CHAPTER - II

MATERIAL AND METHODS
Photo Plate No.1

Seeds of Cv. SB-11
II. MATERIAL AND METHODS:

1. A. Experimental Details:

A field experiment was conducted during 2009-2011 to study the influence of foliar application of salicylic acid and its analogs namely, Acetyl salicylic acid and Sulfosalicylic acid on physiology of groundnut. The experimental site consisted of medium black loam soil. The present work consisted of 12 treatments and the groundnut genotype used was SB-11 (Plate 1). The salient features of groundnut cv. SB-11 are depicted in Table-11.

Table 11. Salient Features of Groundnut Cv. SB-11

<table>
<thead>
<tr>
<th>Sr. No.</th>
<th>Character</th>
<th>Features</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Pedigree</td>
<td>Released in 1955 in Maharashtra, developed by intervarietal cross between selection AH 4218(B) × AH4354</td>
</tr>
<tr>
<td>2</td>
<td>Botanical type</td>
<td>Spanish bunch</td>
</tr>
<tr>
<td>3</td>
<td>Branching habit and Number of branches</td>
<td>n+1, 4 to 5 branches per plant</td>
</tr>
<tr>
<td>4</td>
<td>Growth condition</td>
<td>Kharif and rainfed</td>
</tr>
<tr>
<td>5</td>
<td>Growth habit</td>
<td>Errect</td>
</tr>
<tr>
<td>6</td>
<td>Leaf size and colour</td>
<td>Larger and green</td>
</tr>
<tr>
<td>7</td>
<td>Pods</td>
<td>Small in size with a small beak at the tip</td>
</tr>
<tr>
<td>8</td>
<td>Kernels</td>
<td>2 seeded per pod, small pink in colour</td>
</tr>
<tr>
<td>9</td>
<td>Oil content</td>
<td>About 50%</td>
</tr>
<tr>
<td>10</td>
<td>Plant height</td>
<td>35 cm</td>
</tr>
<tr>
<td>11</td>
<td>Duration</td>
<td>105-110 days</td>
</tr>
<tr>
<td>12</td>
<td>Pod yield</td>
<td>1200-1400 Kg/ha</td>
</tr>
<tr>
<td>13</td>
<td>Disease reaction</td>
<td>a) Leaf spot Susceptible</td>
</tr>
<tr>
<td></td>
<td></td>
<td>b) Rust Susceptible</td>
</tr>
</tbody>
</table>
B. Design and Layout:

The experiment was laid out in Randomized Complete Block Design (RCB) with three replications. The plan of layout of the experiment is recorded in Fig. 9. The size of individual plot was 5×3m with recommended spacing for plants (10 cm) and rows (35-40 cm), consisting of 20 plants.

<table>
<thead>
<tr>
<th>RI</th>
<th>RII</th>
<th>RIII</th>
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</thead>
<tbody>
<tr>
<td>T₁</td>
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<td>T₃</td>
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<tr>
<td>T₂</td>
<td>T₄</td>
<td>T₁₀</td>
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<td>T₁₃</td>
</tr>
<tr>
<td>T₁₃</td>
<td>T₇</td>
<td>T₁</td>
</tr>
</tbody>
</table>

**Fig. 9 Plan of Layout of Experiment**

Each horizontal row represents a block.

The number of blocks is the number of replications (RI-RIII).

There are 13 treatments applied in triplicate (T1-T13)
<table>
<thead>
<tr>
<th>T1</th>
<th>Control</th>
</tr>
</thead>
<tbody>
<tr>
<td>T2</td>
<td>Foliar application of 5 ppm SA</td>
</tr>
<tr>
<td>T3</td>
<td>Foliar application 50 ppm SA</td>
</tr>
<tr>
<td>T4</td>
<td>Foliar application 100 ppm SA</td>
</tr>
<tr>
<td>T5</td>
<td>Foliar application 200 ppm SA</td>
</tr>
<tr>
<td>T6</td>
<td>Foliar application 5 ppm ASA</td>
</tr>
<tr>
<td>T7</td>
<td>Foliar application 50 ppm ASA</td>
</tr>
<tr>
<td>T8</td>
<td>Foliar application 100 ppm ASA</td>
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<tr>
<td>T9</td>
<td>Foliar application 200 ppm ASA</td>
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<tr>
<td>T10</td>
<td>Foliar application 5 ppm SSA</td>
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<tr>
<td>T11</td>
<td>Foliar application 50 ppm SSA</td>
</tr>
<tr>
<td>T12</td>
<td>Foliar application 100 ppm SSA</td>
</tr>
<tr>
<td>T13</td>
<td>Foliar application 200 ppm SSA</td>
</tr>
</tbody>
</table>
2. A. Preparation of Salicylic acid (SA) and Acetyl salicylic acid solutions:

SA and ASA (100 mg) were dissolved in little amount of absolute ethanol separately (Shettel and Blake, 1983) and then added drop wise to de-ionized water (ethanol:water, 1:1000, v/v) (Williams et al., 2003). Then water was diluted from this respective stock solutions with de-ionized water and solutions of SA and ASA in concentrations of (5, 50, 100 and 200 ppm) were prepared. pH of all solutions were adjusted to 4.5 to 5 (Larque-Saadvera, 1978).

B. Preparation of solutions of Sulfosalicylic acid (SSA):

Sulfosalicylic acid is water soluble. Firstly, 100 ppm stock solution of SSA was prepared in water and then from this stock solution, solutions of SSA in concentrations of (5, 50, 100 and 200 ppm) were prepared.

The seeds of groundnut cv. SB-11 were collected from Agricultural Research station, Karad. The seeds were surface sterilized with 1% sodium hypochloride for 2 mins., then washed thoroughly with sterilized glass distilled water. The seeds were transferred for field cultivation (2009-2011). The thirty days old seedlings were sprayed with different concentrations (5, 50, 100, 200 ppm) of SA, ASA and SSA (40-50 ml per plant) in 3-doses by keeping 4 days interval. The fresh leaf samples were collected on 7th day after the last spray of SA, ASA and SSA and washed thoroughly with distilled water for further analysis.

3. Growth Analysis

The influence of foliar application of SA, ASA and SSA on growth characteristics of groundnut were studied. Five plants from each treatment plot were tagged randomly at 35 days, 55 days and at harvest, carefully uprooted and washed thoroughly in running tap water. The water drops from plant surface were removed using blotting paper. Each plant was then analyzed for recording morphological observations and growth parameters.

A. Morphological Characters

a) Root length and Shoot length: Root and shoot length was recorded from randomly
selected plants in each treated plot. Length of each plant was measured with thread and the data was statistically analyzed.

b) **Plant height**: The root length and shoot length of respective plants were added together to find out height of the plant. Root-shoot length and plant height was expressed in cm.

c) **Number of branches per plant**: Number of branches per plant was recorded from five selected plants at 35 and 55 days of plant growth. The mean was taken as number of branches per plant.

d) **Number of leaves per plant**: Number of leaves developed on plant was counted and recorded. From this average number of leaves per plant and standard deviation were calculated.

e) **Leaf area**: The leaf area per plant was recorded by linear regression method using the formula,

\[
\text{Leaf Area (A)} = \text{Length} \times \text{Breadth} \times \text{Factor}
\]

Total leaf area per plant was calculated by multiplying the average leaf area per plant with average number of leaves per plant. From this data average leaf area per plant was calculated.

f) **Fresh weight and Dry weight per plant**: For this purpose, five plants from each treatment were uprooted. The plant samples were separated into root and shoots and fresh weight was measured and recorded in grams on per plant basis. After recording fresh weight, the respective plants were kept in oven separately at 80\(^{0}\)C in hot oven for 72 hours, then dry weights were recorded. Finally dry weights and fresh weights were statistically analyzed.

g) **Disease scoring**: The disease severity was recorded at weekly intervals as per the modified scale given by Subrahmanyan (1990). The response of groundnut plants to given treatments with respect to disease incidence was recorded using the scale as shown in **Fig.10**.
B. Growth Parameters:

a) Absolute Growth Rate (AGR): AGR is the dry matter production per unit time (g/g/day) and was calculated by formula (West et al., 1920),

\[
AGR = \frac{(W_2 - W_1)}{(t_2 - t_1)}
\]

Where,

\(W_1\) = Dry weight of the plant at time \(t_1\)

\(W_2\) = Dry weight of the plant at time \(t_2\)

b) Relative Growth Rate (RGR): RGR was calculated using the formula,

\[
RGR = \frac{\log_{10}(W_2/W_1) \times 2.303}{(t_2 - t_1)} \quad \text{(g/g/day)}
\]

Where,

\(W_1\) = average fresh weight or dry weight (g) at time \(t_1\)

\(W_2\) = average fresh weight or dry weight (g) at time \(t_2\)

\(t_1\) = initial time

\(t_2\) = final time

\(t_2 - t_1\) = is the time interval between two successive growth analysis stages (i.e. 20 days)

2.303 = the conversion factor used to convert the logarithm to the base e to Log10
Fig. 10: The modified 9-point field disease scale for leafspot in groundnut

(Source: P. Subrahmanyam, ICRISAT, personal communication 1990)
c) **Net Assimilation Rate (NAR):** NAR was calculated as suggested by Gregory (1926),

\[
NAR = \frac{(W_2 - W_1)}{(t_2 - t_1)} \times \frac{\log_e A_2 - \log_e A_1}{(A_2 - A_1)}
\]

Where,

\[A_1 W_1 = \text{leaf area (cm}^2\text{)} \text{ and dry weight of the plant (g),}\]

respectively at time \(t_1\)

\[A_2 W_1 = \text{leaf area (cm}^2\text{)} \text{ and dry weight of the plant (g),}\]

respectively at time \(t_2\)

d) **Leaf Area Ratio (LAR):** The LAR (cm² g⁻¹) was calculated using the formula of Radford (1969)

\[
LAR = \frac{\text{Leaf Area (cm}^2\text{Plant}^{-1})}{\text{Total dry weight (g)}}
\]

**C. Yield and Yield Components:** Plants from each treatment and control were harvested at maturity and were used for recording various yield components namely, number of pods, shelling percentage and oil content.

a) **Number of gynophores**

b) **Number of pods**

c) **Shelling percentage:**

\[
\text{Shelling percentage} = \frac{\text{Weight of Kernel (g)}}{\text{Weight of pods (g)}} \times 100
\]
For this, 500 g pods were weighed and shelled.

4. WATER RELATIONS:

A. Leaf Relative Water Content (LRWC):

Two leaves were collected among the young fully expanded leaves of two plants per replicate. Individual leaves detached from the stem were weighed to determine fresh weight (FW). In order to determine the turgid weight (TW) leaves kept floating in distilled water inside a closed petridish. Leaf samples were weighed periodically, after gently wiping the water from the surface with the tissue paper until a steady weight was achieved. Then leaf samples were placed in a pre-heated oven at 70 °C for 48 hrs. in order to determine dry weight (DW). The values of FW, TW and DW were used to calculate LRWC using the equation (Kaya et al., 2003),

\[
LRWC (%) = \left(\frac{FW-DW}{TW-DW}\right) \times 100
\]

B. Osmotic Potential (OP):

Osmotic potential of cell sap was determined by the method described by Janardhan et al. (1975). One g fresh leaves were crushed in mortar with pestle and squeezed through muslin cloth and the volume of filtrate was made to 100 ml with distilled water. The electrical conductivity (EC) of the extract was measured with the help of Electric conductivity meter. Simultaneously, 1 g of leaf material was kept in oven at 60°C for determination of moisture content by substracting the dry weight from fresh weight. The osmotic potential of cell sap was calculated as follows-

\[
OP (\text{bars}) = \frac{EC \times 0.36 \times Df}{0.987}
\]

Where,

\[
EC = \text{Electrical conductivity (mmhos.cm}^{-1}, \text{ at } 25^\circ C \text{ of the extract).}
\]
Df = Dilution factor calculated after moisture correlation

\[ DF = \frac{Volume \ of \ extract \times wt. \ of \ plant \ material}{Moisture \ content} \]

0.36 - Constant for converting EC to OP.

0.987 - Constant for conversion of atm. to bars.

5. PHOTOSYNTHETIC PIGMENTS:

A. Chlorophylls:

The chlorophyll content of young and mature leaves was determined following the method by Arnon (1949). The plant material was homogenized in mortar with pestle and extracted in 80% acetone at 0 to 4°C in dark, with addition of a pinch of magnesium carbonate, to protect and stabilize the chlorophylls. This extract was filtered through Whatman No. 1 filter paper under suction using Buchner’s funnel. The residue was washed thoroughly 2-3 times with 80% acetone, collecting all the washings in the same filtrate. Final volume of the filtrate was made to 100 ml with 80% acetone. Absorbance was read at 663 and 645 nm for chlorophyll ‘a’ and ‘b’, respectively on UV-VIS double beam spectrophotometer, using 80% acetone as blank. The chlorophylls were calculated by using the following formulae-

Chlorophyll ‘a’ = X = 12.7 x A_{663} – 2.69 x A_{645}.

Chlorophyll ‘b’ = Y = 22.9 x A_{645} – 4.68 x A_{663}.

Total chlorophyll (a + b) = Z = 8.02 x A_{663} + 20.20 x A_{645}.

\[ \frac{X}{Y}/Z \times \text{volume of extract} \times 100 \]

Chlorophyll a / b / total = \-----------------------------\---------------------------\---------------------------

(mg.100^{-1}g)  1000 x weight of plant material (g).
B. Carotenoids:

The carotenoid content was estimated from the same extract used for chlorophyll estimation, by recording the absorbance at 480 nm on UV- VIS double beam spectrophotometer, using 80% acetone as blank. The carotenoids were calculated by using the following formula (Kirk and Allen, 1965):

\[ \text{Total Carotenoids} = \frac{A_{480} \times \text{volume of extract} \times 10 \times 100}{2500 \times \text{weight of plant material (g)}} \]

C. Chlorophyll Stability Index (CSI):

The chlorophyll stability index was determined by determining total chlorophyll contents from known quantity (1g) of fresh leaf material and of leaf material kept in oven at 60°C for 2 h. The chlorophyll stability index was calculated by the formula given below:

\[ \text{CSI} = \frac{\text{Total chlorophyll content of oven dried plant material}}{\text{Total chlorophyll content of fresh plant material}} \]

6. TOTAL SOLUBLE SUGARS

Sugar content was estimated from oven dried leaf sample following the method of Nelson (1944). 0.5 g oven dried plant material was homogenized in mortar with pestle and extracted with 80% alcohol. It was filtered through Buchner’s funnel using Whatman No.1 filter paper. The residue on filter paper was washed with 80% alcohol repeatedly. All the washings and filtrate were mixed together. This filtrate was used for estimation of soluble sugars.

From the above filtrate, 20 ml were taken into a conical flask and
hydrolyzed with 2-3 ml conc. HCl in an autoclave at 15 lbs pressure for half an hour. The contents were cooled, neutralized with Na$_2$CO$_3$ and filtered. This filtrate was used for the estimation of total (reducing + non-reducing) sugars. 2 ml of filtrate was taken in 10 ml test tube. 1 ml of alkaline copper tartarate reagent- (4 g CuSO$_4$.5H$_2$O, 24 g unhydrous Na$_2$CO$_3$, 16 g Na-K-tartarate and 180 g anhydrous Na$_2$SO$_4$ were dissolved in distilled water and volume was made to 1000 ml) was added in it. The test tube containing the reaction mixture was subjected to boiling water bath for about 10 min and then cooled to room temperature. 1ml of arsenomolybdate reagent (25g ammonium molybdate in 450 ml distilled water and to this were added 21 ml of conc. H$_2$SO$_4$. This was mixed with solution containing 3 g sodium arsenate dissolved in 25 ml distilled water. The mixture of the solutions was placed in an incubator at 37 °C for 48 hours) was added to test tube and shaken vigorously. The volume of the reaction mixture was made 10 ml with distilled water. A blank was prepared in the same way but without sugar solution. After 10 minutes, the absorbance was read at 560 nm on double beam spectrophotometer (Shimadzu, UV-VIS 190). A standard curve of glucose (0.1mg.ml$^{-1}$) was prepared and the sugar content was calculated.

7. NITROGEN METABOLISM

A. Total Nitrogen

Total nitrogen from the leaves and roots of groundnut plants subjected to different concentrations of SA treatments was estimated according to method of Hawk et al. (1984). Oven dried 1g powdered plant material was taken in Kjeldahl flask with a pinch of micro salt (200g K$_2$SO$_4$ + 5g CuSO$_4$, dehydrated) and to it 5 ml of H$_2$SO$_4$ (1:1) were added. Few glass beads were added to avoid bumping and material was digested on blue flame till it became yellow in colour. The flask was then cooled to room temperature. 15 to 20 ml distilled water were added and with thorough shaking the content was filtered and transferred to volumetric flask and volume of filtrate was made 100 ml with distilled water.

In clean and dry Nessler’s tube 1 ml of plant extract and different concentrations of standard ammonium sulphate solution (0.236 g of ammonium sulphate dissolved in water and few drops of H$_2$SO$_4$ were added. The volume was
made to 1000 ml. This solution contains 0.05 mg of nitrogen per ml.) In blank Nessler’s tube 1 ml distilled water was taken. To this drop of 8% KHSO4 was added and volume was made 35 ml with distilled water. To this 15 ml Nessler’s reagent were added. (Reagent A: 7g KI + 10 g HgI2 in 40 ml distilled water. Reagent B: 10g NaOH in 50 ml distilled water. A and B were mixed in proportion of 4:5 at the time of estimation) the reaction between the sample and the reagent gives the product NH4Hg2I3 which has orange brown colour. This colour was measured after 15 min at 520 nm on double beam spectro photometer. Amount of nitrogen was calculated from the standard curve.

**B. Soluble Nitrogen Fractions**

Oven dried 500 mg leaf material was homogenized in 20 ml 80% alcohol and filtered through Whatman No. 1 filter paper using Buchners funnel. The filtrate was then condensed on boiling water bath upto 1-2 ml. To this about 10 ml of distilled water were added and mixed well. It was filtered through Whatman No. 1 filter paper with frequent washings of the residue on filter paper with distilled water. All the washings and the filtrate were collected and final volume was made to 100 ml with distilled water. From this nitrate content and soluble proteins were estimated.

**a) Nitrate (NO3-) Content**

The method used is based on the reaction between Diphenylamine-H2SO4 reagent and nitrate (Kolhoff and Noponen, 1933). The reaction mixture contained 1ml plant extract, 1ml distilled water and 1.8 ml diphenylamine-H2SO4 reagent (1g diphenylamine 100 ml-1 H2SO4). After vigorous shaking, the blue colour was allowed to develop for 10 min. The absorbance was recorded at 590 nm. Instead of extract, distilled water was used to prepare blank. The standard curve was prepared from 1 M KNO3 and from this, nitrate content was calculated.

**b) Soluble Protein**

The soluble proteins were determined following the method by Lowry et al. (1951). In the assay mixture, 0.5 ml plant extract was diluted to 1ml with distilled water and 5 ml of freshly prepared reagent ‘C’ (50 ml 2% Na2CO3 in 0.1 N
aqueous NaOH mixed with 1 ml 0.5% CuSO₄.5H₂O in 1% Na-K-tartarate) was added to it. After 15 min 0.5 ml Folin Phenol reagent (100g Sodium tungstate mixed with 25g Sodium molybdate dissolved in 700 ml distilled water, 50 ml 85% phosphoric acid and 100 ml concentrated HCl together were refluxed gently for 10 h using water condenser. To this, 150 g Lithium sulphate, 50 ml distilled water and a few drops of bromine water were added. This was boiled for 15 min without water condenser to remove excess bromine. It was cooled and then adjusted to 1N by titrating it against 1 N NaOH) was added. The colour was allowed to develop for 30 min and then absorbance was recorded at 660 nm. Albumin (0.1 mg. ml⁻¹) was used for preparation of standard curve of protein. Blank was prepared with distilled water, reagent ‘C’ and Folin Phenol reagent.

C. Free amino acids:

Free amino acid contents of leaves from treated plants were determined following the method by Moore and Stein (1948). 0.1 g dry powdered plant material was extracted in 10 ml hot 80% ethanol. The extract was filtered through Whatman No. 1 filter paper. The filtrate was collected and used for assay.

The assay mixture containing 0.1 ml of the filtrate, 0.9 ml D.W. and 1 ml ninhydrin solution (0.08 g Stannous chloride dissolved in 50 ml 0.2 M citrate buffer, pH-5 mixed with 2 g Ninhydrin dissolved in 50 ml 2 methoxyethanol and the mixture was filtered) was boiled for 20 min in water bath. After cooling, 5 ml diluent solution (prepared by mixing equal amount of n-propanol and distilled water) was added and shaken vigorously. After 20 min, the intensity of purple colour developed was read at 570 nm on UV-VIS double beam spectrophotometer.

Blank was prepared by using 80% ethanol. Standard curve was obtained by taking different concentrations of standard Leucine (0.1 mg.ml⁻¹). The free amino acid contents were calculated by using the relationship, 1 ml std. Leucine solution = 0.1 mg amino acids.
D. Enzymes Of Nitrogen Metabolism

a) Nitrate Reductase (NR; EC.1.6.6.2):

*In vivo* activity of this enzyme was determined following the method by Jaworsky (1971). The plant material was cut into small pieces of about 0.5 cm$^2$ and incubated in 10 ml incubation medium containing 1ml 1M KNO$_3$, 2ml 5% n-propanol, 5ml 0.2 M phosphate buffer, pH 7.5 and 2ml, 0.5% Triton-X-100, for 1 h in dark. After incubation, 1ml of the reaction medium was taken out for determination of nitrite and was mixed with 1ml each of 1% sulfanilamide in 1M HCL and 0.02% NEEDA. The absorbance was read at 540 nm on double beam spectrophotometer using reagent blank.

The standard curve was prepared with 0.03mM KNO$_2$ (0.0026 mg NO$_2$.ml$^{-1}$) against a mixture of 1ml incubation medium, 1 ml sulfanilamide and 1ml NEEDA as a blank.

b) Nitrite Reductase (NiR; EC.1.6.6.4):

The activity of nitrite reductase was determined following the method by Jaworsky (1971) already described for nitrate reductase except that KNO$_3$ was replaced by 0.3 mM KNO$_2$ in the incubation medium and the incubation was done in light. The amount of KNO$_2$ remained in the incubation medium after the enzymatic activity for 1 h was determined by recording the absorbance of the reaction mixture containing 1ml incubation medium, 1ml sulfanilamide and 1ml NEEDA. The difference between the two readings, one at 0 min and the other after 1 h enzymatic reaction, gave the amount of KNO$_2$ utilized by the enzyme. The standard curve of KNO$_2$ was prepared as described earlier.

For the preparation of blank, 2ml n-propanol, 2ml Triton-x-100 and 6ml 0.2 M phosphate buffer pH 7.5 were mixed well and from this 1ml were mixed with 1ml sulfanilamide and 1ml NEEDA.

c) Glutamate Dehydrogenase (GDH) (E.C. 1.4.1.2):

GDH was studied following the method described by Jain and Srivastava (1981) with slight modifications. 0.5 g leaves were homogenized in 10 ml
ice cold extraction medium containing 120 mM sucrose, 0.6 mM EDTA, 0.3 mM cysteine in 15 mM phosphate buffer, pH 7.5. The extract was filtered through double layered cheese cloth and the filtrate was centrifuged at 5000 g for 15 min at 4°C. All the operations during enzyme isolation were carried out at 0 to 4°C. 0.2 ml enzyme was mixed with 2.8 ml assay mixture containing 4 mM α-ketoglutarate, 30 mM ammonium sulphate and 0.5 Mm NADH in 400 mM phosphate buffer pH 8.1. The reaction was started by adding enzyme and decrease in absorbance at 340 nm following the oxidation of NADH was recorded continuously for every 30 seconds till 4 min on double beam UV spectrophotometer (Shimadzu-190). 0.2 ml distilled water in place of enzyme served as a blank. The soluble proteins in the enzyme extracts were determined following the method of Lowry et al. (1951) described earlier and activity of enzyme was expressed as n moles NADH oxidized min⁻¹ mg⁻¹ protein.

d) Transaminases:

For the extraction and assay of two transaminases namely Aspartate aminotransferase (AsPAT) and Alanine aminotransferase (AlaAT) the method of Harper and Paulsen (1969) was followed with slight modifications. 1g leaves were homogenized in 20 ml ice-cold extraction medium containing 3.3 mM DTT (Dithiothretol) and 0.1 mM Na₂-EDTA (sodium salt of ethylenediaminetetraacetate) in 3.3 mM tris buffer, pH 7.2. The extract was filtered through double layered cheese cloth and centrifuged at 10,000 g for 15 min in cooling centrifuge. The supernatant served as an enzyme source.

i. Aspartate Aminotransferase (AsPAT) (EC 2.6.1.1):

For assay, 0.5ml enzyme was incubated with 1ml assay mixture containing 0.02M L-aspartate and 0.02M α-ketoglutarate in 0.2M potassium phosphate buffer, pH 7.5 (pH was adjusted with 0.2N HCl). After 0 min and 60 min reactions were terminated by adding 1ml DNPH (Dinitrophenyl hydrazine, 0.1g 10⁻¹ ml in 2N HCl). After 15 min 5ml NaOH (0.75N) was added to each DNPH added test tubes followed by 5ml distilled water. The absorbance was read at 504 nm against blank which was prepared with distilled water. The soluble proteins in the enzyme extracts were determined following the method of Lowry et al. (1951). Enzyme
activity is expressed as change in optical density (Δ O.D.) h⁻¹ mg⁻¹ protein.

ii. Alanine Aminotransferase (AlaAT):

For assay 0.5 ml enzyme was incubated with 1 ml assay containing 0.02 M L-alanine and 0.02 M α-ketoglutarate in 0.2 M phosphate buffer, pH 7.5 (pH was adjusted with 0.2N HCl). After 0 min and 60 min reactions were terminated by adding 1 ml DNPH (Dinitrophenyl hydrazine, 0.1g 100⁻¹ ml in 2N HCl). After 15 min 5ml NaOH (0.75N) was added to each DNPH added test tubes followed by 5ml distilled water. The absorbance of pyruvate phenyl hydrazone was read at 504 nm. The soluble proteins in the enzyme extracts were determined following the method of Lowry et al. (1951). Enzyme activity is expressed as change in optical density (Δ O.D.) h⁻¹ mg⁻¹ protein.

8. Leghemoglobin Estimation

Leghemoglobin content in nodules was determined according to the method of Appleby and Bergersen (1980). 500 mg fresh nodules were mixed with 1-3 ml phosphate buffer (pH 7.4) and macerated in a mortar with pestle. Macerate was filtered through two layered cheese cloth. The turbid reddish brown filtrate was centrifuged at 10,000 g for 20 minutes. The supernatant was diluted to 5 ml (4.2 M) alkaline pyridine reagent (0.8 ml NaOH in 50 ml water was added, cooled and 33.8 ml of pyridine was added and diluted to 100 ml with D.W.) was added. This was mixed thoroughly which formed greenish yellow ferric hemochrome. Then hemochrome was divided equally into two test tubes. To one tube few crystals of sodium dithionite were added, stirring without aeration and reading was taken after 5 minutes at 556 nm against a reagent blank. To other tube, few crystals of potassium hexacyanoferrate were added and reading was taken at 539 nm against a reagent blank. Then Lb concentration (mM) was calculated by using formula,

\[
Lb \text{ concentration} = \frac{A_{556} - A_{539} \times 2D}{23.4}
\]

Where, D is the initial dilution
The final values were expressed as µg g\(^{-1}\) fresh weight.

9. Phosphorus Metabolism

A. Phosphorus

Phosphorus was estimated calorimetrically following the method of Sekine et al. (1965).

In test tube 1 ml acid digest, 2 ml 2 N HNO\(_3\) and 1 ml of molybdate and vandate reagent were taken. (A: 1.25 g of ammonium molybdate dissolved in 500 ml 1N HNO\(_3\), B: 25 g of ammonium vandate dissolved in 500 ml distilled water. Then A and B were dissolved in equal volumes). The final volume of reaction mixture was made to 10 ml by adding distilled water. After shaken well reaction mixture was kept for 20 minutes for full colour development. The absorbance of blank reaction mixture containing no phosphorus was read at 420 nm.

By using standard KH\(_2\)PO\(_4\) solution containing 0.025mg phosphorus per ml. the standard curve of phosphorus prepared. The amount of phosphorus in the plant material was calculated using the standard curve.

B. Enzyme ATPase (EC 3.6.1.3)

The activity of enzyme ATPase was determined according to the method described by Todd and Yeo (1964). Extraction of enzyme was carried out according to the method of Weimberg (1970). Five hundred mg leaf tissue from each treatment and control were homogenized in 10 ml ice-cold 0.1 M Tris-HCl buffer (pH-8.0) containing 1 M KCl, 0.01 M EDTA and 0.4 ml 0.2 M β-mercaptoethanol. The homogenate was filtered through 4 layers of muslin cloth and filtrate was centrifuged at 10,000 rpm for 20 minutes. The supernatant served as enzyme source. One ml of supernatant was added to 0.5 ml of 0.01 M CaCl\(_2\) and 0.5 ml 0.003 M ATP. The reaction was carried out at 38°C for 1 hour and was stopped by adding 1 ml of 0.1 M NaOH. In another test tube (0 min.) the assay medium and the enzyme were taken in a similar way and the reaction was immediately terminated. The liberated inorganic phosphorus was estimated by the method of Fiske and Subba Rao (1925). To 1 ml of reaction mixture, 4 ml distilled water, 1 ml 5 N H\(_2\)SO\(_4\), 1 ml 2.5\%
Ammonium molybdate and 0.4 ml ANSA reagent [30 g sodium metabisulphite, 6 g sodium sulphate and 500 mg ANSA (1-amino, 2-napthol, 4-sulphonic acid) were dissolved separately in small quantities of distilled water. All the solutions were combined and volume was made to 250 ml with distilled water. It was allowed to stand overnight and filtered and stored in refrigerator] was added. After 10 minutes the absorbance of developed blue colour complex was measured at 660 nm. The change in OD during 60 min was determined and amount of liberated Pi was calculated with the help of standard curve. The soluble proteins in enzyme extracts were estimated following the method of Lowry et al., (1951) described earlier. The enzyme activity is expressed as µg Pi h⁻¹ mg⁻¹ protein.

C. Enzyme Acid Phosphatase (EC 3.1.3.2)

The activity of acid phosphatase was assayed according to the method of McLachlan (1980). 500 mg leaf tissue from each treatment and control were homogenized in 10 ml of ice-cold 0.1 M Acetate buffer (pH-5). The resultant homogenate was filtered through 4 layers of muslin cloth and the filtrate was centrifuged at 10,000 rpm for 20 minutes. The supernatant served as enzyme source. The assay mixture contained 3 ml of p-nitrophenyl phosphate (0.1 mg p-nitrophenyl phosphate per ml of acetate buffer- pH-5), two ml acetate buffer (pH-5) and 1 ml enzyme. The reaction was allowed to proceed for 30 minutes and then was terminated by addition of 1.5 ml, 1.68 N NaOH. In another test tube (0 min.) the assay medium and the enzyme were taken in a similar way and the reaction was immediately terminated. The optical density of the developed pale yellow colour complex was read at 420 nm. The soluble proteins in enzyme extracts were estimated following the method of Lowry et al. (1951). The enzyme activity was expressed as µ moles p-nitrophenol h⁻¹ mg⁻¹ protein.

D. Enzyme Alkaline Phosphatase (EC 3.1.3.1)

The activity of enzyme alkaline phosphatase was studied following the method of Weimberg (1970). 500 mg of leaf tissue was homogenized in 10 ml ice-cold 0.1 M Tris-HCl buffer (pH-8.0) containing 1 M KCl, 0.01 M EDTA and 0.4 ml 0.2 M β-mercaptoethanol. The resultant suspension was filtered through 4 layered
muslin cloth and filtrate was centrifuged at 10,000 rpm for 20 minutes. The supernatant served as enzyme source. During assay, 0.5 ml crude enzyme was incubated with 1 ml 0.1 M Tris-HCl buffer (pH-7.5), 0.1 ml, 0.05 M MgCl₂, 0.1 ml 0.02 M p-nitrophenyl phosphate and 1.7 ml distilled water at 30°C. Absorbance was measured immediately after addition of enzyme and after 1 hour of incubation at 410 nm. The soluble proteins in the enzyme extract were determined according to the method of Lowry et al. (1951). Enzyme activity was expressed as µ moles p-nitrophenol h⁻¹ mg⁻¹ protein.

10. Secondary Metabolism

A. Polyphenols:

The polyphenols were estimated following the method by Folin and Denis (1915). From the acetone extract used for chlorophyll, 2 ml plant extract was mixed with 10 ml, 20% Na₂CO₃ and the volume was made to 35 ml with distilled water. To this mixture, 2 ml of Folin-Denis reagent (100 g sodium- tungstate together with 20 g phosphomolybdic acid was dissolved in about 800 ml distilled water, to this 50 ml, 85% phosphoric acid was added and the mixture was refluxed for 2.5 h) was added, mixed thoroughly and finally diluted to 50 ml with distilled water. The standard tannic acid solution (0.1mg.ml⁻¹) was used for the preparation of standard polyphenol curve. A blank was prepared without polyphenolics. After development of colour, the absorbance was read at 660 nm on UV-VIS double beam spectrophotometer. Total polyphenols were calculated with the help of std. curve of tannic acid and expressed as mg 100 g⁻¹ fresh weight.

B. Enzyme Polyphenol oxidase (EC 1.10.3.2)

Activity of enzyme polyphenol oxidase was studied according to the method of Mahadevan and Sridhar (1982). 500 mg of plant material was homogenized in 15 ml cold 0.1 M phosphate buffer (pH-6.1). The resultant homogenate was filtered through 4 layers of muslin cloth. The filtrate was centrifuged at 10,000 rpm on cooling centrifuge for 20 minutes. The supernatant served as enzyme source. The assay mixture contained 4 ml 0.1 M phosphate buffer (pH-6.1), 1 ml 0.01 M catechol prepared in 0.1 M phosphate buffer (pH-6.1) and 0.5 ml enzyme
and it was mixed well. The soluble proteins in the enzyme extract were determined according to the method of Lowry et al., (1951). The increase in OD at 30 seconds interval up to 180 seconds at 495 nm was recorded. The enzyme activity was expressed as ΔOD min⁻¹ mg⁻¹ protein.

11. Lipid Peroxidation and Antioxidant Status

A. Lipid peroxidation

Lipid peroxidation was studied following the method described by Cakmak and Horst (1991). 0.5 g leaves of various treatments were homogenized in 10 ml 0.5% thiobarbituric acid (TBA) in 20 % (w/v) trichloroacetic acid (TCA). The extract was heated for 30 min in a boiling water bath. The reaction was then stopped by placing the reaction tubes in ice water. After cooling the flocculent precipitate was removed by centrifugation at 10,000 x g for 5-10 min. Absorbance of the samples along with an appropriate blank was measured at 535 nm and corrected for the non-specific absorbance by subtracting the value obtained at 600 nm. The lipid peroxidation activity is expressed as μ moles MDA.g⁻¹ fresh tissue.

B. Antioxidative and other enzymes

a) Catalase (EC.1.11.1.6)

The activity of catalase was determined by the method described by Sadasivam and Manickam (1991) with slight modifications.

0.5 g fresh plant material was homogenized in 10 ml 0.1 M phosphate buffer (pH 7.0). The extract was filtered through 4 - layered muslin cloth (moistened with phosphate buffer) and the filtrate so obtained was centrifuged at 10,000 x g for 10 min at 0 to 4 °C. The supernatant was used as an enzyme source.

An assay mixture contained 3 ml H₂O₂ phosphate buffer (0.64 ml of H₂O₂ diluted to 100 ml with 0.1M phosphate buffer pH 7.0), 0.2 ml enzyme was mixed immediately and change in optical density was recorded per min at 240 nm. The soluble proteins in the enzyme extract were determined according to the method of Lowry et al. (1951). The enzyme activity is expressed as unit min⁻¹ mg⁻¹ protein.
b) Peroxidase (EC.1.11.1.7)

To study the enzyme peroxidase activity the method of Maehly and Chance (1954) was followed. Enzyme was extracted by homogenizing 0.5g fresh material in 10 ml 0.1 M phosphate buffer pH 7.0. It was filtered through 4- layered musline cloth moistened with phosphate buffer and the filtrate so obtained was centrifuged at 10,000 x g for 10 min at 0 to 4°C. The supernatant was used as an enzyme source.

Enzyme assay mixture contained 2 ml 0.1M phosphate buffer pH 7.0, 1 ml 20 mM guaiacol and 0.5 ml enzyme. The reaction was started by the addition of 0.05 ml H$_2$O$_2$ (20 mM). Change in optical density due to oxidation of guaiacol was recorded after 30 min. at 470 nm. The soluble proteins in the enzyme extract were determined according to the method of Lowry et al. (1951).The enzyme activity was expressed as unit h$^{-1}$ mg$^{-1}$ protein.

c) Superoxide dismutase (E.C. 1.15.1.1)

Superoxide dismutase was determined following the method described by Giannopolitis and Ries (1977), with slight modifications. Enzyme was extracted by homogenizing 0.5 g fresh plant material in 10 ml, 150 mM cold potassium phosphate buffer (pH-7.8) containing 1% PVP, to protect enzyme from the action of polyphenols. Then it was filtered through 4- layered muslin cloth and the filtrate so obtained was centrifuged at 10,000 x g for 20 min at 0- 4°C. The supernatant was used as an enzyme source.

An enzyme assay mixture contained 2 ml potassium phosphate buffer pH-7.8, 0.2 ml methionine (13 mM), 0.1 ml Nitroblue tetrazolium (75µM), 0.5 ml EDTA (0.1 mM), 0.1 ml enzyme and 0.1 ml riboflavin (2M) was added lastly, and immediately, the absorbance was measured at 560 nm on UV-VIS double beam spectrophotometer (Shimadzu-190, Japan). Then the assay mixture was exposed to full sunlight for 30 min and again the absorbance was read at 560 nm. The enzyme activity is expressed as unit h$^{-1}$ mg$^{-1}$ protein.
12. Inorganic Constituents

The leaf and root samples of random sampling were taken, washed thoroughly with glass distilled water and dried in oven at 60\(^\circ\)C for about 7- days till a constant weight was obtained. The dried leaf material was powdered.

A. Preparation of Acid Digest

The plant material was digested following the method of Toth et al. (1948). An acid digest from the oven dried plant material was used for the estimation of inorganic constituents.

500 mg of oven dried powdered material was transferred to 100 ml beaker, to which 20 ml concentrated HNO\(_3\) were added. The beaker was covered with watch glass and was kept till the primary reactions subsided. It was then heated at low temperature till the solid particles completely dissolved. After cooling to room temperature 10 ml of perchloric acid (60 \%) were added to it and mixed thoroughly. It was then heated strongly until a clear and colorless solution was obtained. Heating was stopped when the volume of extract was reduced to approximately 2-3 ml. It was then cooled and transferred quantitatively to 100 ml volumetric flask, then diluted to 100 ml with distilled water. It was kept overnight, and filtered through a dry Whatman No. 1 filter paper next day. The filtrate was used as the source of different inorganic constituents.

B. Estimation of Potassium and Calcium

Potassium (K\(^+\)) and Calcium (Ca\(^{++}\)) concentrations were determined with a flame photometer. Stock solutions of known concentrations 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. The concentrations of K, Ca and Na in acid digested samples were calculated using respective calibration curves.

C. Estimation of Magnesium, Iron and Manganese

Magnesium (Mg\(^{++}\)), Iron (Fe\(^{++}\)) Copper (Cu\(^{++}\)) and Manganese (Mn\(^{++}\)) was analyzed by atomic absorption spectrometer (Perkin-Elmer model-3030) using
acetylene air flame. The light source employed was hollow cathode lamp. The concentration of Mg$^{2+}$, Fe$^{3+}$, Mn$^{2+}$ and Cu$^{2+}$ were react at 285.2 nm, 248.3 nm, 279.5 nm and 324.8 nm respectively.

D. Estimation of Zinc, Boron and Molybdenum

Zinc was determined by autoanalyser. Molybdenum was estimated by using atomic spectrphotometric method at 313.3 nm.

E. Sulfur

The sulfur content was estimated according to the method of Blanchard et al. (1965) with slight modifications. 10ml of acid digested sample was taken in Nesseler’s test tube and to this 1ml of stabilizing reagent [mix 95% ethanol and glycerol in 8:2 ratios (v/v)] and 0.5g of BaCl$_2$ were added and the volume was made 50 ml with distilled water and mixed well. The absorbance was measured at 430 nm against the blank.

The amount of sulfur were calculated by using standard curve of sulfur solution [standard K$_2$SO$_4$ solution (2-14 ppm)] and expressed as mg 100 g$^{-1}$ dry weight.

The values of all macro elements and microelements are expressed in g 100$^{-1}$ g dry tissue.

13. Protein Profile:

The leaf protein profile was prepared following the SDS-PAGE (Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis) technique.

A. Protein Extraction:

Proteins were extracted by using Tris buffer (pH 6.8) at room temperature (1 mg per 20 µl /100 mg per 2 ml extraction buffer). The extract was subjected to centrifugation at 10,000 x g. The supernatant was used as protein source.

Extraction buffer: (pH 6.8)
a) 0.62 M Tris HCl Buffer
b) 2% sodium dodecyl sulphate (SDS)
c) 5% β Mercaptoethanol
   (For 100 ml solution)
   Tris - 7.51 g
   SDS - 2.00 g
   β Mercaptoethanol - 5 ml
   (pH was adjusted by dilute HCl).

B. Preparation of Stock Solutions:

a) Separating gel-
   i. Acrylamide – 2.00 g
   ii. Bis-acrylamide – 25.99 mg
   iii. Distilled – 11.50 ml

b) Stacking gel-
   iv. Acrylamide – 0.6 g
   v. Bis-acrylamide – 9.0 mg
   vi. Distilled water – 16.8 ml.

c) 1% SDS—1 g/ 100 ml D. W.

d) Initiator (1% Ammonium per sulphate solution)
   1 g APS.100 ml$^{-1}$ DW (Every time initiator was freshly prepared).

e) Running gel buffer (pH 8.3)-
   vii. Glycine - 14.11 g
   viii. Tris - 3.00 g
   ix. SDS - 1.00 g
   All these ingredients were dissolved in distilled water, pH was adjusted by dilute HCl and final volume was made to 1 litre.

f) Loading buffer (LB)-
   x. Glycerol - 10.00 ml
xi. Tris HCl Buffer (pH6.8) – 6.25 ml
xii. SDS – 2.00 g
xiii. Bromophenol blue – 10.00 mg
xiv. Distilled water – 12.05 ml.

**g) Actual loading sample/ Constituents:**

i. Loading Buffer (LB) – 1.7 ml
ii. β Mercaptoethanol – 0.3 ml
iii. Distilled water – 4.0 ml.

**h) Stain preparation (Coomassie Brilliant Blue):**

i. Coomassie Brilliant Blue R-250 – 1 g
ii. Methanol – 500 ml
iii. Acetic acid – 100 ml
iv. Distilled water – 400 ml

(Total volume of stain-1 litre).

**i) Washing solution (For 500 ml)-**

i. Methanol - 100 ml
ii. Acetic acid - 50 ml
iii. Distilled water - 350 ml.

**j) Destaining solution (For 500 ml)-**

i. Methanol - 150 ml
ii. Acetic acid - 50 ml
iii. Distilled water - 300 ml.

**k) Gel storage solution (0.05 M NaCl)-**

1.461 g NaCl / 500 ml.

**C) Preparation of working solutions-**

The stock solutions prepared fresh and mixed in following proportion
for the preparation of gel (10%).

<table>
<thead>
<tr>
<th>Sr. No.</th>
<th>Stock</th>
<th>Separating gel</th>
<th>Stacking gel</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>Acrylamide</td>
<td>2 g</td>
<td>0.6 g</td>
</tr>
<tr>
<td>2.</td>
<td>Bis-acrylamide</td>
<td>25.99 mg</td>
<td>9 mg</td>
</tr>
<tr>
<td>3.</td>
<td>Distilled water</td>
<td>11.5 ml</td>
<td>16.8 ml</td>
</tr>
<tr>
<td>4.</td>
<td>1 M Tris HCl Buffer</td>
<td>7.5 ml (pH 8.8)</td>
<td>2.5 ml (pH 6.8)</td>
</tr>
<tr>
<td>5.</td>
<td>1% SDS</td>
<td>0.2 ml</td>
<td>0.2 ml</td>
</tr>
<tr>
<td>6.</td>
<td>TEMED</td>
<td>10 µl</td>
<td>15 µl</td>
</tr>
<tr>
<td>7.</td>
<td>1% APS</td>
<td>0.5 ml</td>
<td>0.75 ml</td>
</tr>
</tbody>
</table>

D) Casting of gel:

The vertical slab gel unit was assembled in casting stand by using .5 mm spacers. The plates were then sealed with cellophane tape and placed in oven (40°C) for 10-15 minutes.

E) Preparation of separating gel:

The separating gel solution was prepared by mixing the ingredients as per the sequence shown in the table. The gel solution was mixed well and poured slowly into the cavity (sandwiched cavity) with the help of pipette to the level of about 4 cm from top. The gel was overloaded by applying about 0.3 ml of water (D/W) or water saturated n-butanol. The polymerization process was carried out for about 20-30 minutes in dark. A sharp liquid gel interface was visible when the gel polymerized.

F) Preparation of stacking gel:

The liquid from separating gel layer was poured off and washed with distilled water. Stacking gel solutions were mixed as per sequence given in the table. Ammonium persulphate and TEMED (N, N, N’, N’- tetramethylethylene diamine) were added by gentle swirling the flask. The cavity was then filled with stacking gel.
solution. The comb was inserted avoiding the air bubble below the teeth of comb (because oxygen inhibits the polymerization process and may cause a local distortion in the gel surface at the bottom of wells). This gel was allowed to set at least for half an hour.

**G) Loading and Running the gel:**

The comb was removed carefully without disturbing any well divider. The wells were rinsed with distilled water and then drained off by inverting the casting stand. The polymerized gel plate was fixed to electrophoresis unit. The lower and upper chambers were filled with running buffer (pH 8.3). In upper chamber the buffer was added till the sandwich gel got immersed into the buffer. The bubbles trapped into the wells were removed by using syringe.

Equal quantities of protein source and sample loading solution were mixed in ephendarf tube. Loading quantity was fixed by pilot experiments i.e. 30, 40, and 50 µl. The 40 µl quantity showed good results. The wells were loaded with 40 µl of sample with micropipette in each well. Power supply was connected to electrophoresis unit. It was set to constant current i.e. 30 mA/1.5 mm thick gel. Power supply was turned off when dye (Bromophenol blue) reached to the end.

**H) Staining and Destaining the gel:**

After complete run, the sandwiches were disassembled and gel was immersed in washing solution for 10-15 minutes. Then it was transferred into staining solution for half an hour. Bands were observed within 10 minutes however, gel was kept overnight for complete staining. It was then destained with destaining solution and was photographed. The band intensity was assessed visually by placing gel over a trans-illuminator for presence or absence of specific bands and recorded as present, prominent and more prominent. The length of marker front and the distance traveled by different protein bands were recorded. The gel was then stored in 0.05 M NaCl solution.

The relative mobility (Rm) and similarity index (SI % of protein bands) were calculated by formulae-
Distance migrated by particular band

\[
\text{Relative mobility } (Rm) = \frac{\text{Distance migrated by particular band}}{\text{Distance migrated by marker front.}}
\]

No. of pairs of similar bands

\[
\text{Similarity Index (SI \%)} = \frac{\text{No. of pairs of similar bands}}{\text{No. of different bands + No. of similar bands}} \times 100.
\]

or Percentage similarity

(Mary and Someswari, 1999)

14. Seed Quality Parameters:

A. Oil content:

Groundnut seeds were ground and oil was extracted for 8 h with diethylether in soxhlet apparatus. Then, the solvent was completely removed under reduced pressure in a rotary evaporator. Oil percentage was determined by weigh difference. The oil content was estimated by Nuclear Magnetic Resonance (NMR) Spectrophotometer against a standard reference sample.

B. GC-FID Analysis of Fatty acid Composition

The dried seeds of groundnut were powdered and the lipids from the seed powder were extracted in petroleum ether using Soxhlet apparatus. To analyze the fatty acids from the oil fractions by gas chromatography technique, the oil was subjected to transesterification to obtain the fatty acid methyl ester. The fatty acid methyl ester fractions were eluted with petroleum ether: diethyl ether = 50: 50 (V/V). The fractions were redissolved in hexane and subjected to GC analysis.

Fatty acid methyl esters (FAMEs) were analyzed by GC-FID (A SHIMADZU GC-17-A gas chromatograph with flame ionization detector). FAMEs were separated on CHROMOPACK WCOT 25mx 0.25 mm ID, 0.2 µm film thickness capillary column using temperature programme from 150 °C with the following conditions: Injector temperature 260 °C, FID temperature 260 °C and the carrier gas –
Helium. The identification of fatty acids was done by comparison with the methyl esters of standard fatty acids.

C. Protein Content:

Protein content of groundnut seeds was determined by using the Kjeldahl N analysis method by taking 4-5 grams of seed samples from each experimental unit. The samples were ground and stored in air tight plastic bottles. Total nitrogen concentration (%) was worked out by using micro Kjeldahl analysis method (Nelson and Sommers, 1980).