MATERIALS AND METHODS

A. PROCUREMENT OF SEEDS :

Seeds of safflower varieties Nira and JSI-7 were brought from Nimbkar Agricultural Research Institute, Phaltan, Dist. Satara.

B. SEED SOWING AND FIELD PRACTICES ::

The plots were made in the Botanical Garden of Botany Department of Shivaji University, Kolhapur. The seeds were sown as per single cropped rabi tracts of Maharashtra. The excess seedlings were thinned within 10 to 15 days in order to maintain desired plant to plant spacing. The distance between two plants was kept 20 cm & distance between two rows was 40 cm. Light irrigation was given after sowing the seeds. Depending on severity of weeds, weeding and interculture operations were prcticed. The second irrigation was given at 35th day after sowing in early elongation stage and third at the 65th day. After that no irrigation was provided. The crop was protected from Aphids by dusting DDT at early stage of growth.

As discussed elsewhere different sowing periods were chosen to have comparative data. First set of plots was sown on 25th of September while second and third sets were sown in October and November respectively on the same date.

C. METHODS :

1. Growth Performance :

In the two safflower varieties, influence of different sowing periods on the growth was examined after first and second month.

After specific period from sowing the seedlings were taken for analysis of growth parameters like root length, shoot length, R/S ratio, height of plant, number of leaves, number of branches, number of buds, fresh weight, dry weight and moisture percentage. After maturity (120-130 days) number of flowers/plant, number of seeds/flower were also recorded in addition to above parameters.

Phenological parameters such as the emergence of leaves, branches, buds and flower heads were recorded from time to time throughout the growth.

2. Biochemical Analysis of safflower leaves :

Various organic and inorganic constituents in the leaves of two safflower varieties growing under the influence of different sowing periods were determined after first month and second month growth.

a) PHOTOSYNTHETIC PIGMENTS :

i) Chlorophylls :

Chlorophylls were estimated using the method described by Arnon (1949). 500 mg of fresh leaves were homogenized in 80 % chilled acetone containing 4 ml. ammonia per liter. A pinch of $MgCO_3$ was added for enhancing the extraction and neutralizing the acids released during extraction. The extract was filtered through Buchner's funnel using Whatman No.1 filter paper. Volume of the filtrate was adjusted to 50 ml with 80% acetone. Then extract was transferred to a conical flask which was wrapped with a black paper to prevent photooxidation of pigments. The absorbance was measured at 663 nm and 645 nm on Shimadzu Spectrophotometer.

Chl.a, Chl.b and total chlorophylls (mg/100 g of fresh leaves) were calculated by using following formulae :

$$\text{Chl.a (X)} = (12.7 \times A_{663}) - (2.69 \times A_{645}).$$

$$\text{Chl.b (Y)} = (22.9 \times A_{645}) - (4.68 \times A_{663})$$

$$\text{Chl.a+b (Z)} = (8.02 \times A_{663}) + (20.2 \times A_{645})$$

$$\text{Chl.a/Chl.b/Chl. (a+b)} = \frac{\text{X/Y/Z} \times \text{Vol. of extract} \times 100}{1000 \times \text{wt. of pl. material (g)}}$$

ii) Carotenoids :

Carotenoid content was estimated by Kirk and Allen's

method (1965). The acetone extract prepared for chlorophyll estimation was used to measure absorbance at 480 nm.

Carotenoids (mg/100 g fresh leaves) were calculated as follows :

$$\text{Total Carotenoids (mg/100 g fr.wt.)} = \frac{A_{480} \times \text{Vol. of extract} \times 10 \times 100}{2500 \times \text{wt. of pl. material (g)}}$$

Where 2500 = average extinction.

b) **TOTOAL POLYPHENOLS :**

The polyphenols were estimated following the method of Folin and Denis (1915). 500 mg fresh leaves were crushed in 80 % acetone and filtered through Buchner's funnel using Whatman No.1 filter paper. The residue, which was present on filter paper was washed several times with 80 % acetone. Final volume of extract was made to 50 ml.Two ml extract was treated with 10 ml of 20% Na_2CO_3 in a Nesseler's tube. The volume was made to 35 ml with distilled water. After adding 2 ml of Folin-Denis reagent, final volume of reaction mixture was adjusted to 50 ml with distilled water. After half an hour absorbance of blue colour was measured at 660 nm using a blank reaction mixture.

A standard curve of polyphenols obtained by using Tannic acid (0.1 mg/ml) was used to calculate the amount of polyphenols.

c) CARBOHYDRATES :

Carbohydrate content was estimated according to the method of Nelson (1944). 1 g of fresh leaves were crushed in 80% alcohol. The extract was filtered through Buchner's, funnel using Whatman No.1 filter paper. The filtrate was condensed on a water bath to about 2 to 3 ml. About 2 g of a mixture of lead acetate and potassium oxalate (1:1) were added with constant stirring. The contents were mixed with 20 ml of distilled water and filtered through Buchner's funnel using Whatman No.1 filter paper. The volume of the filtrate was adjusted to 20 ml with distilled water. It was transferred to a conical flask containing 2 ml conc. HCl. The flask was plugged with a cotton plug and autoclaved for 30 min. under 15 lbs pressure. After cooling to room temperature the contents were neutralized by adding anhydrous Na_2CO_3 and filtered again. The volume of filtrate was recorded and it was used for the estimation of total sugars.

The residue left on the filter paper during the alcoholic extraction was transferred along with the filter paper into a conical flask, containing 5 ml of Conc. HCl and 15 ml distilled water. It was hydrolysed at 15 lbs. pressure for 30 min. and then cooled to room temperature. The contents were neutralized with anhydrous Na_2CO_3 and filtered. The volume of filtrate was recorded. This filtrate was used for the estimation of starch.

Estimation of sugars was carried out colorimetrically by using Arsenomolybdate reagent to determine Cuprous oxide formed in the oxidation of sugars by alkaline copper tartarate reagent.

Extract (0.1 or 1 ml) was heated with 1 ml alkaline Cu-tartarate on a waterbath for 10 min. One ml of Arsenomolybdate reagent was added after cooling and volume was adjusted to 10 ml with distilled water. Absorbance was measured at 560 nm after 10 min. on Shimadzu Spectrophotometer against a blank. Standard curve of carbohydrates obtained by using different concentrations of glucose (0.1 mg/ml) was used to calculate the amount of total sugars and starch present in the leaves.

d) TOTAL SOLUBLE PROTEINS :

Total soluble proteins were determined using Biuret method described by Gornall et al. (1949). When proteins are treated with an alkaline solution of $CuSO_4$, the peptide linkages are broken down giving a violet colour to the solution. This reaction is known as Biuret reaction and was first demonstrated on Biuret which is the product of pyrogenic decomposition of urea.

1 g. fresh leaves was homogenized in 0.14 M cold saline solution. The extract was filtered and centrifuged for 15 min. The supernatant was used as the source of proteins. One ml of extract was mixed with eight ml of Biuret reagent and it was

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incubated at 37°C for 30 min. The absorbance of violet colour developed was measured at 540 nm on Shimadzu Spectrophotometer. At the same time, a set of reaction mixtures containing different concentrations of Std. Casein (20 mg/ml) was prepared to obtain a std. curve of proteins.

e) TOTAL FREE AMINO ACIDS :

Colorimetric method described by Sadasivum and Manickam (1992) was used to determine total free Amino acid content in the leaves.

500 mg fresh leaves were homogenized in 5 to 10 ml 80% ethanol. The extract was centrifuged at ~~full~~ speed (5000 rpm) for 15 min. Supernatant was saved. The leaf extract was again filtered with ethanol, and the supernatant was pooled out. The procedure was repeated once more. The total supernatant was evaporated on a water bath to obtain 10 ml solution. It was used as the source of amino acids.

Estimation was carried out by using Ninhydrin as an oxidizing agent, which decarboxylates the α-aminoacids and gives bluish purple product. 0.1 ml extract was mixed with one ml ninhydrin solution. The volume was adjusted to 2 ml with distilled water and heated on boiling waterbath for 20 min. After cooling 5 ml diluent solution was mixed in it. Absorbance was measured at 570 nm after 15 min.The blank was prepared by using ethanol in place of extract.

Std. curve of amino acid was obtained by using leucine (0.1 mg/ml) for calculating the amount.

f) ASCORBIC ACID :

A titrimetric method given by Sadasivum and Manickam (1992) was used to determine the ascorbic acid content. Ascorbic acid is extracted in metaphosphoric or oxalic acid to lower down the pH and to stabilize its content by preventing catalytic oxidation.

500 mg of fresh leaves were extracted in 4% oxalic acid. The extract was filtered and then centrifuged for 15 min. The supernatant was used as the source of ascorbic acid.

During estimation, ascorbic acid is oxidised to dehydroxy ascorbic acid by reducing 2,6-dichloro phenol indophenol (a blue dye) to a pink coloured solution. Oxalic acid is used as a titrant. The capacity of plant extract to reduce the dye (V_2 ml) is directly proportional to the ascorbic acid content. Std. ascorbic acid (100 µg/ml) is titrated against the dye till the appearance of persistent pink colour. The amount of dye consumed (V_1 ml) is equivalent to the amount of ascorbic acid taken for the titration. The amount of ascorbic acid is calculated by using following formula :

$$\text{Ascorbic acid (mg/100 g)} = \frac{0.5\ \text{mg}}{V_1} \times \frac{V_2}{5} \times \frac{100}{\text{Wt. of the sample (g)}} \times 100$$

g) ELEMENTAL ANALYSIS :

i) Nitrogen :

Nitrogen was estimated colorimetrically following the method given by Hawk et al. (1948).

500 mg of ovendried leaves were digested in a Kjeldahl flask with sulphuric acid (1:1) and a pinch of microsalt (anhydrous $CuSO_4$ and potassium sulphate 1:40) and a few glass bids (to avoid bumping) till a colourless liquid is obtained at the bottom of flask. After cooling to room temperature the contents were diluted with distilled water to adjust 100 ml volume. It was then filtered through Whatman No.1 filter paper and used for the estimationn of nitrogen.

2 ml of filtrate were taken in a Nessler's tube along with a drop of 8% $KHSO_4$ and volume was adjusted to 35 ml with distilled water. Then 15 ml of freshly prepared Nessler's reagent was added and mixed thoroughly. After 15 min. absorbance was measured at 520 nm. A blank solution containing all ingradients except nitrogen source was used to adjust zero absorbance. Std. curve was obtained by using different concentrations of Ammonium sulphate (0.05 mg/ml) in place of filtrate. It was used to calculate the amount of total soluble nitrogen in leaves.

ii) Preparation of extract for other elements :

Extract was prepared using the method of Toth *et al.* (1948). 500 mg ovendried leaves were treated with 20 ml Conc. HNO_3 in a beaker covered with watch glass and kept till the primary reaction is subsided It was then subjected to slow heating on a hot plate to dissolve the plant material. After cooling to room temperature 10 ml of perchloric acid (70 %) were added into the beaker and it was heated again to obtain 2-3 ml clear solution. The extract was then cooled to room temperature, diluted to 100 ml with distilled water and kept over night. Next day it was filtered through Whatman No.1 filter paper and stored at room temperature. This acid digest was used to estimate inorganic elements such as phosphorus, potassium, calcium, sodium, magnesium, iron.

iii) Phosphorus :

Phosphorus was estimated by the method of Sekine (1965). 2 ml acid digest was reacted with 2 ml of 2 N HNO_3 in a test tube. Then 1 ml freshly prepared Molybdate-Vanadate reagent was added and volume was made to 10 ml with distilled water. After 20 min absorbance was measured at 420 nm against a blank. Phosphorus content was calculated by using a Std. curve of phosphorus (0.025 mg/ml).

iv) Potassium, Sodium, Calcium :

K, Na and Ca were estimated flame photometrically

using a Mediflame Photometer. Stock solutions of known concentration in ppm of K as KCl, Na as NaCl and Ca as $CaCl_2$ were prepared. After standardization of the instrument, the readings of samples were recorded in terms of ppm. The ppm concentration was then converted to g per 100 g of dry weight.

v) **Magnesium and Iron :**

These elements were analysed using the acid digest on an atomic absorption spectrophotometer (Perkin-Elmer 3030 model). The readings were recorded in ppm which were converted into grams per 100 g of dry weight.

3. BIOCHEMICAL ANALYSIS OF DEVELOPING SEEDS :

Biochemical analysis of seeds of safflower was made, at different stages during development. Following developing stages were selected.

	Stage	Days after flowering
1)	Young	10
2)	Premature	20
3)	Mature	40
4)	Postmature	60

Seeds at specific stages were collected and analysed for different parameters.

a) **Moisture Percentage :**

Ten seeds were collected at different developing stages.

Fresh weight was recorded and then they were kept in oven at 60°C. Dry weight was recorded after 5-6 days. With the help of fresh & dry weights moisture percentage was calculated.

b) Oil Percentage :

Oil extraction was done using method of Kaufmann (1958) by using petroleum ether and Soxhlet apparatus. Oil percentage was determined using method described by Sadashivum and Manickum (1992). 10 g seeds of different developing stages were pulverized in a mortar and transfered into a filter paper fold. This sample packet was placed into butt tube of Soxhlet extraction apparratus. Extraction was done with petroleum ether, for 6 hours without interruption on a waterbath. After cooling to room temperature, petroleum ether in the flask was evaporated on water bath, and the oil removed from flask was weighed. The oil percentage was calculated using the following formula:

$$\text{Oil percentage} = \frac{\text{Weight of Oil (g)}}{\text{Weight of Sample (g)}} \times 100$$

The oil extracted from the seeds of different developing stages was used for further Qualitative and Quantitative analysis of lipids.

c) Quantitative Analysis of Lipids:

i) Free fatty acid value :

Free fatty acid content is known as the acid value or

number. It is the amount of alkali required to neutralize the free fatty acids present in 1 g sample. It is expressed as oleic acid equivalent and estimated using method given by Plummer (1971).

5 g oil were taken in dry and clean conical flask containing 25 ml neutral alcohol. After shaking vigorously, it was heated on a boiling waterbath. After cooling to room temperature, the contents of flask were titrated against 0.1 N KOH using phenolphthalein indicator. End point was colourless to stable pink. Titre value was recorded and used to calculate free fatty acid value as follows :

$$\text{Free fatty acid value} = \frac{5.611 \times \text{Titre value}}{\text{Weight of sample (g)}}$$

ii) Iodine number of Lipids :

Iodine number is considered as the amount of iodine absorbed by 100 g of oil. Iodine value is considered as a measure of the degree of unsaturation in an oil. Iodine gets incarporated into the fatty acid chain wherever the double bonds exist. Hence the measure of iodine absorbed by an oil gives the degree of saturation. Iodine number was estimated by using method given by Plummer (1971).

10 ml of 2 % oil solution (prepared in chloroform) were

taken into a stoppered bottle and 25 ml of iodine monochloride were added to it. Blank reaction mixture was prepared with the help of chloroform and ICl. The contents of both bottles were mixed well and kept in dark for one hour. After 1 hour the stoppers were washed with 10 ml KI and 50 ml distilled water. The washings were added into the bottle and it was titrated against 0.1 N Sodium thiosulphate, till reaction mixture becomes pale straw in colour. Then 1 ml fresh starch indicator was added into it and titrated further against sodium thiosulphate with constant shaking. End point was blue to colourless. Blank solution was also titrated in similar way. Iodine number was calculated with the help of following formula :

$$\text{Iodine Number} = \frac{12.7 \times (X-Y) \times 100}{0.2}$$

where 1 ml $Na_2S_2O_3$ solution ≡ 12.7 mg of Iodine

X - blank titration reading

Y - sample titration reading

0.2 - amount of oil taken for titration (g).

iii) Saponification Value of Oil :

Saponification value is useful for comparative study of the fatty acid chain length. It is the amount (mg) of alkali required to saponify a definite quantity of oil. It was estimated by using method of Horowitz (1975).

completely moisture free oil (1 g) was taken into flask and 50 ml of alcoholic KOH were added from burette. It was refluxed for 1 hour on a waterbath using condenser. A blank without oil was run simultaneously. During refluxing with alkali glyceryl esters are hydrolysed to form glycerol and potassium salts of fatty acids (soaps). The potassium salts were estimated by titrating the refluxed sample against 0.5 N HCl using phenolphthalein indicator. End point was pink to colourless. Saponification value was calculated by using the formula -

$$\text{Saponification Value} = \frac{28.02 \times (\text{Titre Value of blank} - \text{Titre value of sample})}{\text{Weight of sample (g)}}$$

d) Qualitative Analysis of Lipids :

Qualitative analysis of lipids was carried out using the technique of Thin layer chromatography as described by Mangold and Malins (1960, 1962, 1964). Thin layer plates of uniform thickness were prepared using Silica gel-G (250 mesh, containing $CaSO_4$ binder). The plates were activated in an oven at 110 to 115°C for 1 hour and stored in a dry rack. These plates were used for chromatographic analysis.

For chromatography 1 % oil solution was prepared in

petroleum ether (60-70° bp). Sample (10λ) was loaded with the help of a micropipette. The spots were air dried and then plates were placed in a chromatographic chamber, which was prepared 30 min. before inserting the plates. The chamber was first lined with Whatman No.1 paper on inner three sides and made moist with developing solvent. The solvent system consisted a mixture of petroleum ether (bp 60-70°C) - diethyl ether and acetic acid in proportion of 90 : 10 : 1 (v/v). The plates were run to reach the solvent upto 15 cm from the origin. After development plates were removed and dried in air at room temperature.

Spots on chromatograms were detected by using iodine vapours. Developed plates were placed in to a jar containing a trough filled with crystals of iodine. Within 5-10 min, brown spots started appearing against yellow background on the plates. These spots were identified by comparing their Rf values with Rf values of lipids given by Malins and Mangold (1960) and schematic representation given by Skipski et al. (1965).

e) Estimation of Total Soluble Proteins and Carbohydrates in Safflower Seeds :

Soluble proteins and carbohydrate content was determined in harvested seeds of both the safflower varieties sown in November.

For this estimation, it was necessary to remove the oil

content from seeds. It was carried out by extracting oil using Soxhlet apparatus.

From the residue obtained after complete removal of oil, endosperm and seed coat were separated and used for the estimation.

Total soluble proteins were estimated using Biuret method as per Gornall et al. (1949). Total sugar and starch content of endosperm and seed coat was estimated using method of Nelson (1944).

4. PREPARATION OF REAGENTS :

1. **Folin-Denis Reagent** : (Total Polyphenols)

100 g Sodium tungstate and 20 g phosphomolybdic acid were dissolved in 200 ml distilled water. 50 ml Phosphoric acid (25 %) were added and it was refluxed for 2-3 hours using water condenser. The volume was adjusted to one liter with distilled water. The reagent was stored in an amber coloured bottle.

2. **Somogyi's Alkaline Copper Tartarate Reagent** : (Carbohydrates)

4 g $CuSO_4 .5H_2O$, 24 g anhydrous $Na_2 CO_3$, 16g Na-K-tartarate and 180 g anhydrous $Na_2 SO_4$ were dissolved in one liter distilled water.

3. **Arsenomolybdate Reagent** : (Carbohydrates)

25 g of ammonium molybdate were dissolved in 450 ml. distilled water to which 21 ml concentrated $H_2 SO_4$ were added. 3 g of sodium arsenate were dissolved in 25 ml distilled water. The solutions were mixed well and kept in an incubator at 37° for 48 hours before use. The reagent was stored in an amber coloured bottle.

4. **Biuret Reagent** : (Soluble proteins)

500 mg $CuSO_4.5H_2O$ and 6 g Na-K-tartarate were dissolved in 500 ml of distilled water, 300 ml of 10 % NaOH were added to this solution with swirling. The volume was adjusted to 1000 ml with distilled water.

5. **Ninhydrin** : (Total free Aminoacids)

0.8 g Stannous chloride was dissolved in 500 ml 0.2 M citrate buffer (pH 5.0), 20 g Ninhydrin were dissolved in 500 ml methyl cellosolve. The solutions were mixed well.

6. **Diluent Solvent** : (Total free Aminoacids)

Equal volumes of water and n-propanol were mixed well to prepare a diluent.

7. **DCPIP Solution** : (Ascorbic acid)

45 mg of sodium bicarbonate were dissolved into a small volume of distilled water. 52 mg 2,6 dichloro-

phenol indophenol were dissolved in it and the volume was adjusted to 200 ml with distilled water.

8. **Nessler's Reagent** : (Total Nitrogen)

Nessler's A : 7 g of KI and 10 g of HgI_2 were dissolved in 40 ml distilled water.

Nessler's B : 10 g of NaOH were dissolved in 50 ml of distilled water.

The reagent was prepared by mixing A and B (4:5) at the time of estimation.

9. **Molybdate Vanadate Reagent** : (Phosphorus)

Solution A : 1.25 g of Ammonium vanadate were dissolved in 500 ml of 1 N. HNO_3.

Solution B : 25 g of Ammonium molybdate were dissolved in 500 ml of distilled water.

The reagent was prepared by mixing A and B in equal proportions at the time of estimation.