CHAPTER II

MATERIALS AND METHODS
MATERIALS AND METHODS

MATERIALS

*Plantago ovata* Forskal. and *Trigonella foenum graecum* Linn. were selected to study their response to heavy metal stress.

*Plantago ovata* Forsk.

Vernacular Names
- English: Spogel seed
- Hindi: Isubgol
- Urdu: Isubgol

PLANT DESCRIPTION

Family: Plantaginaceae
Genus: *Plantago*
Species: *P. ovata*

HABIT AND DISTRIBUTION

It is a large genus of about 200 species belonging to the family Plantaginaceae comprised mainly of herbs and under-shrubs and is distributed mostly in the temperate regions, though a few species grow in the tropics as well. About ten species have been reported from India, of which *Plantago ovata* Forsk. is important for its seeds because of their medicinal importance.

*Plantago ovata* is an annual herb, which under cultivation attains a height of 30-40 cm. It is nearly stemless, soft hairy plant with large narrow linear, 7.5 -20 cm. long and about 0.6 cm. broad leaves appearing whorled due to short terete stem. The spikes are 1.2 to 4 cm. long and about 0.5 cm. broad, cylindrical to ovoid in shape and bears between 45-69 flowers. The fruit is an ellipsoid capsule, about 8mm long containing 23mm long, boat shaped, smooth, rosy white seeds. The concave side of the seed is covered with a thin white membrane produced by fusion of outer layer of
ovule together with the inner epidermis, forming the seed coat. The seed epidermis is constituted of polyhedral cells whose walls are thickened by a secondary deposition which is the source of mucilage (Chopra, 1930). Isabgol husk is a membranous covering of the seed, white to light pink in colour. It is 2-3 mm x 0.5 to 1.0 mm in dimension, translucent and odourless. It absorbs moisture and forms a tasteless mucilaginous substance, which constitutes the drug.

CULTIVATION

*Plantago ovata* is a hardy crop and can be grown on a variety of soils, but it grows well on nutrient rich, well-drained loamy soil. Sowing is started by the end of October continuing up to the middle of December. The crop is given 5-8 irrigations till its maturity and is ready for harvesting within three and a half to four months from the date of sowing. The spikes are harvested in March-April, when they turn red. Harvesting is done in the early morning when little dew is present, which prevents seed shedding.

MEDICINAL IMPORTANCE

The seeds of *Plantago ovata* are mainly valued for their mucilaginous husk, though seeds, which contain about 30 percent mucilage, are also used for medicinal purposes.

The mucilage of the *Isabgol* is colloidal in nature and its composition varies with the conditions of preparation. The seeds are mainly composed of xylose, arabinose and galaturonic acid, while rhamnose and galactose have also been reported. Two polysaccharide fractions have also been separated from the mucilage. One fraction of uronic acid is soluble in cold water and on hydrolysis yields D-xylose, an aldobiouronic acid, l-arabinose and insol residues; the other fraction of uronic acid is soluble in hot water forming a highly viscous solution which sets to a gel on cooling and yields on hydrolysis D-xylose, l-arabinose, aldobiouronic acid and traces of D-galactose (Whistler and Bemiller, (1959) Smith and Montgomery, (1959).
In addition, the seeds contain a semi drying fatty oil, small amount of aucubin and tannin and an active principle exhibiting acetyl-cholris like action. The constituent fatty acids of the oil are; linolenic, linoleic, oleic, palmitic, steric and ligonceric acids (Chopra, 1958; Saghavi, 1962; Pendse and Dutt, 1934; Pendse, 1937; Atal et al., 1964).

Isuhgol seeds and husk are mild laxative, emollient and demulcent. It is considered as a safe laxative, particularly beneficial in cases of habitual constipation, chronic diarrhea and dysentery. It does not irritate the intestine and is specifically used when mucous membrane is disturbed by inflammatory infections. Its action is purely mechanical, the husk swells into a jelly like mass with cold water and mechanically stimulates the intestinal peristalsis. Isuhgol preparations are given after colostomy to assist the production of a smooth solid fecal mass. In indigenous system of medicine the seeds are considered diuretic and cooling agents as well as recommended in febrile conditions and during the infection of kidneys, bladder and urethra. A decoction of seed is prescribed in cough and cold. The crushed seeds made to a poultice are applied to the rheumatic and glandular swellings (Chopra, 1958).

Isuhgol mucilage has a remarkable property as a thickener and is, therefore, also used in ice creams in West. The husk in hot water (1.5% w/v) possess, binding properties superior to 10% w/v of starch mucilage and could thus be used in food industry. The dehusked seed is around 69% by weight of the total seed crop and is rich in starch and fatty oil (Atal et al., 1964), and is used as bird feed.

**Trigonella foenum graecum** Linn.

Vernacular names

English: Fenugreek

Hindi/Urdu: Methi
PLANT DESCRIPTION
Family: Leguminaceae
Genus: Trigonella
Species: T. foenum
Sub-species: graecum

HABIT AND DISTRIBUTION
Trigonella foenum graecum Linn. is nearly smooth, erect annual herb. Leaves 2-2.5 cm long, oblong, toothed, flowers 1-2 in number, pods 5-7.5 cm long, 10-20 seeded. It is the native of Punjab and Kashmir and is also found in the Mediterranean region, Europe, Asia, Australia and South Africa. There are about 70 known species of Trigonella. Trigonella foenum gracum is cultivated in various parts of India.

CULTIVATION
The fenugreek can be cultivated twice in a year. It is both sown as rabi (autumn) as well as a kharif (summer) crop. Summer crop is usually sown in the month of May-June and harvested in August-September. On the other hand rabi crop is usually sown in the month of October-November and is ready for harvest only after the winter is over. The yield of kharif crop is usually higher than the rabi crop.

MEDICINAL IMPORTANCE
The leaves and seeds of fenugreek are rich in proteins, carbohydrates, fats and minerals like calcium, phosphorus, manganese, in addition to vitamins like carotene, thiamine, riboflavin, niacin and vitamin C. Moreover, the seeds also contain alkaloids like trigonelline; saponin, nicotinic acid and essential oils.

The seeds are used as tonic, antipyretic, antihelminthic, appetizer, astringent to the bowels and for curing leprosy, vomiting, bronchitis, piles, remove bad taste from the mouth, and in heart disease. The fenugreek seeds are also used as suppurative, aperient, diuretic, emmenagogue; useful in dropsy, chronic cough, and enlargement of
the spleen and the liver. Seeds are also considered as carminative and aphrodisiac. Seeds are considered as antidysentric and rheumatic and the powdered seeds are still used in many veterinary practices.

The leaves are used both internally and externally on account of their cooling properties. Fenugreek is used during pregnancy, lactation, in diabetes, anemia and for many digestive and respiratory infections and disorders. It is also used as antidandruff and as a beauty aid.

EXPERIMENTAL SITE

Botanical Gardan, Jamia Hamdard Complex, New Delhi, was used as experimental site which is situated in the sylvan surrounding of Tughlagabad on the Mehrauli-Badarpur Road with Tughlag Fort in the east, Qutab Minar in the west, Jahan Panah city forest and Alaknanda complex in the north and overlooking hillocks and ridges in the south. The site is picturesque and away from the din of the Metropolis. The serenity, the hillocks, the ridges and the historical remains all around endow the campus that covers an area of about hundred acres of land with an ideal environment for experimental work.

CLIMATE AND WEATHER CONDITIONS

New Delhi is situated within the geographical coordinates of 28.38' N latitude and 77.11' E longitude at an altitude of 228 meters above the mean sea level. The soil is normally loamy and clay loam with pH around 8-9. It has a semi-arid to sub-tropical climate with extremes of hot weather in summer and cold weather in winter. The maximum rainfall of 80-100 cm is recorded in July and August. Winter showers some times are accompanied with high velocity winds and hailstorms. The humidity increases from 45% in June to 85% in July and August. Wind velocity is usually 2m sec. The maximum temperature in summer occasionally exceeds 45 ° C.

EXPERIMENTAL DESIGN

Experiments were carried out in a randomized block design with three to five replicates. The certified seeds of Plantago ovata Forsk. and Trigonella foenum
graecum Linn., procured from Indian Agricultural Research Institute (IARI) New-Delhi, were sown in earthen pots (12cm in diameter) containing 10 kg of garden soil. The soil was surface sterilized. Sludge and farm compost (3kg / pot) was used as manure and mixed thoroughly with the soil at the time of sowing. Seeds were sown in the month of August when the mean monthly minimum temperature was 26°C and mean monthly maximum temperature was 33°C. After a month of seed germination, individual pots were randomly treated with one of the four concentrations (5, 15, 30 and 50 μg/g soil) of cadmium chloride and (25, 50, 100, 200 μg/g soil) of lead acetate. Untreated plants were used as control. De-ionized water was used for irrigation. Plant were sampled at pre-flowering stage (3rd month), flowering (5th month) and post flowering (6th month) stages. Samplings were done in November, January and March. The metal concentrations used per pot were calculated by the following formula.

\[
\text{Total Molecular Weight of the metal} \times 10^6 \times \text{weight of the soil} \times \frac{\text{Total number of pots used with soil}}{\text{Molecular weight of the metal with salt}}
\]

FIELD OBSERVATIONS

The observations were taken at already stated stages of growth and development and following parameters were studied.

MORPHOLOGICAL PARAMETERS

1) Plant height
   i) Root length
   ii) Shoot length
   iii) Plant length

2) Single leaf area

3) Average leaf area

4) Plant dry weight
   i) Root dry weight
   ii) Stem dry weight
iii) Leaf dry weight
iv) Pod dry weight
v) Seed dry weight

5) Number of branches/plant
6) Number of leaves/plant
7) Number of flowers/plant
8) Number of pods/plant
9) Number of seeds/plant

STRUCTURAL PARAMETERS

1) Foliar studies
   i. Length of stomata
   ii. Width of stomata
   iii. Length of stoma
   iv. Width of stoma
   v. Stomatal density
   vi. Length of trichome
   i. Trichome density

2) Root and stem anatomy
   i. Area of cortex
   ii. Area of vasculature
   iii. Area of pith
   iv. Density of vessel element
   v. Length of vessel element
   vi. Width of vessel element
   vii. Area of vessel element
   viii. Length of xylem Fibre
   ix. Width of xylem Fibre
PHYSIOLOGICAL PARAMETERS
1. Photosynthetic rate
2. Intercellular CO₂ concentration
3. Stomatal conductance
4. Stomatal resistance
5. Pigment contents.
   i. Chlorophyll 'a'
   ii. Chlorophyll 'b'
   iii. Total chlorophyll
   iv. Carotenoids

BIOCHEMICAL PARAMETERS
1. Nitrate reductase activity
2. Nitrate content
3. Nitrogen content
4. Total amino acids
5. Proline content
6. Soluble protein content
7. Soluble sugar content
8. Reducing sugar content
9. Total sugar content
10. Peroxidase activity
11. Catalase activity
12. Leaf sap pH

METAL ANALYSIS
Cadmium and lead estimation in root, stem and leaf.
METHODS

The above listed morphological, anatomical, physiological, and bio-chemical parameters were analysed using the following methods.

MORPHOLOGICAL PARAMETERS

The plant height, root length and shoot length were measured in cm. The shoot length was taken as length of plant axis from the ground level to the upper most growing tip of the main axis. For root length, the main tap root was measured from the ground to the tip. Plant height included the length of entire axis, extending from the root tip to the shoot tip. The dry weight of the root, shoot and leaf samples was determined after drying them in an electric oven at 80°C for 48 hours. The dried samples were weighed on an electronic balance, and the dry weight was expressed in gms.

The average leaf area per plant as well as total foliar area of an individual plant was estimated in cm² making use of a leaf area meter (LICOR-Model 3000A, USA).

ANATOMICAL PARAMETERS

Epidermal peels

The epidermal peels were obtained by the method described by Ghouse and Yunus (1972) using hot HNO₃. The peels, processed in the customary ethanol series for dehydration and stained with iron/ aluminium haematoxylin or Bismark brown (Sass, 1958), were mounted in Canada balsam. Stomatal and trichome dimensions were measured under light microscope using ocular and stage micrometer scales.

Microtomy and Microscopy

The collected samples were fixed in FAA (90 ml + 50% alcohol + 5 ml acetic acid + 5 ml formaldehyde for 100ml solution) and then transferred to a mixture of alcohol, glycerol and distilled water (1:1:2) for softening and preservation. The proportion of various tissue components of stem and root were calculated by the method based on the weights of paper cutting of the camera lucida drawing made on a
trace paper of uniform thickness (Ghouse and Iqbal, 1975). In order to study the variation in size of fibers and vessel elements of stem and root, slices of wood taken from the third internode taken from 1cm below the ground were macerated with hot HNO$_3$ until the component cells became free and loose (Ghouse and Yunus, 1972). Of the macerated elements, 10 vessel members and 10 fibers per sample were measured on a random basis with the help of an ocular micrometer scale, fitted to a compound microscope. Twelve to fifteen micron thick transverse sections were obtained on a Reicherts sliding microtome from the third internode of the stem and the root samples of both treated as well as control plants in order to estimate the average width and frequency of vessels. The sections were stained with haemotoxylin and Bismark brown (Johanson, 1940) and dehydrated in ethanol series and mounted in Canada balsam. Microphotography was done on Venox-S microscope (Olympus, Tokyo-Japan)

PHYSIOLOGICAL PARAMETERS

During the field study of control and metal treated plants, the parameters like, net photosynthesis, stomatal conductance, intercellular CO$_2$, and stomatal resistance were measured by clamping individual leaves into the leaf chamber (6000-13 Quarter litre) of the duly calibrated Portable Photosynthesis Measurement system (LICOR-6200, USA). These measurements were taken between 9:00-11:00 am from five leaves of each plant and from five plants of each treatment.

Chlorophyll/Carotenoid estimation

Reagent(s):
Dimethyl sulfoxide (DMSO)

The chlorophyll and carotenoids were estimated by the method of Hiscox and Israelston (1979). The fresh leaf samples were taken and washed with water. After drying the leaves with blotting papers, leaves were cut into small pieces with scissors. 1gm of leaf tissue was taken and put into five test tubes each containing 7ml of
dimethyl sulfoxide (DMSO) and the test tubes were incubated at 65°C temperature in an electric oven for 2 hours. 1ml aliquot was taken from each test tube and the final volume was made up to 4ml by adding 3ml of DMSO. The same was transferred to a cuvette and the absorbance on a spectrophotometer was taken at 480, 510, 645 and 663 nm.

The chlorophyll and carotenoid concentration was calculated using following formulae of Maclachlan and Zakil (1963) and Duxbury and Yantsch (1956).

\[
\text{Chlorophyll 'a' (mg g}^{-1}\text{ fresh weight)} = \frac{12.30 \times D_{663} - 0.86 \times D_{645}}{d \times 1000 \times w} \times V
\]

\[
\text{Chlorophyll 'b' (mg g}^{-1}\text{ fresh weight)} = \frac{19.30 \times D_{645} - 3.6 \times D_{663}}{d \times 1000 \times w} \times V
\]

\[
\text{Total Chlorophyll (mg g}^{-1}\text{ fresh weight)} = \frac{20.2 \times D_{645} - 8.02 \times D_{663}}{d \times 1000 \times w} \times V
\]

\[
\text{Carotenoid (mg g}^{-1}\text{ fresh weight)} = \frac{7.6 \times D_{480} - 1.49 \times D_{510}}{d \times 1000 \times w} \times V
\]

Where

\[V = \text{Volume of the solution taken}\]
\[d = \text{Length of light path (cm)}\]
\[w = \text{Fresh weight of leaves (gm)}\]
\[D_{480} = \text{Absorbance at 480 nm}\]
\[D_{510} = \text{Absorbance at 510 nm}\]
\[D_{645} = \text{Absorbance at 645 nm}\]
\[D_{663} = \text{Absorbance at 683 nm}\]
BIOCHEMICAL PARAMETERS

Nitrate reductase activity (NR-activity)

Reagents:

Phosphate buffer: 3.56 gm. of 0.2 M Na₂HPO₄ were dissolved in 100 ml distilled water to make solution A. In another flask, 3.12 gm. of 0.2 M Na₂H₂PO₄ were dissolved in 100 ml distilled water to make solution B. The solution B was added to solution A so as to get pH 7.0.

Potassium nitrate (KNO₃ 0.4 M): It was prepared by dissolving 4.04g KNO₃ in 100 ml distilled water.

NEDD (0.02 %): Prepared by dissolving 0.02 g NEDD in 100 ml distilled water.

Sulphanalamide (1% in 1N HCl) was prepared by dissolving 1g sulphanalamide in 1N HCl.

1N HCl was prepared by dissolving 8.4ml in 91.6ml DDW.

The methods of Klepper et al., (1971) and Mann et al., (1979) were used to determine nitrate reductase activity. 0.3 gm. of fresh leaf material was chopped into slices of about 3mm size and kept in vials containing 3ml of 0.4M potassium nitrate. In blank samples 3ml of distilled water was added instead of potassium nitrate and all the vials were kept in ice for sometime. The vials were evacuated in a vacuum desiccator for 2-3 minutes, then incubated in dark for 60 minutes in a water bath shaker at 33°C and finally the tubes were placed in boiling water bath for five minutes to stop the enzymatic activity. For developing the colour, 1ml of sulphanalamide, 1ml of NEDD and 4 ml of distilled water was added. The vials were kept in dark for some time for colour development. Finally the absorbance was recorded at 540nm. The corresponding concentration of nitrate reductase was determined against the standard curve prepared by using NaNO₂ solution. The enzymatic activity was expressed on per gram dry weight basis.
Materials and Methods

Nitrate estimation

Reagents:

Acetone (10%): It was prepared by dissolving 10ml acetone in 90 ml distilled water.

Activated Charcoal (0.05 gms)

Catalytic solution: It was prepared by dissolving 31.36 mg of CuSO₄ in 100 ml of distilled water to get solution A, of which 10 ml were taken, to which 10 mg of ZnSO₄ was added. The final volume was made up to 100 ml

NEDD (0.02 %): It was prepared by dissolving 0.02 gm NEDD in 100 ml distilled water.

Sodium hydroxide (NaOH 0.1 N): The same was prepared by dissolving 0.4g NaOH in 100ml distilled water.

Sulphanalamide (1% in 1N. HCl): Prepared by dissolving 1 gm sulphanalamide in 1 N HCl.

1N HCl was prepared by dissolving 8.4ml in 91.6ml DDW.

Hydrazine sulphate: Prepared by dissolving 152 g of hydrazine sulphate in 100 ml distilled water.

The nitrate content was estimated by chemical hydrazine reduction method (Grover et al., 1978). To 0.5gms of fresh leaf material in test tubes, 0.5gm of activated charcoal and 10ml of distilled water was added. The contents were boiled for 2-3 minutes at 100°C. After boiling, the solution in the flasks was filtered through Whatman No2 in a beaker. Residue was again re-extracted two to three times and extract was poured together. After cooling 1ml of aliquot was taken to which 0.5ml of copper sulphate and zinc sulphate solution, 0.25ml 0.1N NaOH, 0.25ml hydrazine sulphate and 1.5ml double distilled water were added in the test tubes, which were incubated for 10 minutes at 33°C. After that, the tubes were kept in ice and 0.5ml acetone was added to the reaction mixture. 1ml of sulphanalamide and 1ml of NEDD were added subsequently to develop colour. The absorbance was recorded at 540nm wavelength using a UV-visible spectrophotometer. The nitrate concentration was
determined against a standard curve prepared by using potassium nitrate solution. The nitrate content was calculated on per gram dry weight basis.

**Nitrogen estimation**

**Reagents:**

**Sulphuric acid**

**Ortho-Phosphoric acid**

**Hydrogen Peroxide (30%):** It was prepared by dissolving 30ml hydrogen peroxide in 70ml distilled water.

**Potassium sulphate**

**Selenium powder**

**Nessler's reagent:** 100 gm of NaOH were dissolved in 400 ml of distilled water to make solution A. In another flask 100 gm of HgI₂ and 70 gm of KI were dissolved in 400ml of distilled water to make solution B. Solution A was then mixed with a cool solution B with continuous stirring. Final volume was made to 1000ml with distilled water and mixture was kept at room temperature for 24h. The solution was then filtered and used.

Nitrogen content was estimated by the method of Linder (1944). The plant material was digested according to a modified version of Kjeldahl procedure. The oven dried samples were ground in a pestle and motor and sieved through 70mm mesh sieve. 1gm. powder was weighed and transferred to 100ml flask. 2ml sulphuric acid and 2ml ortho- phosphoric acid was added and heated at 300-350°C till it turned black. Each digestion tablet contained 1.5g of potassium sulphate and 0.0075g of selenium as catalyst. Samples were cooled and 1 ml of hydrogen peroxide (30%) was added. After further heating, the digested samples turned colourless. To 0.5ml aliquot, 2.0ml Nessler's reagent was added and final volume was made upto 5.0ml by adding 2.5ml double distilled water. The mixture was kept for 20 minutes at room temperature for colour development. The absorbance was taken at 525nm using UV-visible spectrophotometer. The nitrogen content was calculated against the standard curve of
ammonium sulphate. The nitrogen content was expressed on per gram dry weight basis.

**Total amino acids**

**Reagents:**

**Absolute Alcohol**

Glycerol (55%): Prepared by mixing 55 ml glycerol in 100 ml distilled water.

Ninhydrin (1%) solution: Prepared by dissolving 1g ninhydrin in 100 ml of 0.5 M citrate buffer (pH 5.6).

Citrate buffer (0.5M pH 5.6): Prepared by dissolving 21.02 g of citric acid monohydrate in 100ml distilled water to make solution A. In another flask 27.41 g of trisodium acetate was dissolved in 100 ml of distilled water to make solution B. The solution A was added to solution B drop wise to get pH 5.6 for the buffer.

The total amino acid content was estimated by the method of Lee and Takahshi (1966). 0.5 gm. fresh leaf samples were kept overnight in 5ml of absolute alcohol. The used alcohol was discarded and 5ml alcohol was again added and the tissues were ground in a mortar and pestle. The homogenate was centrifuged at 5500 r.p.m for 10 minutes at 4°C. The supernatant was taken and the alcohol was evaporated by boiling the vials in water. To the pellet 10ml citrate buffer was added (0.5M pH 5.6). From this 0.5 ml aliquot was taken to which 1.2ml of 55% glycerol and 0.5 ml of ninhydrin (1% in citrate buffer) was added. The contents were boiled for twenty minutes till bluish purple colour developed. The contents were then cooled and volume was made up to 6ml by adding citrate buffer. The absorbance was recorded at 570nm. The total amino acid concentration was determined against the standard curve prepared by using different concentration of glycine. The amino acid content was expressed on per gram dry weight basis.
Chapter II

Materials and Methods

Proline estimation
Reagents:
Absolute Alcohols
Sulphosalicylic acid (3%): 3ml of sulphosalicylic acid was added in 97ml of distilled water.
Acid ninhydrin: Prepared by dissolving 125g of ninhydrin in a mixture of 30 ml of glacial acetic acid and 20 ml of 6N-orthophosphoric acid. The final volume was made up to 100ml with distilled water.
6-N orthophosphoric acid: It was prepared by adding 38.1 ml of ortho phosphoric acid in 61.9ml of distilled water.
Toluene
Acetic acid

The proline content was estimated by the method of Bates et al., (1973). 0.3 gm. of plant material was homogenised in 10ml of 3% sulphosalicylic acid. The homogenate was filtered and the supernatant was collected. To 2ml supernatant, 2ml acetic acid and 2ml acid ninhydrin was added followed by heating in water bath at 100°C for 1hour. The contents were kept in ice for terminating the reaction. 6ml toluene was added and mixed vigorously. A chromatophore patch appeared at the top. Toluene was used as blank. The absorbance recorded was taken at 520nm. The concentration of proline was determined against the standard curve prepared by using different concentrations of proline. The proline content was expressed on per gram dry weight basis.

Soluble protein estimation
Reagents:
Phosphate buffer: 3.56 gm. of 0.2 M Na₂HPO₄ were dissolved in 100 ml of double distilled water to make solution A. In another flask, 3.12 gm. of 0.2 M Na₂H₂PO₄ were
dissolved in 100 ml double distilled water to make solution B. The solution B was added to solution A so as to get pH 7.0 for the buffer.

Bradford's reagent 100-mg coomassic brilliant-blue G-250 was dissolved in 100ml 90% ethanol. To this solution, 100 ml of 85 % of phosphoric acid were added and stirred well. This was diluted with distilled water to a final volume of 1 litre.

Trichloracetic acid (TCA) (10%) was prepared by dissolving 10 gm. of TCA in 100 ml double distilled water.

Sodium hydroxide (0.1N) was prepared by dissolving 0.4 gm. of NaOH in 100 ml double distilled water.

The soluble protein content was estimated by the method of Bradford (1979). 0.5 gm. of fresh leaf material was homogenized in 5ml of 0.2M phosphate buffer pH 7.5. The homogenate was filtered through muslin cloth and centrifuged at 6000 r.p.m for 10 minutes at room temperature. The supernatant was treated with 10ml of trichloracetic acid and centrifuged at 6000 r.p.m for 5 minutes at room temperature. The pellet obtained was washed 2-3 times with distilled water and dissolved in 1ml of 0.1 NaOH. For reading the O.D, 0.1 ml of aliquot was taken and volume was made up to 1ml with distilled water followed by addition of 5ml of Bradford’s reagent. Similarly blank was prepared by taking 1.0ml of distilled water and 5ml of Bradford’s reagent. O.D was read at 545 nm. The soluble protein concentration was determined by using bovine serum albumin (BSA) as the standard. The protein content was expressed on per gram dry weight basis.

ESTIMATION OF SUGARS

Soluble sugar content

Reagents:

Ethanol (90%) was prepared by adding 10ml of distilled water to 90 ml ethanol.
Phenol (5%) was prepared by adding 95 ml distilled water to 5 ml of phenol.

The soluble sugar content was estimated by the method of Dey (1990). 0.1 gm leaf material was incubated in 10 ml alcohol for 1 hr. at 60°C. The extract was then decanted into a 25 ml volumetric flask and the residue was re-extracted. Final value was made up to 25 ml by adding alcohol. 1 ml of aliquot (containing 20-100 µg sugar) was transferred to a thick walled test tube and 1.0 ml of 5% phenol was added to it and mixed thoroughly by vertical agitation with a glass rod. The test tubes were air cooled and absorbance was measured at 485 nm on spectrophotometer. The corresponding concentration was determined against a standard curve prepared using glucose as the standard. The soluble sugar content was expressed on per gram dry weight basis.

Reducing sugar content
Reagents:
Alkaline copper reagent: 40 g of sodium carbonate, 7.5 g of tartaric acid, 4.5 g of copper sulphate were added and mixed in 400 ml of distilled water. The contents were mixed thoroughly and were further diluted to 1 litre with distilled water.
Phospho molybedate solution: 35 gm. molybedic acid, 5 gm. sodium tungstate were dissolved in 200 ml of NaOH. To it 200 ml of double distilled water was added and then boiled for 20-40 minutes. The volume was reduced to 250 ml. It was cooled and to it 125 ml concentrated phosphoric acid was added. The volume was made up to 500 ml with distilled water.
Saturated solution of lead acetate: Lead acetate was dissolved in a 250 ml beaker containing distilled water till its saturation.
Saturated disodium hydrogen phosphate: Disodium hydrogen phosphate was dissolved in 250 ml beaker containing distilled water till its saturation.
Ethanol (95%): 5 ml distilled water was added to 95 ml ethanol.
Ethanol (80%): 20 ml distilled water was added to 80 ml ethanol.
The reduced sugar content was estimated by Folin-Wu (1988) method. 5 gm. of fresh plant samples were weighed to which 95% ethanol was added and the contents were boiled on hot plate. 80% ethanol was added and the extraction was done twice. The supernatant was collected and the volume was made up to 100 ml with distilled water. From this, 50 ml aliquot was evaporated on hot water bath (100°C) till a small volume was left. The final volume was made to 50 ml by distilled water. The aliquot was treated with 1 ml saturated lead acetate solution and was then filtered into a beaker containing 3 ml of saturated disodium hydrogen phosphate, till precipitation disappeared. The supernatant was collected in a beaker. 5 ml aliquot was taken to which 1 ml alkaline copper reagent was added and the contents were boiled for 10 minutes. To this 2 ml phospho molybdate was added and the volume was made to 10 ml by adding distilled water. The absorbance was taken to 620 nm. The reduced sugar concentrations were estimated by the standard curve of glucose. The reducing sugar content was expressed on per gram dry weight basis.

Total Sugar content

The total sugar was estimated by adding soluble sugar and reduced sugar and was expressed on per gram dry weight basis.

ENZYMATIC ACTIVITY

1 gm fresh leaf samples were weighed and 10 ml of 0.1 M cold phosphate buffer (pH-7.0) containing 5 mM cysteine was added. The tissue was homogenised and the extract was centrifuged at 6000 r.p.m for 30 minutes. The supernatant was taken for quantifying catalase and peroxidase activity.

Peroxidase activity

Reagents:

Extraction solution-0.1 M cold phosphate buffer (pH 7.0) containing 5 mM cysteine.

i) 0.2 M NaOH: Dissolve 0.8 gm. NaOH in 100 ml distilled water
ii) 0.2 M Na₂HPO₄: Dissolve 3.56 gm. Na₂HPO₄ in 100 ml distilled water
Add x ml of 0.2M NaOH to 50 ml of 0.2 M Na2HPO4 and dilute to 100 in following manner.

<table>
<thead>
<tr>
<th>X ml. of 0.2 M NaOH</th>
<th>pH</th>
</tr>
</thead>
<tbody>
<tr>
<td>3.5</td>
<td>5.8</td>
</tr>
<tr>
<td>5.8</td>
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<tr>
<td>30</td>
<td>7.0</td>
</tr>
<tr>
<td>35</td>
<td>7.2</td>
</tr>
<tr>
<td>40</td>
<td>7.4</td>
</tr>
</tbody>
</table>

5-mM cysteine was dissolved (0.006078) in 10-ml buffer.

Phosphate buffer (125 µM): 12.5 ml of phosphate buffer in 100 ml of volumetric flask and diluted up to the mark with distilled water.

Pyrogallol (50µm): Dissolved 0.01261 g of pyrogallol in 100 ml double distilled water (1mM). Take 5 ml of this solution and added 95 ml distilled water. This solution was 50µm.

Hydrogen peroxide solution (50µm): 1.12 ml of H2O2 was dissolved in water and volume made up to 100 ml with distilled water. This solution represented 100 mM. 1 ml of this solution was diluted up to 100 ml to make 1mM solution. 5 ml of this solution were diluted up to 100 ml to get 50 µM solution.

Sulphuric acid (5% v/v) 2ml H2SO4 diluted to 100ml with distilled water.

Diethyl ether

To 1ml aliquot 2ml of 125µM phosphate buffer, 1ml 50µm pyrogallol and 1ml 50M hydrogen peroxide was added and the contents were incubated for five minutes at 25°C. The reaction was terminated by adding 0.5 ml of 5% Hydrogen peroxide. Purpurogallin formed by adding 10ml diethyl ether was extracted and the absorbance was measured at 430 nm. The blank was prepared by terminating the enzyme activity at zero minutes. The enzyme activity was expressed as nmole/min/mg of protein (Polle et al., 1994).

Catalase activity

Reagents:

Phosphate buffer 30 µm (pH 7.0) 29.17 ml of phosphate buffer was diluted to 100 ml of distilled water.
Hydrogen peroxide (100 µM)
Sulphuric acid (2%) 2ml H₂SO₄ was diluted to 100ml by adding 98ml distilled water.
Potassium nitrate (0.02 N)

1 ml aliquot of the above mentioned supernatant was taken to which 2 ml of 30 µM phosphate buffer (pH7.0), 2ml of 100 µM hydrogen peroxide was added followed by incubation at 25°C for 1 minute. After incubation period, 2 ml of 2% sulphuric acid (H₂SO₄ 2% v/v) was added for terminating the reaction and the residual hydrogen peroxide was titrated against 0.02 N Potassium nitrate until a faint pink colour persisted for few seconds. The blank was run likewise, in which enzyme activity was terminated at zero minutes. The absorbance recorded was at 430 nm. The enzyme activity was expressed as picno mole/mint/mg of protein (Koto and Shimizu, 1987).

pH of leaf tissue

1g leaf sample was crushed in 10ml distilled water and centrifuged at 5000 r.p.m. for half an hour (30 minutes) and the pH was determined using a calibrated pH meter.

HEAVY METAL ANALYSIS
Reagents
Nitric acid
Hydrochloric acid
Selenium dioxide: 4.1grms of SeO₂ was dissolved in 50ml of distilled water.

Seventy-two mesh screened dried samples of leaf, stem, root and soil of control and treated plants (1 gm) were digested using nitric acid and hydrochloric acid till it became transparent using hydrogen peroxide as oxidising agent. The digested material was filtered in 100 ml volumetric flask and the volume was made up to 100 ml with distilled water. Heavy metal (cadmium and lead) were determined in each sample through atomic absorption spectrometer (AAS).
STATISTICAL ANALYSIS

The data collected on various morphological, anatomical, physiological and biochemical parameters of the plants grown under control (untreated) and various concentrations of lead acetate and cadmium chloride were statistically analysed through following procedures.

MEAN \(\bar{x}\) = Arithmetic mean or simple mean or the so called average value was computed taking the sum of the number of observations \((x_1 + x_2 + x_3 + \ldots + x_n)\) and dividing it by the total number of observations.

\[
\bar{x} = \frac{x_1 + x_2 + x_3 + \ldots + x_n}{n}
\]

Or

\[
\bar{x} = \frac{\sum x_i}{n}
\]

Where

\(x_1, x_2, x_3, \ldots, x_n\) are the observations
\(n\) = total number of observations
\(\bar{x}\) = arithmetic mean.

STANDARD DEVIATION (S.D)

The dispersion in data was studied making use of standard deviation. Standard deviation or standard range of observations is the positive square root of the average of squared deviation of all the observations about the mean and was calculated as follows for large and small samples.

\[
S.D = \pm \frac{\sqrt{(x-x_1)^2 + (x-x_2)^2 + \ldots + (x-x_n)^2}}{N}
\]

For small samples S.D. = \(N-1\)

\[
S.D. = \pm \frac{\sqrt{(x-x_1)^2 + (x-x_2)^2 + \ldots + (x-x_n)^2}}{N-1}
\]
Where

\[ X = \text{Mean of observations} \]

\[ x_1, x_2, x_3, \ldots, x_n = \text{Number of observations} \]

\[ N = \text{Total number of observations} \]

**PERCENT VARIATION:**

To show and compare relative variability of two or more sets of measurements in entirely different units, percent variation was calculated. The magnitude of variation that was present between the mean of two treatments relative to average of the reference treatment was selected as standard for comparison. The variation was calculated as under:

\[
\% \text{ Variation} = \frac{\bar{X}_{\text{cont}} - \bar{X}_{\text{treated}}}{\bar{X}_{\text{cont}}} \times 100
\]

\[ \bar{X}_{\text{cont}} = \text{Mean of control samples} \]

\[ \bar{X}_{\text{treated}} = \text{Mean of treated samples} \]

**TEST OF SIGNIFICANCE**

It was used to find out whether the observations mean values differ significantly from each other or the difference was merely due to chance and to establish the validity of the result. Student’s t-test was applied in the present study to test significance of differences between two sample means.

The following formula was used to compute t-values, which were compared with expected tabulated values of ‘t’ at particular degrees of freedom. If the calculated ‘t’ value exceed the table value at a particular degree of freedom, the result was considered as significant. If the calculated ‘t’ value was less than the table value at certain degree of freedom, the result was interpreted not significant.
Chapter II

Materials and Methods

\[ t = \frac{\bar{X}_{\text{cont.}} - \bar{X}_{\text{treated}}}{\sqrt{\frac{S.D_{\text{cont.}}}{n_1} + \frac{S.D_{\text{treated}}}{n_2}}} \]

't' = Difference of the sample mean
't' = Standard error of difference

Where

\( \bar{X}_{\text{cont.}} \) = Arithmetic mean of control sample
\( \bar{X}_{\text{treated}} \) = Arithmetic mean of treatment sample
\( S.D_{\text{cont.}} \) = Standard deviation of control sample
\( S.D_{\text{treated}} \) = Standard deviation of treatment sample
\( n_1 \) = No. of observations of control sample
\( n_2 \) = No. of observations of treatment sample

DEGREE OF FREEDOM:

The degree of freedom was calculated as:

\[ \text{D.f.} = n_1 + n_2 - 2 \]

Where \( n_1 \) = number of observations on one sample
\( n_2 \) = number of observations on other sample.