MATERIAL AND METHODS

A Procurement of Seeds :

Seeds of Soybean (Glycine max (Linn) Merrl) variety MACS-13 and JS-335 were obtained from Department of Botany, agriculture College, Kolhapur (India).

B Prepatation of Pots :

Seeds of uniform size were selected and surface sterilized with 0.1% $HgCl_2$ for 1min. and then, were washed repeatedly with distilled water. Surface sterilized seeds were sown in an earthen pots filled with 3 part of soil and 1 part of farmyard manure (FYM) and seedings were allowed to grow in the pots. Similarly the seeds were sown in small field pots (1m x 1m) and were allowed to grow.

C Determination of Plastochron age :

Plastochron Index (PI) of Soybean var. MACS-13 and JS-335, grown in field plots was determined by using the formula of Erickson and Michelini (1957).

$$PI = n + \frac{\log L_n - \log_{10}}{\log L_n - \log L_{n+1}}$$

where,

n = the leaf serial number.

Ln = the length of leaf n longer than 10mm.

Ln+1 = the length of leaf which is just shorter than 10mm.

Further, the age of leaf in plastchron unit, (LPI) was determined by subtracting the leaf number from the plastochron age (PI) of the entire plant (LPI=PI-n)

D Organic Constituents :

Organic constituents such as chlorophylls, carotenoids, nitrogen and proteins, were analysed from young, medium mature and mature leaves of Soybean var. MACS-13 and JS-335, in relation with plastochron age.

Total polyphenol content was determined from leaf, stem and root of MACS-13 and JS-335 at Vegetative, flower initiation, pod formation and maturation stage of growth.

1 Chlorophylls :

Chlorophylls were estimated following the method of Arnon (1949).

0.5 g. fresh material was crushed in morter with pestle and extracted in 80% chilled acetone containing 4 ml. licuor NH_3 per litre in dark. A pinch of $MgCO_3$ was added during crushing. The extract was filtered through Buchner's funnel using Whatman No.1 filter paper. The volume of filtrate was adjusted to 50ml. with 80% acetone. The extract was then transfered to a conical flask. The flask was covered with black paper to retain the activity of chlorophylls. The absorbance was measured at 645 and 663 nm. on Spectrophotometer. Chlorophyll content (mg/100 g fresh wt) was calculated using the following formula.

chl. a + b = (8.02 + A 663) + (20.2xA645) = z

$$\text{Total chlorophyll (mg/100g fresh tissue)} = \frac{z \times \text{Volume of extract} \times 100}{1000 \times \text{weight of plant material (g)}}$$

2 **Carotenoids :**

Total corotenoids were estimated by using the method and formula suggested by Jensen (1978).

The acetone extract prepared for chlorophyll estimation is used for measuring the absorbance at 480mm. for carotenoid determination.

$$\text{Total carotenoids} = \frac{D \times V \times F \times 10}{2500}$$

Where, D = Absorbance at 450 nm.

V = Volume of extract

F = Dilution factor

2500 = Average extinction

$$\text{Dilution factor} = \frac{\text{Total volume of extract}}{\text{moisture content in plant material used.}}$$

3 **Nitrogen :**

Nitrogen was estimated by the method of Hawk et al (1948). 0.5 g dried plant material was taken in kjeldahl's flask containing 10ml 1:1 H_2SO_4 and water. A pinch of microsalt and few glass beads were added to the flask. This was digested on low flame till colourless solution was obtained. Then it was cooled and transferred quantitatively to volumetric flask and volume was made 100 ml with distilled water and filtered through Whatmann No.1 filter paper. From the filtrate 2 ml. of extract

was taken in Nessller's tube to which a drop of 8% $KHSO_4$ was added and volume was made to 35ml with distilled water. Then 15 ml Nessler's reagent (freshly prepared) was added to it.

After 10-15 min. the absorbance was recorded at 520nm on Spectrophotometer (Spectronic 20) The blank contained all the ingradients except nitrogen source.

Standard curve was obtained by using different concentrations of ammonium sulphate (0.1, 0.2, 0.3, 0.4ml.) by using same procedure of nitrogen estimation. The values were expressed in g 100^{-1} g dry weight.

i Preparation of microsalt :

Microsalt was prepared by grinding anhydrous copper sulphate and potassium sulphate in proportion of 1:40 i.e. 0.1 g $CuSO_4$ + 4.0 g $K2SO_4$.

ii Preparation of Nessler's reagent :

a) 7 gm of KI + 1 g of HgI_2 dissolved in 40 ml of distilled water.

b) 10 g of NaOH dissolved in 50 ml of distilled water. Both a and b were mixed immediately before use.

iii Preparation of standard Ammonium Sulphate Solution

$(NH4)_2$ SO_4 was kept in an oven for 10 h and 0.266 g of it was dissolved in water. A few drops of conc. H_2SO_4 were added to it and the volume was made to 1 litre. This contains 0.05 mg of nitrogen/ml.

4. Protein :

Protein content was obtained by multiplying the total nitrogen content by the factor 5.71 recommended for Soybean (Sadasivan and Manikam 1992).

5. Polyphenols :

Polyphenols from leaf, stem and root of Soybean var. MACS-13 and JS-335 were estimated following the method of Follin and Denis (1915). 1g dried powder was crushed in a mortor with pestle using 80% acetone. The extract was filtered through Buchner's funnel using Whatman No. 1 filter paper. The residue on the filter paper was washed several time with 80% acetone and final volume of extract was adjusted to 50 ml. using 80% acetone. 2 ml. of plant extract was taken in a Nessler's tube along with the series of standards (std. tannic acid having concentration 0.1 mg ml^{-1}) to which 10 ml 20% Na_2CO_3 was added. The volume was adjusted to 35 ml with distilled water. Then 2 ml of Folin Denis reagent was added in each test tube and the final volume was adjusted to 50 ml with distilled water. After about 20-30 min. absorbance was measured at 660 nm using reaction blank. Polyphenols were calculated from standard curve of tannic acid and the values are expressed in g 100^{-1} g dry tissue.

1. Preparation of Folin-Denis reagent :

100 g. Sodium tungstate and 20 g phosphomolybdic basic acid were dissolved in 800 ml distilled water. To that 50 ml 80% phosphoric acid was added. The entire mixture ws refluxed for 2h on waterbath using water condenser. After cooling to room temp, the final volume of the mixture was made to 1 litre and stored in an amber coloured bottle at low temperature.

Standard tannic acid was prepared by dissolving 250 mg tannic acid in 250 ml distilled water so as to get the final concentration in the range of 0.1 mg ml^{-1}.

E. Chromatography of Polyphenols :

The polyphenals were extracted by the method suggested by Glass and Bohm (1969). The whole plants of different growth stages viz. vegetative, flower initiation and pod formation stage were uprooted, cleaned dried and powdered. 5g dried powder of a whole plant of each stage was macerated separately in 80% ethenol. The extract was refluxed for 2h on water bath using water condenser and filtered hot through a sintered glass funnel. The solvent was removed under reduced pressure. The residue was triturated in 50 ml hot water. The combined extract was reduced to 3 ml by condensation, centrifuged at 5000 g for 5 min. and supernatant was used for chromatography.

F. Unidimensional paper chromatography of Polyphonols :

Unidimensional paper chromatography was performed using Whatman No. 1 filter paper (size 24 x 14 cm.) with slight modification in the method used by Shetty (1971). Chromatograms were spotted with 10 and 20 µl whole plant extract using micropipette with frequent drying. The solvent system used was n-butanol, acetic acid and water in the proportion of 80:20:44 (v/v). The position of individual phenolic compounds on chromatogram was determined by marking fluorescent area under UV-light as well as under UV-light in presence of Ammonia fumes. Phenolic compounds and flavonoids that could not be detected under UV and UV + NH_3 were detected by deeping the chromatogram in a mixture of 0.3% $FeCl_3$ and 0.3% $K_3Fe(CN)_6$ in equal proportion.

The probable identification of the compounds was made by calculating the Rf values, observing colour under UV and UV + NH_3 and by comparing with Rf values of authentic standards obtained from Dr. P. Neuman, University of Texas, Austin.

G. Quantification of individual phenolic compounds :

Aliquot of plant extract (20 µl each) was spotted on two sets of chromatographic paper. One set was used for detecting the spots under UV, UV + NH_3 and in a mixture of 1:1 0.3% $FeCl_3$ + 0.3% K_3 $Fe(CN)_6$. The other set was used to mark the area of separated phenolic compounds by over lapping it on the first set. The marked area of separated phenolic compounds on the second set of chromatogram were cut with a sharp razer blade and the individual cut portions were transferred in a Nessler's tube containing 6 ml 80% ethanol and eluted by boiling it for 5 min. till the smell of ethanol get vanished. After removal of the paper and cooling the tubes at room temperature, 1 ml 20% Na_2CO_3 and 0.2 ml Folin-Denis reagent were added. The volume was made to 10 ml with distilled water. The absorbance of the colour intensity was measured at 660 nm. on double beam spectrophotometer (Shimadzu). Paper blanks of comparable size were taken as background colour. The calibration curve was prepared with tannic acid. The values of the individual phenolic compounds were recorded as µg $spot^{-1}$.

H. Oxidative enzymes :

1. IAA oxidase :

The activity of an enzyme IAA oxidase was measured by using the extraction and assay procedure given by Mahadeven and Sridhar (1982) with slight modification.

a) **Extraction :**

2 g randomly sampled and cleanly washed fresh leaf material of different plastochron age was harvested and pre chilled at 4°C for 1 h and macerated in pre-chilled morter with pestle in 15 ml cold McIlvaine's buffer (PH 4.8). The homogenate was filtered through 4 layered muslin cloth and filterate was centrifuged at 5000 x g for 10 min at 4°C. The supernatant was used for enzyme assay.

b) **Assay :**

The activity of IAA oxidase was determined by measuring the residual amount of IAA in the reaction mixture containing 2 ml McIlvaine's buffer (PH 4.8), 0.5 ml $MnCl_2$ (0.05M), 0.5 ml 2, 4 Dichlorophenol (0.01M), 1 ml IAA (0.01M) and enzyme in a total volume of 5 ml. The reaction mixture was incubated at 30°C in a waterbath and at an interval of 15 min. 1.5 ml Salper reagent was added and the intensity of stable pink colour developed was measured at 535 nm.

2. **Polyphenol Oxidase (E.C. 1 10.3.2)**

The activity of an oxidative enzyme polyphenol oxidase was studied spectrophotometrically by using the extraction and assay procedure suggested by Mahadevan and Sridhar (1982) with slight modification to suit our laboratory conditions.

a) **Extraction**

1 g randomly sampled and cleanly washed leaf material was cut into small pieces and extracted in 15 ml cold 0.1 M phosphate buffer (pH 6.1) in pre-chilled mortar with pestle.

The homagenate was filtered through 4 layers of muslin cloth and centrifuged at 5000 g at 4°C. The supernatant was used for assaying the enzyme activity.

b) Assay :

In order to score the activity of polyphenol oxidase the oxidation of catechol was measured from the reaction mixture containing 2 ml phosphate buffer (PH 6.1), 0.5 ml enzyme extract and 1 ml. 0.01 M Catechol at 495 nm. The change in the absorbance between the first 30 sec. and 150 Sec, of incubation at room temperature (27°C) was measured. The control reaction was maintained with heated enzyme.

I Phenylalanine Ammonia Lyase (PAL) :

Activity of an enzyme phenylalanine ammonia lyase was assayed according to slightly modified method of Bopp and Murrach (1980) and Mahadvan and Sridhar (1982).

1. Extraction :

0.5 g cleanly washed fresh leaf material of different plastochron age was homogenised in 0.025 M cold Sodium Borate buffer containing 0.01 M mercaptoethanol (PH 8.8) and filtered through 4 layered muslin cloth and the filtrate was centrifuged at 10,000 x g for 15 min. The supernatant was used as a source of an enzyme.

2. Assay

The assay mixture consists of 0.5 ml enzyme, 1 ml 0.025 M Sodium Borate buffer (PH 8.8), 0.3 ml 0.01 M L-phenylalanine and 1.2 ml distilled water. The assay mixture was incubated at 30 °C for 15 min to allow for an initial non enzymatic decrease

in absorbance (which is usually detected in control reactions containing boiled enzyme or no substrate). The absorbance of the assay mixture was measured at 290 nm at 20 min interval for 1 hr. A reaction mixture without either L - phenylalanine or enzyme extract was used as the control.

J. Enzyme Chlorophyll :

Enzyme chlorophyll was determined by measuring the absorbance of a clear filtrate obtained by mixing 1 ml. enzyme extract + 8 ml. pure acetone + 1 ml. extraction medium at 652 nm. by the method reported by Mahadevan and Sridhar 1982.

K. Enzyme Protein :

The enzyme protein was determined by using phenol cataicaeu reagent by the method of Lowery et al (1951).