# CHAPTER-II

# MATERIAL AND METHODS

A) **Material** :

In all about 60 improved groundnut varieties have been released by different states for general cultivation. During the period from 1968 to 1981, (when All India Co-ordinated Research Project on Oil seeds (AICORPO) came into existence) as many as 29 improved varieties have been released which include varieties like M-145, TMV-10, Jyoti, JL-24 (phule-pragati) etc. These varieties fall under three habit groups such as Bunch (Spanish/Valencia), Semispreading (Virginia Bunch) and spreading (Virginia Runner). For present investigation a promising cultivar of groundnut JL-24 was selected.

JL-24 (Phule pragati) is a cultivar of groundnut belonging to botanical group valencia and habit group Bunch. It is a selection from EC 94943. It was released in the year 1978 in Maharashtra from Jalgaon Centre. It is early maturing variety ( 90-100 days) having compact bearing of pods. The leaves are dark green. The pods are smooth and 2 to 3 seeded, seeds are non-dormant. Seeds bear rose colour testa which is susciptible to pests. The average pod yield of this variety is 1800 kg $ha^{-1}$ with shelling percentage 75% and seed oil content 50%.

The pods of groundnut CV JL-24 were locally purchased. Shelling was done periodically as and when required.

B) **Methods** :

In the present investigation the presowing soaking treatment described by Salim and Todd (1968) is followed with some modification. Fifty grams of groundnut seeds were soaked in 250 ml, 100 ppm solutions of CCC and Kinetin prepared in distilled water and acetone for 4 hours. (CCC was soluble in water, its solution was prepared in small amount of water and then diluted with acetone and water while kinetin was dissolved in small volume of sodium hydroxide solution and then diluted with acetone and water). Then seeds were air dried till the original weight was maintained. Untreated seeds were used as control. The groundnut plants were raised in polythene troughs of uniform dimensions (54 cm x 28 cm x 17 cm). The troughs were filled up with garden soil and compost in the proportion 3:1 (v/v).

Ten seeds from each treatment were sown at a depth of 2 cm in specifically labelled troughs. Two sets of such troughs were maintained. One was used as a control. The troughs were regularly watered with 1 litre of tap water per day. After uniform growth of seedlings only four seedlings in each tube were maintained in order to avoid over crowding and shadowing. The watering was done regularly for one month. After one month of plant growth one set was subjected to 10 days water stress by witholding the water supply for ten days, while other set used as control was regularly watered. On 10th day of stress

groundnut plants were analysed to find out the influence of presowing soaking treatment on various physiological parameters associated with drought resistance. The experiment was performe in triplicate and the values of mineral nutrients, organic constituents, enzyme activity and stomatal parameter depicted in Chapter 'Results and Discussion' represent average of three determinations.

a) Inorganic constituents :

The inorganic constituents were determined from oven dried plant parts. The plant parts were subjected to acid digestion following the standard method of Black (1965). Accurately weighed (0.5 g or less) plant material was taken in a 100 ml clean corning beaker and to it 10 ml of acid mixture was added. (Acid mixture was prepared by mixing 75 ml $HNO_3$ + 15 ml $H_2SO_4$ + 30 ml $HClO_4$). The beaker was kept in fuming hood for 1 hour till the primary reaction subsided. Then beaker was subjected to heating first on low flame and then vigorously till clear solution remained. It was cooled and transferred to 100 ml volumetric flask and volume was made to 100 ml with distilled water. Then it was filtered through a dry Whatman No.1 filter paper. The filtrate was used for the estimation of inorganic constituents such as $Na^+$, $K^+$, $Ca^{+2}$, $Mg^{+2}$, $Mn^{+2}$, $Fe^{+3}$, $P^{+5}$. Of these $Ca^{+2}$, $Mg^{+2}$, $Mn^{+2}$, $Fe^{+3}$ were estimated on Atomic absorption spectrophotometer at common facility centre, Shivaji University, Kolhapur. $Na^+$ and $K^+$ were estimated

flame photometrically following the standard procedure. For flame photometric estimation of $Na^+$ and $K^+$ stock solutions of known concentration in parts per million (ppm) of K (K of KCl) and Na (of NaCl) were prepared. The standard curve was obtained by using various concentrations (2, 4, 6, 8, 10 ppm) of $Na^+$ and $K^+$ in ppm. From these standard curves the concentrations of $Na^+$ and $K^+$ in the acid digest were calculated..

Phosphorus :

Phosphorous was estimated according to the method of Sekine et al. (1965). Phosphorus was estimated colorimetrically because it gives yellow colour reaction with molybdate vandate reagent. Phosphorus can be estimated colorimetrically by comparing the colour intensity of known standards with colour intensity of sample.

In clean, labelled test tube 1 ml and acid digest was taken. To it 2 ml of 2 N $HNO_3$ and 1 ml of molybdate vandate reagent (A : 1.25 g of ammonium molybdate were dissolved in 500 ml N $HNO_3$. B : 25 g of ammonium vandate were dissolved in 500 ml distilled water. Then A and B were mixed in equal volumes) were added. The volume of reaction mixture was made to 10 ml with distilled water. The reaction mixture was shaken well and kept for 20 minutes for full colour development. Absorbance was read at 420 nm using blank reaction mixture containing no phosphorus.

Standard curve for phosphorous was prepared using various concentrations of phosphorous (0.025, 0.05, 0.1, 0.2, 0.4 mg phosphorous) and standard $KH_2PO_4$ solution containing 0.025 mg phosphorous/ml. By using the standard curve the amount of phosphorous in the plant material was calculated.

b) Organic constituents :

i) Moisture percentage : Moisture percentage in various plant parts was determined from the difference between fresh weight and dry weight (weight taken after drying the plant material in oven at 80°C till a constant weight was obtained).

ii) Relative water content : Relative water content is the water content of a tissue expressed as a percent of the water content of the fully turgid tissue. It was determined by the method of Slatyer (1967).

25 leaf discs were punched out and their initial weight ($W_1$) was recorded accurately. The leaf discs were then transferred to a petridish containing distilled water. They were immersed in petridish until they become fully turgid and water saturated. (This period was of 4 hours). Then leaf discs were removed and their weight in turgid condition was recorded (Turgid weight $W_2$). Then the discs were allowed to dry in oven at 80°C for four days and then dry weight ($W_3$) was recorded.

The relative water content was calculated by using following formula.

$$\text{Relative water content (RWC)} = \frac{W_1 - W_3}{W_2 - W_3} \times 100$$

Where,

$W_1$ : weight of fresh leaf discs.

$W_2$ : weight of turgid leaf discs.

$W_3$ : weight of oven dried leaf discs.

iii) Carbohydrates :

Carbohydrates were estimated according to method of Nelson (1944). Fresh leaves (0.3 g) were extracted with 80% ethanol. Then extract was, filtered through Buchnner's funnel by using filter paper (Whatman No.1). The filtrate was collected and used for the estimation of sugars while the residue was used for the estimation of starch. The filtrate thus collected was condensed to 3-5 ml on water bath in porcelain dish. Then decolorizing agents lead acetate and potassium oxalate (1 g : 1 g) were added and thoroughly mixed with condensed filtrate. Then about 15 ml of distilled water was added to it and filtered through filter paper (Whatman No.1). The residue was washed for three times with distilled water. The final volume of aliquot was measured and noted down. 20 ml of this extract (aliquot) was hydrolysed with 2 ml cone HCl in 150 ml conical flask with a bored cork by autoclaving at 15 lbs

pressure for half an hour. The contents were cooled, neutralized with sodium carbonate and filtered. This filtrate was used for the estimation of total sugars.

The insoluble residue of starch along with filter paper was transferred to 150 ml conical flask. Then 50 ml distilled water and 5 ml conc. HCl were added. The conical flask was corked with bored cork and contents were hydrolysed by autoclaving at 15 lb. pressure for half an hour. The contents were cooled, neutralized with sodium carbonate and filtered through filter paper (Whatman No.1). The final volume was measured and noted down. This filtrate contains reducing sugars produced as a result of hydrolysis of starch. These sugars were estimated.

The appropriate quantity (0.5 ml) of extract prepared for estimation of total sugars and starch was taken separately in 10 ml marked test tubes. In other such test tubes different concentrations (0.1 ml, 0.2 ml, 0.3 ml, 0.4 ml and 0.5 ml) of standard glucose solutions were taken (standard glucose solution contains 0.1 mg glucose $ml^{-1}$ distilled water) 1 ml of Somogyi's alkaline copper tartarate reagent (4 g of $CuSO_4$, 5 $H_2O$; 24 g of anhydrous $Na_2CO_3$; 16 g of Na-K-tartarate (Rochella salt) and 180 g of anhydrous $Na_2SO_4$ dissolved in 1 litr. of distilled water) was added to each test tube. All test tubes containing mixtures were kept in water bath for about 10 mimutes. Afterwards they were cooled to room temperature and 1 ml of arsenomolybdate

reagent (25 g of ammonium molybdate { $(NH_4)_6 MO_7 O_{24}$, 4 $H_2O$} was dissolved in 450 ml of distilled water. To it was added 21 ml of conc. $H_2SO_4$ to this solution, then, was added 3 g of sodium arsenate ($Na_2 HASO_4$, 7 $H_2O$) dissolved in 25 ml water. All ingradients of reagent were mixed well and reagent was kept in incubator at 37°C for 24 hours before use. The reagent was stored in brown bottle) was added to each test tube with the help of burette. The contents of each test tube were then diluted to 10 ml with distilled water. The blank was prepared by the same way but without any sugar solution. After 10 mimutes absorbance of each reaction mixture was read at 560 nm on (double beam spectro photometer UV-190) shimadzu. The standard curve was used for calculating the amount of sugars in various samples. The values are expressed as g 100 $g^{-1}$ dry tissue.

iii) RNA content :

RNA content in leaf tissue was determined by using the method of Legocka and Szweykowska (1981). 0.5 g leaves were homogenized in 10 ml of 10% TCA (0.4°C) in a chilled mortor with pestle. Then extract was centrifuged at 1000 x g for 10 minutes. After washing the pellet 3 times with cold 10% TCA it was

extracted with 5% (w/v) perchloric acid at 85°C for 15 minutes on water bath and then the absorbance of the extract was measured at 260 nm on Shimadzu double beam spectrophotometer-UV-190. Standard curve of RNA was prepared from different concentrations of RNA (0.1 mg/ml of 5% (w/v) perchloric acid). The values expressed are mg 100 $g^{-1}$ dry tissue.

iv) Free proline :

Free proline was estimated from various plant parts (viz. leaf stem and roots) according to method of Bates et al., (1973). 0.5 g fresh plant material was homogenized in 10 ml of 3% sulfosalicylic acid. After complete homogenization it was filtered through Buchnner's funnel by using Whatman No.1 filter paper. Then filter paper was washed repeatedly with small amount of 3% sulfosalicylic acid. The filtrate was collected and volume was made to 20 ml with 3% sulfosalycylic acid. 0.5 ml extract was taken in clean dry test tube and 2 ml of acid ninhydrin reagent was added to it. (Acid ninhydrin was prepared by warming 1.25 g ninhydrin in 30 ml glacial acetic acid and 20 ml of 6 M phosphoric acid with agitation cooled and stored at 4°C). Then 2 ml of glacial acetic acid was added to it. The reaction was allowed to continue for about 1 hour by keeping test tubes in boiling water bath at about 100°C. At the same time reaction mixtures for standard proline curve were prepared by taking different concentrations of proline

(0.1 ml, 0.2 ml, 0.4 ml, 0.8 ml). Standard proline solution contains 0.1 mg proline $ml^{-1}$ of sulfosalicylic acid. After 1 hour the reactions in all test tubes were terminated by keeping them in ice bath. Then 4 ml of tolune were added to each test tube with vigorous shaking for 15 to 20 seconds. The reaction mixtures were brought to room temperature and absorbance of toluene chromophore layer was read at 520 nm using toluene blank. Toluene chromophore layer was pipetted out with the help of vaccupipette. The standard curve of proline was used for the determination of proline in various samples. The values are expressed as mg 100 $g^{-1}$ dry tissue.

v ) Organic Acids (TAN) :

Titratable Acid Number (TAN) values was estimated according to the method of Thomas and Beevers (1949). The plant material (leaves) was washed with distilled water and blotted to dryness. Then it was cut into small bits. 1 g plant material was accurately weighed and put in boiling distilled waterin a 150 ml beaker and boiled for half an hour. Then it was allowed to cool to room temperature and filtered through cheese cloth. The volume of extract was made to 25 ml with distilled water. 10 ml of extract was titrated against N/40 NaOH using phenolphthalein as an indicator. Before this titration NaOH was standardized against N/40 oxalic acid using the same indicator. Titratable acid number is helpful in determining the acidity of the plant

material. It represents the number of ml of decinormal alkali (NaOH) required to neutralize acids present in 100 g of the plant material. The values in this experiment are expressed on dry weight basis. TAN was calculated by using following formula.

$$TAN = \frac{\text{Volume of oxalic acid taken for titration}}{\text{Titration reading (ml)}} \times \frac{\text{Total Vol.of extract}}{\text{wt. of plant material in g}} \times \frac{\text{Extract reading (ml)}}{\text{Vol.of extract taken for titration.}} \times \frac{100}{4}$$

vi ) Ascorbic acid content :

Ascorbic acid content in plant leaves was estimated following the method of Åberg. (1958). The material was washed and cleaned with distilled water and immediately blotted to dry. It was weighed accurately (3 g) and cut into 2 to 3 mm pieces. Then pieces were placed in mortor and oxalic acid (0.4% w/v) was added at the rate of 4 ml/g tissue. Leaves were thoroughly crushed for about 5 minutes and the extract was filtered through 2 layers of cheese cloth. Then it was centrifuged at 1000 g for 20 minutes. The volume of supernatant was made to 15 ml (1 g tissue in 5 ml oxalic acid) with oxalic acid reagent (0.4% w/v). Ascorbic acid in extract was estimated by visual titration method based on reduction of 2,6,dicholrophenol indophenol dye.

Standard ascorbic acid solution was prepared by adding 50 mg of ascorbic acid to 50 ml of 0.4 percent oxalic acid solution in 250 ml volumetric flask and finally volume was made to 250 ml with oxalic acid. One ml of this solution contains 0.2 mg of ascorbic acid.

Indophenol reagent was prepared as follows. 150 ml of glass distilled water was added to 200 ml volumetric flask. Then 50 mg of sodium 2,6,dichlorophenol indophenol was added to it. The flask was gently heated in a hot water bath to dissolve the dye. Then 42 mg of $NaHCO_3$ were added. The flask was allowed to cool. After cooling the volume of solution was made to 200 ml with glass distilled water and the reagent was stored in dark glass bottles at 2°C. For standardization of Indophenol reagent 5 ml ascorbic acid solution was taken in white porcelain dish and then it was titrated against the indophenol dye kept in burette until the solution became pink. (The pink colour persisted for at least 15 seconds).

After standardization of indophenol reagent 5 ml of plant extract (prepared as described earlier) was titrated against standardized indophenol reagent as above.

The ascorbic acid content of the extract was calculated by using formula -

$T \times S \times D/A \times 100/W$ = mg of ascorbic acid 100 $g^{-1}$ fresh tissue.

Where,

I = ml of indophenol reagent used in the titration.

S = mg of ascorbic acid reacting with 1 ml of the reagent.

D = Volume of the extract in ml.

A = The aliquot titrated in ml.

W = The weight of the sample in grams.

The value of ascorbic acid so obtained was further corrected on the basis of moisture content in the fresh tissue and expressed on dry weight basis.

C) Enzymes and - SH - content.

i) Nitrate Reductase (E.C. 1.6.6.2) :

Activity of nitrate reductase was determined following the in vivo method of Jaworski (1971). The plant leaves were washed with distilled water and then immediately blotted to dry. 0.5 g of leaves were accurately weighed and incubated in the medium containing 1 ml 1 M $KNO_3$. 2 ml 5% n-propanol, 5 ml 0.2 M phosphate buffer (pH = 7.5) and 2 ml 0.5% Triton-x-100 in sealed tubes for one hour in dark. After incubation period 1 ml of reaction medium was taken out and mixed with 1 ml 1% sulfanilamide in 1 M HCl and 1 ml 0.02% NEEDA (N-1-Naphthylethylene diamide dihydrochloride). The absorbance was read at 540 nm on Shimadzu double beam spectrophotometer UV-190.

Standard curve was prepared with 0.03 mM $KNO_3$ (0.0026 mg

$KNO_3$ $ml^{-1}$ distilled water) while mixture of 1 ml incubation medium, 1 ml sulfanilamide and 1 ml NEEDA was used as a blank.

The activity of NR is expressed on dry weight basis in terms of $\mu g$ $NO_2$ liberated $g^{-1}$ dry tissue $h^{-1}$.

ii) Sulphydryl groups :

The content of sulphydryl groups was determined following the method of Ellman (1959). Plant material (leaves) was washed with distilled water and blotted to dryness. Then it was cut into small bits 0.2 g material was accurately weighed and crushed in 10 ml phosphate buffer (pH 7). Extract was filtered through Buchner's funnel using Whatman No.1 filter paper. The reaction mixtures were prepared for 0 minutes and 5 minutes. Zero minutes reaction mixture contained 2.3 ml of phosphate buffer (pH-7), 0.2 ml of 1 N NaOH and 0.5 ml of extract. To this reaction mixture 0.02 ml of 5-5'-dithiobis (2-nitrobenzoic acid) (DTNB) was added. (It was prepared by dissolving 39.6 mg of 5-5'-dithiobis (2 nitrobenzoic acid) in 10 ml phosphate ($\mu$ = 0.1) buffer pH=7). The absorbance was read for 0 minutes and 5 minutes reaction mixtures at 412 nm on Shimadzu double beam spectrophotometer UV-190. The sulphydryl content was calculated by using formula.

$$Co = \frac{A}{\epsilon} \times D$$

Where,

Co = Original concentration.

A = Absorbance at 412 nm.(Difference between 5 min. and 0 min. reading).

$\epsilon$ = Extinction coefficient = 13600/m/cm.

D = Dilution factor.

$$D = \frac{\text{Volume at 5 minutes (after addition of DTNB)}}{\text{Volume at 0 minutes (without addition of DTNB)}}.$$

The results are expressed on dry weight basis taking into consideration the moisture content in the leaf material.

d) Chlorophylls and carotenoids :

Chlorophylls were estimated following the method of Arnon (1949) while the method of Kirk and Allen (1965) was followed for the estimation of carotenoids. The fresh leaves were washed with distilled water and blotted to dryness. Exactly 0.25 g leaves were weighed. They were cut into small bits and crushed in mortor with pestle in 80% chilled acetone in cold room. The extract was filtered through Buchnner's funnel using Whatman No.1 filter paper. The residue was washed with 80% acetone. Then filtrate was collected and the volume was made to 25 ml with 80% acetone. The absorbance for chlorophylls was read at 663 nm and that for carotenoids was read at 480 nm.

Chlorophylls and carotenoids were calculated by using the following formulae :

Chlorophyll a = 12.7 x A 663 - 2.69 x A 645 = X

Chlorophyll b = 22.9 x A 645 - 4.68 x A 663 = Y

Chlorophyll a (mg 100 $g^{-1}$ fresh tissue) =

$$= \frac{X \times \text{Vol.of extract} \times 100}{1000 \times \text{weight of plant material in grams}}$$

Chlorophyll b (mg 100 $g^{-1}$ fresh tissue) =

$$= \frac{Y \times \text{Vol.of extract} \times 100}{1000 \times \text{weight of plant material in grams}}$$

Carotenoids (mg 100 $g^{-1}$ fresh tissue) =

$$= \frac{A\ 480 \times \text{Vol.of extract} \times 10 \times 100}{2500 \times \text{weight of plant material in grams}}$$

Chlorophylls and carotenoids (mg 100 $g^{-1}$ fresh tissue) were calculated and then the values were expressed on dry weight basis taking into consideration the moisture content in leaves.

e) Stomatal behaviour :

The stomatal behaviour was studied from leaves on identical position in plants of various treatments by using autoporometer model steady state porometer II 1600 LICOR (U.S.A.). With the help of this instrument percentage relative humidity, light intensity in quantum, Diffusive resistance in terms of Sec/cm, Transpiration rate in terms of $\mu g/cm^2/sec$ and flow rate in terms of $cm^3/sec$ of both leaf surfaces were determined at 11.30 a.m.