6. MATERIALS AND METHOD

Well planned exploration trips were conducted to the study area so as to cover all the seasons and all the types of forest areas, viz. Ever Green Forest, Grass Land and Shola Forests of Poochipara and Valakkadu sections of Silent Valley National Park. Random sampling method was adapted for collecting soil and root samples from the dominant plants.

The following steps are involved in the collection, isolation and multiplication of AM fungi:

i. Collection of samples

ii. Isolation from soil

iii. Trap culturing

iv. Mass multiplication of individual species

v. Inoculation for Mass Multiplication and Identification

vi. Preparation of Permanent Slides

vii. Estimation of colonization from root samples

i. Collection of samples

In Poochipara section: five ever green forests, five grass lands and in Valakkadu section: three ever green, five grass lands and three shola forest were selected as a study site. Four to five spots in each forest types were selected for collecting the soil samples. Soil sample collected after scraping out the top soil, about 500 gm of soil around the roots of dominant plants was dug with the help of a trowel to the depth of 15 cm and collected in clean polythene cover. All the soil samples collected from each spot was mixed homogenously, roots were separated, washed thoroughly in tap water so as to remove the soil particles attached to them and fixed in Formalin –Acetic acid – Amyl alcohol (FAA) and 500 gms of composit soil was collected in fresh polythene covers.

ii. Isolation from soil (Gerdi. & T.H. Nicolson, 1963)

1. 100 gms of soil mixed in 300 ml of distilled water in a beaker and shaken thoroughly to get uniform suspension.

2. All the heavier particles allowed to settle down
3. The suspension was decanted through series of sieves (750 µm, 250 µm, 75 µm & 45 µm sieves) placed one above other. The residues in the beaker was re-suspended in water and decanted. This process repeated for five times to separate the grit, sand and heavy organic particle in the beaker.

4. Residue collected from all the sieves by flushing distilled water and filtered it through a filter paper. Filter paper placed on a suitable petri dish and observed under stereomicroscope for spore count and to separate mycorrhizal spores by using a sterile needle. Healthy spores were isolated, selected and used for the inoculation of the culture pots where Sorghum and Maize seeds were to be sown.

iii. Trap culturing

Pot culture technique was used for trapping VAM fungi and increasing their spore numbers for further studies

A soil sample contains propagules of several VAM fungi; however, it is well known that species can only be clearly recognized from a spore which has all typical morphological features for identification. These spores are used to reproduce the fungi in pot cultures. Hence, VAM fungi species have not sporulated at time of sampling (which often cannot be known) or if only few spores of species are available from a soil sample, Gerd. & Trappe’s (1974) –inoculated pot culture‖ method reproduces the indigenous VAM population on trapping the VAM fungi and increases their numbers.

1. Clean earthen pots were filled with one kg sterilized soil and sand mixture (1: 1) on top of which about 200-250 gm of the field soil sample was spread
2. Another layer of 0.5kg sterilized soil is spread on field sample layer.
3. Sorghum and Maize seeds were surface sterilized and sown in the prepared pots
4. When the potted plants attained the age of three months, mycorrhizal spores were isolated from the roots and soil by using wet sieving and decanting method.
5. Mother cultures of the original native Mycorrhizal fungi were maintained in the shade house of TBGRI.

iv. Mass multiplication of individual species

Isolation of spores was done directly from field sample or from trap pot cultures. Spore separation started when the trap plant attained 4- 6 months after their establishment; isolation was periodically repeated until trap pot cultures were at least 12 months old. From
every isolate, spores were morphologically identified and used for the mass multiplication to understand more about it.

v. **Inoculation for Mass Multiplication and Identification** (Mertz et al., 1979)

1) Spores which showed similarity in size and shape were isolated and grouped separately by using stereo microscope.
2) These isolated spores were surface sterilized by using 0.1% mercuric chloride solution
3) Pots were filled by using equal amount of sterilized soil and sand.
4) Surface sterilized Sorghum and Maize seeds were selected, smeared with mycorrhizal spores and sown in the prepared pots
5) When the potted plants attained the age of three months, mycorrhizal spores were isolated from the roots and soil.
6) Mother cultures of the original native Mycorrhizal fungi were maintained in the shade house of TBGRI.

vi. **Preparation of Slides**

1) A drop of distilled water placed on a clean slide, the isolated spores were placed in it, allowed to dry.
2) Place a drop of PVA mountant on clean glass slide, spread it gently, place the spores carefully on it, add two more drops of mountant, allow the mountant to set for a few minutes to become more viscous and then place the cover slip over it gently by avoiding air bubbles.
3) Label the slide and allow it to dry for 3-4 days.
4) Seal the cover slip along the edges by nail polish to prevent an entry of air bubbles.

vii. **Estimation of colonization from root samples**

The following procedure has been adapted here:

1. The roots in FAA were washed gently and thoroughly in tap water by keeping the external mycelium intact.
2. Placed them in beaker containing 10% KOH solution
3. Beaker (containing root samples) placed in hot water bath at 60°C for 10-15 minutes
4. The root samples rinsed thoroughly in tap water
5. Dipped in alkaline H₂O₂ for 10-20 minutes until roots are bleached,
6. Rinsed thoroughly in tap water
7. Placed in the beaker containing 1% HCl for 3-4 minutes and the acidic nature maintained.
8. Transferred material to the beaker containing 0.01% acid fuschin or 0.05% tryphan blue.
9. Observed under microscope for the percentage of root colonization.

viii. **Soil analysis**
Soil samples were analyzed for the concentration and presence of nutrients like Nitrogen, phosphorus and Potassium in the soil testing laboratory, Wayanad-Manathavady.

ix. **Collection of Climatological data**
Climatological data collected from meteorological stations of Poochipara and Valakkadu section office of the Silent valley National park.