MATERIALS AND METHODS

A] Material

For the present investigation groundnut varieties S.B – 11 and W – 55 were selected due to their following characters. **Plate No. 1.**

SB– 11 :-

This variety was released in 1965 in Maharashtra. It was produced by an intervarietal cross between selection A H. 4218 (B) X, A H 4354. By habit this variety is bunch type. The height of this variety 30-35 cm. Branching habit is generally n+1. Leaflet colour is pale green and size is larger, Pod sizes are small but with a small beak developed at the tip and reticulation on the pod are not so prominent. Kernel is with 1-2 seeded. Kernels are small, pink in color. Oil percentage is about 40%. Flowering duration is 55-60 days. Days for maturity are 105–110 for this variety. The variety is cultivated in both Kharif as well as summer season. Under ideal condition it yields about 1200-1400 Kg/ha.

W – 55 :-

This variety was released by Western Agri Seeds Ltd, Gujarat .It is research variety and type of variety is G_2 Variety .The height of this variety is 15-16 inches. Leaflet colour is darkgreen and size is larger. Flowering duration is witnin 35 days .Kernal is with 2 seeded which matures between 95-100 days. Oil percentage is 40% and under ideal condition it yields about 1200-1500 Kg per Acre.

B] Methods:-

The seed samples of variety SB-11 and W-55 were collected from Agricultural Research Station, Karad.

The healthy seeds were sorted out and surface sterilized with 20% Sodium Hypochlorite (4 %) for 10 minute followed by through washing with distilled water. Twenty seeds were placed on whatman filter paper. No- 1 in sterilized Petri dishes in BOD incubator 30^0 c for germination in 15 ml of various solutions. Aluminium toxicity studies involve treatments of seeds with solution of Aluminium sulphate containing 10 ppm, 50 ppm and 100 ppm of aluminium. The investigation covered different stages of germination from 24 hrs to 120 hrs.

1) Germination Studies :-

The emergence of radical from seed coat was acknowledged as criterion for germination and accordingly germination performance was evaluated. The seedling

growth were analyzed for various growth parameters including germination percentage, seedling growth. Germination percentage as well as seedling growth was recorded in both the selected cultivars of groundnuts under the aluminium toxicity.

2) Moisture :-

Accurately weighed 5gm of sample prepared in moisture dish, which is dried previously in oven and accurately weighed Dish is placed in over for 4 hours at 105 $\pm 1^0$C and cool in the desiccators and weight process is repeated of drying cooling and weighing at 30 minute intervals, till difference between two consecutive weighing is less than one milligram Also lowest weight is recorded. Moisture is calculated in percentage by using formula.

$$\text{Moisture percentage} = \frac{\text{Initial Wt. - Final Wt.}}{\text{Initial Wt.}} \text{ X } 100$$

3) Qualitative determination of Al tolerance :-

The relative aluminium tolerance in different crops with the use of different stains (hematoxylin, Molybdenum etc) was investigated by some workers such as McLean and Gillbert (1927), Bennet *et al.*, (1985), Rincon and Gonzales (1992) and Ownby (1993). This is the most rapid and simple assay method for determination of Al tolerance.

Five-day old seedling of groundnut treated with 10, 50 and 100 ppm of Al, were subjected to hematoxylin stain, according to Polle *et al.*, (1978) with slight modification.

These Seedling first rinse with deionized water, then rinse with 1 mM K_2PO_4 for 30 min (pH 4.5). The roots of intact seedling of all treatment were rinsed in deionized H_2O for 30 min, then transferred to 60 ml of hematoxylin solution for 1 min. The roots were then rinsed in the ionized H_2O for 2 min, and transferred to fresh stannous chloride solution. (1 ml of concentrated HCL containing 0.1 g $SnCl_2$, H_2O, diluted with 99 ml deionized H_2O) in 10-15 min, after which the seedlings were transferred to deionized H_2O until photographed.

4] Oil :-

Seeds like groundnut, are rich in oil. The germination groundnut is accompanied by breakdown of large amount of fat stored in endosperm. Fat which are

Plate 1
Seeds of groundnut cultivators
W - 55 and SB - 11

esters of glycerol. When in liquid form that is called oil. In groundnut oil is present as reserve food for further development of embryo. The methyl esters of FA present in the oil were prepared by the method of Morrison and Smith, (1964).

2 gms of plant material taken in continuous extraction apparatus with diethyl ether for 18 hrs. Ether is removed by distillation, followed by blowing with a steam of air with the flask on a boiling water bath and dry in an oven at 110 ± 1^0 C till the loss in mass between two successive weighing is less than 2mg.Residue shake with 2-3 ml of diethyl ether at room temp, allow to settle and decant the ether. Repeat the extraction until no more of the mass between successive weighing is less than 2mg. Lowest mass is noted and calculated by formula.

$$\text{Oil \%} = \frac{100 \text{ X } (M_1\text{-}M_2)}{M}$$

5) Carbohydrate Metabolism:-

The seedlings at each stage of germination from 24 h, to 120 h, were washed with distilled water, blotted to dryness and were kept in oven at 60^0 c for 10 days for drying. The dried material was powdered and soluble sugars were estimated following the method of Nelson (1944).

The soluble carbohydrates were exacted from 0.5 g oven dried material with 80% neutral alcohol. The extract was filtered through Buchner funnel employing whatman No.1 filter paper. The filtrate thus obtained was condensed on water bath to about 5 ml. To this 2.3 g lead acetate and potassium oxalate (1:1) were added for decolourization. After decolourization 50 ml of distilled water were added and aliquot was filtered. The volume of filtrate was measured and it served as an extract (20 ml) was hydrolyzed with 2 ml conc. HCL at 15 lbs. atmospheric pressure for half an hour. The content was cooled, neutralized with addition of an hydrous sodium carbonate and filtered. The volume of filtrate was measured. This filtrate was used for the estimation of total [reducing and non-reducing] sugars.

The reducing sugars from both the filtrates were estimated by determining the reducing power by employing arsenomolybdate reagent introduced by Nelson (1944), for the colorimetric determination of cuprous oxide formed in the oxidation of sugars by alkaline copper reagents. For this 0.5 ml aliquots were taken in test-tubes. To this required amount of D.W. was added to make final volume 1 ml. In case of blank instead of filtrate or standard glucose distilled water was added to begin with reaction.

One ml of somogy's alkaline copper tartarate reagent (4g $cuso_4$, $5H_2O$, 24g anhydrous Na_2CO_3, 16g Na-K tartarate and 180g anhydrous Na_2SO_4 dissolved in 1 litter distilled water.) was added in each test-tube water bath for 10 minutes. After cooling to room temperature 1 ml arsenomolybdate reagent [25g ammonium molybdate in 450 ml water to which 21 ml conc. H_2SO_4, were added followed by 3g sodium arsenate (Na_2HASO_4, $7H_2O$) dissolved in 25 ml distilled water. These ingredients were mixed well and digested for 48h at 37° c in incubator before use] was added to each reaction mixture which were further diluted to 10 ml with distilled water. After 10 minutes absorbance was read at 560 nm on spectrophotometer.

In order to prepare a calibration curve different concentration of standard glucose solution (0.1 mg/ml) were taken in another set of test-tubes and they were allowed to react with alkaline copper tartarate and arsenomolybdate reagent in the similar manner and the amount of sugars in the plant material was calculated. Values of soluble sugars are expressed as mg g^{-1} dry tissues.

6] Enzymes :-

All the enzymes were studied using *in vitro* technique. For the exaction of most of the enzyme seedlings form different treatments along with control from each germination stage (24, 48, 72, 96 and 120 h respectively). Were selected and they were made free from seed coats. The extraction and assay of enzymes were performed in the following.

a] Enzyme - Lipase (EC.3.1.1.1) :-

For extraction and study of Lipase activity titrimetric methods of Wolf (1968) and Chinoy *et al.*, (1969) were followed. Five gms of germinating seeds from each treatment were taken and seed coat was removed. The seed material was repeatedly homogenized in 50 ml of chilled acetone. This was filtered through two layers of cheese cloth. The oil free residue left on the cheese cloth served as the source of enzyme lipase. For study of this enzyme Triacetin was employed as substrate, 1 ml of triacetin was allowed to react with 500 ml of enzyme in 10 ml of 0.1 M phosphate buffer pH 7.5 in conical flask. The conical flask was continuously shaken for 60 minutes and the reaction was terminated by addition of mixture of 30 ml ethyl alcohol and 10 ml solvent ether. The contents of conical flask containing free fatty acids liberated due to lipase action were titrated against 0.1 N NaOH using phenolphthalein indicator. Simultaneously in another conical flask a similar reaction mixture was

taken but the reaction was terminated immediately following addition of enzyme (0 min). The difference in 60 min and 0 min titration readings was considered as a measure of enzyme activity and expressed as ml alkali consumed min^{-1} g^{-1} fresh tissue.

b] Enzyme - Peroxidase (EC.1.11.1.7) :-

Peroxidase activity was assayed according to method of Kondo and Morita (1951) as described by Horigunchi (1988). The seed coat was removed and 1g of germinating seeds from all the treatments at different stages of germination (24 to 120 h) were homogenized in 10 ml of 1 (1/15 M) Phosphate buffer (pH 6.8) and filtered through 4 layers of muslin cloth. The filtrate was centrifuged at 10,000 rpm for 20 minutes at 4°c and supernatant was used as source of enzyme.

The reaction mixture contained 5 ml of 1/15 M Acetate buffer (pH 5), 0.5 ml of 0.1% Guiaicol, 1 ml enzyme extract, 2 ml distilled water, 0.5 ml of 0.08% H_2O_2 were mixed and incubated at 30°c. After 15 minutes of incubation, 1 ml of 1N H_2SO_4 was added to stop the reaction. In another test-tube the reaction mixture was taken in the similar manner and the reaction was immediately terminated by addition of 1N H_2SO_4. The difference in absorbance at 470 nm of '0' minute and '15' minutes reaction mixture was determined with the help of shimatzu double bean spectrophotometer. The soluble protein in the enzyme extract was assayed according to method to Lowry *et al.,* (1951). The unit of enzyme calculated by the method described by Bergmeyer (1974). The enzyme activity is expressed as units hr^{-1} mg^{-1} protein.

7) Estimation of Nitrogen:-

Total Nitrogen in digest was determined by micro – Kejldahl method. It was estimated following the method of Hawk *et al.,* (*1*948). The oven dried powdered plant material (0.1 g) was taken in Kjeldahl flask with a pinch of microsalt (200 gm K_2SO_4 + 5 gm $CuSO_4$, dehydrogenase) and to it 5 ml H_2SO_4 (1:1) was added. Few glass beads were added to avoid bumping and the material was digested on low flame .After complete digestion a faint yellow solution was obtained which cooled to room temperature, transferred to volumetric flask and diluted to 100ml with distilled water. In Nessler's tube 1ml of plant extract and different concentration of standard Ammonium Sulphate solution. Solution of Ammonium Sulphate dissolved in distilled water and few drops of H_2SO_4 were added. The volume was made 1000 ml with distilled water. This solution contains 0.05 mg of Nitrogen per ml were taken. In

control tube 1 ml distilled water was taken. To this one drop of 8% $KHSO_4$ was added and volume was made 35 ml with distilled water, to this 15 ml Nessler's reagent was added (Reagent A:7 gm KI + 10 gm $HgCl_2$ in 40 ml distilled water, Reagent B:10 gm NaOH in 50 ml H_2O, A and B are to be mixed in proportion of 4:5 at the time of estimation).

The reaction between the sample and the reagent gives the product $NH_4Hg_2I_3$ which has orange brown colour .This colour was measured after 15 minutes at 520 nm on double beam spectrophotometer.(Shima dzu UV.190).

8) Estimation of organic Phosphorus :-

Organic phosphorus fraction Groundnut seeding were estimated according to method of Racob and Terry (1992) by subtracting inorganic phosphorus content.

Total phosphorus was estimated following method of Sekine *et al.*, (1965). 2 ml of acid digest (in perchloric acid and nitric and prepared as described earlier), was pipetted in test tube to which 2 ml of molybdate vanadate reagent (A=1.25 g of ammonium molybdate were dissolved in 500 ml 1N HNO_3, 'B'= 25 g of ammonium vanadate in 500 ml distilled water. Equal volumes of A and B were mixed), were added. Then final volume in each test tube was adjusted to 10 ml with distilled water. The ingredients were mixed well and kept for 20 minutes. The yellow colored molybdate venadate reagent was measured at 420 nm. Spectrophotometrically using a reagent blank. A standard curve of phosphorus was prepared by taking different concentrations of standard phosphorus solutions an (standard 'P' solution was prepared by dissolving 0.11 gm of monobasic potassium phosphate in distilled water and by adjusting the volume to one liter. This solution contained 25 ppm phosphorus), following the same procedure, the amount of phosphorus in the plant material was calculated with the help of this standard curve.

For estimation of inorganic phosphorus content 500 mg of seeding sample was extracted with 10 ml 20% (w/v) acetic acid. The ip content was measured with Fiske and Subbarrow (1925) method described earlier.

9) Inorganic Mineral Status :-

The peanut seeds were germinated in Hogland nutrient medium containing Aluminium as stress factor.

Two liters of Hogland nutrient medium (1/2 strength) containing following ingredients –

1M $KMNO_3$	- 6 ml
1M Ca $(NO3)_2$	- 4 ml
1M M_9SO_4	- 2 ml
1M $(NH_4)_2$ NPO_4	- 1 ml
5% Iron tartarate	- 1 ml
Micronutrient	- 1 ml

[2.86 g H_3BO_3, 1.81 g $MnCl_2$, $4H_2O$, 0.11 g $ZnCl_2$, 0.05 g $CuCl_2$, $2H_2O$ and 0.05 g Na_2MoO_4, $2H_2O$]

Micronutrient solution is as final volume one liter was prepared by adding above ingredients in 100 ml of distilled water.

Seedling were harvested after 120 h of germination in different concentration of aluminium. They were washed thoroughly with distilled water and dried in oven at 60^0c till a constant weight is obtained. The seedling material was powdered.

a] Preparation of Acid Digest :–

For the estimation of different inorganic constituents an acid digest was prepared by the method of Toth *et al.,* (1948). The oven dried seedling were powdered and 500 mg of powdered plant material was transferred to 150 ml capacity beaker to which 20 ml of concentrated HNO_3 were added. The beaker was covered with watch glass and was kept till the primary reactions subsided. It was then heated slowly to dissolve solid particles. After cooling to room temperature 10 ml perchloric acid (72%) were added to it and mixed thoroughly. It was then heated strongly until a clear and colourless solution (about 2-3 ml) was obtained. While heating the liquid was not allowed to dry. It was then cooled and transferred quantitatively to 100 ml capacity volumetric flask, then diluted to 100 ml with distilled water and kept overnight. Next day it was filtered through dry whatman filter paper No.– 44 (ashless) and the filtrate was used as the source of different inorganic constituents.

b] Estimation of K, Ca, Mg, Mn, Cu, Zn, Mo :-

Sodium, Potassium, Calcium were estimated flame photometrically. Stock solutions of known concentration in parts per million (ppm) of 'K' in KCl (10 to 50 ppm), Ca in $CaCl_2$ (10 to 200 ppm) and 'Na' in NaCl (1 to 10 ppm) were used for calibration curves from these calibration from these calibration curves, the concentrations of K, Ca, Na respectively in acid digested samples were calculated.

Molybdenum is estimated by using Automic spectrophotometric method, at 313.3 nm. The acid digest extract was used to estimated Ca, Mg^{2+}, Fe^{3+}, Zn, Mn^{2+} and Mo on atomic absorption spectrophotometer [Perkin–Elmer Model - 3030] using acetylene air flame. The light source employed was hollow cathode lamp. The concentration of Ca, Mg^{2+}, Fe^{3+}, Mn^{2+} and Zn, Mo were read at 422.7 nm, 285.2 nm, 248.3 nm, 231.9 nm and 279.5 nm, 313.3 nm respectively.