

CHAPTER - II

MATERIAL AND METHODS

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A) Material :

In the present investigation attempts have been made to study the effect of water stress and salinity (NaCl treatment) on the two safflower (Carthamus tinctorius, L.) varieties, as Local and JLSF-88 (Figure-1).

The local variety is obtained from local market while seeds of JLSF-88, a released variety of safflower, obtained from oil seed specialist, Agriculture Research Station, Jalgaon-425 001. Though the number of released varieties are available the JLSF-88 is taken for the present investigation because JLSF-88 gave more yield (1772 Kg/ha) than the check Bhima (1605 Kg/ha) and NRS-209 (1663 Kg/ha) the data obtained from Agriculture Research Station, Jalgaon.

B) Methods :

B.1. Water Stress Studies :

The healthy seeds of two safflower varieties (Carthamus tinctorius, L) Local and JLSF-88, were sorted out and surface sterilized with 0.1 % HgCl₂ solution. Then seeds are sown in earthen pots (size : 30cm X 45 cm) filled with garden soil supplemented with farm yard manure in the proportion of 3:1 in the last week of October. After one month of establishment the plants were thinned to 5 healthy plants per pot. The

two replicates or sets were kept for each cultivar. At the time of heading stage, water stress was given for four and eight days by withholding water from the pots. The control plants were watered regularly. The control and water stress plants were harvested to study the effect of water stress on the growth parameters, organic constituents, inorganic constituents and the activity of a few enzymes.

a) Growth Parameters :

From each set 5 plants were carefully uprooted, cleaned well with distilled water, surface dried and used for analysis. The parameters selected for growth analysis were total plant height, shoot length, root length, shoot/root ratio, leaf area and leaf thickness.

b) Organic constituents :

i) Moisture and Relative Water Content (RWC) :

Moisture :

The healthy leaves of control as well as stressed safflower plants were taken and cleaned well with distilled water, surface dried and weighed. Then the leaves were dried at 80°C in an oven till constant weight obtained. The moisture percentage can be calculated by using following formula :

$$\text{Moisture percentage} = \frac{\text{Fresh wt.} - \text{Dry wt.}}{\text{Fresh wt.}} \times 100$$

RWC (Relative Water Content) :

The clean, washed and surface dried leaves of control as well as stressed plants were taken. The leaf discs were prepared by punching leaf lamina and weighed. Then the same leaf discs were kept for 4 hours in distilled water and weighed. Later on these discs were dried at 80°C in an oven till constant weight obtained. The RWC can be calculated by using the following formula :

$$\text{RWC} = \frac{\text{Fresh wt.} - \text{Dry wt.}}{\text{Turgid wt.} - \text{Dry wt.}} \times 100$$

ii) Titrateable Acid Number (TAN) :

TAN was estimated by the method of Thomas and Beevers (1949)✓. The plant leaf material was cut into small pieces and weighed for 1 g. It was boiled with distilled water for half an hour, then cooled and aliquot was titrated against N/40 NaOH using phenolphthalein as an indicator. NaOH was standardized against N/40 oxalic acid using the same indicator. Titrateable acid number (TAN) represents the number of ml. of decinormal NaOH required to neutralize the acids present in 100 g of fresh tissue. It was estimated by using following formula :

$$\text{TAN} = \frac{\text{Volume of oxalic acid taken for titration}}{\text{Titration reading of NaOH}} \times \frac{\text{Total volume of extract}}{\text{Wt. of plant material in g}} \times \frac{\text{Extract titration reading}}{\text{Volume of extract taken for titration}} \times \frac{100}{4}$$

iii) Total Polyphenols :

Polyphenols were estimated according to the method of Folin and Denis (1915). Polyphenols from fresh leaves of safflower were extracted in 80 % acetone and filtered through Whatman filter paper No.1 using Buchner's funnel under suction. Polyphenols were extracted repeatedly from residue. The volume of filtrate was made to 50 ml. 0.5 ml of filtrate was taken in a 50 ml marked Nessler's tube. In other such tubes, the different concentrations e.g. 0.5, 1, 2, 3 and 4 ml of standard polyphenol solution (Tannic acid, 0.1 mg ml^{-1}) were taken. Then 10 ml of 20 % Na_2CO_3 were added to each tube to make the medium alkaline. 2 ml of Folin-Denis reagent (100 g of sodium tungstate and 20 g of phosphomolybdic acid dissolved in 200 ml distilled water were mixed with 25 % phosphoric acid. This was refluxed for 2.5 hours then cooled to room temperature and diluted to 1 litre with distilled water) were then added to each Nessler's tube and finally the volume was made to 50 ml with distilled water. A blank was prepared without polyphenols. The ingredients were allowed to mix thoroughly well. After some time the optical density (OD) of each mixture was read at 660 nm. Polyphenols were

calculated from the calibration curve of standard tannic acid.

iv) Chlorophylls :

The chlorophylls were estimated by the method of Arnon (1949). Chlorophylls were extracted in 80% acetone from 1 g of the plant material. The extract was filtered through Buchner's funnel using Whatman filter paper No.1. Residue was washed repeatedly with 80% acetone, collecting the washings in the same filtrate. The volume of the filtrate was made to 100 ml with 80% acetone. Extraction was carried out in dark and in cold conditions. The absorbance of the filtrate was read at 663 nm and 645 nm.

Chlorophylls in $\text{mg } 100 \text{ g}^{-1}$ fresh tissue were calculated by using following formulae :

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

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

$$\text{Total Chlorophylls} = 8.02 \times A_{663} + 20.2 \times A_{645} = 'Z'$$

$$\begin{array}{l} \text{Chlorophyll 'a' or} \\ \text{'b' or total} \\ \text{chlorophylls} \\ \text{(mg } 100 \text{ g}^{-1} \text{)} \end{array} = \frac{X/Y/Z \times \text{Volume of extract} \times 100}{1000 \times \text{Wt. of the plant material in g}}$$

c) Inorganic Constituents :

i) Preparation of acid digest (Extract) :

The leaf material of random sampling was taken,

cleaned well in distilled water and dried at 80°C in an oven till constant weight obtained. This oven dried material was taken for the estimation of different inorganic elements by following the method of Toth et al., (1948).

0.5 g of oven dried powdered material was transferred to a 150 ml beaker to which 20 ml con. HNO_3 were added. The beaker was covered with watchglass and kept till the primary reaction subside. It was then subjected to slow heating to dissolve solid particles completely. After cooling to room temperature, 10 ml of 60% perchloric acid were added and mixed thoroughly. It was then heated strongly and vigorously until a clean and colourless solution reduced to about 2-3 ml. While heating the liquid was not allowed to dry. It was then cooled and transferred quantitatively to a 100 ml volumetric flask and volume was made to 100 ml with distilled water and kept overnight. Next day it was filtered through a dry Whatman filter paper No. 44 (ashless) and the filtrate was used for the estimation of different inorganic elements.

ii) Estimation of Sodium and Potassium :

Sodium and potassium were estimated Flame photometrically following the standard procedure. The standard solutions of known concentrations in parts per million (ppm) of Na^+ in NaCl (1 to 10 ppm) and K^+ in KCl (1 to 50 ppm) were used for calibration curves. From these calibration curves the unknown concentrations of Na^+ and K^+ in the acid digest

samples were calculated.

iii) Estimation of Calcium, Magnesium, Iron, Copper, Zinc and Manganese.

The acid digest extract was used to estimate Ca^{2+} , Mg^{2+} , Fe^{3+} , Cu^{2+} , Zn^{2+} and Mn^{2+} elements on atomic absorption spectrophotometer (Perkin-Elmer Model-3030) using acetylene air flame. The light source employed was hollow cathode lamp. The concentrations of Ca, Mg, Fe, Cu, Zn and Mn were read at 422.7 nm, 285.2 nm, 248.3 nm, 324.8 nm, 213.9 nm, and 279.5 nm respectively (Perkin-Elmer, 1973).

iv) Estimation of Chlorides :

For estimation of chlorides, method described by Imamul Huq and Larher (1983) with slight modification was used. Estimation was done using Chapman and Pratt's (1961) method. The chlorides were extracted in boiling distilled water. After cooling the extract was filtered through a layer of cheese cloth. The filtrate was collected in 25 ml volumetric flask and final volume was made with distilled water. From this 10 ml of extract was taken for titration against standardized 0.05 N AgNO_3 .

A few drops of 25% acetic acid solution were added to the filtrate until the pH of the solution was 6 to 7. Then a few drops of 1 % potassium chromate solution were added and titrated against standardized 0.05 N AgNO_3 (Dissolve

8.5 g AR grade AgNO_3 in 1000 ml distilled water) until the first permanent reddish brown colour appears and noted the burette readings.

(1 ml of 0.05 N AgNO_3 = 1.775 mg Cl^-).

d) Enzymes :

Enzyme Peroxidase (EC 1.11.1.7) and Acid phosphatase (EC 3.1.3.2) were isolated from the fresh leaves. The isolation of enzymes were done at low temperature (0 - 4°C)

1) Peroxidase :

Peroxidase from fresh plant leaves was determined by following the method described by Maehly (1954). The enzyme was extracted by homogenizing the plant material (0.5 g) in 10 ml ice cold water. It was then filtered through two layered cheese cloth and the filtrate was centrifuged for 15 minutes at 500 rpm at 0 to 4°C and supernatant was used as an enzyme source. Enzyme assay mixture contained 2 ml of 0.1 M phosphate buffer (pH 7) 1 ml of 20 mM guaiacol and 0.5 ml of enzyme. The reaction was started by addition of 0.04 ml of 10 mM H_2O_2 . The change in optical density (OD) due to oxidation of guaiacol was recorded per minute at 470 nm on spectrophotometer with frequent stirring of the reaction mixture with glass rod. Enzyme activity is expressed as change in $\Delta \text{OD min}^{-1} \text{g}^{-1}$ fresh tissue.

ii) Acid phosphatase :

The enzyme was isolated from fresh leaves following the method of McLachlan (1980). The enzyme was prepared by homogenizing 0.5 g of plant leaves in 10 ml of 0.1 M acetate buffer (pH 5) with a mortar and pestle. The extract was filtered through the muslin cloth already moistened with acetate buffer and the filtrate was centrifuged at full speed of 500 rpm for 10 minutes. The supernatant was stored at 0 to 4°C and used as an enzyme source.

Enzyme assay mixture contained 3 ml of p-nitrophenyl phosphate (0.1 mg ml^{-1}), 2 ml of 0.1 M acetate buffer (pH-5) and 1 ml of enzyme. Enzymatic reaction was initiated by the addition of enzyme and was stopped by the addition of 1.5 ml of 1.68 N NaOH. Yellow coloured complex, ^{p-nitrophenol} produced as a result of reaction between enzymatic breakdown of p-nitrophenyl phosphate and NaOH, was estimated spectrophotometrically at 400 nm. The enzyme activity was expressed as change in OD $\text{hr}^{-1} \text{ g}^{-1}$ fresh tissue.

B-2) Salt Tolerance Studies :

The healthy seeds of two safflower (Carthamus tinctorius, L.) varieties such as Local variety and JLSF-88, were sorted out and surface sterilized with 0.1 % HgCl_2 solution. They were sown in earthen pots (size: 30 cm X 45 cm) filled with garden soil supplemented with farm yard manure

in the proportion of 3:1, in the last week of October. After one month of establishment the plants were thinned to 5 healthy plants per pot and two replicates or sets were kept for each cultivar. Then plants were subjected to 0.025 %, 0.05%, 0.1%, 0.2% and 0.4% sodium chloride. The control plants without NaCl treatment were watered regularly. At the time of heading stage the plants were harvested and employed for analysis of effect of NaCl treatment on growth parameters, organic and inorganic constituents and the activity of a few enzymes.

a) Growth parameters :

Growth parameters like total plant height, shoot length, root length, shoot/root ratio, leaf thickness and leaf area were studied by following the methods described earlier in water stress studies.

b) Organic constituents :

i) Moisture and RWC :

Methods described already in water stress studies.

ii) TAN

The estimation of TAN was done by the method of Thomas and Beevers (1949) as described already in water stress studies.

iii) Polyphenols

Polyphenols were estimated according to Folin and

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Denis (1915) method which has been already described in water stress studies.

iv) Photosynthetic pigments :

Chlorophylls - The chlorophylls were estimated according to the method described by Arnon (1949) which has been already described in water stress studies.

Carotenoids : Carotenoids were extracted by crushing the fresh leaves in 80% acetone. Procedure is similar to that of chlorophylls described earlier and carotenoids were estimated spectrophotometrically at 480 nm by following the method of Kirk and Allen, (1965). Total carotenoids were estimated using the following formula of Liaaen-Jensen and Jensen (1971).

$$C = D \times V \times F \times \frac{10}{2500}$$

Where,

C = Total carotenoids in mg

D = Optical density,

V = Total volume in ml.

F = Dilution factor.

2500 = Average extinction.

c) Inorganic Constituents :

The analysis of inorganic constituents namely Na⁺,

K^+ , Ca^{2+} , Mg^{2+} , Fe^{3+} , Cu^{2+} , Mn^{2+} , Zn^{2+} and Cl^- from the leaves of control and NaCl treated plants were done according to the procedure described in earlier section of water stress studies.

d) Enzymes :

1) Peroxidase :

The activity of peroxidase enzyme was determined by following the method of Maehly (1954) described in water stress studies.

2) Acid phosphatase :

The activity of acid phosphatase enzyme was determined by the method of McLachlan (1980) which has been already described in water stress studies.