2.1 Soil sample
Soil samples were collected from the rhizosphere of various kinds of plants, which are of high economic/medicinal value, from different localities in and around Kottayam Dist, Kerala to study the natural occurrence of mycorrhizal fungi. Soil was dug out with a trowel to a depth of 15 cm after scraping away the top layer of soil.

Root samples along with surrounding soil were taken in polythene bags and stored at 4°C until they were processed further. The root samples were preserved in Formalin Alcohol Acetone (FAA) in the ratio of 90:5:5 (v/v/v) before clearing and staining.

2.1.1 Soil pH determination
pH of the soil samples was determined in 1:2.5 v/v soil: water solution in a Philip's pH meter.

2.1.2 Estimation of Mycorrhizal colonization
Mycorrhizal colonization was expressed using the following formula. Giovanetti & Mosse, (1980)

\[
\text{Percent colonization} = \frac{\text{No: of root segments with VAM}}{\text{Total No: of root segments examined}} \times 100
\]

2.2 Estimation of Growth rate
The growth rates of the plants were assessed using standard parameters like shoot and root length, the fresh and dry weights of root and shoot etc.

a). **Shoot length**: The height of the main shoot was measured from the ground level to the tip of the terminal bud.
b) **Root length**: The length of the main root from the collar to the tip was measured.

c) **Fresh and Dry weight of Root and Shoot**: The root and shoot portions of the plants were separated. The root portion was washed gently in running tap water to remove all the adhering soil particles. Both root and shoot were pressed in folds of filter paper to remove excess moisture. The fresh weight was determined and the samples were kept in a hot air oven at 70°C for one day as we got a constant value and taken the dry weight.

d) **Nodule number and weight**: Plants were removed carefully from the bags with their root system and nodules intact, after 30, 60, and 120 days of growth. The nodules with roots were washed, separated and counted. The fresh and dry weights of the nodule were also taken.

e) **Estimation of Relative mycorrhizal dependency** (Plenchette et al., 1983)

The relative mycorrhizal dependency (RMD) was calculated by the following formula

\[
RMD = \left( \frac{\text{Total dry weight of mycorrhizal plant} - \text{Total dry weight of non-mycorrhizal plant}}{\text{Total dry weight of mycorrhizal plant}} \right) \times 100
\]

2.3 **Estimation of Nitrogen, Phosphorus and Potassium**

2.3.1. **Determination of Nitrogen (Microkjeldahl method)** Karthikakutty Amma (1989)

Nitrogen present in the sample in organic form was converted to inorganic form (ammoniacal) by digestion with concentrated sulphuric acid in the presence of mercuric oxide- potassium sulphate mixture. Mercuric oxide was added as catalyst and potassium sulphate to raise the boiling point of sulphuric acid from 330°C to 420°C (Digestion was carried out between 360°C and 420°C). Ammonia, which was fixed as Ammonium
sulphate, liberated by adding an excess of caustic alkali was determined by distilling off the liberated Ammonia in to Boric acid solution. Along with caustic alkali; sodium thiosulphate was also added to decompose the mercuri- ammonium compound formed.

**Preparation of Sample Digest**

Digestion was carried out in a Microkjeldahl digestion set.

**Reagents**

1. Concentrated sulphuric acid
2. Potassium sulphate
3. Mercuric oxide

**Procedure**

Transferred 500mg of the sample (previously dried out 105°C for six hours) in to a digestion flask. Added 1.9± 0.1g of potassium sulphate and 40±10 mg of mercuric oxide. Carefully added 2.4ml of concentrated sulphuric acid through the sides of the digestion flask.

Heated the flask gently until frothing ceases. Continued heating more strongly until the solution was cleared. Heated for 30 minutes more. Allowed to cool. Added 10 ml of distilled water and warmed to dissolve the solute material. A blank using reagents was run similarly. Nitrogen in the Sample Digest was determined by

Microkjeldahl distillation method.

**Reagents**

1. Boric acid 4% solution in water. Dissolve 4g of boric acid in 100ml of water.

2. Sodium hydroxide: Sodium thiosulphate solution. Dissolve 50g of sodium hydroxide and 5g of hydrated thiosulphate in 100ml of water.
3. Methyl red- Bromocresol green indicator. Mix five parts of 0.2% alcoholic methyl red solution.

4. Hydrochloric acid 0.02N: 17.8 ml of concentrated hydrochloric acid was diluted to 10 litres

**Procedure**

Steamed out the distillation unit for 20 minutes. Transferred the digested sample into the distillation flask. Added 10 ml of sodium-hydroxide, sodium-thiosulphate mixture into 5 ml of boric acid solution containing 2-3 drops of methyl red –bromo cresol green indicator. Collected 20 ml of distillate and titrated against 0.02N hydrochloric acid. The end point was the appearance of a gray colour. Distilled the blank digest also and titrate value obtained was subtracted from the test values.

**Reagents**

1. Methyl orange indicator 0.1% in water.
2. Reference solution: 80 ml of CO₂ free water with three drops of methyl orange indicator.
3. Sodium carbonate solution 0.02N.

Dried sodium carbonate (A.R., anhydrous) at 120°C for two hours. Cooled in a desiccator. Weighed 1.06 g to a 100 ml volumetric flask and made up to the mark with distilled water (This gives a 0.2 N solution). 20 ml of this solution was diluted to get 0.02 N solution.

**Procedure**

40 ml of 0.02 N sodium carbonate solution was taken in a 250 ml conical flask. Added 3 drops of methyl orange indicator and titrated with the hydrochloric acid to be standardized until the colour begins to deviate from water tint reference solution- equivalent point has not been reached now. Boiled the solution gently for two minutes to expel carbon dioxide.
Cooled and titrated against the acid until the colour was barely different from water tint of indicator. Strength of acid was calculated from the equation:

\[
\text{Normality of acid } N_2 = \frac{V_1 \times N_1}{V_2}
\]

Where \(V_1\) - Volume of sodium carbonate

\(N_1\) - Normality of sodium carbonate

\(V_2\) - Volume of acid

From the normality of acid, the percentage of nitrogen was calculated.

**Calculation**

Since 1ml of 1 Normal acid is equivalent to 14 mg of nitrogen Karthikakutty Amma, (1989).

Percentage of nitrogen in the sample =

\[
\frac{\text{Volume of acid used} \times \text{Normality} \times \text{Equivalent weight of N}}{\text{Weight of sample in mg}} \times 100
\]

**2.3.2 Estimation of Phosphorus and Potassium using Autoanalyser**

Autoanalyser is an instrument that utilizes continuous flow technique to perform quantitative analysis. The system is usually composed of a combination of four separate modulus, which are interconnected by tubings. The modulus are:

1. Sampler
2. Proportionating pump
3. Measurement module, and
4. Recorder

In some autoanalysers there will be another module for reagents.
Working of Autoanalyser

Liquids were pumped from appropriate reagent container and from the sampler and are drawn in to the manifold where they were mixed, heated (if necessary) and diluted. Flame photometer and optical filters were used in the measurement module so that only a narrow band of wavelength was used to determine the concentration of elements in the sample. The concentration was determined against a reference. Each output signal drives a pen across a strip of moving chart paper to graphically display the analytical results in a series of peaks.

Estimation of Phosphorus

Reagents

Standard

Stock 1000ppm phosphorus

Dissolved 5.0368g sodium dihydrogen phosphate (NaH$_2$PO$_4$.2H$_2$O) and made up to one litre in a volumetric flask.

100ppm Phosphorus

Pipetted 25 ml of 1000ppm solution in to 250 ml standard flask and made up to 250 ml with distilled water.

Working standards

Pipetted 20 ml and 10 ml of 100ppm solutions in to 100ml standard flask and added 5 ml 1:1 HCl made up to 100ml with distilled water.

Ammonium molybdovandate solution

Solution A

Dissolved 20g ammonium molybdate in distilled water.

Solution B

Dissolved 1g ammonium metavandate in water
To solution B, added 40ml of conc. Sulphuric acid. Added solution A to B. Made up to 1 litre after adding 1ml of aerosole.

**Procedure**

Accurately weighed out the sample (dried at 105°C for 6 hours) in to a silica dish. The ash was prepared in a furnace at 500-550°C for half an hour. Allowed to cool and moistened the ash with distilled water, keeping a watch glass over the dish and passing a jet of water through the spout. Carefully added 5ml of 1:1 HCl and digested for 1 hour, in a water bath. After cooling, transferred the contents of the dish to a 100ml standard flask and made up to 100ml. This was used for the determination of phosphorus.

The setting up of the instrument is same as in the case of nitrogen. The calibration curve for phosphorus obtained for 20ppm and 10ppm, standards were used to compare.

For 20ppm the amount of phosphorus = \( \frac{20}{50} \times 100 \times \frac{100}{\text{weight}} \times \frac{1}{103} \)

**Estimation of Potassium**

**Reagents**

1. Lithium nitrate stock solution

   Dissolved 9.86g lithium nitrate in distilled water and made up to one litre.

   Lithium nitrate- lanthanum chloride solution

   Dissolved 6g lanthanum chloride heptahydrate in water. Added 10ml lithium nitrate stock solution and one ml Brij35, made upto one litre.

   Brij 35 solution: One ml Brij 35 diluted to one litre with water
**Standards**

Dissolved 1.906g of AR potassium chloride in one litre of water to get a stock solution of 1000ppm.

Pipetted 50ml of 1000ppm solution in to 250ml standard flask. Made up to 250ml. this will be 200ppm.

Calibration curve obtained by using 70ppm and 50ppm was used to compare.

**Calculation**

Let $R$ be the value obtained from the graph for the sample.

$$\% K = R \times \frac{50}{50} \times \frac{100}{\text{weight}} \times \frac{100}{103}$$

**2.4 Estimation of Biochemical constituents**

**2.4.1 Estimation of Proteins**

Protein estimation was performed by the method of Lowry, *et al.* (1951)

**Lowry, et.al. Method**

**Reagents**

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sodium hydroxide solution</td>
<td>0.1 N</td>
</tr>
<tr>
<td>Sodium carbonate solution</td>
<td>2% in 0.1 N</td>
</tr>
<tr>
<td>Sodium potassium tartrate solution</td>
<td>1%</td>
</tr>
<tr>
<td>Copper Sulphate solution</td>
<td>0.5 %</td>
</tr>
<tr>
<td>Alkaline copper reagent</td>
<td>A mixture of 50 ml sodium carbonate solution and 0.5 ml of each of copper sulphate solution and sodium potassium tartrate solution.</td>
</tr>
</tbody>
</table>
Folin's phenol reagent : 1:1 dilution with distilled water.

Standard protein solution : 100 mg % in 0.1 NaOH.

Procedure

Pipetted out 0.2 ml extract to the test tube and added 1.8 ml of sodium hydroxide solution and 5 ml of alkaline copper reagent. Shaken well and kept the mixture for 15 min. After 10 min, 0.5 ml of Folin's phenol reagent was added and mixed well. The mixture was kept for another 30 minutes. The optical density was measured at 675nm, in a spectrophotometer. The system devoid of sample was used as the blank. The concentration of protein was determined by comparing the absorbance got with a standard curve prepared.

2.4.2 Estimation of total Chlorophyll

Chlorophyll estimation was performed according to the method of Arnon (1949)

Reagent

Ice cold 80% aqueous acetone.

Procedure

1. Weighed 1 gm of finely cut and well-mixed representative sample of leaf in to a clean mortar.

2. The tissue was grinded to a fine pulp with the addition of 20 ml of 80 % acetone.

3. Centrifuged at 5000 r p m for 5 min, and transferred the supernatant to a 100ml volumetric flask.

4. Again the residue was grinded with 20 ml of 80 % acetone, centrifuged and transferred the supernatant to the same volumetric flask.
5. Repeated this procedure until the residue becomes colourless. Washed the mortar and pestle thoroughly with 80 % acetone and collected the clear washings in the volumetric flask.

6. The volume was made up to 100ml with 80 % acetone.

7. The absorbance was read at 645, 663 and 652 nm against the solvent (80% acetone) blank.

**Calculation**

Calculated the amount of chlorophyll present in the extract as mg chlorophyll per g tissue using the equation.

\[
\text{Chl.a} = 12.7 \times \frac{A_{663}}{1000} - 2.69 \times \frac{A_{645}}{1000} \times W
\]

\[
\text{Chl.b} = 22.9 \times \frac{A_{645}}{1000} - 4.68 \times \frac{A_{663}}{1000} \times W
\]

\[
\text{Total Chlorophyll} = 20.2 \times \frac{A_{645}}{1000} + 8.02 \times \frac{A_{663}}{1000} \times W
\]

2.4.3. Estimation of total Carbohydrates

Total carbohydrate in the sample was estimated by Anthrone method. Hedge & Hofreiter, (1962)

**Materials**

1. 2.5 N HCl

2. Anthrone reagent: Dissolved 200mg anthrone in 100 ml of ice cold 95 % H₂SO₄.

3. Standard glucose- Stock- Dissolved 100 mg of glucose in 100 ml water.

   Working standard: 10 ml of stock diluted to 100 ml with distilled water
Materials and Methods

Procedure

1. Weighed 1g of the sample in to a boiling tube

2. Hydrolyzed the sample by keeping it in boiling water bath for three hours with 5 ml 2.5 N HCl and then cooled it to room temperature.

3. Neutralized it with solid sodium carbonate until the effervescence stopped.

4. The volume was made up to 100 ml and then centrifuged.

5. Collected the supernatant and took 0.5ml aliquot for analysis.

6. Prepared the standard by taking 0, 0.2, 0.4, 0.6, 0.8 and 1ml of the working standard. Sample with out standard served as blank.

7. The volume of all the tubes, including the sample tube was made up to 1ml by adding distilled water.

8. Then added 4ml 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. A standard graph was drawn by plotting concentration of the standard on the x-axis versus absorbance on the y-axis.

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

Calculation

Amount of carbohydrate present in 1g of the sample

\[
\text{mg of glucose} \times 1000 = \frac{\text{mg of glucose}}{\text{volume of test sample}}
\]
2.4.4. Estimation of total Reducing Sugars

The amount of total reducing sugars in the leaf samples was determined by the method of Nelson-Somogyi (1952)

**Materials**

Alkaline Copper tartrate

(A) Dissolved 2.5g anhydrous sodium carbonate, 2g sodium bicarbonate, 2.5g potassium sodium tartrate and 20g anhydrous sodium sulphate in 80 ml water and made up to 100ml.

(B) Dissolved 15g copper sulphate in a small volume of distilled water. Added one drop of sulphuric acid and made up to 100 ml.

Mixed 4 ml of B and 96 ml of Solution A.

Arsenomolybdate Reagent - Dissolved 2.5 g ammonium molybdate in 45 ml water. Added 2.5 ml Sulphuric acid and mixed well. Then added 0.3 g disodium hydrogen arsenate dissolved in 25 ml of water. Mixed well and incubated at 37˚C for 24 to 48hrs.

Standard glucose solution – Dissolved 100mg glucose in 100 ml distilled water.

Working standard – 10 ml of stock solution diluted to 100 ml with distilled water. (100-µg/ml)

**Procedure**

1. Weighed 1g of the leaf sample and extracted the sugar with hot 80% ethanol twice (5ml each time)

2. Collected the supernatant and evaporated it by keeping it on water bath at 80˚C.

3. Added 10 ml water and dissolved the sugars.

4. Pipetted out aliquots of 0.1 ml to a test tube.
5. Pipetted out 0.2, 0.4, 0.6, 0.8 and 1 ml of the working standard solution in to a series of test tubes.

6. The volume in both sample and standard tubes were made up to 2ml with distilled water.

7. A blank was prepared by pipetting 2ml-distilled water in a separate tube.

8. Added 1ml of alkaline copper tartrate reagent to each tube.

9. Placed all the tubes in a boiling water bath for 10 minutes.

10. The tubes were cooled and then added 1ml of arsenomolybdolic acid reagent to all.

11. The volume in each tube was made up to 10 ml with water.

12. After 10 minutes, the absorbance of blue colour was read at 620nm.

13. From the standard graph drawn, calculated the amount of reducing sugar present in the sample.

**Calculation**

Absorbance corresponds to 0.1 ml of test = X mg of glucose.

\[
10 \text{ ml contains} \quad \frac{X}{0.1} \times 10 \text{ mg glucose}
\]

2.5. **Estimation of Proline** (Bates et al, 1973)

**Materials**

Acid- Ninhydrin- Warmed 1.25g Ninhydrin in 30ml glacial acetic acid and 20ml 6m phosphoric acid, with agitation until dissolved. It was stored at 4˚C.

3% aqueous Sulpho salicylic acid.

Glacial acetic acid

Toluene

Proline- standard
**Procedure**

1. 1g of plant material was extracted by homogenizing in 10ml of 3% aqueous Sulphosalicylic acid

2. The homogenate was filtered through whatman No: 2 filter paper.

3. 2 ml of the filtrate was taken in a test tube and added 2ml of glacial acetic acid and 2ml of acid-ninhydrin.

4. Heated the tubes, in a boiling water bath for 1hr.

5. Terminated the reaction by placing the tubes in ice bath.

6. Added 4 ml of toluene to the reaction mixture and stirred well for 20-30 sec.

7. Separated the toluene layer and warmed to room temperature.

8. The intensity of red colour was measured at 520 nm.

9. Using pure proline, a series of standards were run and prepared a standard graph.

10. From the standard graph the amount of praline in the test sample was calculated.

**Calculation**

The proline content on fresh weight basis was expressed as

\[
\mu \text{ moles per g tissue} = \frac{\mu g \text{ proline/ml}}{115.5} \times \text{ml toluene} \times \frac{5}{\text{g. sample}}
\]

where 115.5 is the molecular weight of proline.

2.6. **Antioxidant Enzyme Systems**

2.6.1. **Estimation of Super oxide Dismutase**

Super oxide dismutase in tissues was determined using the method of Kakkar *et al*, (1984).
Reagents

Sodium pyrophosphate buffer: 0.052M (pH 8.3)
Tris- HCl buffer : 0.0025 M (pH 7.4)
Sucrose : 0.25 M
Phenazine methosulphate : 186µ M
Nitro blue tetrazolium : 300 µ M
NADH : 780 µ M
Glacial acetic acid
n- butanol

Procedure

The sample supernatant was dialyzed against 0.0025M Tris HCl buffer (pH 7.4) over night before using for enzyme assay. Assay mixture contained 1.2 ml of the sodium pyrophosphate buffer, 0.1 ml of phenazine methosulphate, 0.3 ml nitroblue tetrazolium, 0.2 ml NADH and 1.2 ml of the enzyme source. Reaction was initiated by the addition of NADH. Incubated at 30˚ C for 90 sec and stopped the reaction by the addition of 1 ml of glacial acetic acid. Reaction mixture was shaken vigorously with 4.0 ml of n-butanol. The mixture was allowed to stand for 10 min. and was centrifuged. The upper butanol layer was taken out. Colour intensity of the chromogen in butanol was measured at 560 nm, against n- butanol blank. A system devoid of enzyme served as control. Protein estimation was carried out on the same enzyme source by the method of Lowry, et. al.(1951)

2. 6.2. Estimation of catalase

Catalase level in tissues was determined using the method of Cohen et al, (1970)
Reagents

Phosphate buffer : 0.067 M (pH 7.0)

H$_2$O$_2$ : 30 l/ 10 ml buffer.

Procedure

To 30µl sample supernatant, added 3 ml of buffer and 0.75 ml of H$_2$O$_2$. Change in OD was measured at 240 nm at 0 sec, 30 sec, 60 sec respectively. The control system devoid of tissue extract was used as blank.

2.6.3. Estimation of Reduced glutathione (GSH)

GSH was estimated by the method of Beutler and Kelly, (1963)

Reagents

Phosphate buffer : 0.2M, P$^\text{H-8}$

TCA : 5%

Ellman’s reagent : 19.8 mg DTNB/ 100ml of 0.1% sodium citrate.

Standard glutathione solution : 10 mg GSH 100 ml H$_2$O

Procedure

Tissue was homogenized in phosphate buffer (0.2M, PH-8). From this 0.5ml was pipetted out and precipitated with 2ml of 5% TCA. 1ml of the supernatant was taken after centrifugation and 0.5 ml of Ellman’s reagent and 0.3ml of phosphate buffer were added. The yellow colour developed was read at 412nm. A series of standards were treated in a similar manner along with a blank containing 3.5 ml of buffer.

The amount of glutathione was expressed as mg/100g tissue.

2.6.4. Estimation of Glutathione S transferase (GST)

Glutathione- S- transferase in tissues was determined using the method of Beutler, (1986)
**Materials and Methods**

**Reagents**

Phosphate buffer : 0.5 M (pH 6.5)  
CDNB : 25 mM in 95% ethanol  
GSH : 20mM

**Procedure**

The reaction mixture containing 200µl phosphate buffer, 20µl CDNB, and 730µl distilled water were taken in the control tubes and 200µl phosphate buffer, 20 µl CDNB and 680 µl distilled water were taken in the test sample tubes. Then the tubes were incubated at 37˚C for 10 min. After the incubation added 50µl of GSH in both the set of tubes. After mixing well, added 50µl of tissue extract in the test sample tubes. Absorbance was noted at 340 nm for 5 minutes in a quartz cuvette of 1cm length path in a spectrophotometer.

2.6.5. Estimation of Ascorbic acid

Ascorbic acid was estimated by the method of Stanley et al, (1979)

**Reagents**

Trichloroacetic acid- 5% and 10% solution

2,4-Dinitrophenyl hydrazine/ thiourea/copper (DTC): added 0.4g thiourea, 0.05g CuSO4.5 H2O, and 3.0g 2,4-dinitrophenyl hydrazine and brought to a total volume of 100ml with 9N H2SO4. 65% H2SO4.

**Procedure**

1 ml of the homogenate was added to ice cold 10% TCA, mixed thoroughly, and centrifuged for 20 min at 3500g. 0.5 ml of the supernatant was mixed with 0.1 ml of DTC and incubated for 3hr at 37°C. Then 0.75 ml of ice cold 65% H2SO4 was added and mixed well. The solutions were allowed to stand at room temperature for an additional 30 min. Absorbance was determined at 520nm. Standards should be made in 5% TCA and range from 0 to 20µg/ml.
2.7. Estimation of Nitrogenase

Nitrogenase was estimated by gas chromatography (Turner and Gibson, 1980)

Materials
Gas Chromatograph with Flame Ionization Detector (FID)
Airtight syringes.
Conical flasks (100 ml) with small mouth to fit serum caps
Acetylene Gas
Ethylene Gas
GLC operating conditions- Carrier gas- Nitrogen/ Helium/ Argon with a flow rate 30 to 45 ml
Gas for detector- Hydrogen and Air
Column- Propane N, R, T and Q or Silica gel

Procedure
1. Removed the plants from the soil without disturbing the root nodules
2. Excised the roots with nodules
3. The root system was placed in a 100 ml conical flask and sealed with a rubber septum/ serum cap
4. 10 ml of air was removed from the flask with an airtight syringe
5. Injected 10 ml acetylene into the flask
6. Incubated for 30 to 60 min. at room temperature
7. Removed 1 ml gas mixture from the flask with an airtight syringe
8. Injected the gas mixture into a pre-conditioned GLC
Materials and Methods

9. The acetylene and ethylene peaks were noted and the ethylene peak height was measured.

10. At the end of the experiment, the nodules were detached and their dry weights were taken.

11. For standard-injected 10µg (Z) of pure ethylene into a 100ml sealed conical flask. Removed 1ml gas and injected it into the same GLC and measured the ethylene peak height.

Calculation

Standard amount of ethylene (E) in µ mol =

\[
\frac{0.0466 \times 2 \mu l}{\text{Peak height in m.m} \times \text{attenuation}}
\]

Amount of ethylene produced in µmol in the sample =

\[E \times \text{peak height of sample ethylene in m.m} \times \text{attenuation}\]

Activity of nitrogenase = \(n\) mol or µ mol ethylene per unit time

(Unit sample may be g dry weight nodules)

2.8. Estimation of Phosphatase (Sadasivam and Manickam, 1996)

Acid Phosphatase (ACP) and Alkaline Phosphatase (ALP)

Materials

Sodium Hydroxide : 0.085N

Substrate solution for ACP : Dissolved 1.49 g EDTA, 0.84 g Citric acid and 0.03 g p-nitrophenyl phosphate in 100 ml water and adjusted the pH 5.3

Standard : Weighed 69.75 mg p-nitrophenol and dissolved in 5 ml water
Enzyme extract: 1 g of fresh tissue was homogenized in 10 ml of ice cold 50 mM citrate buffers (pH 5.3) in a pre-chilled mortar and pestle. Filtered through four layers of cheesecloth. Centrifuged the filtrate at 10000 rpm for 10 min. The supernatant was used as enzyme source.

Substrate solution for ALP: Dissolved 375 mg glycine, 10 mg magnesium chloride, 165 mg p-nitrophenyl phosphate in 42 ml of 0.1N sodium hydroxide and diluted to 100 ml pH-10.5.

Enzyme extract: 1 g of fresh tissue was homogenized in 10 ml of ice cold 50 mM glycine NaOH buffer (pH 10.4) in a pre-chilled mortar and pestle. Filtered through four layers of cheesecloth and centrifuged at 10000 g for 10 min. The supernatant was used as enzyme source.

Procedure (common to both ACP & ALP)

1. Incubated 3 ml of substrate solution at 37°C for 5 min.
2. added 0.5 ml enzyme extract and mixed well
3. Removed immediately 0.5 ml and mixed it with 9.5 ml of sodium hydroxide 0.085N- this corresponds to zero time assay (blank)
4. Incubated the remaining solution (substrate + enzyme) for 15 min. at 37°C
5. 0.5 ml of sample was drawn and mixed it with 9.5 ml NaOH solution.
6. The absorbance of blank and incubated tubes was measured at 405 nm.
7. 2 to 1.0 ml (4 to 20 mM) of the standard was taken and diluted to 10 ml with NaOH solution. The colour was read and a standard graph was drawn.

**Calculation**

Specific activity is expressed as m moles p-nitro phenol released per min. per mg protein.

**2.9 Statistical Analysis**

It was done using two and three factorial analysis of variance. The programme was developed in FORTRAN. The C.D values, P=0.05 was used to separate the treatment means in all tests. ANOVA for variable measures by INSTAT were also done. The values are the average of six values in each case ±SD. Control (group I) was compared with treatments for all the statistical analysis.