EXPERIMENT I: EFFECTS OF HEAVY METALS ON GROWTH AND METABOLISM OF MAIZE AND WHEAT SEEDLINGS (PETRIPLATE EXPERIMENT):

Maize (Zea mays L. var. Ganga Safed-2) and Wheat (Triticum aestivum L. var. Sonalika) were selected for the study. Heavy metals namely copper, cadmium and mercury in the form of their chloride salts CuCl₂, CdCl₂ and HgCl₂ were used as a source of heavy metals. The following concentrations of each heavy metal were prepared.

(i) 50 µg/ml, (ii) 100 µg/ml, (iii) 150 µg/ml, (iv) 200 µg/ml, (v) 250 µg/ml and (vi) 300 µg/ml DW.

The uniform graded 10 seeds of maize and wheat were germinated in sterilized petriplates (9 cm D) lined with Whatmann filter paper no.1. The media for germination were DW (control) and above mentioned concentrations of each metal separately. The experiment was carried out at 28±2°C under laboratory conditions. The percentage germination was recorded after 24h in case of wheat and after 48h in case of maize. The effects of heavy metals on growth and metabolism were studied at the intervals of 24h. In case of maize the data were recorded on completion of 48, 72, 96 and 120h. Wheat seedlings were studied after 24, 48, 72 and 96h. The details are as follows:
A. STUDY ON SEEDLING GROWTH:

Twenty seedlings from each treatment were studied for elongation, fresh weight and dry weight. The elongation of root and shoot was recorded. Mean was calculated and expressed as cm/seedling. Root shoot ratio was also calculated. Embryo and endosperm from the seedlings were separated. Their fresh weights were recorded and expressed as mg/seedling. The embryo and endosperm were dried at 80°C for 48h, then dry weights were recorded and expressed as mg/seedling. The percent moisture was calculated using the following formula:

\[
\text{Percent moisture} = \frac{\text{Fresh weight} - \text{Dry weight}}{\text{Fresh weight}} \times 100
\]

The effects of heavy metals on seedling growth was evaluated through Relative Root Growth (RRG) and it is calculated according to the formula given by Ouzounidou et al. (1992). The formula is given below:

\[
\text{RRG} = \frac{\text{Mean length of longest roots in toxic solution}}{\text{Mean length of longest roots of control}} \times 100
\]

Relative Growth Rate (RGR) was another parameter for studying the effects of heavy metals on seedling growth. Relative growth rate is determined as differences between Naperian logarithms of fresh weight of successive days.
samples. The formula of Relative Growth Rate (Blackmann, 1919) is as follows:

\[ RGR = \log_e W_1 - \log_e W_2 \]

where \( W_0 \) = initial fresh weight of seedlings,
\( W_1 \) = successive fresh weight of seedlings.

B. STUDY ON METABOLISM:

The embryo and endosperm in triplicates of DW and heavy metal treated seedlings were analysed for the following enzymic activities and metabolite contents:

1. Carbohydrate metabolism:
   i) \( \alpha \)-amylase and \( \beta \)-amylase activities
   ii) Starch content (endosperm)
   iii) Invertase activity
   iv) Non-Reducing and reducing sugar contents

2. Protein metabolism:
   v) Protease activity
   vi) Protein content
   vii) Total amino acid content
   viii) Proline content

3. RNA Metabolism:
   ix) RNAse activity
   x) RNA content
4. **Phosphatases:**
   
   xi) Acid phosphatase activity

5. **Oxidative enzymes:**
   
   xii) Peroxidase activity
   
   xiii) Polyphenol oxidase activity

6. **Phenolic substances:**
   
   xiv) Total phenol content.

   For enzymatic activities, embryo and endosperm were crushed in ice-cold DW and centrifuged at 2000 RPM. Supernatant was the source of enzyme. The enzymic activities were expressed on the basis of protein. The following is the method for enzymatic protein determination.

**Enzyme protein:**

Enzyme protein was estimated by the method of Lowry *et al.* (1951). To 1ml aliquot, 4ml 12.5% sodium carbonate and 1ml 0.1% copper sulphate were added, incubated for 30 minutes and 0.5ml folin-phenol reagent was added. After incubating for 10 minutes optical density (OD) was recorded at 660 nm on Systronics 106 spectrophotometer. The following regression formula was calculated by using known concentrations of casein.

\[ X = 236.6Y - 35.22 \]

where \( Y \) = optical density.

The protein content was expressed as \( \mu g/mg \) fresh weight.
1. Carbohydrate Metabolism:

1) α-Amylase & β-Amylase Activities:

These activities were determined by estimating total amylase activity and α-amylase activity. Total amylase activity was determined using the method of Paleg et al. (1962) and it was as follows:

1.0 ml enzyme aliquot was mixed with 1.0 ml citrate buffer (pH 5) and 1.0 ml of 1% starch solution and incubated for 30 minutes at room temperature. The blank had 1.0 ml of DW instead of the enzyme extract. The enzyme activity was stopped by adding 0.5 ml of cold iodine solution (2.5g of iodine and 25 g of potassium iodide in one litre of 0.05N HCl). The final volume was made (20 ml) and OD was read at 600 nm. The difference in OD between blank reaction i.e. starch hydrolysed was calculated using the regression formula

\[ X = 331.49Y - 2.34 \]

Amylase activity was expressed as μg starch hydrolysed/30 min/mg protein.

α-amylase activity was determined by killing the β-amylase. For this, enzyme aliquot was heated at 70°C for 20 minutes then it was used for determination of α-amylase. Same procedure was used for the determination and calculation of enzyme activity. The value of β-amylase activity was calculated by subtracting the value of α-amylase activity from the value of total amylase activity.
11) Starch content:

The method of Malik and Singh (1980) was used for estimation of starch. The plant tissue was boiled in 80% alcohol, homogenized and sugars were removed by centrifugation. The residue was dissolved in 10 ml DW and boiled for 45 minutes in water bath at 100°C. Tubes were cooled and volume was made upto 10 ml with DW and centrifuged. Starch was estimated by taking 1 ml aliquot + 1 ml citrate buffer (0.02 M, pH 5) + 1 ml I₂KI and incubated for 5 minutes at room temperature. Final volume was made to 20 ml with DW. OD was recorded at 600 nm. The amount of starch (mg/g dry wt) was calculated using the regression

\[ X = 331.49Y - 2.34 \]

iii) Invertase activity:

Invertase activity was determined by the method of Hatch and Glasziou (1963). To 1 ml enzyme aliquot, 1 ml acetate buffer (pH 4.8) and 1 ml 0.25% sucrose (250 mg sucrose in 100 ml acetate buffer pH 4.8) was added. Blank had 1 ml acetate buffer instead of 0.25% sucrose. This was incubated for one hour at room temperature. Enzyme activity was stopped by adding 2 ml 5% perchloric acid and volume was made 10 ml. 1 ml of this mixture was taken to develop colour by Nelson-Somogyi method (Wharton and McCarty, 1972). (Method is mentioned under the method for reducing and total sugars). OD was taken at 540 nm on Systronics 106 spectrophotometer. By using the regression equation
amount of reducing sugars released by invertase activity was calculated and expressed as \( \mu g \) glucose liberated/h/mg protein.

iv) Reducing and Non-reducing sugar contents:

Weighed plant material (oven dried) was boiled in 80% ethanol for 4 to 5 minutes, homogenized with sand and centrifuged. Residue was again extracted with 5 ml ethanol. After centrifugation, the ethanol was evaporated and the residual sugars were dissolved in a fixed volume (20 ml) of distilled water.

From the 20 ml of the extract, 10 ml each was taken for reducing sugars and total sugars. To the extract of total sugars, 3 ml 1N HCl (8.75 ml of conc. HCl + 91.25 ml distilled water) was added and kept in boiling water bath for 20 minutes to hydrolyse non-reducing sugars. It was cooled and neutralized by adding 3 ml 1N NaOH. 1 ml of 25% lead acetate and 1 ml of 25% sodium carbonate were added in both the sets and volume was made 20 ml, then it was filtered. 1 ml of the aliquot was taken for sugar estimation by the method of Nelson-Somogyi (Wharton and McCarty, 1972).

To 1 ml of the above aliquot was added 1 ml of Nelson-Somogyi reagent (Nelson reagent A: 12.5 g sodium carbonate, 12.5 g sodium potassium tartarate, 10 g sodium bicarbonate
and 100 g sodium sulphate were dissolved and volume was made up to 500 ml with distilled water and Nelson reagent B: 15 g copper sulphate in 100 ml distilled water and 2 drops of conc. H$_2$SO$_4$).

Nelson reagent was prepared by mixing 50 parts of A with 2 parts of B. Tubes were capped with glass marbles and heated in a water bath at 100°C for 20 minutes then cooled rapidly. 1 ml arsenomolybdate reagent (25 g ammonium molybdate dissolved in 400 ml distilled water. To this was added 21 ml conc. H$_2$SO$_4$ + 3 g sodium arsenate in 25 ml DW and volume was made to 500 ml) was added and shaken thoroughly for 5 minutes to dissolve the red ppt. Final volume was made to 25 ml with distilled water. OD was taken at 540 nm on Systronics spectrophotometer 106. By referring to the regression equation of glucose,

\[ \text{X} = 426.67Y - 15.25 \]

amount of reducing sugar and total sugars were calculated, and expressed as mg/g dry weight. Non-reducing sugar was calculated from this data.

2. Protein metabolism:

v) Protease activity:

The method described by Penner and Ashton (1967) was followed for protease activity. The reaction mixture containing 2 ml of enzyme extract, 1 ml of 0.5% casein solution (pH 7.0) and 3 ml of 0.1M phosphate buffer (pH
7.0), was incubated for one hour at room temperature. Then 2 ml of the reaction mixture was mixed with 2 ml of 15% TCA. After 20 minutes, the precipitated protein was discarded by centrifugation. To 1 ml of enzyme aliquot, 1 ml of DW, 4 ml of 0.5N NaOH and 1 ml of folin-phenol reagent were added. After 30 minutes, the OD was recorded at 660 nm on spectrophotometer. The regression formula was made by reacting known amounts of tyrosine with folin-phenol reagent and activity was expressed in terms of μg tyrosine liberated/h/mg protein.

\[ X = 232.14Y - 0.71 \]

vi) Protein content:
Protein was determined using the method of Lowry et al. (1951). Weighed amount of oven-dried plant material was ground in 80% ethanol and extracted twice. The residue was first washed with cold 5% perchloric acid (to remove sugars and soluble protein fractions), centrifuged and secondly, it was washed with mixture of ethanol : ether : chloroform in the ratio of 2:1:1 (to remove acid soluble fractions and lipids) and centrifuged. The protein fraction was dissolved in 1N sodium hydroxide and kept for one hour and centrifuged. The supernatant was made upto 5 ml with sodium hydroxide and used as an aliquot. To 2 ml of the above aliquot was added 5 ml of Lowry reagent C (prepared by mixing 50 ml reagent A which is 2% sodium carbonate in 0.1N NaOH and 1 ml of reagent B which is 0.5% copper sulphate in
1% sodium potassium tartarate) and incubated at room temperature for 30 minutes. The colour was developed by adding 0.5 ml folin phenol reagent. After 10 minutes, OD was read at 660 nm on Systronics 106 Spectrophotometer. The following regression equation was prepared by using known concentration of casein.

$$X = 236.6Y - 35.22$$

The protein content was expressed as mg/g dry weight.

vii) Total amino acid content:

The content of amino acids was determined following the method of Harding and McClean (1916). The reaction system contained 0.5 ml of ethanol extracted from the material, 1 ml 10% pyridine and 1 ml of 2% ninhydin reagent was stoppered and heated in a water bath at 100°C for 30 minutes. Violet blue colour was developed. Later it was cooled and diluted with distilled water to a final volume of 10 ml. OD of the violet blue colour was read at 570 nm on spectrophotometer. The following regression formula was prepared using isoleucine as standard.

$$X = 413.42Y - 19.23$$

The amino acid content was expressed as mg/g dry weight.

viii) Proline content:

The method described by Bates et al. (1973) was followed to estimate free proline. Weighed oven dried plant
material was extracted in 3% sulphosalicylic acid. After centrifugation, 2 ml of the aliquot was added to 2 ml of acid ninhydrin reagent (1.25 g of ninhydrin dissolved in 30 ml of glacial acetic acid and 20 ml of 6M phosphoric acid) and 2 ml of glacial acetic acid. The tubes were stoppered and the reaction mixture was allowed to boil in water bath at 100°C for 1 hour and the reaction was terminated in ice-bath. Then the reaction mixture was extracted against 6 ml toluene with a separating funnel. The absorbance of the toluene extract was read at 520 nm against toluene blank. The proline content was calculated using the regression equation

\[ X = 49.776Y - 0.286 \]

Proline was expressed as mg/g dry weight.

3. RNA Metabolism:

ix) RNAse activity:

RNAse activity was determined by the modified method of McDonald (1955). 1 ml of 0.4% RNA in 0.2M acetate buffer pH 5.0 (30 ml of 0.2M acetic acid i.e. 1.2 ml acetic acid in 100 ml DW and 70 ml of 0.2M sodium acetate i.e. 2.7 g in 100 ml DW) was added to 0.5 ml enzyme extract and incubated for 30 minutes at 37°C. Reaction was stopped by adding 1 ml of uranyl acetate (0.75% in 25% perchloric acid) and centrifuged for 5 min. 1 ml of supernatant was mixed with 5 ml orcinol reagent (10 ml of 1% orcinol, 40 ml of conc. HCl and 1 ml of 10% FeCl₃) and boiled for 8 minutes in waterbath.
at 100°C. The mixture was cooled and OD was read at 660 nm on Spectrophotometer 106 against DW blank instead of enzyme. Difference between blank and reaction was noted and RNAse activity was calculated and expressed as μg RNA utilized/30 min/mg protein. The following regression formula was used:

$$X = 99.588Y + 1.376$$

x) RNA content:

The content of RNA was determined by the method of Markhan (1955). A known weight of the plant material was crushed in 80% ethanol and centrifuged. The supernatant was discarded and the residue was first washed with 5% perchloric acid to remove acid soluble substances and again with a mixture of ethanol : ether : chloroform (2:1:1) to remove lipids. The residue was then dissolved in 1M TCA (cold) and centrifuged. Finally the residue was dissolved in 0.3M KOH and incubated for 42h at 37°C. After incubation the mixture was centrifuged and the pH of the supernatant collected was adjusted to 3. Then final vol. 10 cc was made with 0.3M KOH.

To a known volume of aliquot (2 ml), 6 ml of orcinol reagent was added and heated at 100°C for 10 minutes. The mixture was then cooled and OD was read at 660 nm on spectrophotometer. Control for the reaction was made with 2 ml DW instead of RNA aliquot and 6 ml of orcinol reagent. The following regression formula was made with known amount
of RNA.

\[ X = 99.588Y + 1.376 \]

RNA was expressed as mg/g dry weight.

4. Phosphatases:

xi) Acid phosphatase activity:

Acid phosphatase (APase) activity was determined by the method of Murray and Collier (1977). To 0.2 ml enzyme extract, 0.6 ml of 4-nitrophenolphosphate (5 mM in 0.05M acetate buffer pH 5.0) was added and incubated for 30 minutes at 37°C. Reaction was stopped by adding 4 ml 0.1N NaOH (0.4 g NaOH in 100 ml DW). In control 0.2 ml DW was taken in the place of enzyme. The OD of the yellow colour developed due to the liberated p-nitrophenol was read at 420 nm against blank. The acid phosphatase activity was calculated referring to the standard concentration of p-nitrophenol and expressed as \( \mu \)g p-nitrophenol liberated/30 min/mg protein using the following regression:

\[ X = 67.794802Y - 1.022901 \]

5. Oxidative enzymes:

xii) Peroxidase activity:

George (1953) and Maehly (1954) method was employed to assay peroxidase activity. The reaction mixture having 2 ml enzyme aliquot, 2 ml 0.2M phosphate buffer (pH 7.0) and 2 ml 20 mM guaicol reagent (0.22 ml guaicol in 100 ml distilled water, was prepared 24 hours before carrying out
the estimation) was taken in a cuvette. The cuvette was placed in the spectrophotometer 106. The wavelength was adjusted to 470 nm and OD was noted. 2 drops of 10 μM hydrogen peroxide (0.4 ml H$_2$O$_2$ of 20 volumes in 9.6 ml distilled water) was added to the reaction mixture in the cuvette and again the OD was noted after 30 seconds. The difference in OD before and after adding H$_2$O$_2$ to the reaction mixture was used to calculate peroxidase activity. Peroxidase activity was expressed as OD of colour developed/min/mg protein.

xiii) Polyphenol oxidase activity:

Polyphenol oxidase activity was determined by the method of Kar and Mishra (1976). For this, 0.5 ml enzyme extract was mixed with 2 ml phosphate buffer (pH 7.0), 2 ml of pyrogallol (50 μM = 12.6 mg in 100 ml DW) and incubated at room temperature for 5 minutes. Similarly blank had 0.5 ml enzyme extract and 4 ml phosphate buffer. OD was taken at 420 nm. Polyphenol oxidase activity was calculated and expressed as OD/10 min/mg enzyme protein.

6. Phenolic substances:

xiv) Total phenol content:

It was determined using the method of Farks and Kiraly (1962). The reaction mixture containing 0.5 ml of the ethanol extract, 1 ml of 20% sodium carbonate and 0.5 ml folin phenol reagent was heated in a water bath for 10 minutes, cooled and diluted to a fixed volume of 5 ml with
distilled water. It was filtered using Whatmann filter paper No.1 to remove the precipitates. The OD of the blue coloured filtrate was read at 660 nm. Phenol content was expressed as mg/g dry weight. The following regression formula was prepared using gallic acid as standard:

\[ X = 96.05Y + 10.03 \]

EXPERIMENT II: EFFECTS OF HEAVY METALS ON GROWTH AND METABOLISM OF MAIZE AND WHEAT SEEDLINGS (POT EXPERIMENT):

The experiment was carried out in earthen pots (16 cm D) filled with refined, thin, acid washed silica sand (Agarwala and Sharma, 1976). Before sowing the seeds, the following concentrations of copper chloride, cadmium chloride and mercuric chloride separately were mixed throughly with sand.

(i) 50 mg/kg sand, (ii) 100 mg/kg sand, (iii) 150 mg/kg sand, (iv) 200 mg/kg sand, (v) 250 mg/kg sand and (vi) 300 mg/kg sand.

The sand without addition of any heavy metal was considered as control. Uniform graded 15 seeds of maize and wheat were sown in each pot separately and 5 pots were kept for each treatment. Necessary irrigation was done. Experiment was carried out at 28±2°C under laboratory conditions. On completion of 6, 8 and 10 days, 20 seedlings at random were studied for their growth and metabolism.
A. STUDY ON SEEDLING GROWTH:

The elongation of root and stem was recorded from 20 seedlings. Mean was calculated and elongation was expressed as cm/seedling. Leaf number was also noted and expressed as no/seedling. Root, stem, leaf and endosperm of 20 seedlings were separated and their fresh weight and dry weight were recorded as per method described in Expt. I. Percent moisture was calculated on fresh weight basis. RRG and RGR were calculated as described earlier.

B. STUDY ON METABOLISM:

The uppermost fully unfolded leaf, in triplicates, of 6, 8 and 10 days old control and treated seedlings were analysed for enzymic activities and metabolites. The biochemical parameters were same as studied in Expt. I.

C. STUDY ON PHOTOSYNTHETIC PIGMENTS:

The photosynthetic pigments using the method of Arnon (1949) were determined in replicates from 10 days old control and heavy metal treated maize and wheat seedlings.

ESTIMATION OF PHOTOSYNTHETIC PIGMENTS:

Weighed fresh leaf material is crushed in 80% acetone (80 ml acetone + 20 ml DW) with a pinch of sand. The homogenate is filtered using Whatmann No.1 filter paper and the filtrate was made upto a specific volume. The absorbance of the chlorophyll suspension was read on a spectrophotometer (Systronics-106) at 480, 510, 645 and 663
The following formula were used to calculate the quantity of photosynthetic pigments.

Chlorophyll 'a' (mg/g fr wt) = 12.7 (D663) - 2.69 (D645)
Chlorophyll 'b' (mg/g fr wt) = 22.9 (D645) - 4.68 (D663)
Total chlorophyll (mg/g fr wt) = 20.2 (D645) - 8.02 (D663)
Carotenoids (mg/g fr wt) = 7.6 (D480) - 1.49 (D510)

where D = Optical Density at

STATISTICAL ANALYSIS:

For finding out the significance of heavy metal treatments on metabolism, the data on biochemical parameters of both the experiments were subjected to Analysis of Variance using Fisher's method (1954). Critical difference at 5% was also calculated.