
CHAPTER—II

MATERIAL AND METHODS

A. MATERIAL :

The seeds of Trigonella foenum graecum L. (Fenugreek, Methi) and Amaranthus blitum L. Var oleracea Hooker (Tambada Pokla) of reputed companies were collected from the local market.

They were sown in the well ploughed plots of m² in the fields of the university campus. The seedlings were raised under normal conditions of temperature, light and irrigation, without the addition of any fertilizer. Plants were well irrigated every alternate day. They were harvested at the time of their peak vegetative growth. (After about 20 - 25 days growth).

The harvested plants were thoroughly cleaned, washed with distilled water and surface dried. Some plants from the same harvest were treated with 50mM NaCl solution. For this the intact plants were immersed in NaCl solution for 10 min and surface dried.

The following were the samples prepared according to their method of storage and subjected to storage in refrigerator.

The temperature maintained was $5^{\circ} \pm 2^{\circ}$ C

1. Material covered with polyethylene bag.
2. Material uncovered.
3. Sprayed (NaCl immersion) material covered with polyethylene bag.
4. Sprayed material uncovered.

Some plants from the same harvest were also kept outside, in the laboratory, at normal temperature and light conditions, covered with polyethylene bag.

5. Material covered with polyethylene bag. (normal temperature).
6. Sprayed material covered with polyethylene bag. (normal temperature).

The plant material was analysed for various physiological parameters, after 48 and 96 hours of storage. Everytime, freshly harvested material was used as a control for analysis. Experiment was carried out in triplicate.

The plant material was analysed for moisture level, chlorophyll, carotenoid, carbohydrate, polyphenol, soluble protein, proline, nitrate and ascorbic acid contents. Changes in the level of peroxidase, catalase and nitrate reductase as influenced by storage were also investigated. Absorption spectrum of photosynthetic pigments was also determined.

Chlorophylls, carotenoids, polyphenols, soluble proteins, proline, nitrate and ascorbic acid contents, moisture level, absorption spectrum of photosynthetic pigments were estimated from the fresh material, while, carbohydrates (total sugars and starch) were determined from oven dried (control as well as treated or stored material) material. Enzymes were prepared from fresh plant material.

B. METHODS :

a. Moisture :

5 g of the plant material (root, stem and leaves separated) was subjected to oven at about 60° C temperature for 8 days. The difference between the fresh weight and constant dry wt was recorded and used to calculate moisture content (%).

b. Photosynthetic pigments :

i. Chlorophylls :

Chlorophylls (a, b and a + b) were estimated from acetone extract, following the method by Arnon (1949). For estimation, randomly sampled leaves were homogenized with a pinch of Mg CO₃ and extracted with 80% acetone. The extract was filtered through Whatman No 1 filter paper using Buchner's funnel under suction.

Final volume of the filtrate was made to 100 ml with 80% acetone. Absorbance was read at 663 nm for chlorophyll 'a' and 645 nm for chlorophyll 'b' on double beam spectrophotometer (Shimadzu, Japan).

Chlorophyll 'a' Chlorophyll 'b' and total Chlorophylls were calculated using the following equation of Mackinney - Arnon (1949).

$$\text{Chlorophyll 'a'} = 12.7 \times A_{663} - 2.69 \times A_{645} = 'x'$$

$$\text{Chlorophyll 'b'} = 22.9 \times A_{645} - 4.68 \times A_{663} = 'y'$$

$$\text{Total Chlorophylls} = 8.02 \times A_{663} + 20.20 \times A_{645} = 'z'$$

$$\text{Chlorophyll a/b/total} = \frac{X/Y/Z/ \times \text{volume of extract} \times 100}{(\text{mg } 100^{-1} \text{ g fr. wt}) \quad 1000 \times \text{weight of plant material (g)}}$$

Values are expressed in $\text{g } 100^{-1} \text{ g dry wt.}$

ii. Carotenoids :

Carotenoids were estimated from the same acetone extract of chlorophylls, according to the method of Kirk and Allen (1965). Carotenoids were recorded by the absorbance reading at 480 nm on double beam spectrophotometer (Shimadzu, Japan). Carotenoids were calculated with the following formula.

$$\text{Total Carotenoids (mg. } 100^{-1} \text{ g fr wt)} = \frac{A_{480} \times \text{volume of extract} \times 10 \times 100}{2500 \times \text{weight of material in g.}}$$

Values are expressed as g 100^{-1} g dry wt.

iii. Absorption spectrum of photosynthetic pigments :

Absorption spectrum was determined by reading the absorbance of acetone extract of the plant material at various wave lengths (Mackinney, 1938), from 340 to 740 nm with a difference of 10 nm each time, on double beam UV - VIS spectrophotometer (shimadzu, Japan). The graph of absorbance against wave length was drawn to obtain smooth curve of absorption spectrum of photosynthetic pigments.

c. Carbohydrates :

Estimation of total sugars and starch was done by Nelson (1944) method.

For extraction of total sugars and strach from root, stem and leaves, oven dried material was used.

i. Extraction of sugars :

0.5 g oven dried powdered material was homogenized in 40 ml of 80% alcohol. The extract

was filtered through Whatman No 1 filter paper under suction. The filtrate so collected was condensed on water bath under reduced pressure to about 2 - 3 ml in evaporating dish. The filtrate was cooled and to it was added 1 g each of lead acetate and potassium oxalate, and the volume was made to 40 ml with distilled water. It was filtered through Whatman No. 1 filter paper under suction. 3 ml concentrated HCl was added to the collected filtrate, which was hydrolysed for 15 min at 15 lbs pressure. The filtrate was allowed to cool and was neutralised with Na_2CO_3 . It was again filtered through Whatman No. 1 and the final volume of the filtrate was made to 70 ml with distilled water.

ii. Extraction of starch :

Residue obtained out of first filtration was used for the extraction of starch. To the above residue 4 ml conc. HCl and 40 ml distilled water were added. It was hydrolysed for 15 min under 15 lbs pressure, and later allowed to cool. It was neutralized with Na_2CO_3 and filtered through Whatman No. 1 filter paper, and the final volume of the filtrate was adjusted to 60 ml with distilled water.

For assay, 0.2 ml filtrate for soluble sugars and 0.1 ml filtrate for starch were mixed separately with 1 ml Somogyi's alkaline copper tartarate

solution (4 g of $\text{Cu SO}_4 \cdot 5\text{H}_2\text{O}$; 24 g of anhydrous Na_2CO_3 , 16 g - K tartarate and 180 g of anhydrous Na_2SO_4 dissolved in 1000 ml of distilled water). It was boiled in the waterbath for 10 min, then allowed to cool to room temperature. To this was added 1 ml of Nelson's arsenomolybdate reagent (25 g of $[(\text{NH}_4)_6 \text{Mo}_7\text{O}_{24} \cdot 4\text{H}_2\text{O}]$ dissolved in 450 ml of water, were mixed with 21 ml of conc. H_2SO_4 . Then 3 g of $\text{Na}_2\text{HAsO}_4 \cdot 7\text{H}_2\text{O}$ dissolved in 25 ml of water were added to the above mixture). The contents were diluted to a total volume of 10 ml and the absorbance was read at 560 nm on a spectrophotometer (Milton Roy and Co.).

For the standard curve, standard glucose solution (0.1 mg ml^{-1}) at different concentrations was taken and the procedure was repeated. Blank was prepared in the same manner without adding glucose standard. Sugar and starch contents were calculated by using the following formula.

Sugar and Starch =

$$\frac{\text{Absorbance reading} \times 0.1 \times \text{volume of Extract} \times 100}{1000 \times \text{volume of extract taken for estimation} \times \text{Absorbance reading for 1ml Std glucose} \times \text{wt of plant material (g)}}$$

Value are expressed as $\text{g } 100^{-1} \text{ g dry wt.}$

d. Polyphenols :

Polyphenols were estimated following the method by Folin and Dennis (1915). The acetone extract prepared for estimation of chlorophylls was used as a source of polyphenols. For estimation of polyphenols the following ingredients were thoroughly mixed and the absorbance of each reaction mixture was recorded at 660 nm on spectronic 20 equipped with red filter (Milton Roy and Co.).

10 ml of the extract was mixed with 10 ml of 20% Na_2CO_3 (20 g of Na_2CO_3 in 100 ml distilled water). The volume was made to 35 ml with distilled water. To this was added 2 ml of Folin - Dennis reagent (mixture of 100 g sodium tungstate and 20 g phosphomolybdic acid in about 800 ml distilled water, 50 ml 85% orthophosphoric acid were added and refluxed for 2 hours). Final volume was made to 50 ml with distilled water. Standard polyphenol solution was prepared from tannic acid (0.1 mg ml^{-1}). Std. polyphenol (tannic acid) solution at different concentration was taken to prepare standard curve.

Polyphenols were calculated by the following formula.

Polyphenols (g 100⁻¹ g fresh wt) =

$$\frac{\text{Absorbance reading for plant extract} \times \text{Std tannic acid} \times \text{volume of extract} \times 100\text{g}}{\text{ml}^{-1} (\text{mg}) \times \text{wt of plant material} \times 1000}$$

Values are expressed as g 100⁻¹ g dry wt.

e. Soluble proteins :

Soluble proteins were estimated from roots, stem and leaves by the method of Lowry et al. (1951). Plant material was extracted in distilled water and filtered through Whatman No 1 filter paper under suction. Final volume of the extract was made to 20 ml with distilled water.

For assay 0.05 ml of extract was taken to which was added 5 ml of reagent 'c' (reagent 'c' was prepared from 50 ml of reagent 'a' : 2% Na₂CO₃ in 0.1 N aqueous NaOH, mixed with 1 ml of reagent 'b' : 0.5% CuSO₄ · 5H₂O in 1% sodium tartarate). It was allowed to remain for 15 min at room temperature. To it was added 0.5 ml of Folin - Phenol reagent [mixture of 100 g sodium tungstate mixed with 25 g sodium molybdate, 700 ml distilled water, 50 ml 85% phosphoric acid and 100 ml conc HCl, was refluxed gently for 10 hours using water condenser. To this 150 g lithium sulphate, 50 ml distilled water and a few drops of bromine water were

added. The mixture was boiled for 15 min and later cooled. The mixture was adjusted to 1 N by titrating it against 1N NaOH and the final volume was made to 10 ml. The assay mixture was kept for 30min at room temperature to develop colour. Absorbance was read at 660 nm on spectronic 20 (Milton Roy and Co.) by using red filter. Blank was prepared with distilled water, reagent 'c' and Folin - phenol with final volume 10 ml as above.

Std protein was prepared from albumin (10 mg in 100 ml distilled water). Standard protein solution of different concentrations was used to prepare standard curve of protein. The colour was allowed to develop for 30 min and the absorbance was read at 660 nm.

Proteins were calculated from the formula

$$= 4 \times \frac{B}{A} = g \ 100^{-1} \ g \ \text{fresh wt}$$

'B' - extract reading, 'A' - reading for std. protein solution.

Values are expressed as $g \ 100^{-1} g$ dry wt.

f. Proline :

Free proline in different parts of the plant (roots, stem and leaves) was estimated following

the method of Bates et al. (1973). The material was homogenized in 10 ml of 3% sulfosalicylic acid and then filtered through Whatman No 1 filter paper. 1 ml of the filtrate was mixed with 2 ml acid ninhydrin reagent (mixture of 2.5 g ninhydrin, 60 ml glacial acetic acid and 40 ml 6 M orthophosphoric acid, heated for 5 - 10 min) and 2 ml acetic acid. The contents were boiled for 1 h at about 100° C on boiling water bath. It was rapidly cooled in ice - bath. 4 ml of toluene were then added, mixed rapidly and absorbance of toluene chromophore at room temperature was recorded at 520 nm against toluene blank. Standard curve of proline (0.1 mg ml⁻¹) was prepared taking different concentrations such as 0.2, 0.4, 0.6 and 0.8 ml of proline.

Proline content was calculated by the formula

$$\text{Proline (mg } 100^{-1} \text{ g fresh wt)} = \frac{200 \times \text{Reading of 1 ml extract}}{\text{Absorbance reading for 1 ml Std proline}}$$

The values are expressed in g 100⁻¹ g dry wt.

g. Nitrate :

Nitrate content was estimated following the method put forth by Cataldo et al. (1975) 0.5 g of plant material was extracted in cold distilled water, and filtered through double layered muslin cloth. The

volume of the filtrate was made to 20 ml. The filtrate was centrifuged at 5000 x g for 10 min and the supernatant was used to determine the nitrate content.

The assay mixture contained :

0.1 ml extract + 0.8 ml 5% (w/v) salicylic acid in conc H₂SO₄. After 30 min, 19 ml 2 N NaOH was added slowly to adjust the pH to 12.

The sample was allowed to cool and the absorbance was read at 410 nm.

Blank was prepared as follows.

1 ml distilled H₂O + 0.8 ml Salicylic acid in conc H₂SO₄ + 19ml 2 N NaOH

Nitrate content was calculated as follows

$$\frac{A \times 50(\text{mg NO}_3) \times \text{volume of Extract} \times 1}{\text{Std reading} \times \text{volume of extract taken} \times \text{wt of plant material}} = \text{mg NO}_3 \text{ g}^{-1} \text{ fr. wt.}$$

Values are expressed as mg NO₃ g⁻¹fresh tissue.

h. Ascorbic acid :

Ascorbic acid was estimated following the method described by Dhopte and Phadnawis (1989) 0.5

g of the leaves were homogenised in 2% oxalic acid (20 g oxalic acid in 1000 ml distil water) The aliquot was filtered through muslin cloth and the final volume was made to 50 ml with 2% oxalic acid.

Standardization :

The burette was filled with the dye 2, 6 - Dichlorophenol indophenol (100 mg 2, 6 -DCPIP was dissolved in 250 ml warm distilled water). 10 ml of standard ascorbic acid [10 mg ascorbic acid dissolved in 100 ml 2% oxalic acid] was slowly titrated against 2,6 - DCPIP until a pink colour was obtained, which persisted for few seconds. The first appearance of the pink colour was taken as an end point. The ml of dye consumed for 10 ml of ascorbic acid solution were equivalent to 1 mg of ascorbic acid.

Analysis of the sample :

10 ml of aliquot was titrated against the dye. The volume of the dye consumed was noted as 'y' ml. 'x' = 10 ml of std ascorbic acid = 'x' ml 2 - 6 -DCPIP (std reading). Ascorbic acid was calculated by the following formula.

$$\frac{Y}{X} = \% \text{ AA (ascorbic acid)}$$

Values are expressed as $\text{mg } 100^{-1} \text{ g dry wt.}$

1. ENZYMES :

i) Hydroxyperoxidases :

1) Peroxidase (E.C. 1.11.1.7) :

Peroxidase was determined by the method described by Maehly (1954).

0.5 g plant material (roots and leaves) was extracted with cold distilled water in a chilled mortar with pestle. It was filtered through two layers of cheese cloth. Final volume of the filtrate was made to 20 ml with distilled water. The filtrate was centrifuged at $5000 \times g$ for 10 min and the supernatant was stored at $0-4^{\circ}\text{C}$ temperature. The supernatant served as the source of an enzyme peroxidase.

Assay mixture for peroxidase was prepared by mixing.

a) 2 ml phosphate buffer (pH 7.0) [0.2 M NaH_2PO_4 195 ml + 0.2 M Na_2HPO_4 . 305 ml were mixed and diluted to 1000 ml with distilled water].

b) 1 ml - 20 mM Guaiacol [2.5 ml guaiacol in 1000 ml hot distilled H_2O].

c) 0.05 ml enzyme preparation.

The assay mixture was taken in a cuvette and zero absorbance was adjusted at 470 nm. Then 0.04 ml H₂O₂, 10 mM [0.6 ml 30% H₂O₂ solution in 1000 ml distilled water] was mixed thoroughly with the help of a thin clean glass rod and absorbance was read at 470 nm after 30 and 60 sec. Blank was prepared by mixing 2 ml phosphate buffer (pH. 7.0), 1 ml guiacol and 0.05 ml enzyme. Enzyme activity was calculated by using the formula.

$$\frac{(30 \text{ Sec. reading} \times 2 + 60 \text{ Sec reading})}{2} = 'X'$$

$$[('X') \times 800] = \Delta \text{ O. D. min}^{-1} \text{ g}^{-1} \text{ fresh tissue.}$$

Values are expressed as $\Delta \text{O. D. min}^{-1} \text{ g}^{-1}$ fresh tissue.

2. Catalase (EC. 1.11.1.6) :

Activity of this enzyme was determined, following the method described by Herbert (1955).

0.5 g plant material was extracted in cold distilled water with final volume to 20 ml. It was filtered through muslin cloth and centrifuged at 5000 x g for 10 min. The supernatant served as the source of enzyme catalase.

The assay mixture for the enzyme was prepared as follows.

1 ml of 0.45 MH_2O_2 / 10 mM H_2O_2 [0.6ml H_2O_2 in 100 ml distilled H_2O].

+

2 ml phosphate buffer, pH 7.8, 0.1 M.

+

0.5 ml enzyme preparation.

After 5 min the reaction was terminated by adding 5 ml 1 N H_2SO_4 . Then 1 ml 10% KI [10 g KI in 1000 ml distilled water with a drop of Ammonium molybdate] was added. Liberated iodine was titrated against 0.01 N $\text{Na}_2\text{S}_2\text{O}_3$. (2.482 g in 1000 ml distilled H_2O) Starch was used as an indicator. [1g starch in 90 ml hot distilled H_2O]

Blank was prepared by mixing. 1 ml H_2O + 2 ml phosphate buffer pH 7.8 + 5 ml H_2SO_4 + 1 ml KI + a drop of starch.

Catalase enzyme activity was calculated by using the formula.

$$\frac{(B-A) \times 340 \times \text{volume of extract}}{B \times 5(\text{min}) \times \text{Extract taken for estimation}} = \frac{\text{Mg } \text{H}_2\text{O}_2 \text{ broken}}{\text{min}^{-1} \text{g}^{-1} \text{ fresh tissue.}} \times \text{wt of plant material (g) taken}$$

Where B = Blank reading (absorbance)

A = Extract reading (absorbance)

Values are expressed as $\mu\text{g H}_2\text{O}_2$ broken $\text{min}^{-1}\text{g}^{-1}$ fresh tissue.

ii) Nitrate reductase [NR, E.C. 1.6.6.2] :

Activity of this enzyme was determined following the method described by Jaworski (1971).

0.5 g of leaves and roots were incubated separately in 10 ml assay mixture containing 1 ml - 1M KNO_3 , 2 ml 5% n - propanol, 5 ml 0.2 M phosphate buffer - H 7.5 and 2 ml 0.5% Triton ~~X~~-100.

The material was incubated for an hour in the dark under anaerobic conditions. After an hour, 1 ml of reaction medium was taken out and mixed with 1 ml - 1% sulfanilamide (1 g in 100 ml 1 M HCl) and 1 ml 0.02% NEEDA (Naphthylethylene diamine dihydro chloride) [20 mg in 100 ml distilled H_2O].

The colour was developed with the reaction mixture. Absorbance was read at 540 nm on UV - VIS double beam spectrophotometer (Shimadzu - 190). Standard curve was prepared with 0.03 mM KNO_2 , while

mixture of 1 ml assay mixture, 1 ml sulfanilamide and 1 ml NEEDED served as blank.

Nitrate reductase was calculated using the formula Enzyme reading x 150.

$$= \frac{10 \times \text{enzyme reading} \times 16.2 \mu\text{g NO}_2}{\text{Std A reading (2.16)} \times \text{wt. of plant material (g) (0.5)}}$$

Activity of enzyme is expressed as $\mu\text{g NO}_2 \text{ h}^{-1} \text{ g}^{-1}$ fresh tissue.