CHAPTER III

MATERIALS AND METHODS

3.1.0. Field Studies
3.1.1. Number of Soils
3.1.2. Details of Soils
3.1.3. Collection of Soil from the Field

3.2.0. Laboratory Studies
3.2.1. Determination of Soil Colour
3.2.2. Determination Soil Texture
3.2.3. Determination of Soil pH
3.2.4. Estimation of Organic Carbon
3.2.5. Determination of Electrical Conductivity
3.2.6. Estimation of Available Phosphorus in Soil
3.2.7. Estimation of available Potassium in Soil
3.2.8. Estimation of exchangeable Calcium and Magnesium
3.2.9. Spore count of AM, enumeration from the Soil
3.2.10. Isolation and Identification
3.2.11. Multiplication of AM Fungi in Host Plant

3.3.0. Pot culture Studies
3.3.1. Preparation of Pot
3.3.2. Sowing of Seeds
3.3.3. Post Harvest Observations
3.3.4. Microbiological Parameters
3.3.5. Analysis of Soil
3.3.6. Analysis of Plant Sample
3.3.7. Biochemical Assay of Soil Samples
3.3.8. Estimation of Nutrients in Plant Samples
3.3.9. Biochemical Assay on Plant Samples.

3.3.9.1. Estimation of Total Protein
3.3.9.2. Estimation Total Carbohydrate
3.3.9.3. Estimation of Starch Content
3.3.9.4. Estimation of Proline Content
3.3.9.5 Estimation of Phospholipid

3.3.10. Uptake Studies
3.3.11. Economics
3.3.12. Statistical Analysis
MATERIALS AND METHODS

To meet the objectives of the studies as indicated in the introduction, three types of studies were undertaken viz.,

(i) Field studies (survey works)

(ii) Laboratory studies

(iii) Pot culture experiments

The methodology of each type of experiment and the details of conduct of each field experiment, analysis of soil and plant samples, and the procedures followed are given in this chapter.

3.1.0 FIELD STUDIES

Survey works were carried out to study the best soil of Kerala suited for the experiments based on the quantity of AM fungal population present in the soil. The details of experiment were as follows.

3.1.1 Number of soils – Three

3.1.2 Details of the soils

<table>
<thead>
<tr>
<th>Details of soil</th>
<th>Date of collection</th>
<th>Location and district</th>
<th>Address of the farmer</th>
</tr>
</thead>
<tbody>
<tr>
<td>S1</td>
<td>13.4.04</td>
<td>Vackavu, Kizhakkampadam, Palghat Dt. India</td>
<td>Mr. Krishnankutty S/o Aaru Vackavu, Kizhakkampadam, Palghat Dist. Nenmara Block Survey No. 834 Crop Cow pea Area 50 cents</td>
</tr>
</tbody>
</table>
### 3.1.3. Collection of soils from the field

The field to be sampled was went around and surveyed. Variations in slope, colour, texture, management and the cropping pattern were recorded and demarcated the field accordingly in to uniform areas.

Located different spots of sampling in a zig zag manner in the field. In each spot scraped away the surface litter and surface sample was taken from the “v” shaped plough depth. Like wise samples were collected from 10-20 spots in the field depending upon the area.

Uniform slice sample was taken from each spot. Collected the spot samples in to a piece of clean paper or cloth in shade and mixed thoroughly. Collected the representative sample by quartering method i.e., mixed well and divided in to four equal parts and rejected the two opposite quarter samples. (Clarson, 2002,a). Mixed the remaining two portions and repeated the procedure as many times as necessary.
times as necessary to arrive at the desired size of sample. The collected samples were allowed to dry under shade. Filled up the case history sheet of the sample completely and put inside the sample bag and labelled it. The sample bag was free of contamination. The samples so collected were brought to the laboratory for further analysis.

The physical and chemical properties of the soil were studied at Agro Clinic and Research Centre, Kottayam; based on procedures outlined by Clarson (2002,b). Separate samples were also collected for undertaking the Microbiological studies.

These samples were processed and preserved in separate sterile containers without any contamination. Each sample was numbered and labelled neatly and kept in the refrigerator for further studies.

**3.2.0. Laboratory studies**

**3.2.1 Determination of soil colour (By quick method)**

In most of the soil testing laboratories for the quick determination of soil colour this method is being followed. Soil colour being an easily visible characteristic, it could be determined by seeing its colour. This visible method gave an indication for giving right type of agronomic practices along with the manural recommendation in the soil testing laboratories.

**3.2.2 Determination of soil texture (By feel method)**

Texture refers to the relative percentage of soil separates namely sand, silt and clay. The relative percentage of these soil
separates in the fields are almost infinite in all possible combinations. Therefore it is necessary to establish limits of variations among the separates so as to group them in to textural classes. Based on different combinations of sand, silt and clay, soil textures were determined.

Handful of soil was taken and moistened with water. The soil was rubbed between the thump and fingers. The texture of the soil was felt and recorded accordingly.

3.2.3. Determination of soil pH (Potentiometric method)

Principle

A glass electrode in contact with H+ ions of the solution under test acquires an electrode potential which depends on the concentration of H+ ions. This is measured potentiometrically against some reference electrode which is usually a calomel electrode. The potential difference between glass electrode and calomel electrode is expressed in pH units.

Twenty gm soil was weighed and transferred to 100 ml beaker. Added 40ml distilled water (1: 2 ratio). Stirred it well with glass rod and allowed it to stand for half an hour with intermittent stirring, adjusted the pH meter with standard buffer solutions (4.0 and 9.2). Washed the electrodes with a jet of water and carefully wiped with a piece of filter paper. Immersed the electrodes in the beaker containing soil water suspension and operated the switch to ‘ON’. The meter
automatically displayed the pH value of the sample which were recorded. The rating of soil is as follows.

pH <6.5 – Acidic

pH  6.5 – 7.5 – Neutral

pH > 7.5 – Alkaline

**3.2.4 Estimation of organic carbon (By Walkley and Black)**

**Titration method**

**Principle**

Soil organic matter is oxidized under standard conditions with excess of potassium dichromate in H$_2$SO$_4$ solution and the excess dichromate is determined by titration with standard ferrous sulphate solution using diphenylamine as indicator.

Exactly 0.5 – 1.0 gm of finely ground soil was weighed (passing through 0.2 mm non ferrous sieve, 80 meshes/ inch) in to a 500 ml conical flask. Pipetted out 10 ml of IN. potassium dichromate solution in to the flask. The flask was shaken to mix the dichromate with the soil. Then added 20 ml of con. Sulphuric acid and gentle shakings were given for one minute to ensure complete contact of the reagents with the soil. Left the flask to stand for 30 minutes. Then added 200 ml of distilled water, 10 ml of phosphoric acid and 1 ml of diphenyl indicator. Titrated the contents of the flask against 0.5 N ferrous sulphate solution till the contents attained a green colour. This change in colour from blue to green being occurred suddenly,
the end point of the titration was carefully followed and located the end point with great care.

The following procedure was adopted to locate the exact end point of the colour change. Titrated with ferrous sulphate solution by addition of 0.5 ml ferrous sulphate each time and when the colour changes to green add 0.5 ml of 0.1 N dichromate solution and restored the blue colour. Then added the ferrous sulphate solution drop by drop till the colour suddenly changed to green.

**Calculation**

Organic carbon percentage = \( \frac{(V_1 - V_2) \times 0.003 \times 100}{W} \)

Where

- \( V_1 \) – volume of IN Pottassium dichromate
- \( V_2 \) – \( \frac{1}{2} \) the volume of 0.5N Ferrous sulphate
- \( W \) - weight of the soil taken.

Rating of organic carbon is as follows:

- Up to 0.5 % OC = low
- 0.5 – 7.5% OC = Medium
- More than 0.75% = High

**3.2.5 Determination of electrical conductivity**

**Principle**

The electrical conductivity in a solution increase with the amount of soluble salts of that solution. The measurement of conductivity is based on Ohm’s law. That is \( I = \frac{E}{R} \) where \( I \) is the current in Amperes, \( E \) is the electromotive force (e.m.f) in volts and ‘\( R \)’
is the resistance in ohms. Since conductance is the reciprocal of resistance it is expressed in mhos. Since the relative concentration of electrolyte in soils is very low, measurement of specific conductance are usually reported in milli- mhos.

Twenty gm of soil was weighed into a 100 ml beaker. Added 40 ml of distilled water and stirred it well. Allowed to stand for half an hour. Switched on the conductivity meter. Checked the instrument with saturated calcium sulphate solution and 0.01 N KCl solution (EC 2.2m.mhos/cm and 1.4m.mhos/cm, respectively) before measuring the sample. Washed the electrode with distilled water. Immersed them into the soil suspension or sucked the supernatant solution in to the electrode bulb. The multiplier switch was set up at an intermediate position and rotated the main dial control until the magic eye of the null indicator was at its full width. The readings of the scale at this position multiplied by the value of multiplier switch position indicated the electrical conductivity. Multiplied this by the cell constant value noted on the cell itself recorded as specific conductivity.

3.2.6. Estimation of available phosphorus in soil

In most soil testing laboratories Olsen’s sodium bicarbonate or Bray’s No. 1 or No. 2 are used for the extraction of available phosphorus based on soil pH value. Bray’s method was used in the present studies for the estimation of available P in it.
**Principle**

This method is based on the reduction of the molybdophosphoric and complex by ascorbic acid in the presence of antimony potassium tartarate. The blue colour developed is stable for 24 hrs and has less interfering substances.

**Preparation of Reagent**

Dissolved 12 gm of ammonium molybdate reagent in 250 ml of distilled water. In another 100 ml of distilled water, dissolved 0.2908gm of antimony potassium tartarate. Added both of these dissolved reagents to 1000 ml of 5 N.H₂SO₄ (148 ml H₂SO₄ /litre) and mixed thoroughly and made up to 2 litre with distilled water. The solution is heat and light sensitive. This is Reagent A, dissolved 1.056gm of ascorbic acid in 200 ml of reagent A and mixed well. This is reagent B and prepared fresh reagents as and when required.

**Procedure**

Pipetted out 5 ml of aliquot, Bray solution in a 25ml volumetric flask, acidified with 5N H₂SO₄ to pH 5.0. This was done using paranitrophenol indicator, which gave yellow colour at pH 5.0. After adjusting the pH, diluted to 20 ml with distilled water and added 4ml of reagent B. waited for 10 minutes and read the blue colour intensity in calorimeter using 660 nm filter. Prepared a standard curve. From the graph, the calculation was made for available P and expressed as mg/100 gm of soil.
3.2.7 Estimation of available potassium in soil

Among the different forms of soil K, the exchangeable K is the chief source of available potassium in the soil. In certain cases potassium may be available from the fixed form. Various reagents have been proposed from time to time to estimate the available soil potassium. The most widely adopted method is extracting potassium by using neutral normal ammonium acetate which is an estimate of water soluble and exchangeable potassium. The potassium extracted by different extractants is determined with the help of flame photometer.

By extraction with neutral normal ammonium acetate

Principle

The K ions in the exchangeable sites are replaced with ammonium ions and leached from the soils. The K ions in the solution is then determined in the flame photometer. The principle underlying the flame photometry is that a large number of elements when excited in a flame, emit radiation at characteristic wavelengths. It measures the emission intensity in proportion to the concentration of particular ion in the solution.

Weighed 5gm of the soil and transferred it into a 100ml polythene shaking bottle. Added 25 ml of neutral normal ammonium acetate solution and shook in a mechanical reciprocating shaker for 5 minutes. Filtered through Whatman No. 40 filter paper and collected the filtrate in a dry test tube. Measured the amount of K in the
filtrate in the flame photometer and calculated the available K (mg/100 gm soil) from the standard graph.

**Preparation of standard curve from available potassium**

Dissolved 1.9068gm of pure dry potassium chloride in ammonium acetate and made up to one litre with the same reagent. This gave 1000ppm of potassium. Made up 10 ml of the 1000ppm K solution to 100ml with any reagent which gave 100ppm of K. From this prepared the working standards of potassium of 5,10,15,20 and 25ppm by making up 5,10,15,20 and 25ml of the 100ppm K solution to 100 ml with the reagent. Set the flame photometer. Aspirated the standard solutions and prepared a standard graph relating to the concentration of potassium.

**Calculation**

Weight of soil = 5gm

Volume of extractant = 25 ml

\[
\text{Available K (mg/100)} = \frac{\text{Graphreading (ppm)}}{10^6} \times \frac{25 \times 2 \times 10^6}{5}
\]

= ppm x 10

**3.2.8 Estimation of exchangeable (available) Calcium and Magnesium (Versante method)**

**Principle**

The method is based on that calcium, magnesium and number of other ions form stable complexes with Versene (Ethylene Diamine Tetra Acetic acid disodium salt) at different pH levels. Copper, Zinc,
Iron, Manganese may interfere in the estimation of Calcium and Magnesium, if present in higher amounts. Their interference is prevented by the use of 2% sodium cyanide solution.

1. Exchangeable ‘Ca’ alone

Pipetted out 10 ml of the ammonium acetate leachate into a porcelain basin. Added 1.5-2.0 ml of 10% sodium hydroxide solution and about 0.1 gm of murexide indicator. Titrated this against 0.02 N versante solution. (Dissolved 3.8 gm of EDTA in 1 litre distilled water and standardized this against 0.02 N calcium solution) until the colour changed from red to violet.

Calculation

\[
m.e\%\ of\ exchangeable\ calcium = TV \times 0.02 \times \frac{250}{10} \times \frac{100}{W} \times \frac{100}{(100 - M)}
\]

Where, \( W \) is the weight of soil (10gm)

\( M \) is the moisture percent.

2. Exchangeable Ca + Mg

Pipetted out 10ml of the ammonium acetate leachate in a porcelain basin, added 5 ml to buffer solution (ammonium chloride + ammonium hydroxide), followed by 0.5 gm of Erichrome black T indicator. Titrated this against 0.02 N versante solution till the colour changed from red to blue.

Calculation

\[
Ca + Mg\ (m.e\%) = TV \times 0.02 \times \frac{250}{10} \times \frac{100}{W} \times \frac{100}{(100 - M)}
\]
Where, W is the weight of soil (10gm)

M is the moisture percent.

3. Exchangeable Mg alone

Ex. Mg is estimated by subtracting the exchangeable Ca alone from exchangeable Ca+ Mg values.

i.e., m.e % of exchangeable Mg= m.e% of (Ca+ Mg) – m.e% of Ca alone.

3.2.9 Spore count of AM enumeration from the soil

Arbuscular mycorrhizal population was estimated in the soil sample preserved for the bioassay by wet sieving and decanting method of Gerdemann and Nicolson (1963). About 50 gm soil was suspended in 200ml of lukewarm water in a 500 ml beaker and stirred thoroughly. The contents were decanted through one mm, 450µ, 250µ, 105µ and 45µ sieves placed one below the other. The residue was re-suspended in fresh water and the process was repeated, till one litre suspension was passed through above series of sieves. The spores were collected by observing under a stereomicroscope. These were transferred to a nylon mesh with pore size of about 45µ. Quantitative estimation was done by counting the number of spores isolated under the same microscope. The separated spores were maintained in sterile, distilled water in a refrigerator after surface sterilizing with 200 ppm streptomycin for 15 min. the
spores were identified based on the characters for different taxa of order Glomales (Schenck & Perez, 1988).

3.2.10 Isolation and identification

Characterization studies of 4 genera isolated from soils were conducted. Predominantly *Glomus*, *Gigaspora*, *Acaulospora*, *Scelerocystis* were isolated from the samples.

These 4 genera identified, due to the following reasons.

**Glomus sp.** : Sporocarp 1–10 spored, globose to ellipsoid upto 1mm diameter peridium of loosely interwoven irregularly branched hyaline, septate hyphae 2-12 μ diameter, the walls up to 0.5 μ thick, irregularly anastomising to form a thin network enclosing the chlamydospores entirely in completely or with some spores unenclosed. Endocarpic and ectocarpic spores similar. Chlamydospores yellow to brown, globose ovoid, or some what irregular. 105-310 × 110-305μ with one or occasionally two funnel shaped bases 20-30μ diameter divided from subtending hyphae by a curved septum; walls 2-7 μ thick, with a thin often barely perceptible hyaline outer membrane, and a thick brownish yellow inner layer.

**Acaulospora sp.** : Single globose spore developed laterally on hyphae below capital swelling. Hyphal apex is inflated and with maturity of the spore the stalk cell collapses. Spores are with reticulate wall, mature ones measure 100 – 300 μm. Multiple wall layers are present
with outer thick layer over one or more thin layers. The wall colour is yellowish brown.

**Gigaspora sp.:** Spores are globose to subglobose measuring 200-500 μm at maturity with smooth surface having multiple wall layers with thick outer layer over two or more thin separable inner layers. Subtending bulbous hyphae are orange brown with a diameter of 30 – 50μm.

**Sclerocystis sp.:** Chlamydospores are formed in sporocarps with spores arranged in a single layer around a central plexus of global hyphae. Individual spores measuring 90-120 μm are light yellow in colour.

### 3.2.11- Multiplication of AM fungal spp in host plant

The following AM fungal spp were used for the study.

Name of the species.

1. *Glomus mosseae*

2. *Glomus microcarpum var. microcarpum*

3. *Glomus fasciculatum*

The above three AM fungi were procured from, CTCRI, Trivandrum. They were multiplied in host plant. Maize (*Zea mays*) after estimating the spore count of each one; by quantitative estimation method (Daniel and Skipper, 1982). One ml of each culture was pipetted in to a nematode counting slide and spores/ml
was calculated by counting the number of spores present in a portion of the slide and multiplied it with the dilution factor.

**3.2.11.1. Multiplication of AM fungi in host plant by pot culture**

Three plastic pots were purchased and made four holes in it. About eight kg of vermiculite was mixed thoroughly with ½ kg of *Glomus spp*, and filled each in each pot and labelled the pots.

Pots were placed in order to get sufficient sunlight and wetted them with tap water. About 60 numbers of maize seeds (purchased from Agrobiotech, Poovanthuruthu, Kottayam, were treated with a fungicide (Dithane). It was prepared dissolving 2gm dithane in 500 ml tap water. Seeds were kept in this solution for about three hours. After fungicide treatment, seeds were taken out and, twenty numbers of seeds in each pot were sown, after making channels of 3cm depth, and were irrigated gently. Observed the pots for germination and irrigated daily. On the 9th day gap filling was done.

On the 30th day height of the plants were taken and photos were taken. On the 60th day harvested the plants after taking the height of the plants. Various observations were carried out like root length, root biomass, shoot biomass, Total biomass, AM spore count, (based on the procedure outlined earlier) colonisation in roots were done. Root infection was calculated by the method of Phillips and Hayman (1970). The roots were washed gently in tap water. The roots were cut in to 1 cm size and bits were immersed in 10% KOH solution to clear the host cytoplasm and nuclei for stain penetration,
and autoclaved it at 15 lb/sq inch pressure for 20min. The KOH solution was then poured and the roots were rinsed in tap water thrice or until no brown colour appeared in the rinsed water. The roots were acidified for 3-4 minutes with 2 percent HCl for proper staining. The acid was then poured out without rinsing with water and the root bits were stained with 0.05 percent trypan blue in lactophenol and boiled for about 10 minutes. These root bits were examined under binocular microscope. About 50 root bits for each replication were observed to determine AM fungal colonization percentage.

Percentage of AM colonization = \[
\frac{\text{Number of root bits with infection} \times 100}{\text{Total number of root bits examined}}
\]

Root samples and culture of AM (Vermiculite + Spores of AM fungi) were collected. Root bits were finely chopped and mixed with culture of AM and allowed to dry in shade. Shoots of host plant also allowed to dry in shade. Root bits and culture after drying were collected and stored for inoculum of Test crops. Dry weight of shoot and root of host plants were taken separately. Total dry biomass was calculated and the loss in weight was also calculated. The dried leaves of host plants were analyzed for N.P.K, Ca and Mg based on procedures outlined by Clarson (1989).

Permanent slides of infected roots and spores were prepared by the procedure described by Schenck and Perez (1988). Transferred spores with a little water to microscopic slides, after evaporation of
the water a few drops of poly vinyl alcohol lactophenol (PVL) was poured to the site where the spores were placed before mounting the cover glass. PVL permanent mounting medium, was kept for hardening for 1-2 days, and observed under a compound microscope.

**3.3.0 Pot Culture Studies**

3.3.1: A test crop, cowpea (*Vigna unguiculata*) was selected for pot culture studies, to find out the effectiveness of AM fungi on test crop. Soil sample collected from Thrikkodithanam, Kottayam district was selected for studies. Twenty four large pots having 30 cm height were selected for pot culture experiments. Experiment was conducted in a completely randomized block design. Each pot was filled with seven kg unsterilised soil, and the soil was neutralized with 20g CaCO$_3$/pot. The 24 pots were arranged in three rows with eight pots in each row. Eight treatments were replicated thrice. Added sand to each pot to the make volume to 8.0 kg. Moistened each pot with water after bringing the soil in neutral. The experimental details are as follows.

1. Number of Treatments - Eight
2. Number of Replication - Three
3. Design - CRD (Completely randomized block design)
4. Number of AM species - Three
5. Duration - Six months
Cowpea seeds were procured from State Seed Farm, Mannuthy, *Rhizobium* culture were from Agrobiotech, Poovanthuruthu, Kottayam and AMF spp. From CTCRI, Thiruvananthapuram.

The eight pots in the first row were labelled as RITI- RIT8 and in the second row as R2 TI- R2T8 and III row as R3TI- R3T8.

About 10 gm of AM sp. 1 (*Glomus mosseae*) was added to T2 and T6 pots of all the three replications. 10 gm of AM sp. 2 (*Glomus microcarpum*) was added to T3 and T7 pots all the three replications. 10 gm of AM sp. 3 (*Glomus fasciculatum*) was added to T4 and T8 pots of all the three replication, and 5g *Rhizobium leguminosarum* to T5, T6, T7 and T8 pots of all the three replications. Mixed well the contents of all the 24 pots and poured one cup full of water so as to level the surface of soil in each pot. Placed the pots above bricks so as to drain the water properly. Cowpea seeds of 25 g were mixed with

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Structure</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Soil + Test crop (Cowpea seeds, 12 in number</td>
</tr>
<tr>
<td>2</td>
<td>Soil + Test crop + AM sp. 1</td>
</tr>
<tr>
<td>3</td>
<td>Soil + Test crop + AM sp. 2</td>
</tr>
<tr>
<td>4</td>
<td>Soil + Test crop + AM sp. 3</td>
</tr>
<tr>
<td>5</td>
<td>Treatment 1 + <em>Rhizobium</em></td>
</tr>
<tr>
<td>6</td>
<td>Treatment 2 + <em>Rhizobium</em></td>
</tr>
<tr>
<td>7</td>
<td>Treatment 3 + <em>Rhizobium</em></td>
</tr>
<tr>
<td>8</td>
<td>Treatment 4 + <em>Rhizobium</em></td>
</tr>
</tbody>
</table>
5g *Rhizobium* and kept in rice water (starch water) on the previous day for about 6 hrs and drained off, and kept the seeds on a plastic sheet for drying and kept for sowing. Then kept another set of untreated seeds for sowing (without *Rhizobium*).

### 3.3.2 Sowing of the Seeds

Made channels (three horizontal and Two vertical) of about 1-2 cm depth and 5-8 cm apart in each pot. Two seeds were sown in each 12 holes made in the channels. So that 24 seed/pot were sown. Of these T1, T2, T3, T4 pots were with untreated cowpea seeds and T5, T6, T6 and T8 pots were with *Rhizobium* treated seeds. Sowing of the seeds were conducted as described earlier. All the pots were irrigated on alternate days. Seeds were started to germinate from the third day onwards; and competed within a week. No. of seeds germinated were recorded in each treatment of each replication. 200 gm of garlic was ground, added four litres of water and the mixture was sprayed on to leaves to prevent the pest infestation. Irrigated the plants on alternate days.

Thinning of the plants were done so that only 12 plants were kept in each pot. Height of the plants were taken on 16th day of sowing; from all the plants. Neem leaves were chopped and also applied to each pot for the prevention of pest and disease.

On 26th day, 40 gm of *Pseudomonas fluorescens* was dissolved in 2 litre of water and sprayed to all the plants for the protection of plants from all casualties.
On the 30\textsuperscript{th} day, height of plants were taken. Treatments like T2, T3, and T6 plants were exhibited initial bud and tendril formation. Flowering on T3, T4, T6, T7 and T8 were observed on 43\textsuperscript{rd} day. On 45\textsuperscript{th} day, heights of the plants were again taken. Plants were irrigated on all days. Pods were found to be developed in T4 plants on 45\textsuperscript{th} day itself, followed by T7, T3, T8, T6 and finally in T5 plants. Neem oil at the rate of 10 ml/litre was proposed and sprayed in to each pot; to prevent the entry of pests if any.

Matured pods were collected from each pot then and there, stored separately in paper bags for further studies. Height of the plants were again taken on 60\textsuperscript{th} day. Irrigated the plants on all days and height of the plants were again taken on 75\textsuperscript{th} day. Irrigation was continued up to 100\textsuperscript{th} day and then stopped. On 120\textsuperscript{th} day, the plants were harvested. Soil, root and shoot samples were collected separately from each treatment for further studies. Photos at different stages were also taken from the experimental plants.

3.3.3 Post Harvest Observations:

Shoot length, root length, root biomass, shoot biomass and total biomass of the harvested plants were measured and recorded separately. The mean value of each parameter were calculated and recorded in their respective unit value.

Roots of each treatment was washed with water to remove the adhering soil particles, weighed and expressed in grams.
The dry weight of shoot (oven dry basis) was measured for each treatment of each replication; and mean value were calculated and recorded in grams.

The total biomass was calculated by adding root biomass, shoot biomass together with pod weight. Number of root nodules were recorded from each treatment of each replication, and the value were calculated.

### 3.3.4 Microbiological Parameters

Spore count of the post harvest soil was estimated quantitatively by wet-sieving and decanting method (Gerdemann, and Nicolson, 1963) as per the procedure outlined earlier. Root infection percentage of AM was calculated by the method of Phillips and Hayman (1970). Colony count of *Rhizobium* of post harvest soil was calculated by culturing the soils of each treatment on yeast extract mannitol agar medium as per the procedure adopted by Dubey and Maheswari (2004).

YEM agar medium was prepared with 1% Congored and 0.002% Amphotericin, triplicate. The contents were autoclaved and poured in to sterile petridishes. Five sterile flasks were taken for 8 treatments. Transferred 90 ml sterile water aseptically in to each flask. Ten gram soil from each treatment was transferred in to flask (1) containing sterile water (90 ml) it gives dilution of 1:10 (10⁻¹). The flask was gently shaken for 10 minutes by using an electric shaker to get a homogenous soil suspension. Transferred 10 ml soil suspension
10\(^{-1}\) dilution in to flask (2) containing 90 ml sterilized water to get dilution 10\(^{-2}\). Mixed the suspension gently similarly serially transferred 10 ml soil suspension from 10\(^{-2}\) dilution to flask (3) containing 90 ml water to get the final dilution of 10\(^{-3}\). Mixed the suspension gently. Similarly serially transferred 10 ml suspension from 10\(^{-3}\) dilution to flask (4) containing 90 ml water to get dilution of 10\(^{-4}\). From there to flask (5) containing 90ml water to get a final dilution of 10\(^{-5}\). Aseptically poured 1 ml soil suspension from 10\(^{-5}\) dilution in to culture plate, for each treatment in three replication. Gently rotated the plates so as to spread the soil suspension on the medium. Incubated the plates at 28\(^{\circ}\)c for 48 hrs. Observed the bacterial colonies which were white and gummy in appearance. Counted the number of colonies using a colony counter and the values were multiplied with dilution factor. Average of three replications were taken for each treatment.

Identified the colony by characters and Gram’s staining. Gram negative bacilli were observed.

**3.3.5 Analysis of Soil**

Soil was analyzed for finding physical and chemical properties in Agro clinic and Research Centre, Kottayam based on procedures outlined by Clarson, (2002,a) as described earlier.
3.3.6 Analysis of Plant Samples of Cowpea

Root samples, shoot samples, leaf samples, grain and husk samples were analysed for total nutrients based on the procedures outlined by Clarson (1989).

Total Yield

Total yield was calculated by counting the number of pods collected. Measured the weight of pods. Again measured the weight of grains and husk separately and calculated average values. From these, mean value, the ratio of Pod to grain was also calculated. Total yield in respect to gain alone was worked out in Kg/ha.

3.3.7 Biochemical Assay: On Soil Samples

The following biochemical properties of the post harvest soil samples were analyzed based on the procedures outlined in the following paragraphs.

1. Acid phosphatase
2. Alkaline phosphatase
3. Estimation of Total phospholipids

3.3.7.1 Estimation of Acid phosphatase (by Lowery et al., 1954)

Principle:

The enzyme phosphatase hydrolyses p-nitrophenol phosphate. The released p-nitrophenol is yellow in colour in alkaline medium and is measured at 405 nm. The optimum pH for acid and alkaline phosphatases are 5.3 and 10.5 respectively.
(i) Acid Phosphatase

Materials

Sodium hydroxide 0.085 N

Substrate Solution

Dissolve 1.49 g EDTA, 0.84g citric acid and 0.03 g p. nitrophenyl phosphate in 100 ml water and adjust the pH to 5.3.

Standard

Weighed 69.75 mg p. nitrophenol and dissolved in 5.0 ml distilled water. (100 mM)

On gram soil sample was homogenized in 10 ml of ice cold 50 mM citrate buffer (pH 5.3) in a pre chilled pestle and mortar. Filtered through flour layers of cheese cloth: Centrifuged the filtrate at 10,000 rpm for 10 min. used the supernatant as enzyme source.

Procedure:

Incubated 3 ml substrate solution at 37°C for 5 min. Added 0.5ml of enzyme extract and mixed well. Removed immediately 0.05 ml and mixed it with 9.5 ml of sodium hydroxide 0.085 N. This corresponded to the blank. Incubated the remaining solution (Substrate +enzyme) for 15 min at 37°C. 0.5 ml sample was drawn and mixed it with 9.5 ml sodium hydroxide solution. Measured the absorbance of blank and incubated tubes at 405nm. 0.2 ml to 1 ml (4 to 20 mM) of the standard was taken, diluted to 10.0 ml with NaOH solution. Read the colour and the standard curve was drawn.
Specific activity was expressed as m moles p-nitrophenol released per min. per mg protein.

3.3.7.2. Estimation of Alkaline Phosphatase

One gram of soil was homogenized in 10 ml of ice-cold 50 mM glycine NaOH buffer at pH 10.4.

Substrate Solution

Dissolved 375 mg glycine, 10 mg magnesium chloride, 165 mg p-nitrophenyl phosphate in 42 ml of 0.1 N sodium hydroxide and diluted to 100 ml. Adjusted pH to 10.5. The other procedures followed were similar to Acid phosphatase.

3.3.7.3 Estimation of Total Phospholipids
(by Zilver Smith and Davis, 1950)

Ten gram of soil was extracted in 2:1 chloroform methanol after keeping for 24 hours at room temperature. It was filtered and the residue was washed with chloroform methanol 2:1 at least 3 times. To the filtrate in a stoppered tube distilled water was added, mixed and allowed to stand in the fridge overnight. The aqueous layer was removed with Pasteur pipette. The washed lower layer of chloroform was evaporated to dryness and the residue was dissolved in a known volume of chloroform. From this, aliquots were used for lipid analysis

Reagents

(a) 5N H$_2$SO$_4$

(b) 2.5% Ammonium molybdate

(c) ANSA- 1 Amino – 2 naphthol – 4 sulfonic acid
Procedure

Five ml of the lipid extract was pipetted in to a Kjeldahl’s flask and evaporated to dryness. 1ml of 5N H\textsubscript{2}SO\textsubscript{4} was added and digested in a digestion rack till it became light brown. One or two drops of 2N HNO\textsubscript{3} was added and digested again till it became colourless. The Kjeldahl’s flask was cooled. 1 ml of water was added and heated in a boiling water bath for 10 minutes. 1ml of 2.5% ammonium molybdate and 0.1 ml ANSA were added to this. The volume was made up to 10 ml with distilled water and absorbance was measured at 660 nm within 10 min. The amount of phospholipid was determined by multiplying the amount of phosphorus with the conversion factor 25.9. The standard used was KH\textsubscript{2}PO\textsubscript{4}.

3.3.8 Estimation of Nutrients in plant samples

3.3.8.1 Estimation of Nitrogen in Plant Tissue (Baruah, 1977)

Principle

The method of determination, essentially involves three successive phases.

1. Digestion of organic material to convert N in to NH\textsubscript{3}.
2. Distillation of the digested materials
3. Titration of the distilled product

Digestion of the organic material is accomplished by boiling the sample with con. H\textsubscript{2}SO\textsubscript{4} in the presence of certain catalysts (Hg, Cu or Se) which accelerate the speed of digestion and salts (K\textsubscript{2}SO\textsubscript{4} or
Na$_2$SO$_4$) which raise the digestion temperature. The organic materials decomposes in to several components.

\[ C \rightarrow CO_2, \ O \rightarrow H_2O, \ N \rightarrow NH_3 \]

N is transformed into NH$_3$ during digestion and because of the excess H$_2$SO$_4$, it remains in the form of ammonium sulphate

\[ 2NH_3 + H_2SO_4 \rightarrow (NH_4)_2SO_4 \]

NH$_3$ was determined by distillation, following a strong alkalanisation. The NH$_3$ gas, so liberated is absorbed in a known volume of boric acid, the quantity of which is determined by back titrating it with a standard acid.

**Equipment and Apparatus**

Kjeldahl digestion assembly; Kjeldahl’s Flask, Ammonia distillation assembly, conical flask or beaker, pipette.

**Reagents**

1. Con. H$_2$SO$_4$ and 0.1 N H$_2$SO$_4$

2. Granulated Zn

3. Digestion mixture. Mix 10g of CuSO$_4$.5H$_2$O (oven dried) 3g HgO and 1g Se. powder by grinding in a mortar.

4. Potassium sulphate

5. 30% NaOH solution

Boric acid indicator solution: Dissolved 20 g boric acid, H$_3$BO$_3$ (AR), in about 900 ml of hot water cooled and added 20 ml of a mixed indicator solution (prepared by dissolving 0.1 g bromocersol green and 0.07g methyl red in 100 ml of ethanol) Added 0.1 N NaOH
solution drop wise, until the colour is reddish purple, and then diluted to one litre with distilled water.

**Procedure**

**Digestion:** Weighed 1.0 g sample of homogenized plant tissue in a kjeldhal flask. Added 25 ml of Con. H₂SO₄, about 20 ml distilled water, 1.0 g of digestion mixture and 10 g of K₂SO₄, placed the flask in digestion unit. Digested the content of flask at low heat to prevent frothing. After about 15-20 minutes gradually raised the heat until the content became clear, and coloured pale green or blue.

**Distillation:** Cooled the contents, added about 200 ml of distilled water and swirled the flask for about 2 minutes, and took the supernatant liquid in to a distillation flask. Added about 50 ml of water to the digestion flask, and took the water extract in to a distillation flask. Repeated this process for at least 4 times. Added about 150 ml of 30% NaOH slowly along the side of the distillation Flask. Added two pieces of Zn to prevent superheating of the soil extract. Distilled NH₃ in to 25ml of boric acid indicator solution, contained in a conical flask, and placed the flask below the condenser, so that the discharging tube of condenser was immersed in the boric acid indicator solution. When no more NH₃ is received (tested with a wet red litmus paper-not turning blue) stopped the distillation and proceed for titration.
**Titration**

Ammonium tetraborate formed during distillation, was back titrated with 0.01N H$_2$SO$_4$, releasing boric acid with the formation of (NH$_4$)$_2$SO$_4$. The disappearance of blue colour just indicated the end point of the titration. Conducted a blank, without the plant material, to avoid contamination and to ensure precision.

**Observation and Calculation**

- a. Wt. Of the plant material - 1 gm
- b. Normality of H$_2$SO$_4$ - 0.1
- c. Volume of H$_2$SO$_4$ used in sample titration - $S$ ml
- d. Volume of H$_2$SO$_4$ used in blank titration - $B$ ml
- e. M eq of H$_2$SO$_4$ used in sample titration - 0.1$xS$
- f. M eq of H$_2$SO$_4$ used in blank titration - 0.1$xB$
- g. M eq of H$_2$SO$_4$ actually used in sample titration – 0.1$x(S-B)$
  
  (1 ml of 0.1 N H$_2$SO$_4$ (=0.1 M eq H$_2$SO$_4$) = 1.4 mg N= 0.0014 gm N)
- h. M eq. Of n per gram of plant material = (S-B) $x$ 0.1$x$ 0.0014
- i. M eq Of N/100 of plant material = (S-B) $x$ 0.1$x$0.0014$x$ (100/1)

**3.3.8.2 Estimation of plant issue phosphorus by vanadomolybdate method (Baruah, 1997)**

**Principle**

A suitable portion of the plant digest was quantitatively transferred in to a volumetric flask and allowed to react with vanadomolybdate in an acidified solution. A characteristic yellow chromogen of the vanadomolybdo phosphoric system is formed, the
intensity of which is measures calorimetrically, using an appropriate filter.

**Equipment and Apparatus**

Photoelectric colorimeter, Volumetric flask (50 ml), pipette.

**Reagents**

1. **Vanadomolybdate reagent:** It is prepared by mixing the following 2 solutions

   **Solution A:** Dissolved 25g of ammonium molybdate \((NH_4)_6MO_7O_{24}.4H_2O\) in 400 ml of warm distilled water and cool.

   Solution B: Added slowly 1.25 g of ammonium meta vandate to 300 ml of boiling water, and cooled added to it 250 ml con. HNO\(_3\) and cooled. Poured solution B in to a 1 litre volumetric flask and added solution A. Mixed well and diluted the volume with distilled water.

2. **Phosphate standard:** Dissolved 0.2195 g of KH\(_2\)PO\(_4\) and diluted to 1 litre. This solution contains 50ppm P. used this stock solution for the preparation of working standards of P, required for drawing the standard curve of P.

**Procedure**

Five ml (which contained 1-15 ppm of P) of the ash solution was taken in a 50 ml volumetric flask. Added 10 ml of nitric acid-molybdate-Vanado mixture, diluted to volume, and mixed thoroughly. The colour developed fully in about 30 minutes which is stable for even 2 months at high ‘P’ concentrations; at P concentrations of 5 ppm, it is stable for only 2 weeks. Read the intensity of yellow colour
formed, on a photoelectric colorimeter at a wavelength of 450 m\(\mu\) filter.

**Preparation of Standard Curve**

Pipetted out 0, 1, 2, 4, 6, 8, 10 ml of 50ppm solution separately in 50 ml volumetric flasks. Added 10 ml of nitric acid-molybdate vandate mixture and proceed for the development of colour in the same way as in the procedure described above.

**Observation and Calculations**

a. Weight of the plant material = 1 gm

b. Volume of the plant digest made = V ml

c. Volume of aliquot taken for analysis = VI ml

d. Volume made upto = 50 ml.

**Phosphorous**

e. Transmittance (%) as read from the colorimeter = T

f. Ppm of P as read from the std. Curve against T= Y

\[ \text{g. Ppm of 'P' in the given plant material} = Y \times \frac{50}{V} \times \frac{V}{V_i} \]

**3.3.8.3 Estimation of Potassium and Calcium in Plant Tissue**

Plant tissue potassium and calcium were determined by Flame photometer following the method described by Jackson (1967).

**Principle**

Potassium when excited in Flame emit radiations of lilac colour. The emission intensity is measured through the flame
photometer which is proportional to concentration of potassium present in the tissue sample.

The % were calculated using the formula

\[
K\% = \frac{\text{ODValue} \times 100 \times \text{Dilution}}{10 \times 0.5 \times \text{Aliquot} \times 10000}
\]

\[
\text{Ca}\% = \frac{\text{ODValue} \times 100}{5 \times 0.5 \times 10000}
\]

### 3.3.8.4 Estimation of Mg in Plant Tissue

The tissue element Mg was measured in Perkin-Elmer-2380 atomic absorption spectrometer using the method proposed by Ure, (1983). The % was obtained by using the formula.

\[
\text{Mg}\% = \frac{100 \times \text{Dilution} \times \text{ODValue}}{0.5 \times \text{Aliquot} \times 10000}
\]

### 3.3.9 Biochemical Assay on Plant Samples

The following biochemical parameters of the cowpea grains were analysed based on the procedures outlined in the following paragraph.

(i) Total protein of Grain

(ii) Total carbohydrate of Grain

(iii) Total starch of Grain

(iv) Proline content of Grain

(v) Phospholipid of Grain
3.3.9.1 Estimation of Total Protein Content (By Lowry’s Method
(Lowry et al., 1951) of Grain

Principle

The blue colour developed by the reduction of the phosphomolybdic, phosphotungstic components in the Folin ciocalteau reagent by the amino acid tyrosine and tryptophan present in the protein plus the colour developed by the biuret reaction of the protein with the alkaline cupric tartarate are measured in the Lowry’s method.

Materials: 2% sodium carbonate in 0.1 N sodium hydroxide (Reagent A)

- 0.5% copper sulphate (CuSO₄. 5H₂O) in 1% potassium sodium tartarate (Reagent B)

- Alkaline copper solution: Mixed 50ml of A and 1 ml of B prior to use. (Reagent C)

- Folin ciocalteau Reagent (Reagent D): Refluxed gently for 10 hrs a mixture consisting of 100 g sodium tungstate 25g sodium molybdate, 700ml water, 50ml of 85% phosphoric acid, and 100ml of con. HCl in a 1.5 L flask. Added 150 g lithium sulphate, 50ml water and a few drops of bromine water. Boiled the mixture for 15 min without condenser to remove excess bromine. Then it was cooled, diluted to 1 L and filtered.
- **Protein solution (Stock std.)**
  Weighed accurately 50 mg of bovine serum albumin (Fraction V) and dissolved in distilled water and made upto 50ml in a standard flask.

- **Working standard:** Diluted 10ml of the stock solution to 50 ml with distilled water in a standard flask one ml of this solution contains 200 \( \mu \)g protein.

**Procedure: Extraction of Protein from Sample**

Extraction was usually carried out with buffer used for enzyme assay. Weighed 500mg of the sample and ground well with a pestle and mortar in 5-10 ml of the buffer. Centrifuged and used the supernatant for protein estimation.

**Estimation**

1. Pipetted out 0.2, 0.4, 0.6, 0.8 and 1 ml of the working std into a series of test tubes.
2. Pipetted out 0.1 ml and 0.2 ml of the sample extract in two other test tubes.
3. Made up the volume to 1 ml in all the test tubes. A tube with 1 ml of water serves as the blank.
4. Added 5 ml of reagent C to each test tube including the blank. Mixed well and allowed to stand for 10 min.
5. Then added 0.5 ml of reagent D, mixed well and incubated at room temp in the dark for 30 min. Blue color was developed.
6. Took the readings at 660nm
Drawn a standard graph and calculated the amount of protein in the sample.

Expressed the amount of protein in mg/g or 100 g sample.

3.3.9.2 Estimation of Total Carbohydrate Content of Cowpea Grains

(Hedge and Hofreiter, 1962)

**Principle:**

Carbohydrates are first hydrolysed to simple sugars with dil HCl. In hot acid medium glucose is dehydrated to hydroxymethyl furfural. This forms green colour with anthrone at 630nm.

**Materials**

1. 2.5 N HCl
2. **Anthrone reagent:** Dissolved 100 mg anthrone in 100ml of ice cold 95% H₂SO₄. Prepared fresh before use.
3. **Standard of glucose:** Stock Dissolved 100 mg in 100 ml water.
   Working standard-10 ml of stock diluted to 100 ml with distilled water.

**Procedure**

1. Weighed 100 mg of the powdered grains into a boiling tube.
2. Hydrolysed by keeping it in a boiling water bath for three hours with 5 ml of 2.5 NHCl and cooled to room temperature.
3. Neutralised it with solid sodium carbonate until the effervescence ceases.
4. Made up the volume to 100 ml and centrifuged.
5. Collected the supernatant and took 0.5ml and 1 ml aliquots for analysis.

6. Prepared the standards by taking 0, 0.2, 0.4, 0.6, 0.8 and 1 ml of the working standard.

7. Made up the volume to 1 ml in all the tubes including the sample tubes by adding distilled water.

8. Then added 4 ml of anthrone reagent.

9. Heated for eight minutes in a boiling water bath.

10. Cooled rapidly and read the green to dark green colour at 630nm.

11. Drawn a standard graph by plotting concentration of the standard on the X-axis versus absorbance on the Y-axis.

12. From the graph calculated the amount of carbohydrate present in the sample tube.

**Calculation**

Amount of carbohydrate present in 100mg of the sample

\[
= \frac{\text{Mg of glucose} \times 100}{\text{Vol. of test sample}}
\]

3.3.9.3 Estimation of Starch Content of Cowpea Grains by Anthrone Method (Hedge and Hofreiter, 1962)

Starch is an important polysaccharide. It is the storage form of carbohydrate in plants abundantly found in roots, tubers, stems, fruits and cereals. Starch which is composed of several glucose molecules, is a mixture of two types of components namely amylose
and amylopectin. Starch is hydrolysed into simple sugars by dilute acids and the quantity of simple sugars is measured calorimetrically.

**Materials**

Anthrone: Dissolved 200 mg anthrone in 100 ml of ice-cold 95% sulphuric acid.

80% ethanol

52% perchloric acid

Standard glucose-Stock 100 mg in 100 ml water.

Working standard- 10 ml of stock diluted to 100 ml with water.

**Procedure**

1. 0.1 to 0.5 g of the sample was homogenized in hot 80% ethanol to remove sugars. Centrifuged and retained the residue. Washed the residue repeatedly with hot 80% ethanol till the washing do not gave colour with anthrone reagent. Dried the residue well over a water bath.

2. To the residue added 5 ml of water and 6.5 ml of 52% perchloric acid.

3. Extracted at 0°C for 20 min Centrifuged and saved the supernatant.

4. Repeated the extraction using fresh perchloric acid. Centrifuged and pooled the supernatants and made up to 100 ml.

5. Pipetted out 0.1 or 0.2 ml of the supernatant and made up the volume to 1 ml with water.
(6) Prepared the standards by taking 0.2, 0.4, 0.6, 0.8 and 1 ml of the working standards and made up the volume to 1 ml in each tube with water.

(7) Added 4 ml of anthrone reagent to each bath.

(8) Heated for eight minutes in a boiling water bath.

(9) Cooled rapidly and read the intensity of green to dark green colour at 630nm.

Calculated the glucose content in the sample using the standard graph and multiplied the value by a factor 0.9.

3.3.9.4 Estimation of Proline Content of Cow Pea Grains (Bates et al., 1973)

Principle

During selective extraction with aqueous sulphosalicylic acid proteins are precipitated as a complex. Other interfering materials are also presumably removed by absorption to the protein sulphosalicylic acid complex. The extracted proline is made to react with ninhydrin in acidic conditions (pH 1.0) to form the chromophore (red colour) and read at 520 nm.

Materials

- Acid Ninhydrin. Warmed 1.25g ninhydrin in 30 ml glacial acetic acid and 20 ml 6M phosphoric acid, with agitation until dissolved. Stored at 4°C and used within 24 hrs.
- 3% aqueous sulphosalicylic acid
- Glacial acetic acid
Procedure

Extracted 0.5gm of grains by homogenizing in 10 ml of 3% aqueous sulphosalicylic acid. Filtered the homogenate through Whatman No. 2 filter paper. About 2 ml filtrate was taken in a test tube and added 2 ml of glacial acetic acid and 2 ml of acid ninhydrin. Heated it in the boiling water bath for one hour. Terminated the reaction by placing the tube in ice bath. Added 4ml toluene to the reaction mixture and stirred well for 20-30 seconds. Separated the toluene layer and warmed to room temperature. Measured the red colour intensity at 520 nm. Run a series of standard with pure proline in a similar way and prepared a standard curve. Found out the amount of proline in the test sample from the standard curve.

Calculation

Expressed the proline content on fresh weight basis as follows:

$$\mu \text{ moles per grain} = \frac{\mu g \text{ proline/ml} \times \text{ml toluene}}{115.5} \times \frac{5}{\text{g sample}}$$

where 115.5 is the molecular wt of proline.
3.3.9.5 Estimation of total phospholipid of cowpea grain

(By Zilver Smith and Davis, 1950)

Cowpea grains were ground thoroughly and about one gram of the powder was extracted in 2:1 chloroform methanol after keeping for 24hrs at room temperature. It was filtered and the residue was washed with chloroform methanol 2:1 at least three times. To the filtrate in a stoppered tube distilled water was added mixed and allowed to stand in the fridge overnight. The aqueous layer was removed with a Pasteur pipette. The washed lower layer of chloroform was evaporated to dryness and the residue was dissolved in a known volume of chloroform. From this aliquots were used for lipid analysis.

Reagents

(a) 5N H$_2$SO$_4$

(b) 2.5% Ammonium molybdate

(c) ANSA

Procedure

0.5ml of the lipid extract was pipetted into a kjeldahl’s flask and evaporated to dryness. One ml of 5N H$_2$SO$_4$ was added and digested in a digestion rack till it became light brown. One or 2 drops of 2N HNO$_3$ was added and digested again till it became colourless. The kjeldahl’s flask was cooled. 1ml of water was added and heated in a boiling water bath for 10 minutes. One ml of 2.5% ammonium molybdate and 0.1ml of ANSA were added to this. The volume was
made upto 10ml with distilled water and absorbance was measured at 660nm within 10min. The amount of phospholipid was determined by multiplying the amount of phosphorous with the conversion factor 25.9. The standard used was KH$_2$PO$_4$.

### 3.3.10 Uptake Studies

Uptake of the nutrients viz. N, P and K were calculated. Uptake of N, P and K by roots, shoots, grains and husks were calculated for each treatment by multiplying the content of the nutrients (expressed in percentage) with the respective yields and the results were expressed in Kg ha$^{-1}$. From these values total uptake of N, P and K by Cowpea plant was calculated by adding respective values of root, shoot, grain and husk together and that also were expressed in Kg ha$^{-1}$.

### 3.3.11 Economics

Economics studies on the yield and profit were carried out; using the market rate of cowpea, calculated the income in rupees and increase income over control; for various treatments under study.

### 3.3.12 Statistical Analysis

All the data generated in the studies were analysed statistically to find out the real effects due to various treatments. Analysis of variance was calculated as suggested by Panse and Sukhatme (1967). From the analysis of variance table, the critical difference values were worked out at five percent probability level as outlined by Gomez and Gomez (1984).