CHAPTER 2

Materials and Methods
The entire study was conducted in three phases. In the first phase, the objective was to identify under nursery conditions, the most efficient species of AMF for Neem in terms of growth and Pi uptake in the perspective of the climatic conditions prevalent in Gandhinagar, Gujarat.

In the second phase, a field trail was laid down to assess the performance of different AMF species under field conditions. This was to compare the results obtained from the nursery trial against field trial with same set of AMF species.

In the third phase, efforts were made to judge the benefits of two of the important bio-fertilizers - vermicompost versus AMF association on growth of Neem. Attempts were made to co-relate different morphological and biochemical parameters with AMF association and vermicompost application as well as with their cumulative effect.

2.1 **PHASE I**

An experiment was conducted to screen the most efficient species of AMF (*Glomus* sp.) for best growth, vigour and phosphorous uptake of the important tree species – *Azadirachta indica* A. Juss (Neem) under nursery conditions.

2.1.1 **Experimental site**

The study was conducted at the forest nursery of Basan Research Range, Gandhinagar, Gujarat (Latitude: 23° 13’ N Longitude: 72° 41’E, altitude- 80 m a.s.l.; Fig 8). Plants were grown using a 14 h day / 10 h night cycle. The average maximum temperature and average minimum temperatures were 34°C and 22.5°C respectively during the phase I studies.

2.1.2 **Plant material**

For conducting the first phase of experimental trial, seeds of Neem were procured from Basan Research Range, Forest Department, Gujarat. Seeds were collected from Candidate Plus Trees (CPT) of Gandhinagar Research Range. Forest Department, Gujarat has identified phenotypically superior trees of important tree species throughout the Gujarat State and marked them as Candidate Plus Trees. A number has been assigned to each of them.
2.1.3 **Fungal material**

Pure cultures of five species of *Glomus* were obtained from Arid Forest Research institute (AFRI), Government of India, Jodhpur, Rajasthan. Pure cultures of *Glomus aggregatum* and mixed inoculum (consortium) were prepared from rhizosphere soil samples collected from nearby plot of Neem plantation at Basan research Range (Fig 9). The composition (Schenck and Perez, 1987) of consortium was *Glomus* (79%), *Gigasspora* (8%), *Sclerocystis* (5.3%), *Scutellospora* (5%), *Acaulospora* (2.7%) (Fig 10).
Figure 10: Spores of different species of AMF; a: *Glomus agrregatum*; b: *Glomus constrictum*; c: *Glomus mosseae*; d: *Acaulospora sp.*; e: *Scutellospora sp.*; f: *Scutellospora sp.*; g: *Sclerocystis dusii*; h: *Sclerocystis rubiformis*;
2.1.4 Soil preparation

Soil used to culture of AMF was prepared by mixing garden soil with sand in 1:1 (v/v) ratio. Such sand-soil mixture was then sterilized by autoclaving at 121°C and 15 psi pressure for 60 minutes thrice (at an interval of one day).

2.1.5 Culture and inoculum production

Trap culture

The rhizosphere soil samples collected from the sampling plot of neem planation were brought to the laboratory. Such soil sample was mixed with sterilized sand-soil mixture (1:1; v/v) and planted with a suitable trap crops like maize. Maize was chosen as the nurse crop because it is mycotrophic and highly responsive to mycorrhizal propagules regardless of low propagule densities (Liasu and Shosanya, 2007). After 4 months, spores were isolated from the potting mix by wet sieving and decanting technique of Gerdemann and Nicolson (1963) as discussed in section 2.9.

The indigenous mycorrhizal endophytes isolated from the trap culture were used for preparing starter culture. The spores collected were examined under stereo-zoom microscope. The spores were mounted on a glass slide in lacto-phenol. AM spores were identified with the help of Manual for Identification of VA Mycorrhizal Fungi (Schenck and Perez, 1990) and web based database (Morton, INVAM).

About 500 spores were surface sterilized with Chloramine-T (0.2 %) and Streptomycin (0.02 %) for 15 minutes and washed in sterile distilled water (4 times). Such spores were then used for initiating the culture of AMF. Morphologically similar spores were picked up for preparing the culture of one species.

The starter culture was prepared by soil-funnel technique (Nicolson, 1967). The funnel technique (Fig 11) ensures that growing roots are colonized by AMF. In this method the glass funnel was filled 3/4th with autoclaved soil-sand (2:1; v/v) mixture and the end of the funnel was plugged with glass wool. The funnel was kept over the conical flask which was filled with sterilized water so as to touch the funnel end. Spores suspension was spread over and covered with thin layer of soil. The seeds of Zea mays (maize) were sown and covered with soil. Prior to sowing, seeds were also surface sterilized by 0.1 % HgCl₂ for 2 minutes and then washed thoroughly 6 times with sterilized distilled water. After 21 days of inoculation, roots were examined for AM fungal infection.
On successful colonization of roots, the shoots of maize seedlings were discarded and the soil substrate containing hyphae, spores and root bits were mixed, air dried and used as the inoculum. Inoculum thus prepared through the soil-funnel technique was mixed with sterilized sand-soil mixture (1:2; v/v). The seeds of *Zea mays* (maize) were sown for further bulking by pot culture.

The AMF from AFRI, Jodhpur were multiplied as pot culture using sterilized sand-soil mixture (1:2; v/v) and *Zea mays* as the host in a greenhouse conditions (Fig 12). In pot culture technique, 3/4th of the pot was filled with autoclaved soil-sand (2:1 v/v) mixture and then the starter culture obtained from AFRI, Jodhpur or produced by funnel technique was then spread over the sand soil mixture as 2-3 cm thick layer. Such layer is then covered with a thin layer of autoclaved sand-soil mixture. Pots were seeded with maize and finally covered with autoclaved sand-soil mixture. The pot cultures were then maintained in the greenhouse for four months. Sporulation occurs during the first three months,
but it is during the fourth month when plant shoots (and roots) have ceased to grow and carbon gets more repartitioned for sporulation rather than mycorrhizal development (Trap cultures, INVAM).

Plants were irrigated with sterilized water as per the requirement. After 120 days of growth, shoots of *Zea mays* were discarded and the substrate containing hyphae, spores and root bits were air dried and used as the inoculum. Infective propagules (IP) present in each culture were estimated adopting the most probable number (MPN) method as outlined by Porter (1979).

### 2.1.6 Seedling production

Collected Neem seeds free from any visible defect were sown in plastic germination trays filled with sterilized sand-soil mixture (1:2; v/v). Sand-soil mixture was sterilized by autoclaving as mentioned earlier thrice at an interval of one day. Seeds germinated within 8 days and the seedlings were maintained there for another 3 days. At the end of 11 days the healthy seedlings of uniform length (2.5 cm) were selected and transferred to root trainers of 150 cc capacity containing a mixture of nursery soil: sand: compost (2:1:1; v/v). The soil pH was 8.4, EC- 0.24 mS/cm, organic carbon- 0.29 % (Walkley and Black 1934). It contained 12.5 mg/kg available P (Olsen 1954), 180.35 mg/kg available K (Richards, 1954), and an indigenous AMF population of 206 spores/100g of soil and an inoculum potential of 900 infective propagules/g soil. The seedlings were maintained for 75 days.

### 2.1.7 Soil analysis

The soil, used in the root trainers and poly-bags for raising the seedlings with AMF association, was analysed for physical and chemical parameters. Soil analysis was conducted prior to the initiation of inoculation experiment.

*Preparation of soil sample for analysis*

The soil for analysis in the laboratory was first spread on a thick paper for air drying. Coarse concretions, stones and pieces of roots, leaves and other undecomposed organic residues were removed. Large lumps of moist soil were broken into smaller ones. Soil was shifted during drying to expose all surfaces. The soil was crushed gently in porcelain mortar - pestle and sieved through a 2mm sieve. Crushing was continued until the soil retained on the sieve contained no aggregate. The material larger than 2 mm was discarded. The soil was mixed well and weighed for analysis.
Determination of pH

The soil pH reflects whether the soil is acidic, neutral, Basic or alkaline. Before determining the soil pH, the pH meter was calibrated by using two buffer solutions of different pH values, 4.0 and 7.0. First of all the temperature of the solution was measured and the temperature knob was adjusted. Then the combined electrode rod was dipped in pH 7.0 buffer solution, actual pH was checked at measured temperature and adjusted/calibrated with buffer knob. Then the combined electrode was dipped in pH 4.0 buffer solution and adjusted with sensitivity Knob. These were repeated until pH meter gave correct reading of both the test buffer solutions.

Procedure followed for determining soil pH in a 1:1 (soil: water) suspension (McKeague, 1978; McLean, 1982) is:

50g air dried soil (<2-mm) was weighed into a 100ml glass beaker. 50ml DI water was added, mixed well with glass rod and allowed to stand for 1 hour. The suspension was stirred every 10 minutes during this period. The combined electrode was lowered in the suspension (3cm deep). Reading was recorded after 30 seconds. The combined electrode was then removed from the suspension and rinsed thoroughly with DI water in a separate beaker, and excess water was carefully wiped with tissue paper.

Determination of electrolytic conductivity of soil

Salinity is a soil property referring to the amount of soluble inorganic salt in the soil. Electrical conductivity (EC) is the most common measure of soil salinity and is indicative of the ability of an aqueous solution to conduct an electric current. The principle by which EC-meters measure conductivity is simple, two plates are placed in the sample, a potential is applied across the plates (normally a sine wave voltage), and the current is measured. Conductivity (G), the inverse of resistivity (R), is determined from the voltage and current values according to Ohm's law.

\[ G = \frac{1}{R} = \frac{I}{V} \]  

(ampere/volts or mho)

Since the charge on ions in solution facilities the conductance of electrical current, the conductivity of a solution is proportional to its ion concentration. Conductivity measurements are temperature dependent. The degree to which temperature affects conductivity varies from solution to solution and can be calculated using the following formula:
\[ G_t = G_{\text{cal}} \left( 1 + \alpha(T - T_{\text{cal}}) \right) \]

where: \( G_t \) = conductivity at any temperature \( T \) in °C, \( G_{\text{cal}} \) = conductivity at calibration temperature \( T_{\text{cal}} \) in °C, \( \alpha \) = temperature coefficient of solution at \( T_{\text{cal}} \) in °C.

The basic unit of conductivity is the siemens (S), formerly called the mho. Since cell geometry affects conductivity values, standardized measurements are expressed in specific conductivity units (S/cm) to compensate for variations in electrode dimensions. Specific conductivity (\( C \)) is simply the product of measured conductivity (\( G_t \)) and the electrode cell constant (\( L/A \)), where \( L \) is the length of the column of liquid between the electrode and \( A \) is the area of the electrodes.

\[ C = G_t \times \left( \frac{L}{A} \right) \quad \text{(S/cm)} \]

If the cell constant is 1 cm\(^{-1} \), the specific conductivity is the same as the measured conductivity of the solution. Although electrode shape varies, an electrode can always be represented by an equivalent theoretical cell.

Plants are detrimentally affected, both physically and chemically, by excess salts in soils including high levels of exchangeable sodium. Salinity is an important laboratory measurement since it reflects the extent to which the soil is suitable for growing a crop/tree species. The electroconductivity was measured with the help of EC meter (ELICO Conductivity Meter CM 180) from the soil water mixture (1: 2 soil: water ratio; w/v) after minutes of shaking. Before taking the reading the EC meter was calibrated with 0.01 N KCl solution which should give a reading of 1.413 dS/m at 25 °C. An adjustable temperature coefficient in the meter allows compensation for temperature changes in the test solution. Reading was taken by adjusting the temperature at the measured value and thereby inserting the conductivity cell into the soil-water solution.

*Determination of organic carbon/available nitrogen*

Easily oxidizable organic carbon (OC) and mineralizable N are largely used as measure of available nitrogen in Indian soils. Organic carbon was estimated by the modified Walkley-Black method (Walkley and Black 1934).
Organic carbon by wet digestion

Soil organic matter represents the remains of roots, plant materials and soil organisms in various stages of decomposition and synthesis, and is variable in composition. Though occurring in relatively small amounts in soils, organic matter (OM) has a major influence on soil aggregation, nutrient reserve and its availability, moisture retention, and biological activity.

Organic matter (OM) in the soil was oxidised with a mixture of potassium dichromate ($K_2Cr_2O_7$), concentrated $H_2SO_4$ and utilizing the heat of digestion of $H_2SO_4$. Unused $K_2Cr_2O_7$ was back titrated with ferrous ammonium sulphate [FeSO$_4$. (NH$_4$)SO$_4$. 6H$_2$O]. This process gives actual measurement of oxidizable organic carbon. The data obtained is converted to percentage organic matter using a constant factor, assuming the OM contains 58% organic carbon. However, as this proportion is not constant, it is preferable to report results as oxidizable organic carbon, or multiplied by 1.334 as organic carbon.

Preparation of reagents

A. Standard potassium dichromate solution- $K_2Cr_2O_7$ (0.1667 M = 1.00 N)
   - Potassium dichromate was dried in an oven at 105 º C for 2 hours, and cooled in a desiccators (silica gel), and stored in a tightly stoppered bottle.
   - Exactly 49.04g of reagent grade K2Cr2O7 was dissolved in distilled water and brought to 1 L volume with DI in volumetric flask.

B. Sulphuric Acid ($H_2SO_4$), concentrated (98%, Sp. Gr. 1.84)

C. Orthophosphoric acid, $H_3PO_4$ (85%)

D. Ferrous ammonium Sulphate solution [FeSO$_4$. (NH$_4$)SO$_4$. 6H$_2$O].-
   - 196.1 g of reagent grade [FeSO$_4$. (NH$_4$)SO$_4$. 6H$_2$O] was dissolved in about 800ml distilled water and 20ml of conc. $H_2SO_4$ was added to it, mixed well and then diluted to 1 L with distilled water in a volumetric flask.

E. Diphenylamine Indicator ($C_6H_5$)$_2$NH-
   - 0.5g of diphenylamine was dissolved in a mixture of 20ml water and 100ml of conc. $H_2SO_4$

Procedure followed

1. 1g of air dried soil was placed in a dry 500 ml Erlenmeyer flask.
2. 10ml of 1 N dichromate solution was added. The flask was swirled gently and kept on an asbestos sheet.
3. 20ml of conc. H$_2$SO$_4$ was added rapidly using a dispenser.
4. The flask was swirled (2 to 3 times) and kept on the hot sheet for 30 minutes.
5. About 200 ml of distilled water was mixed followed by 10 ml of ortho phosphoric acid using a dispenser. The mixture was allowed to cool.
6. Then 10-15 drops (1 ml) of diphenylamine indicator was added and mixed well.
7. The mixture was titrated with 0.5 M ferrous ammonium sulphate solution until the colour changes from violet blue to green.
8. Two blanks, containing all the reagents but no soil were prepared and treated exactly the same way as the soil suspensions.

If the burette reading was 0-4 ml, it was repeated with less soil. When it was 17ml or higher it was repeated with more soil.

**Formulation**

Organic carbon (%) = \( \frac{10(B-V)}{B} \times \frac{0.003 \times 100}{\text{wt. of soil (g)}} \)

Where B = volume (ml) of ferrous ammonium sulphate solution required for blank titration

\[ V = \text{volume (ml) of ferrous ammonium sulphate solution required for titration of soil sample} \]

Actual organic carbon (%) = Organic carbon estimated \( \times \) 1.3. There was incomplete oxidation of organic matter in this procedure. The organic carbon was multiplied by 1.3 on the assumption that there is 77% recovery.

Organic matter (%) = Actual Carbon (%) \( \times \) 1.724

The Van Bemmelen factor of 1.724 was used because organic matter contains 58 % C.

**Estimation of available phosphorous**

Because of its significance as a major nutrient coupled with the fact that it is widely deficient in the alkaline –calcareous soil, phosphorous was estimated in the experimental soil by the described process (Olsen et al. 1954). Sodium bicarbonate (NaHCO$_3$) solution extracts exchangeable or surface adsorbed Al-P, Fe-P, calcium phosphates and other phosphates.

**Preparation of reagents**

A. Sodium Hydroxide Solution (NaOH), 5N
• 200 g of sodium hydroxide was dissolved in enough DI water and the solution was transferred to a 1-litre volumetric heavy walled Pyrex flask, allowed to cool. The volume was made to 1 litre with DI water.

B. Sodium bicarbonate (Olsen’s reagent) 0.5M NaHCO₃, pH 8.5.
• 42 g of sodium bicarbonate was dissolved in 900 ml DI water. The pH was adjusted to 8.5 with 5 N NaOH solution. Finally, the volume was made to 1-L with DI water.

C. Sulfuric acid solution (H₂SO₄), 5 N
• 148 ml of concentrated sulphuric acid (in fume hood) was diluted to 1-L volume with DI water

D. p-nitrophenol Indicator, 0.25 % w/v

E. Reagent A
• 12 g of ammonium-molybdate [(NH₄)₆Mo₇O₂₄.4H₂O was dissolved in 250 ml of DI water.
• 0.2908 g antimony potassium tartrate (K₅SbO₆C₄H₄O₆) was dissolved in 100 ml of DI water.
• Both these two solutions were added to a 2-L volumetric flask, and 1-L of 2.5 M H₂SO₄ was added to the mixture.

The solution was thoroughly mixed, diluted to 2-L volume with DI water and stored in a Pyrex glass bottle (amber coloured) in a cool place.

F. Reagent B
• 1.056 g of L-ascorbic acid (C₆H₈O₆) was dissolved in 200 ml of Reagent A, and mixed. This reagent was prepared fresh (as and when required).

G. Standard stock solution
• 2.5 g potassium dihydrogen phosphate (KH₂PO₄) was dried in an oven at 105°C for 1 hour, cooled in desiccator, and stored in a tightly stoppered bottle.
• 2.197 g dried potassium di-hydrogen phosphate was dissolved in DI water to 1-L volume. This solution contains 500 ppm P (stock solution).
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- 50 ml stock solution was diluted to 250 ml by DI water. This solution contains 100 ppm P (diluted stock solution).
- A series of standard solutions were prepared from the diluted stock solution as:
  5, 10, 15, 20 and 25 ml diluted stock solution diluted to 500 ml. These solutions contained 1, 2, 3, 4, and 5 ppm P, respectively.

Procedure
1. 5 g air-dried soil was transferred to 250-ml Erlenmeyer flask and 100 ml of 0.5 M sodium bicarbonate solution was added.
2. The flask was closed with a rubber stopper and was kept on shaker (30 minutes). One flask containing all chemicals but no soil was used as blank.
3. The solution was filtered through a Whatman No. 40 filter paper, and 10 ml clear filtrate was pipetted out in a 50 ml volumetric flask.
4. Acidification was done with 5 N sulphuric acid to make pH 5.0 by taking 10 ml of 0.5 M NaHCO₃ solution and determining the amount of acid required to bring the solution pH to 5.0, through P-nitrophenol indicator (colour change was from yellow to colourless). The required acid was added to all the sample solutions. Adding 1 ml 5 N H₂SO₄ was adequate to acidify each 10 ml NaHCO₃ extract.
5. DI water was added to about 40-ml volume, 8 ml Reagent B was added, and brought to 50 ml volume.

6. A standard curve was prepared as follows:
   a. 2 ml of each standard (1 - 5 ppm) were pipetted out and proceeded as for the samples.
   b. A blank was made with 10 ml 0.5 M NaHCO₃ solution, and proceeded as for the samples.
   c. The absorbance of blank, standards, and samples was read after 10 minutes at 882 nm wavelength.
7. A calibration curve was prepared for standards, with absorbance against the respective P concentrations.
8. P concentration in the unknown samples was read from the calibration curve.

Formulation for extractable phosphorus in soil

\[
\text{Extractable P (ppm)} = \frac{A}{W} \times \frac{50}{V}
\]
Estimation of available potassium

Along with N and P, potassium (K) is also of vital importance in determining the productivity of soil. Most soils contain relatively large amounts of total K (1 - 2%) as components of relatively insoluble minerals. However, only a small fraction (about 1%) is present in a form available to plants, i.e., water-soluble and exchangeable K. Soils of arid and semi-arid areas tend to be well supplied with K. Nevertheless, extractable-K, or exchangeable plus water-soluble K, is often considered as the plant-available fraction. Water-soluble K tends to be a large proportion of the extractable K fraction in drier-region soils.

Extractable potassium

This fraction of soil K is the sum of water-soluble and exchangeable K. The method uses a neutral salt solution to replace the cations present on the soil exchange complex. Therefore, the cation concentrations determined by this method are referred to as exchangeable for non-calcareous soils. For calcareous soils, cations are referred to as exchangeable plus soluble (Richards, 1954).

Reagents

A. Ammonium acetate solution (NH₄OAc), 1 N

- 57 ml of concentrated acetic acid (CH₃COOH) was added to 800 ml DI water, and 68 ml of concentrated ammonium hydroxide (NH₄OH) was mixed and allowed to cool.
- pH was adjusted to 7.0 by acetic acid or ammonium hydroxide, and volume was brought to 1L with DI water.

B. Standard stock solution

- 3 g potassium chloride (KCl) was dried in an oven at 120°C (1 – 2 hours) and cooled in a desiccator, and stored in a tightly stoppered bottle.
- 1.907 g of dried potassium chloride was dissolved in DI water to 1 L volume. This solution contained 1000 ppm K (stock solution).
- A series of Standard solutions were prepared from the stock solution as: 2, 4, 6, 8, 10, 15 and 20 ml of stock solution were diluted to 100-ml final volume by DI water or 1 N
ammonium acetate solution. These solutions contained 20, 40, 60, 80, 100, 150, and 200 ppm K, respectively.

**Note**
Standard solutions for measuring soluble-K should be prepared in deionized water, but for measuring extractable-K the standards should be made in ammonium acetate solution.

**Procedure**
5 g air-dried soil was placed into a 50-ml centrifuge tube, 33 ml ammonium acetate solution was added. The tubes were kept on a shaker (5 minutes). The tubes were stoppered with a clean rubber stopper.

1. The samples were centrifuged (2000 rpm) until the supernatant was clear (10 minutes). The extract was collected in a 100-ml volumetric flask through a filter paper to exclude floating soil particles. This process was repeated for two more times and the extracts were pooled.
2. The combined ammonium acetate extracts was diluted to 100 ml with 1 N ammonium acetate solution.
3. The K in the samples was measured by flame photometer using potassium filter after necessary setting and calibration of the instrument.
4. A series of suitable potassium standard solution having different concentration of K was run and read in flame photometer and calibration curve was drawn by plotting the readings against different concentrations of K.
5. Potassium (K) concentration was calculated according to the calibration curve.

**Formulation**

\[
\text{Extractable K (ppm)} = \text{ppm K (from calibration curve)} \times \frac{A}{Wt}
\]

Where: A = Total volume of the extract (ml)
Wt = Weight of air-dry soil (g)

2.1.8 **AMF inoculation**
Two months old seedlings were inoculated with different AMF inocula. On the basis of their abundance in the soil types, the *Glomus* species was used as AMF isolates for this phase of nursery experimental trial. Each seedling in the root trainer was inoculated near the root zone at
the rate of 10,000 infective propagules. Same amount of sterile inoculum (which had been autoclaved thrice at 121°C and 15 psi pressure for 60 minutes at an interval of one day), was applied to the control (non-inoculated with AMF) seedlings. The experiment was laid out in completely randomized design (CRD). The experiment was conducted in greenhouse at Basan nursery, Gandhinagar and comprised of following eight treatments including control. There were four replicates for each treatment (Table 1). Irrigation was followed as per the requirement.

Table 1: Selected AMF as treatment

<table>
<thead>
<tr>
<th>Serial number</th>
<th>Treatment code</th>
<th>Inoculum</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>T1</td>
<td><em>Glomus intraradices</em></td>
</tr>
<tr>
<td>2</td>
<td>T2</td>
<td><em>Glomus reticulatum</em></td>
</tr>
<tr>
<td>3</td>
<td>T3</td>
<td><em>Glomus fasciculatum</em></td>
</tr>
<tr>
<td>4</td>
<td>T4</td>
<td><em>Glomus mosseae</em></td>
</tr>
<tr>
<td>5</td>
<td>T5</td>
<td><em>Glomus constrictum</em></td>
</tr>
<tr>
<td>6</td>
<td>T6</td>
<td><em>Glomus aggregatum</em></td>
</tr>
<tr>
<td>7</td>
<td>T7</td>
<td>Mixed inoculum (consortium)</td>
</tr>
<tr>
<td>8</td>
<td>T8</td>
<td>Control</td>
</tr>
</tbody>
</table>

Initially 768 seedlings (Number of seedlings for the tree species × treatment × replication: 24 × 8 × 4 = 768 plants) were considered for inoculation experiment. At the end of 75 days, seedlings were transferred from root trainers to polybags containing 1.5 kg of same nursery soil: sand: compost mixture (Fig 13) and maintained under ambient conditions with normal irrigation in the greenhouse for the study. Each bag contained one seedling. The bags were shuffled/rearranged every 15 days to ensure uniform growth conditions.

2.1.9 Data collection

Data were recorded for different parameters like -

*Plant height*

Plant height was measured for all the experimental plants [15 (Number of plants in each treatment) ×8 (Number of treatments) × 4 (number of replication for each treatment) = 480]. Plant height was measured from soil surface to the growing tip of the plant. It was recorded quarterly after plantation.
Collar diameter
Collar diameter was measured 1 cm above the soil surface using vernier callipers for all the experimental plants (480 plants). This parameter was recorded quarterly after plantation.

The experiment was terminated at 270 days after plantation (DAP) and the plants were harvested. At harvest, observations on morpho-physiological parameters like plant fresh weight, plant dry weight, root colonization, spore density and phosphorous uptake were recorded.

Plant fresh weight
All the experimental plants were measured for fresh weight.

Plant dry weight
Plant dry weight was determined after drying the plant sample at 60 °C to a constant weight in a hot air oven. The constant weight was then recorded.

Estimation of spore density
The AM fungal spores were isolated from the root-zone soil by wet sieving and decanting technique (Gerdemann and Nicolson, 1963). The AM spores collected in the filter paper were then examined under stereo - zoom microscope and the spore number (spore density) was estimated. The details of the process are as follows –

Isolation of AM fungal propagules from soils
The soil was passed through a 2 mm sieve for removing foreign debris such as leaves and stones. For isolation of AM fungal propagules from the soil wet sieving and decanting technique (Gerdemann and Nicolson, 1963) was used. In this method, 100 g soil were taken in 1L conical flask. Soil was mixed with 200 ml of water and shaken thoroughly to form uniform suspension. The suspension was kept for 90 minutes to settle soil particles at the bottom. Soil particles being heavier than spores get settled to the bottom in the flask while the spores got separated being light floating on the water. The water containing spores was then passed through a series of sieves of various microns (53 – 710 µm, Fig. 14). Most of the suspension was decanted through 710 µm sieve. The root pieces retained in 710 µm were examined for attached hyphae, spores and sporocarps under a microscope. The residue was re-suspended in more water and decanted again in the same way. The residue up to 250 µm was examined for sporocarps and large spores. Residues collected in each sieve were thoroughly washed with fine jet of water to remove soil particles. The spores were then collected from the sieve in a
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beaker along with water. Such water containing spores was filtered through filter paper—Whatman no.42 (15 cm diameter) in funnel. Filter paper containing spores was spread over the petridishes and examined under Stereo Zoom microscope (Leica Combistereo). Semi permanent slides were prepared by mounting the spores in lactophenol or polyvinyl lactophenol. The photographs were taken by Nikon Optiphot-2 compound microscope.

**Spore population (enumeration)**
Sucrose centrifugation technique as described by Jenkins (1964) was employed for the isolation of AM fungal spores. Soil samples were passed through 500 μm sieve to separate the litter. The sieved soil was mixed with water and the suspension was transferred in 50 ml centrifuge tube. The suspension was centrifuged at 4500 rpm (5 minutes). The supernatant was decanted and the pellet was re-suspended in sucrose solution (1.3 M) and again centrifuged for 1 minute (3800 rpm). The spores were passed through 53 μm sieve and the contents rinsed with water several times to remove sugar. The AM fungal spores were collected on filter paper with the help of funnel. The filter paper was placed on a glass petri-plate and the spores were counted with appropriate magnification. For easy counting, the circular Whatman filter paper was divided into eight compartments by drawing seven vertical lines from the centre towards the periphery with a pencil. The spore density was expressed in terms of the number of spores per 100 g of soil.

The AMF spores were identified on the basis of colour, size, shape, surface, nature of spore cell wall and hyphal attachment with the help of synoptic keys (Schenck and Perez, 1987; Raman and Mohankumar, 1988).

**Percentage Root colonization**
For estimating root colonization, roots of AMF treated seedlings were separated from soil samples and processed for investigating development of vesicle, arbuscules, spores and hyphae. The roots were cut into 1 cm pieces and immersed in 10% potassium hydroxide solution at 90 °C (15-20 minutes). The KOH solution cleared host cytoplasm and nuclei, and readily allowed stain penetration. The KOH solution was drained and the samples were washed with distilled water till brown colour disappeared. Alkaline H₂O₂ (30%) was added to the samples and treated for 8 minutes or till the roots were bleached. The samples were again rinsed with distilled water to remove H₂O₂. One per cent HCl was added to the samples and again rinsed with distilled water to remove HCl. These samples were stained in 0.05% trypan blue following the Phillips and Hayman
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(1970) procedure for rapid assay of endomycorrhizal association. Total 100 root pieces were selected randomly and mounted in lactophenol and examined under compound microscope. The percentage of root colonization was determined by the following formula (Giovannetti and Mosse, 1980):

\[
\text{Percentage root infection} = \frac{\text{Number of root segments infected}}{\text{Total number of root segments examined}} \times 100
\]

**Phosphorus estimation**

Grinded and sieved (< 2 mm) plant samples were digested in tri-acid mixture (Jackson, 1973). 0.5 g plant sample was placed in 100 ml chilled conical flask and 5 ml of tri-acid mixture (HNO₃: H₂SO₄: HClO₄; 9:2:1) was added. The digestion was carried out at 200 °C for complete digestion. The final volume was made up to 50 ml with DI water. For phosphorus estimation, 10 ml of digested and diluted sample was placed in 50 ml volumetric flask, 20 ml distilled water and 15 ml mixture (dilute HNO₃, ammonium molybdate (5%) and ammonium metavanadate (0.25%) in equal proportions; 1:1:1) were mixed. The final volume was made up to 50 ml with DI water and the yellow colour intensity was read at 485 nm using UV-VIS Spectrophotometer (El Digital Spectrophotometer, Model: 301E)

2.1.10 **Statistical analysis**

The data so generated was subjected to one – way Analysis of Variance (ANOVA). Based on the outcome of ANOVA, post-hoc analysis was performed in the form of Duncan’s Multiple Range Test (DMRT) (Duncan, 1955) to separate the means. Linear regression analysis was used to assess the relationship between AMF colonization, growth parameters and phosphorous uptake.

2.2 **PHASE II**

To assess the effects of association of different species of AMF on growth and adaption of Neem under field conditions, a trial was conducted in natural field conditions in Lekawada, Gandhinagar.

2.2.1 **Site location and environmental variation**

The study was conducted at a plot in Lekawada village, of Gandhinagar district, Gujarat (Latitude: 23° 13’ N Longitude: 72° 41’E, altitude - 80 m a.s.l.). The Gandhinagar district has multi seasonal climate with an average precipitation of 667 mm. Minimum temperature records 7.5 degree
centigrade in winter and maximum temperature records 45 degree centigrade in summer.
[Gandhinagar District Panchayat, Gujarat Government, 2008-09]

2.2.2 Field preparation
Before laying down the trial, the field was prepared. Plot (2.9 hectare) was cleared of pre-existing vegetation and weeds. Such operation helped in creating favourable growing conditions for neem tree seedlings as well as in facilitating seedling planting operations. On inspection, the site conditions were uniform all over the selected area. Soil preparation was as per the routine practice including termite treatment with monocrotophos.

2.2.3 Soil analysis
Before laying down the field trial, field soil was analysed for soil pH, EC, soil organic carbon, Phosphorous and Potassium levels. Detailed methodologies for such analyses have been described as in section 2.7.

2.2.4 Planting of seedlings
6 months old Neem seedlings from the nursery trial of phase I fortified with AMF were transferred to the prepared field at Lekawada. Experiment was designed as randomized complete block design with eight treatments with four replications (seven mycorrhizal treatment and control; Table 1). The details of the design were as follows:

<table>
<thead>
<tr>
<th>Units</th>
<th>Number</th>
</tr>
</thead>
<tbody>
<tr>
<td>Blocks</td>
<td>32</td>
</tr>
<tr>
<td>Plants / block</td>
<td>9</td>
</tr>
<tr>
<td>Total plants</td>
<td>288 (9 x 4 x 8)</td>
</tr>
<tr>
<td>Spacing</td>
<td>3.0 x 3.0 m</td>
</tr>
<tr>
<td>Size of pit</td>
<td>30 x 30 x 30 cm</td>
</tr>
</tbody>
</table>

The treatments were same as that of phase I of nursery trail. Control plants received no mycorrhizal inoculum. The transfer to the field was carried out in July, 2007. Immediately, after transplantation, the seedlings were irrigation. Later on due to the onset of monsoon and adequate rain, no artificial irrigation was required.


2.2.5 Data acquisition

Initial and quarterly data for shoot length of AMF fortified seedlings were recorded for one year. After one year, data on root colonization, survival percentage, phosphorous concentration, chlorophyll concentration in leaves was compiled.

2.3 PHASE III

The III phase of the study was conducted to assess plant growth and biochemical composition of selected plant when supplied with vermicompost in isolation as well as in combination with AMF association in greenhouse container media or soil.

2.3.1 Study site

The present study was conducted in the polyethylene greenhouse at the Forest nursery of Basan Research Range, Gandhinagar, Gujarat (Latitude: 23° 13' N Longitude: 72° 41'E, altitude- 80 m a.s.l.) under normal day length (14 hours). The average maximum temperature and average minimum temperatures were 30 °C and 20 °C respectively during the phase III of the studies.

2.3.2 Soil analysis

The autoclaved soil-sand mixture used in the study was analysed for pH, EC, organic carbon, available Phosphorous and available Potassium. The soil analysis was carried out at three different times during the tenure of the phase III trial, on initiation, at 60 DAP and 150 DAP. This is to investigate the role of vermicompost and AMF association on soil properties and corresponding plant growth. The detailed procedure followed for such analysis has been described earlier in section 2.7.

The soil was also analysed for micronutrients like Copper (Cu), Iron (Fe), Zinc (Zn) and Manganese (Mn). Though required by plants in much smaller amounts than the major plant nutrients (like N, P, K), micronutrients are, nevertheless, equally essential for plant growth. Solubility of micronutrient cations decreases with an increase in soil pH. Micro-nutrient analysis was carried out by with the help of Atomic absorption spectro-photometer in the laboratory.
Figure 15: Different stages for seedling preparation of Neem for phase III: a. a germination chamber; b. a tray with germinated seedlings; c. seedlings being transferred to root trainers; d. set of root trainers with planted seedlings; e. poly-bags filled with sand-soil mixture; f. seedlings being transplanted to poly-bags from root trainers
2.3.3 Soil Preparation

Prior to the initiation of the greenhouse experiment the nursery soil was mixed with sand (2:1; v/v) and such mixture was sterilized by autoclaving at 121°C and 15 psi pressure for 60 minutes (thrice) at an interval of one day to eliminate indigenous AMF.

2.3.4 Seedling production

Seeds of Neem were collected from candidate plus tree of Gandhinagar Research Range of Gujarat State Forest Department. Such seeds, free from any visible defect, were germinated for all treatments in the plastic tray filled with the sterilized mixture of sand: soil (Fig 15a,b). All seeds got germinated within 8 days following sowing. Germinated healthy seedlings of uniform length (2.5 cm) were selected and transferred from germination tray to root trainers (150 cc capacity) containing sterilized soil: sand mixture (Fig 15c). Seedlings for all treatments were maintained there for 30 days in the greenhouse under natural photoperiod.

2.3.5 Procurement and culture of inoculum

Association with Glomus intraradices showed better performance of seedlings in terms of growth parameters in the first phase of nursery trial. Moreover G. intraradices is known as a generalist fungus as it associates with a number of plant species and is found in many ecosystems (Öpik et al, 2003; Scheublin et al, 2004). Therefore, AMF fungal species of Glomus intraradices was used for mycorrhizal inoculation in this phase of the study. Starter culture of G. intraradices was procured from CMCC/TERI, New Delhi. It contained spores and other propagules of the designated arbuscular mycorrhizal fungi at the rate 10,000 propagules / 100 g of soil: sand based inoculum. Such culture was bulked with sterilized sand: soil mixture in 15 cm diameter pot culture using Zea mays as the host plant for 120 days. Seeds were surface-sterilized with 75% (v/v) ethanol for 1 min, followed by 10% (w/v) sodium hypochlorite solution (5 minutes) and then washed four times with sterilized distilled water.

Autoclaved DI water was used for watering plants in such culture pots. At the end of 120 days the shoots were discarded and pot was left in the greenhouse for one week without watering thereby allowing the soil to dry and spores to mature (Fig 16a). The soil substrate containing hyphae, spores and root bits was mixed thoroughly, air dried and used as the inoculums (Fig 16b).
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Figure 16: a. a culture pot of *zea mays* with AMF inoculum; b. AMF inoculum prepared for application to seedlings

Figure 17: a. Vermicompost beds; b. earthworms used for vermicomposting

Figure 18: Seedlings of vermicompost and mycorrhizal treatments at 150 DAP.
2.3.6 Production of vermicompost

Vermicompost (VC) was prepared from plant waste (nursery based) materials in Basan in brick-based vermicompost bed (Fig 17a). For vermicompost production, a layer of plant waste was spread over the bed. This is called the bedding material. A layer of FYM (Farm Yard Manure) was spread over the bedding material. Again a layer of plant waste was spread. Water was sprinkled to maintain humidity. *Eisenia fetida*, earthworms were released in the bed at a density of about 2000/m² (Fig 17b). These earthworms were then covered with some waste material and left for 35 days for vermicomposting. The FYM and plant waste materials act as the food material for the earthworm. Water was sprinkled as per the requirement to maintain humidity in the range of 30-40% and temperature 20°C-30°C. To avoid direct exposure from sunlight and birds, the vermicompost beds were covered with iron mesh. After 35 days, small, round brown soil granules were noticed on the upper surface of the bed. Sprinkling of water was stopped which caused the worm to move downward to the lower levels and the vermicompost was scrapped easily from the upper surface. The vermicompost prepared was subjected to analysis to determine its pH, OC, N, P and K.

2.3.7 Greenhouse experiment

The present study comprised of four treatments. Total number of seedlings produced, were equally distributed into four groups. Four replicates were prepared for each treatment according to a completely randomized design. The 40 days old Neem seedlings (15d) were subjected to Mycorrhizal inoculation (group 3 and 4). Each of the seedlings of group 3 and 4 received the sand: soil based inoculum of *Glomus intraradices* at the rate of 10,000 infective propagules near the root zone in the root trainer to facilitate root colonization. Seedlings of group 1 and 2 received the same amount of autoclaved inoculum as group 1 (control) and group 2. The plants were kept in the greenhouse undisturbed for another 15 days.

AT 55 DAP, all the seedlings were transferred to the polybags of 1.5 kg capacity. Seedlings that received treatment 1 (autoclaved inoculum) and treatment 4 (mycorrhizal inoculum) were transferred to the polybags filled with sterilized soil: sand mixture (2:1; v/v). While those received treatment 2 (autoclaved inoculum) and treatment 3 (mycorrhizal) were transferred to polybags filled with soil: vermicompost (2:1:1.8; v/v). Vermicompost used in the study was produced in the Basan Research Range, Gandhinagar. Soil filtrate was added to all the poly-bags to ensure uniform microbial and chemical background for all the treatments.
So the details of the treatments are as follows-

Table 3: Treatments and groups

<table>
<thead>
<tr>
<th>Code</th>
<th>Treatments</th>
</tr>
</thead>
<tbody>
<tr>
<td>T1</td>
<td>Soil: sand (2:1)</td>
</tr>
<tr>
<td>T2</td>
<td>Soil : sand : vermicompost (2:1:1.8)</td>
</tr>
<tr>
<td>T3</td>
<td>Soil : sand : vermicompost (2:1:) with AMF (<em>Glomus intraradices</em>)</td>
</tr>
<tr>
<td>T4</td>
<td>Soil : sand with AMF (<em>Glomus intraradices</em>)</td>
</tr>
</tbody>
</table>

All the seedlings in the poly-bags were kept in the greenhouse for another 3 months. At the end of 5 months (at 150 DAP) the experiment was terminated and plants were harvested (Fig 18). Eight plants from each treatment were harvested randomly for the biochemical study. Roots and shoots were separated and then subjected to different morpho-physiological and bio-chemical analysis.

**2.3.8 Morphological and physiological studies**

*Plant height*

All the experimental plants were screened for plant height. Plant height was recorded from soil surface to the growing tip of the plant. The data of 1st observation was recorded at 15 Days after plantation (DAP) and then consecutively at an interval of 15 days till the termination of the experiment.

*Leaf number*

Number of compound leaves was counted for all the experimental seedlings. Observations were recorded from 15 DAP - 150 DAP. The number of fresh leaves (excluding very tiny new emerging leaf) was recorded.

*Collar diameter*

Collar diameter was measured at 1 cm above soil surface using vernier callipers. This parameter was recorded at the termination of the experiment, just before harvest of experimental seedlings.

*Total Leaf area*

This was estimated with image analysis using MATLAB and the details are given in the respective chapter (chapter 3).
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Total tap root length
The length of the main tap root was measured for harvested plants from each treatment on every month from 30 DAP to 150 DAP.

Total root length
This was estimated with image analysis using MATLAB and the details are given in the respective chapter (chapter 3).

Fresh weight
At harvest adhered soil particles were removed completely from roots before their fresh weight were measured. Shoot, root, and total biomass of harvested plants per treatment were determined.

Plant dry weight
After recording the fresh weight, the roots were washed under running tap water until they were free of soil particles. Each plant material was oven-dried at 60 °C until constant weight was achieved. The constant weight was then recorded.

2.3.9 Biochemical analysis
The effects of Mycorrhizal inoculation separately and in combination with vermicompost, on various biochemical parameters of Neem were studied. The biochemical parameters that were considered for analysis in the Phase III were as follows:

Estimation of Chlorophyll and Carotenoids
The contents of chlorophyll a (Chl_a), chlorophyll b (Chl_b), chlorophyll a +b (Chl_total) and carotenoids (Car_x+c) in leaves were determined by the spectrophotometer (EI Digital Spectrophotometer, Model: 301E).

Procedure
1 g finely cut fresh leaves (mid vein discarded) were grinded in a mortar with 20 ml of 80% acetone (pre-chilled). The suspension was centrifuged at 5000 rpm (7 minutes) and the supernatant was transferred to a 100 ml measuring cylinder. The residue was again grinded with 20 ml of 80% acetone and centrifuged at 5000 rpm (7 minutes). The supernatant was transferred to the same measuring cylinder. The procedure was repeated until the residue became colourless.
The volume was made upto 100 ml with 80% acetone. The extract was mixed well and absorbance was measured at 470, 645 and 663 nm against the solvent blank (80% acetone).

The concentration of chlorophyll a/b and carotenoids was calculated by the following formulae:

Chlorophyll a ($Chl_a$ in mg/g tissue) = $12.7(A_{663}) - 2.69(A_{645}) \times \frac{V}{1000 \times W}$

Chlorophyll b ($Chl_b$ in mg/g tissue) = $22.9(A_{645}) - 4.68(A_{663}) \times \frac{V}{1000 \times W}$

Total Chlorophyll ($Chl_{total}$ in mg/g tissue) = $20.2(A_{645}) + 8.02(A_{663}) \times \frac{V}{1000 \times W}$

Where $A$ = Absorbance at specific wavelength,
$V$= Final volume of the extract in 80 % acetone.
and $W$ = Fresh weight of leaf tissue taken for the extraction of the pigments.

The concentration of Carotenoids was deciphered by the following equation,

Carotenoids ($Car_{(x+c)}$ in mg/g tissue) = $(1000A_{470} - 1.82Chl_a - 85.02Chl_b) \times \frac{V}{198(1000 \times W)}$

**Total protein**

Total protein in the leaves and roots was estimated (Lowry et al 1951).

a) **Extraction of protein**

100 mg plant material was homogenized in 5 ml of cold 10% trichloro-acetic acid (TCA) with 1 ml of distilled water. The homogenate was centrifuged (4400 rpm for 15 minutes) and supernatant was discarded. The pellet was dissolved in 10ml of 0.3N NaOH, left for 20 minutes at room temperature. An aliquot (0.1 ml) was used for the estimation of protein.

b) **Estimation of protein**

0.1 ml of the aliquot was taken in a test tube. Distilled water was added to it to make the final volume 1 ml. Similarly, 1ml of distilled water was taken as blank. 5 ml of alkaline copper reagent (freshly prepared by mixing 50 ml of reagent A + 1 ml of reagent B) was added to both blank and test samples. After 15 minutes, 0.5 ml of 1N of Folin- ciocalteau reagent (commercially available solution is 2N, it was diluted to make 1 N, with distilled water) was added. The absorbance was read at 750 nm. The amount of soluble protein present in the plant material was obtained using regression formula generated through a curve using standard protein - BSA: $Y = 0.372X + 0.0074$
where $Y$ is the concentration of the protein and $X$ is the absorbance; and the protein content was expressed as mg protein.

**Extraction of enzymes (Khare et al. 2010 and Sukhada 1992)**

Enzymes were extracted by homogenizing 1 g of detached root tissue in a mortar at 4°C using 20 ml of Phosphate buffer (0.1M KH$_2$PO$_4$, at pH 6.6). The homogenate was filtered through muslin cloth and the filtrate was centrifuged at 10,000 rpm (10 minutes) using cooling centrifuge (REMI). The supernatant was used for the assay for acid and alkaline phosphatase.

**Assay for alkaline phosphatase**

The enzyme extract (0.2 ml) was incubated in test tube in a mixture of 0.5 ml of 0.25 M Tris - HCl buffer (pH 9.8) and 1.5 ml of 1mg ml$^{-1}$ p-nitrophenyl phosphate (PNP) at 37°C. After 30 minutes, 5ml of 0.1N NaOH was added to each of the test tubes and absorbance at 410 nm was measured in a spectrophotometer (make). Appropriate controls were included and activity was expressed in terms of n moles of PNP hydrolysed per hour per gram fresh weight of the tissue.

**Assay for acid phosphatase**

The enzyme extract (0.2 ml) was incubated in test tube in a mixture of 0.5 ml of 0.25 M Sodium acetate buffer (pH 6.0) and 1.5 ml of 1mg ml$^{-1}$ p-nitrophenyl phosphate (PNP) at 37°C. After 30 minutes, 5ml of 0.1N NaOH was added to each of the test tubes and absorbance at 410 nm was measured in a spectrophotometer (Systronics Spectrophotometer 166). Appropriate controls were included and activity was expressed in terms of n moles of PNP hydrolysed per hour per gram fresh weight of the tissue.

**Estimation of total sugars and reducing sugars (Nelson and Somogyi, 1944)**

100 µg plant material was homogenized in 5 ml of 80% alcohol using a pinch of purified sand and centrifuged at 10000g for 10 minutes and the supernatant was stored. Residue was re-extracted with 5 ml of 80% alcohol. The supernatant were pooled and used for estimation of reducing sugars and total sugars.

For total sugars, 1ml of extract was taken and 1ml of 1N H$_2$SO$_4$ was added to hydrolyze non-reducing sugars and kept in boiling water bath (30 minutes). Tubes were cooled, and 1 drop of methyl red indicator was added and neutralized by adding drop-wise few ml of 1N NaOH. 1 ml of Nelson - Somogyi (NS) reagent was added and tubes were capped with glass marbles and kept in
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water bath at 100°C (20 minutes) and cooled rapidly. 1 ml of arseno-molybdate reagent was added and the tubes were frequently shaken for 5 minutes to dissolve the red precipitate. Final volume was made to 20ml with distilled water. Absorbance was read at 540 nm on spectrophotometer (Systronics Spectrophotometer 166). The blank was prepared in the same manner with aliquot being replaced with 80% ethanol. The amount of total sugars were calculated and expressed in terms of mg/g fresh weight.

1 ml aliquot was mixed with 1 ml NS reagent and the tubes were kept in boiling water bath at 100°C (20 minutes). The tubes were cooled rapidly and 1 ml of arseno-molybdate reagent was added and tubes were shaken vigorously to dissolve the red precipitate. The volume was made to 20 ml with distilled water. The absorbance was recorded at 540 nm.

Nutrient analysis
At the completion of the experiment both leaf and roots samples were collected from all treatments. The leaves and root samples were randomly selected but were apparently of normal health, without insect injury and disease symptoms. Twenty leaves per tree per treatment per replication were collected, placed in labelled Zip lock bags and brought to the laboratory. Finer roots were harvested from each tree and brought to the laboratory for analysis. They were subjected to the elemental analysis for quantification of phosphorous, nitrogen, potassium, calcium, and magnesium.

Sample preparation
The leaves were lightly washed with tap water and rinsed with DI water. Similarly, the collected roots were washed thoroughly with tap water and rinsed with DI water. The leaves and roots were then air dried under the shade to remove the surface moisture. Finally they were placed in labelled perforated paper bags and kept in the oven for 48 hours at 65°C. The oven dried root and leaf samples were grinded to fine powder by an electric stainless steel grinder (Phillips HL1606). This powder was then stored in properly labelled air tight glass/plastic bottle for further analysis.

Determination of total nitrogen by Kjeldahl method (Tandon, 2004)
Total nitrogen in leaf and roots were determined by Kjeldahl method. This method involves the conversion of nitrogen in biological materials into (NH₄)₂SO₄ by digestion with H₂SO₄ followed by distillation of NH₃ in an alkaline medium. The ammonia is collected in sulphuric acid of known strength (0.05 N) which is titrated with standard sodium hydroxide solution.
**Procedure**

0.5 gram of oven dried leaf/root powder was transferred to a Kjeldahl digestion flask and digestion mixture was added to it. The digestion mixture consisted of the following: 1 g of a well-ground mixture of 20 g CuSO$_4$.5H$_2$O and 1 g Selenium. To one part of this mixture 10 parts of K$_2$SO$_4$ were added. One g of this catalyst/salt mixture is added to the digestion flask. K$_2$SO$_4$ raises the boiling temperature of H$_2$SO$_4$, CuSO$_4$ and Selenium, accelerates the rate of digestion. Samples were slightly heated and 2.5ml of 30% hydrogen peroxide was added to the digestion flask. Addition of H$_2$O$_2$ prior to high temperature helps in destroying organic material and minimizing foaming. 10 ml concentrated H$_2$SO$_4$ and a few glass beads were added. The contents were left as such for 30 minutes. The digestion flasks were then placed on heater and mixture was heated gently at first until all the water is removed and charring is completed. The heat was then gradually increased so that the solution is brought to constant boiling with slight bubbling. Finally the mixture turned into transparent liquid. After complete clearing, boiling was continued gently for another 15-20 minutes. By this time whole of the nitrogen gets converted into (NH$_4$)$_2$S0$_4$. The flask was allowed to cool, about 25 ml distilled water was added, the contents was transferred to a 100 ml volumetric flask and the volume made up with DI water.

**Distillation**

Ten ml of the digest was transferred to vacuum jacket of micro-kjedahl distillation apparatus. In a conical flask, 10 ml of 4% boric acid solution was taken containing bromocresol green and methyl red indicator, to which the condenser outlet of the flask was dipped. After adding the aliquot, the funnel of the apparatus was washed with 3 ml of DI water and 10 ml of 40% NaOH solution was added.

Five ml aliquot was distilled to the flask containing 10 ml of boric acid. On completion of distillation, the boric acid was titrated against 0.005 N H$_2$SO$_4$. Blank was run simultaneously and titration was carried out to the end point as those of samples. The nitrogen content in plant samples was calculated as:

- Weight of sample = 0.5g
- Normality of H$_2$SO$_4$ = N/200
- Volume of Digestion = 100ml
- Aliquot taken = 5ml
- Titration Value = Sample titration value - Blank Titration Value
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Estimation of potassium (K)
For estimating potassium in the plant sample, it was first digested. The digestion for estimation of elements like K, Ca, Mg was done according to the method described by Yoshida et al (1976).

Procedure

Preparation of standard stock solution
The standard stock solution was prepared in the following way:
1.9069g of analytical grade KCl was dissolved in DI water and the volume was made upto 1 litre. This solution contained 1000 ppm K. 1000 ppm K solution was diluted 10 times (10 ml in 100 ml final volume) to prepare 100 ppm K solution. Final standard solutions of 0, 1, 3, 5 ppm were prepared from 100 ppm K solution.

Preparation of standard curve
The standard curve was prepared using the standard stock solutions. To prepare the standard curve, the instrument was set at the highest concentration of 5 ppm using a standard filter. The manufacturer specified the linear range of K to be 5 ppm. From the standard curve, the K in the plant sample was calculated.

Digestion of plant sample
One gram oven dried plant material was transferred to 100ml beaker and 10 ml of tri acid mixture comprising of HNO3: HSO4: HClO4 (9:2:1) was added to it. The content of the beaker was mixed by swirling. It was covered with watch glass and left as such for about 2 hours till the initial reactions subsided. The beakers were heated on hot plate gently until the solid material disappeared, then heated vigorously until the production of fumes ceased. The contents were further evaporated until the volume got reduced to 1.5 ml. It was then removed from hot plate and cooled to ambient temperature. Then distilled water was added to it and the entire content was transferred to a 100ml volumetric flask. The volume was made up to the mark and it was used for analysis of potassium.

Estimation of K by flame photometer
Potassium present in the digested plant material was then determined by flame photometer. Flame photometry is based on the principle that atoms of some specific elements take energy from flame
and get excited to the higher orbit. Such atoms release energy of a wavelength which is specific for that element and is proportional to the concentration of atoms of that element. So for estimating potassium the test sample solution containing potassium was introduced to the flame photometer. A fine aerosol was formed and the atoms got excited by taking energy from flame created by mixture of liquid petroleum gas mixed with air. The emitted radiation was of several wave lengths. It was therefore passed through specific filter to isolate radiation of specific wave length.

The standard solutions containing known amount of potassium were introduced first into the flame photometer and the reading was recorded and standard curve was prepared thereby. The test samples were then read by flame photometer using filter for K. The concentration of Potassium in the test sample was then measured by comparing the radiation emitted by a known standard with that of the sample.

\[ \text{Estimation of the amount of K} \]
\[ K \text{ in } \% = R \times \frac{5}{100} \times \frac{100}{\text{Sample}} \times \frac{100}{1000000} \]

Where 5 ppm K = 100 R

\[ \text{Determination of calcium and magnesium} \]
\[ \text{Calcium (Ca)} \]

\[ \text{Standard stock solution} \]

To 2.247 g of primary standard calcium carbonate, 5ml of DI water was added. A minimum volume of HCl (approximately 10 ml) was added drop-wise to effect complete solution of CaCO3. It was then diluted to 1 litre with DI water. That gave 1000 ppm Ca. 10 ml of this solution was diluted to 100ml to get 100 ppm Ca.

\[ \text{Ca in } \% = R \times \frac{100}{\text{Sample}} \times \frac{100}{1000000} \]

\[ \text{Magnesium (Mg)} \]

\[ \text{Preparation of Standard Stock solution} \]

1.0 g magnesium ribbon was dissolved cautiously in a minimum volume of (1+1) HCl. It was diluted to 1 litre with 1% (v/v) HCl.
10.141 g MgSO₄·7H₂O was dissolved in 200 ml of de-ionized water and the volume was made up to 1 litre. This will give 1000 ppm Mg. The acid digested plant material was diluted and fed to the atomic absorption spectrometer for Ca and Mg.

**Micronutrient analysis**
Micro-elemental determinations of Copper (Cu), Iron (Fe) and Zinc (Zn) in leaves and roots of different treatments were made with atomic absorption spectrophotometer.

**Preparation of sample for estimation of Fe, Cu and Zn**
1g oven dried plant samples are digested using acid mixture and made up to 100 ml using DI water on cooling.

**Preparation of standard stock solution for Fe**
(i) 1 gm of pure iron wire was dissolved in 50 ml of (1+1) analytical grade HNO₃. It was diluted to 1 L with DI water.
(ii) 7.022g of analytical grade (NH₄)₂Fe(SO₄)₂·6H₂O was dissolved in 400 ml DI water. 5 ml of concentrated H₂SO₄ was added and the volume was made up with DI water. This gave a stock solution of 1000 ppm iron. From this, solution of 100 ppm was prepared and this was used for preparation of final standard solutions.

\[
Fe \text{ in ppm} = R \times \frac{100}{\text{sample}}
\]

Similar calculation was followed for Cu and Zn.

**Preparation of standard stock solution for Cu**
(i) 1 g of copper metal was dissolved in a minimum possible volume of (1+1) HNO₃.
(ii) It was further diluted to 1 L with 1% (v/v) HNO₃.
(iii) 3.929 g CuSO₄·5H₂O was dissolved in distilled water and the volume was made up to 1000 ml. This gave a solution of 1000 ppm Cu. 10 ml of this solution was diluted to 100 ml to get 100 ppm solution of Cu.

**Preparation of standard stock solution for Zn**
(i) 0.5 g of metallic zinc was dissolved in a minimum volume of (1+1) HCl and diluted to 1 L with 1% (v/v) HCl
(ii) 4.398 g of ZnSO$_4$.7H$_2$O was dissolved in distilled water and the volume was made up to 1000 ml. This gave a solution of 1000 ppm zinc. 10 ml of this solution was diluted to 100 ml to get 100 ppm zinc solution. The final standard solutions were prepared from this 100 ppm solution.

The concentration of Zn, Cu, Fe in leaves and roots were estimated in more or less the same way by AAS.

### 2.3.10 Derived parameters

Several derived parameters were analyzed to gain further insight at the plant-AMF interaction and the pros and cons of vermicompost treatment. Following derived parameters are considered for the present study:

**Leaf area ratio (LAR)**

The leaf area ratio, $LAR = \frac{\text{leaf area (cm}^2\text{)}}{\text{total plant dry weight (g)}}$

**Specific leaf area, specific root length and ratio of root length to leaf area**

Specific leaf area (SLA) is defined as the ratio of total leaf area to the total dry biomass. Specific root length (SRL) is defined as the ratio of total root length to the total dry biomass. Root length to leaf area (RLLA) ratio is defined as the amount of root length grown per unit total leaf area of the plants.

**Root to shoot ratio (dimensionless)**

This quantity was determined for both fresh weight and dry weight of plants.

**Modified seeding quality index (MSQI) in g plant$^{-1}$**

This is defined as:

$$MSQI = \frac{DW_{\text{total}}}{H} + \frac{DW_{\text{shoot}}}{RL} + \frac{DW_{\text{root}}}{DL}$$

Where, $DW$, $H$ and $RL$ represent dry weight (g plant$^{-1}$), plant height (cm) and total root length (cm) respectively.
Microbial inoculation effect
This is to quantify the efficacy of AMF inoculation in terms of the biomass produced and is defined as:

\[
\frac{\text{[Dry weight of AMF inoculated plant (T3 and T4) – Mean dry weight of control plant]} \times 100}{\text{Dry weight of AMF inoculated plant (T3 and T4)}}
\]

Vermicompost treatment effect
This is to quantify the efficacy of vermicompost treatment in terms of the biomass produced and is defined as:

\[
\frac{\text{[Dry weight of vermicompost treated plant (T2 and T3) – Mean dry weight of control plant]} \times 100}{\text{Dry weight of vermicompost treated plant (T2 and T3)}}
\]

Bio-volume index
This is a measure of the total volume of a seedling and is estimated by multiplying the square of the diameter by the plant height \((D^2H)\). Thus volume index correlates well with the aboveground biomass (Hatchell et al, 1985).

2.3.11 Fungal parameters

Estimation of spore density
The AM fungal spores were isolated from the root-zone soil by wet sieving and decanting technique of Gerdemann and Nicolson (1963). The AM spores collected in the filter paper were then examined under stereo zoom microscope and the spore number (spore density) was estimated. The details of the process have been described in phase I.

Staining and photography of spores
The soil samples collected from root zones of seedlings receiving T4 and T4 are processed for isolation of AMF spores. The isolated spores are picked up from the filter paper on slide and stained in slides with help of PVLG and Melzer’s reagent. Some of the spores are fixed in lactophenol.
Preparation of Polyvinyl-Lacto-Glycerol (PVLG) reagent

The PVLG was prepared in the following way

Reagent Used
- Distilled water - 100 ml
- Lactic acid – 100 ml
- Glycerol – 10 ml
- Polyvinyl alcohol (PVA) – 16.6 g

Procedure
All ingredients were mixed in a dark bottle before adding the polyvinyl alcohol. PVA powder was mixed to the other liquid ingredients. It was then placed in a hot water bath (70-80°C). The PVA got dissolved slowly and by 4-6 hours a clear solution of PVLG was prepared. It was incubated overnight in the hot water bath before using.

Preparation of Melzer’s reagent

Reagent Used
- Chloral hydrate - 100g
- Distilled water – 100 ml
- Iodine – 1.5 g
- Potassium iodide – 5 g

Procedure
All the ingredients were mixed well for preparing Melzer’s Reagent. Melzer's reagent was then mixed with PVLG in a volume ratio of 1:1 and the mixture was used for staining spores and mounting it on slides. The spores mounted on the slides were then examined under the compound microscope. Photographs of the spores were taken with the help of microscope (Magnus MLXi) and digital camera (Olympus E 450). These facilities are provided by GSBTM, Gandhinagar. Some of the photographs were also taken at Botany Department, Gujarat University.

Root colonization percentage
For examining root colonization, roots of seedlings receiving different treatments were separated from soil samples and processed to investigate the mode of colonization by AMF in terms of
development of vesicle, arbuscules, spores and hyphae. The AM fungal infection was examined by using compound microscope. The details of the process have been described in phase I.

2.3.12 Statistical analysis
The final data was subjected to one – way Analysis of Variance (ANOVA). Based on the outcome of ANOVA on all data, post-hoc analysis had been performed in the form of Duncan’s Multiple Range test (DMRT) (Duncan, 1955) at p≤0.05 level of significance to separate the means. Linear regression analysis was used to assess the relationship between AMF colonization, growth parameters and phosphorous uptake.