CHAPTER - III

MATERIAL AND METHODS

Seed Source and preparation

Seeds of *Vigna radiata* (L.) Wilczek Var. Vamban – 2 commonly called green gram of the family Fabaceae were procured from the Seed Centre, Sivakasi. Healthy seeds were surface sterilized with 0.1% sodium hypo chloride (Na$_2$HCO$_3$) (w/v) for a minute, washed with running tap water followed by distilled water and used to find out the effects of various concentrations of mercury and aluminium on the morphometric, biochemical and enzymatic characteristics of it under stress condition.

Preparation of the soil

The soil for raising the cultivars was prepared by mixing red soil, black soil and sandy soil in the ratio of 1 : 1 : 1. The prepared soil was solar sterilised for 5 days. It was then analysed for its physico-chemical properties (Table-1) following the methods mentioned in Table-2.

The analysed soil was taken in earthen pots of size 30×33 cm for about two-third of their height (5 kg of soil per pot).

Experimental design

The aqueous heavy metal solutions of mercuric chloride and aluminium chloride were prepared in different concentrations such as 2 mM, 4 mM, 6 mM, 8 mM and 10 mM (w/v) and stored individually in containers with proper labelling. The earthen pots filled with the prepared soil were also labelled in accordance with the different concentrations of both the metals. In each pot, ten surface sterilized seeds of *Vigna radiata* (L.)
Wilczek were sown and wetted with different concentrations of the prepared metal solutions. A set of plants treated with water was treated as control. From the day of emergence of the first pair of leaves, the plants were wetted on alternate days with the respective concentration of both metal solutions. This practice was continued for 20 days till the plants in the pot treated with maximum concentration started withering. A set of five replicates was maintained for each concentration.

**Choice of bioadsorbents and preparation**

The dried and powdered biomass of *Sargassum wightii* and *Padina commersonii* were tested for metal bioadsorption for the removal of mercury and aluminium from the aqueous solutions. The algae mentioned above were collected from the seashore of Thoothukudi, brought to the lab, washed repeatedly with running water and tap water, shade dried, pulverised, got in powder form and stored in plastic containers.

**Land plant biomass**

Land plants such as *Datura metel* L. and *Typha angustifolia* were collected locally, shade dried, powdered and used to remove the toxic effect of the metals mentioned already.

**Organic amendments**

As Farm Yard Manure (FYM) and vermicompost are commonly used as amendments in conditioning contaminated soil, in the present study also they were used as organic amendments.
Treatment of metal solutions with algal bioadsorbents

Various amounts of each seaweed dry powder such as 2 g, 4 g and 6 g was dissolved in 100 mL of 6 mM solution (The concentration at which toxicity was found to be at optimum level based on LSD analysis Zar, 1984) of mercuric chloride and aluminium chloride, mixed thoroughly, taken in conical flasks and kept under mechanical shaking overnight. This was then filtered and the filtrate was applied to the experimental plants on every alternate day for the number of days for which the plants were treated with untreated metal solutions (20 days). After 20 days of treatment, the plants were analysed in terms of morphometric, biochemical and enzymatic characteristics to understand the absorptive properties of algae.

Treatment of metal solutions with land plant bioadsorbents

The procedure adapted to remove the metal toxicity using algal bioadsorbents was followed in total for the bioadsorbents of land plants as such.

Application of organic amendments

To the soil filled in the earthen pots (each approximately 5 kg capacity), 750 gm of farm yard manure and vermicompost were added (150 gm of FYM / vermicompost / kg soil) separately, mixed thoroughly for uniform distribution, wetted with 6 mM concentrated solution of aluminium chloride and mercuric chloride and the seedlings of *Vigna radiata* (L.) Wilczek. were treated for 20 days. After this period, the morphometric, pigment, biochemical and enzymatic characteristics were analysed.
Morphometric characteristics

For the analysis of the morphometric characteristics, ten seedling were taken from both experimental and control sets and the observation is the average value of ten seedlings along with the standard error.

Length of root

Ten seedlings, each from the control and experimental plants were uprooted with no damage to the root and the length of the root was measured with the help of a meter scale.

Length of shoot

The length of the shoot of the randomly selected ten seedlings of experimental and control plants was measured with the help of a meter scale.

Measurement of leaf area

The total leaf area of each plant was computed and expressed in cm². The leaf area of the harvested leaves was measured by conventional graphical method.

Measurement of fresh weight

The fresh weight of the seedlings was obtained using an electronic balance soon after harvest. Care was taken to avoid wilting of plants.

Measurement of dry weight

The seedlings were dried in hot air oven at 80°C over night after which the dry was measured in an electronic balance to determine the dry weight.
Biochemical characteristics

For all the biochemical analysis, experiment for each character was done five times and the average of it was taken in to account for estimation for both control and treated plants.

Pigment characteristics

Estimation of chlorophyll

To quantify the total chlorophyll from leaves, fresh leaves were deveined and cut into small bits. From the pooled leaf bits, a sample of 100 mg was weighed. The leaf bits were homogenized in 100% acetone using a mortar and pestle. The homogenate was centrifuged at 4000 rpm for 5 minutes at room temperature. Extraction with 100% acetone was repeated till the pellet becomes pale-yellow or white in colour. The supernatant was used for the estimation of photosynthetic pigments. The absorbance was measured at 662 nm, 645 nm and 470 nm for chlorophyll \(a\), \(b\) and carotenoids, respectively using a Systronics spectrophotometer model no:106.

The amount of chlorophyll \(a\), chlorophyll \(b\) and total chlorophyll was calculated using the formulae of Wellburn and Lichtenthaler (1984).

\[
\text{Chlorophyll } a \text{ (mg / L)} = 11.75 \times A_{662} - 2.35 \times A_{645}
\]

\[
\text{Chlorophyll } b \text{ (mg / L)} = 18.61 \times A_{645} - 3.96 \times A_{662}
\]

Total chlorophyll = chlorophyll \(a\) + chlorophyll \(b\)

The carotenoid content was calculated using the following formula:

\[
\text{Xanthophyll + Carotenes} = \frac{1000 \times A_{470} - 2.27C_a - 8.14C_b}{227}
\]
\[ C_a = \text{Total chlorophyll a} \]
\[ C_b = \text{Total chlorophyll b} \]

**Estimation of anthocyanin**

Two hundred mg of leaf samples was incubated in 200 mL of extraction medium. It consists of methanol, distilled water and HCl in the ratio of 50:20:1, respectively. The incubation was extended to 48 hours at 4°C in dark condition with agitation. After 48 hours, the solution was collected and the optical density was measured at 630 and 657 nm. The absorbance value of anthocyanin content was estimated by Mancinelli *et al.*, (1973) method.

\[ \text{Absorbance of Anthocyanin} = A_{530} - 0.3 \times A_{657} \]

**Estimation of total soluble sugar**

One hundred mg of leaf samples was ground in 20 mL of distilled water using mortar and pestle. The homogenate was filtered through two layers of cheese cloth and the filtrate was spun at 3000 rpm for 5 minutes. The pellet was discarded and the supernatant was taken. Three mL of Trichloroacetic acid (TCA) was added to the supernatant. It was thoroughly mixed and kept in ice for 10 minutes. This mixture was centrifuged at 3000 rpm for 5 minutes. The pellet was discarded and the supernatant was used as test solution. An aliquot of 0.1 mL of test solution was taken in a test tube and to this, 0.9 mL of distilled water and 4 mL of anthrone reagent were added. The solution was mixed thoroughly using a cyclomixer and the tubes were kept in boiling water for 10 minutes. Glucose content was measured using standard value (Jayaraman, 1981).
Estimation of protein

The total soluble protein was estimated by Lowry’s Method (Lowry et al., 1951). Fresh leaf samples (100 mg) were ground in 10 mL of distilled water using mortar and pestle. The homogenate was spun at 3000 rpm for 5 minutes. The supernatant was taken and the pellet was discarded. To the supernatant, 1 mL of ice-cold 10 % (w/v) trichloroacetic acid was added and kept in ice for 10 minutes. The extract was centrifuged at 5000 rpm for 10 minutes. The pellet was dissolved in 0.1 N NaOH and used as the test solution.

Reagents for protein estimation

A) CuSO₄ : 0.5% - Solution A

B) Sodium-Potassium tartarate : 1% - Solution B

C) Na₂CO₃ solution in 0.1 N NaOH : 2% - Solution C

The mixture of 0.5 mL of A and 0.5 mL of B with 4.9 mL of solution C is known as alkaline copper reagent. An aliquot of 0.1 mL of test solution was taken in a test tube and 0.4 mL of distilled water, 0.5 mL of freshly diluted (1:1) folin phenol reagent and 5.5 mL of alkaline copper reagent were added. Contents in the tube were mixed immediately and left undisturbed for 10 minutes for the development of blue colour. The absorbance was measured at 650 nm with a Systronics Model 106 spectrophotometer with alkaline copper reagent as blank. The protein content was calculated from a standard graph of protein constructed with bovine serum albumin (BSA) as standard protein.
**Estimation of free amino acids**

Free amino acids were estimated by ninhydrin assay (Jayaraman, 1981) method. The leaf material (200 mg) was ground in 10 mL of ethanol. The homogenate was centrifuged at 5000 rpm for 3 minutes. The pellet was discarded and the supernatant was used as the test solution. To 1 mL of the test solution, 3 mL of distilled water and 1 mL of ninhydrin reagent were added and mixed thoroughly. After mixing, the test tube was kept in boiling water bath for 10 minutes. Then the tube was cooled down to room temperature and 1 mL of 50 % ethanol was added. The absorbance was measured at 550 nm using proper blank. Blank solution consisted of 4 mL of distilled water, 1 mL of ninhydrin and 1 mL of ethanol. The amino acid content was estimated from standard curve prepared with glycine as amino acid source.

**Estimation of proline**

Proline is quantitatively measured by the method of Bates et al. (1973). Fresh leaf material (200 mg) were ground in 10 mL of 3 % sulfosalicylic acid and filtered through a four layered cheese cloth. The volume of the filtrate was noted. To 2 mL of extract, 2 mL of 2 % acid ninhydrin (2 % ninhydrin in the mixture of 6 M orthophosphoric acid and warm absolute glacial acetic acid) and 2 mL of glacial acetic acid were added and kept in a water bath at 100°C for 1 hour. After an hour, the test tube containing solutions were transferred to ice for 10 minutes. Four mL of toluene was added to each solution in the test tube and was thoroughly mixed with the help of cyclomixture. Then, the test tube was allowed to stand for 10 minutes. Coloured proline solution in the test tube was taken out and the absorbance was read at 520 nm. The proline accumulation in the leaf was estimated with the help of a standard proline curve.
**Estimation of leaf nitrate**

The nitrate content of the leaf tissue was determined by the method of Cataldo *et al.* (1978). Fresh leaf material (100 mg) was taken and ground with 10 mL phosphate buffer (pH 7.0). This phosphate buffer contains 0.2 M potassium dihydrogen orthophosphate at pH 7.5. The total volume of the extract was noted down for calculation. Then, the extract was incubated in a water bath for 15 minutes at 100°C. Then the extract was centrifuged at 3000 rpm for 15 minutes. The supernatant was taken for the assay of nitrate contents. To 0.2 mL of supernatant, 0.8 mL of 2% salicylic acid and 19 mL of 2 N NaOH were added. The test tubes were kept in room temperature for 10 minutes for the development of yellow colour. Then, the absorbance was measured at 410 nm. The nitrate content was estimated with the help of a standard nitrate curve.

**In vivo Nitrate Reductase Activity**

*In vivo* Nitrate Reductase (NR) activity was assayed according to Jaworski (1971) method with modification. Fresh leaf material (100 mg) was incubated in scintillation vials containing 5 mL of incubation medium composed of,

- 100 mM KH$_2$PO$_4$ – KOH buffer pH 7.5
- 200 mM KNO$_3$
- 1% (v/v) n-Propanol
- 0.1% (v/v) Triton X 100
Incubation was carried out in dark for one hour at room temperature with occasional shaking. Aliquots of 0.5 mL was taken from the vials and analysed for nitrite after 1 hour incubation.

To 0.5 mL of incubation mixture, 1.5 mL of distilled water, 1 mL of 3 % sulphanilamide and 1 mL of N-1-N (Napthyl ethylene–diamene dihydrochloride) were added in quick succession. Fifteen minutes were allowed for colour formation and absorbance was measured at 540 nm. The nitrite was estimated with the help of a standard nitrite curve.

**Preparation of enzyme extract**

The leaves of experimental plants weighing about 1 g was ground in 5 mL of 100 mM phosphate buffer (pH 6.0) and filtered through a three layered cheese cloth and spun at 3000 rpm for 30 minutes. The supernatant obtained was served as the source for crude enzymes such as peroxidase and catalase.

**Estimation of peroxidase activity**

To assay peroxidase activity, the enzyme extract was added to pyrogallol which gets oxidized to a coloured derivative in the presence of hydrogen peroxide (1% (v/v)). The amount of purpurogallin formed during the reaction was assayed spectrophotometrically (Addy and Goodman, 1972).

To 3 mL of pyrogallol phosphate buffer (0.058 M pyrogallol dissolved in 0.1 M phosphate buffer pH 6.0), an aliquot of 0.1 mL of enzyme extract was added. Then, absorbance was set to zero at 420 nm. To this, 0.5 mL of H₂O₂ (1% (v/v)) was added. Then, the content was thoroughly mixed and the absorbance was measured using systronics model no. 106 spectrophotometer. The difference in the absorbance at an interval of
20 seconds for a period of 3 minutes was measured. The peroxidase activity was expressed as moles of H\textsubscript{2}O\textsubscript{2} reduced per unit enzyme per unit time.

**Estimation of catalase activity**

To assay the catalase activity, 3 mL of phosphate buffer was added to 1 mL of H\textsubscript{2}O\textsubscript{2} and 1 mL of enzyme extract (Kar and Mishra, 1976). The reaction mixture was incubated at 25°C for 1 minute. The reaction was terminated by the addition of 1 mL of H\textsubscript{2}SO\textsubscript{4}. The reaction mixture was titrated against 0.01 N KMnO\textsubscript{4}. The end point was the persistence of pink colour at least for 15 seconds. The catalase activity was expressed in micro moles H\textsubscript{2}O\textsubscript{2} catalysed per unit time per mg protein.

**Analysis of mercury and aluminium in plant material**

The heavy metal accumulation of the experimental plants was analysed at the end of the plant life. Mercury and Aluminium concentrations in plants were analysed using the method of Baker *et al.*, (1994). The plant sample as a whole was washed, dried in oven at 160°C for 40 minutes and digested in a mixture of nitric acid and perchloric acid (10:1). Then the solution was centrifuged at 5000 rpm for 5 minutes and double filtered with Whatmann filter paper no.4 and the filtrate was analysed for mercury and aluminium concentration by Atomic Absorption Spectrometry (Shimadzu Model AA – 6300), available in the Science Instrumentation Center of Ayya Nadar Janaki Ammal College (Autonomous), Sivakasi, Tamilnadu.

**STATISTICAL ANALYSIS**

For morphometric characteristics, the average value of ten independent samples, and for biochemical and enzymatic characteristics the
average value of five findings were considered. The data were reported as mean ± SE and the per cent activity was represented in the parentheses.

Statistical analysis (One way ANOVA – Tukey test) was done using the statistical package, Origin – version 7.0.