degree of dispersion of individual species in an area and usually expressed in terms of percentage occurrence. It was studied by sampling the study area at several places at random and recorded the name of the species that occurred in each sampling units. It is calculated by the equation:
Materials and Methods

Frequency (%) = \text{Number of quadrats in which the species occurred} \times 100 \over \text{Total number of quadrats studied}

3.5.2 Importance Value Index

This index is used to determine the overall importance of each species in the community structure. In calculating this index, the percentage values of the relative frequency, relative density and relative dominance are summed up together and this value is designated as the Importance Value Index or IVI of the species (Curtis, 1959).

(a) Relative density

Relative density is the study of numerical strength of a species in relation to the total number of individuals of all the species and can be calculated as:

\[
\text{Relative density} = \frac{\text{Number of individual of the species}}{\text{Number of individual of all the species}} \times 100
\]

(b) Relative frequency

The degree of dispersion of individual species in an area in relation to the number of all the species occurred.

\[
\text{Relative frequency} = \frac{\text{Number of occurrence of the species}}{\text{Number of occurrence of all the species}} \times 100
\]

(c) Relative dominance

Dominance of a species is determined by the value of the basal cover. Relative dominance is the coverage value of a species with respect to the sum of coverage of the rest of the species in the area.

\[
\text{Relative dominance} = \frac{\text{Total basal area of the species}}{\text{Total basal area of all the species}} \times 100
\]

The total basal area was calculated from the sum of the total diameter of immerging stems. In trees, poles and saplings, the basal area was measured at breast height (1.5m) and by using
the formula $\pi r^2$; but in case of herbaceous vegetation it was measured on the ground level by using calipers.

3.6 Plant Analysis

Plant samples were collected from the same sites as the soil samples. Plant identification was confirmed by the Forest Research of India, Dehradun. At least three to five individuals of all plant species were randomly collected within the sampling areas. Fresh plant materials were washed thoroughly with the tap water, cleaned with distilled water and then separated into roots and shoots. All plant parts were oven dried at 72°C for 72 h and then ground to powders. For total metal concentrations in the plant components 0.5 g of plant samples were digested. Heavy metal concentrations in plants were also determined by the method as outlined by Gupta (2000). This method has been explained above, in detail. The total metal concentrations were measured by a flame atomic absorption spectrophotometer (AAS Hitachi Z-6100). To assess the analytical precision, three analytical replicates of each sample, an appropriate standard reference material (from Sigma-Aldrich Company) and a reagent blank were performed in each analytical batch.

3.7 Plant Cultivation, Treatments and Samplings

3.7.1 Pot culture experiment

The effect of heavy metals on sunflower and marigold plants under various concentrations of heavy metals was studied using pot culture. The pot culture experiments were conducted using earthen pots. The soil was collected from non-contaminated fields. The soil was alluvium with sandy loam texture. Before the analysis of some physico-chemical characters of soil, the soil samples were air dried, sieved through a 2mm mesh and homogenized. Then 5.0 kg of air dried soil was placed in each pot and fertilized with NPK. Nitrogen was supplied in the soil as ammonium sulphate (150 mg kg$^{-1}$), phosphorus as potassium dihydrogen phosphate (70 mg kg$^{-1}$) and potassium as potassium sulphate (100 mg kg$^{-1}$) before sowing the seeds. The seeds of marigold (Tagetes erecta, cultivar, Pusa basanti) and sunflower (Helianthus annuus L.) were sown in prepared pots in the month of November. Before sowing seeds were sterilized in 0.1% HgCl$_2$ and soaked in water overnight, and then sown into pots. Soil for HM pots was contaminated with five levels of Cr, viz., 50, 100, 250
and 500 mg Cr kg\(^{-1}\) soil and five levels of Zn, viz. 50, 250, 500 and 1000 mg Cr kg\(^{-1}\) soil. Soil for HM pots was also contaminated with nine levels of binary combinations of Cr and Zn, viz., (1) 50 mg Zn + 50 mg Cr, (2) 50 mg Zn + 100 mg Cr, (3) 50 mg Zn + 250 mg Cr, (4) 250 mg Zn + 50 mg Cr, (5) 250 mg Zn + 100 mg Cr, (6) 250 mg Zn + 250 mg Cr, (7) 500 mg Zn + 50 mg Cr, (8) 500 mg Zn + 100 mg Cr and (9) 500 mg Zn + 250 mg Cr kg\(^{-1}\) soil. The treatments were designed after scanning of literature (Shanker et al., 2005). These metal concentrations were supplied in the form of potassium dichromate (a source of Cr-VI), Zinc sulphate (a source of Zn) dissolved in double distilled water (DDW). Plants without metal treatments served as control. The soil contamination was performed before sowing by adding the correct amount of heavy metals dissolved in distilled water to the soil and mixing it throughout. The prepared pots were placed in field conditions to grow the plants in natural environment. The seeds were germinated at the end of November 2009 to reach a plant density of 3 plants per pot. Distilled water and natural precipitation were used as water sources for plants. Biomass, Cr and Zn metal concentrations and activity of antioxidant enzymes was estimated in roots and shoots in 30, 60, 90 and 120 days old plant. Plants were harvested after 120 days of treatment.

### 3.7.2 Dry Weight of the Plant Parts

Plants were cut at the root–shoot and the petiole–stem junctions in order to divide them into root, stem and leaves. The separated plant parts were dried separately in a hot air oven at 65\(^\circ\)C ± 2\(^\circ\)C for 72 h. The samples were weighed on an electronic top pan balance (Sartorius BL–210S, Germany) to obtain the dry weight of roots, stem, and leaves independently, which was expressed in g per plant.

### 3.7.3 Heavy metal analysis

Cr and Zn metals were analysed at interval of 30, 60, 90 and 120 days in different parts of plants. Method of heavy metal analysis in plants has been explained above.

### 3.8 Enzymatic studies

#### 3.8.1 Preparation of cell free extract

Plant material (roots and leaves separately) was taken and washed with cold distilled water and dabbed dry with several folds of filter paper. Extraction conditions were
standardized with respect to molarity and pH of buffer to achieve maximum extraction of enzymes in leaves and roots. All the steps of extraction were carried out at 0-4°C. The tissue was macerated in chilled pestle and mortar in the presence of 5 ml 0.1 M phosphate buffer (pH 7.5). The homogenate was centrifuged at 10,000 x g for 20 min at 4°C. The supernatant was carefully decanted and used as the crude enzyme preparation.

3.8.2 Enzyme assays

3.8.2.1 Catalase (EC 1.11.1.6)

Catalase activity was determined by the procedure of Sinha (1972). The reaction mixture (1.0 ml) consisted of 0.5 ml of 0.2 M phosphate buffer (pH 7.0), 0.4 ml of 0.2 M hydrogen peroxide and 0.1 ml of properly diluted enzyme extract. After incubating at 37°C for 3 min, the reaction was terminated by adding 3 ml mixture of 5% (w/v) potassium dichromate and glacial acetic acid (1:3 v/v) to the reaction mixture. The tubes were heated in boiling water bath for 10 min. A control was run under similar conditions where enzyme extract was added after stopping the reaction. After cooling the tubes, absorbance of test and control was measured at 570 nm. The amount of residual H$_2$O$_2$ in the reaction mixture was determined by subtracting the absorbance of test samples from that of control. One unit of enzyme activity is defined as the amount of enzyme which catalyzed the oxidation of 1 mmole H$_2$O$_2$ per minute under assay conditions.

3.8.2.2 Superoxide dismutase (EC 1.15.1.1)

The activity of superoxide dismutase was measured by the method of Nishikimi et al. (1972) as modified by Kakkar et al. (1984). The assay mixture contained 50 mM Tris-HCl buffer (pH 8.3), 6.2 μM phenazonium methosulfate (PMS), 30 μM nitro-blue tetrazolium (NBT), 52 μM NADH and varying amount of cell free extract. The reaction was started by the addition of NADH. After incubation at room temperature for 90 seconds, the reaction was terminated by the addition of 1 ml glacial acetic acid and its absorbance was recorded at 560 nm against blank which was without NADH. The reaction mixture without extract gave maximum reduction of NBT. One unit of SOD activity was defined as the amount of enzyme required for 50% inhibition of NBT reduction under assay conditions.
3.8.2.3 Ascorbate peroxidase (EC 1.11.1.11)

The enzyme activity was determined following the oxidation of ascorbic acid (Nakano and Asada, 1981). The reaction mixture (2.7 ml) contained 2.25 ml of 100 mM phosphate buffer (pH 7.0), 0.2 ml of 0.5 mM ascorbate, 0.2 ml of 0.1 mM H$_2$O$_2$ and 0.05 ml of enzyme extract. The reaction was initiated by the addition of H$_2$O$_2$. The decrease in absorbance at 290 nm was recorded spectrophotometrically which corresponded to oxidation of ascorbic acid. The enzyme activity was calculated using the molar extinction coefficient of 2.8 mM$^{-1}$ cm$^{-1}$ for ascorbic acid. One enzyme unit is defined as 1 umole of ascorbic acid oxidized per min at 290 nm.

3.8.2.4 Glutathione reductase (EC 1.6.4.2)

Method of Halliwell and Foyer (1978) was followed for measuring the enzyme activity. The reaction mixture consisted of 2 ml of 0.1 M phosphate buffer (pH 7.5), 0.1 ml of 5 mM oxidized glutathione (GSSG), 0.1 ml of 0.2 mM NADPH and 0.1 ml enzyme extract in final volume of 2.3 ml. The decrease in absorbance at 340 nm due to oxidation of NADPH was monitored. Non-enzyme oxidation of NADPH was recorded and subtracted from it. An extinction coefficient of 6.22 mM$^{-1}$ cm$^{-1}$ for NADPH was used to calculate the amount of NADPH oxidized which corresponded to GR activity. One enzyme unit was defined as 1.0 umole of NADPH oxidized per minute.

3.9 Statistical analysis

Statistical analysis was done by using Microsoft excel and Graphpad prism 5.0 software. All the experiments were conducted in triplicates. The obtained data were statistically analysed for the mean ±S.D. and differences in values for control and treated plants were analyzed by means of Dunnett’s multiple comparison test and Tukey’s test through one way ANOVA taking p≤ 0.05 as significant level. Further, data was subjected to two way ANOVA to determine the effect of treatment conditions, time period and their interaction on various parameters.

52