3 MATERIALS AND METHODS

3.1 Distribution and isolation of VAM

3.1.1 Collection of soil samples

Soil samples were collected from sixteen locations of rubber growing area (Fig. 1) to study the natural distribution of VAM fungi. Soil was dug out with a trowel to a depth of 15 cm after scraping away the top 1 cm layer of soil. Samples were collected from 20 different places in each area, pooled and homogenised. Representative samples were taken in polythene bags, labelled and stored at 2°C until they were processed further. From this, 10 lots of 50 g soil were wet sieved for taking spore count and isolation of spores (Hayman, 1982).

3.1.2 Determination of soil pH

pH of the samples was determined in 1:2.5 (v/v) soil:water solution in a Philips pH meter.

3.1.3 Enumeration of VAM spore population

The spores were collected by wet sieving as detailed by Gerdemann and Nicolson (1963). A quantity of 50 g of soil was suspended in 200 ml of luke-warm water. Heavier particles were allowed to settle for a few seconds and the suspension was decanted through a 710 μm sieve to remove the larger particles of organic matter. The residue was resuspended in more water and sieving was repeated. The
suspension that passed through this sieve was saved and stirred to resuspend all particles. The heavier particles were allowed to settle for a few seconds and the liquid decanted through 250 μm sieve. The suspension that passed through this sieve was again collected and the sieving was repeated using 105 μm sieve and 45 μm sieve. The larger particles of organic matter were caught on the top sieves of higher pore size. The soil particles and spores collected in 105 μm and 45 μm sieves were taken in 100 ml conical flasks separately. The suspension in each flask was shaken thoroughly and allowed to settle for 30 seconds. The spores present in these suspensions were trapped on a nylon mesh, with 45 μm pore size placed on a marked petridish and the number of spores were counted by observing under a stereoscopic microscope.

3.1.4 Identification of spores

Spores of common species of VAM were identified using synoptic keys to the genera and species of Zygomycetous mycorrhizal fungi by Trappe (1982) and photographic slide collection illustrating features of the endogonaceae by Hall and Abbott (1981).

3.1.5 Isolation of VAM fungi

Spores of fungi were taken in water and they were picked up with a fine capillary pipette under a dissecting microscope using fine needles to separate organic matter. Thirty different spore types were isolated based on their morphology and they were surface sterilised using 2 per cent chloramine T and streptomycin sulphate (200 μg ml⁻¹) for 20 minutes and washed in several changes of sterile water. A single spore was placed in the neck region of a funnel assembly (Plate 1) filled with
sterilised sand and established seedlings of *Sorghum bicolor* (Nicolson, 1967). The seedlings maintained in the funnel for 4 weeks were transferred to mud pots containing sterilised 1:1 sand soil mix and maintained in a glass house. After two months a portion of the soil was removed and checked for purity. Pure isolates of selected VAM were multiplied under sterile condition. *P. phaseoloides* plants raised under similar conditions were also inoculated by VAM fungi for confirming their efficacy.

Soil sand mix (1:1 w/w) in mud pots (15 cm diameter) was steam sterilised for 2 h for two consecutive days and used for this purpose.

### 3.1.6 Establishment of stock plants (Hayman, 1982)

The root system of uniformly well infected sorghum seedlings together with the adhering soil were finely chopped and used as the starter inoculum. Sterile soil in pots were inoculated with 5-10 per cent of the starter inoculum as a layer of two inches below the soil level and surface sterilised seeds of *S. bicolor* were planted. The seedlings were periodically watered with sterile water for 90 days. Rock phosphate 0.75 g and urea 0.25 g bag⁻¹ were applied after 25 days of establishment.

### 3.1.7 Establishment of *P. phaseoloides*

To break dormancy, seeds of *P. phaseoloides* were taken in a glass beaker and added concentrated sulphuric acid just sufficient to form a coat over the seeds and kept at constant stirring for 10 minutes. It was then washed thoroughly with tap water to remove acid.
Figure 1. Sites of soil sample collection
Plate 1  Funnel assembly for VAM isolation studies
Bradyrhizobium sp. isolated from *P. phaseoloides* was grown in yeast extract mannitol broth (Annexure 1) for 3 days and mixed with the acid treated seeds of *P. phaseoloides*. Such treated seeds were pellatised with required quantity of calcium carbonate and planted in soil taken in polythene bags.

### 3.2 Selection of VAM

The relative efficiency of 11 different VAM fungi representing four genera on root colonisation, nodulation, nitrogenase activity, biochemical contents and growth of *P. phaseoloides* was studied under sterile conditions and the competitive ability of VAM fungi was confirmed under unsterile conditions too.

#### 3.2.1 Sterile condition

The experiment was conducted in polythene bags of 15 cm diameter using steam sterilised sandy loam soil of pH 5.2 containing total nitrogen 0.26 per cent, available potassium 2.74 mg g\(^{-1}\), available phosphorus 0.225 mg g\(^{-1}\) and organic carbon 1.7 per cent. Each poly bag contained 4 kg of soil. Eleven VAM fungi obtained from the earlier experiment were used. A quantity of 15 ml of inoculum containing about 300 spores and infected roots (85%) was uniformly distributed below 2 cm of top soil in polythene bags. The treatments included are

- \(T_1\) Inoculated with *Gigaspora calospora*
- \(T_2\) Inoculated with *Sclerocystis* sp.
- \(T_3\) Inoculated with *Glomus monosporum*
- \(T_4\) Inoculated with *G. boreale*
- \(T_5\) Inoculated with *G. macrocarpum*
Inoculated with \textit{G. epigaeum}

Inoculated with \textit{G. multicule}

Inoculated with \textit{G. flavisporum}

Inoculated with \textit{G. fasciculatum}

Inoculated with \textit{Acaulospora scrobicularu}

Inoculated with \textit{A. laevis}, and

Uninoculated control.

Acid treated \textit{P. phaseoloides} seeds inoculated with \textit{Bradyrhizobium} sp. were sown in polythene bags. Five plants were maintained per bag. The treatments were replicated thrice in RBD design. The bags were irrigated with sterile water periodically to maintain field capacity and harvested 50 days after sowing.

\textbf{3.2.2 Studies under unsterile condition}

The experimental conditions were the same as that of sterile condition except sterilisation. The experiment was repeated with soil containing an indigenous population of 140 VAM spores in 50 ml. In this study tap water was used for watering the plants. The plants were harvested after 50 days of growth and observation were made for various parameters as given below.

\textbf{3.2.2.1 Shoot length}

The height of the main shoot was measured from the ground level to the tip of the terminal bud.
3.2.2.2 Root length

The length of the main root from collar to the tip was measured.

3.2.2.3 Dry weight

The root and shoot portions of the plants were separated. The root portion was washed gently to remove all the adhering soil particles in running tap water. Both the portions were gently pressed in folds of filter paper to remove excess moisture. The fresh weight was determined and the samples were wrapped in paper and kept in a hot air oven at 105°C for 24 h, removed, cooled in desiccator and reweighed.

3.2.2.4 Nodule number and dry weight

After 50 days of growth in the polythene bags the number of nodules and dry weight of nodules were determined. Plants were removed carefully from the bags with their root system and nodules intact. The nodules with roots were washed, separated and counted. Samples were dried in an oven at 105°C till constant dry weight was attained.

3.2.2.5 Per cent of mycorrhizal infection (Phillips and Hayman, 1970)

The roots were cut into 1 cm bits, washed gently in tap water without disturbing the external mycelium. The samples were heated to about 90°C for 1 h in 10 per cent potassium hydroxide solution in a waterbath. It was rinsed four times in tap water and acidified by immersing for five minutes in 2 per cent hydrochloric acid. The acid was poured off and added 0.05 per cent cotton blue in lactophenol.
The roots were boiled in this stain for three minutes. The stain was poured off and added with lactophenol and kept overnight to destain the host tissues and examined under a microscope for mycorrhizal infection.

Mycorrhizal colonisation was expressed using the following formula,

\[
\text{Per cent colonisation} = \frac{\text{Number of root segments with VAM}}{\text{Total number of root segments examined}} \times 100
\]

The root segment was considered mycorrhizal even if one of the three structures, i.e., hyphae, arbuscules or vesicles was present.

3.2.2.6 VAM spore count

The spores were collected by wet sieving and decanting method and spore counts were taken as described earlier.

3.2.2.7 Ethyl alcohol extraction of plant materials (Chandramohan et al., 1967)

Leaves of the plants were collected, chopped and used for ethyl alcohol extraction after removing excess moisture by blotting them between folds of filter paper. Exactly 1 g of the chopped material was plunged into 20 ml of boiling 80 per cent ethyl alcohol, extracted for 5 minutes on a hot waterbath and cooled in running tap water. The material was homogenised by grinding in a porcelain mortar with pestle and squeezed through two layers of cheese cloth. The residue was reextracted with ethyl alcohol and the extracts pooled. The final volume was adjusted to 20 ml with 80 per cent ethyl alcohol. The residue after drying was used for the estimation of starch.
3.2.2.8 Quantitative estimation of total phenols

Total phenols were estimated by employing Folin-Ciocalteu reagent (Bray and Thorpe, 1954) (Annexure 2).

Folin-Ciocalteu reagent was diluted with equal quantity of water. One ml of this reagent was added to 1.0 ml of the alcohol extract in a 25 ml marked boiling tube followed by a 2 ml of 20 per cent sodium carbonate and the mixture was heated in a boiling waterbath for exactly one minute. The blue colour was diluted to 25 ml with glass distilled water. Reagent blank was maintained with 1.0 ml of distilled water instead of ethyl alcohol extract. The percentage of light transmittance was determined in a ‘Spectronic-20’ colorimeter at 725 nm. Total phenols were calculated from a standard curve plotted using catechol.

3.2.2.9 Quantitative estimation of ortho dihydroxy phenols

Ortho dihydroxy phenols were estimated by the method described by Johnson and Schaal (1952) employing Arnow's reagent. To 1 ml of the alcoholic extract in a 25 ml marked boiling tube, 1 ml of 0.5 N hydrochloric acid, 1 ml of Arnow’s reagent prepared by dissolving 10 g of sodium nitrate and 10 g of sodium molybdate in 100 ml of glass distilled water and 2 ml of 1 N sodium hydroxide were added. The volume was raised to 25 ml with distilled water and the light pink colour was read in the ‘Spectronic-20’ colorimeter at 522 nm. Reagent blank contained 1 ml of distilled water in the place of ethyl alcohol extract. Ortho dihydroxy phenols were calculated from a standard curve prepared using catechol.
3.2.2.10 Determination of reducing sugars

Reducing sugars content in the alcohol extract was determined by Nelson's (1994) method.

To 1 ml of alcohol extract in a 25 ml marked boiling tube, 1 ml of mixture of reagent 'A' and 'B' (Annexure 2) prepared by mixing 25 parts of reagent 'A' with 1 part of reagent 'B' was added. The mixture was heated for 20 minutes in a boiling waterbath, cooled in tap water and 1 ml of the Arsenomolybdate reagent was (Annexure 2) added. The solution was thoroughly mixed and diluted to 25 ml with glass distilled water. Reagent blank contained 1 ml of distilled water in the place of ethyl alcohol extract. The resulting blue colour was read in a 'Spectronic-20' colorimeter at 497 nm. Glucose was used as standard and the results are expressed as glucose equivalent.

3.2.2.11 Determination of non-reducing sugars

Non-reducing sugars present in the alcohol extract were first hydrolysed to reducing sugars (Inman, 1962) and then estimated. Exactly 1 ml of the alcohol extract was taken in a boiling tube and evaporated to dryness on a waterbath. One ml of glass distilled water and 1 ml of 1 N sulphuric acid were added to the residue. The mixture was hydrolysed by heating at 49°C for 30 minutes over a waterbath. The solution was neutralised with 1 N sodium hydroxide using methyl red indicator.

Total sugars content of the hydrolysed samples was estimated by the Nelson's method. Non-reducing sugars were calculated by substrating the reducing sugar value from that of total sugars and were expressed in glucose equivalents.
3.2.2.12 Determination of amino nitrogen

Amino nitrogen was determined by the ninhydrin method of Moore and Stein (1948).

To 1 ml of the alcohol extract in a boiling tube, 1 drop of methyl red indicator was added and the extract was neutralised with 0.1 N sodium hydroxide, if necessary. To this solution 1 ml of ninhydrin reagent was added, mixed thoroughly by shaking and aluminium caps were placed on the tubes. The mixture was heated for 20 minutes in a waterbath. The tubes were removed cooled in running tap water, 5.0 ml of diluent solution was added and the contents thoroughly mixed. The purple colour of the solution was read in a ‘Spectronic-20’ colorimeter at 475 nm. Blanks consisted of 1 ml of distilled water in the place of alcohol extract. Amino nitrogen was calculated from the standard graph prepared using glutamic acid.

3.2.2.13 Quantitative estimation of starch

Starch in the samples was estimated by the method of Sumner and Somers (1949).

Two hundred mg of finely powdered 80 per cent alcohol insoluble residue, dried in an oven at 60°C for two consecutive days, were placed in a glass stoppered 100 ml Erlenmeyer flask. Three ml of 6 N hydrochloric acid were added to the flask and steamed in an autoclave at 110°C for 1 h. The flasks were cooled and the solution was neutralised by using 1 N sodium hydroxide. The volume was raised to 25 ml with distilled water. An aliquot of 1 ml was withdrawn and glucose was estimated by Nelson’s (1944) method. The amount of starch was determined by multiplying the amount of estimated glucose by the factor 0.9.
3.2.2.14 Extraction and estimation of chlorophyll (Arnon, 1949)

A quantity of 1 g fresh leaves were cut into small pieces and homogenised in a mortar with excess acetone, with a pestle. The supernatant was decanted and filtered on a Buchner funnel through Whatman No. 42 filter paper. Sufficient quantity of 80 per cent acetone was added and repeated the extraction. The filtrates were pooled and the volume was made to 100 ml in a volumetric flask. The absorbance of the extract at 645 and 663 nm was determined for determination of total chlorophyll.

The chlorophyll content was calculated on a fresh weight basis employing the following formula,

\[
\text{Total chlorophyll (mg/g)} = \frac{20.2 \times A_{645} + 8.02 \times A_{663}}{a \times 1000 \times w} \times V
\]

where,

- \( a \) - length of path light in the cell
- \( v \) - volume of the extract in ml, and
- \( w \) - fresh weight of the sample in g

3.2.2.15 Determination of nitrogen

Total nitrogen in the sample was determined by microkjeldahl method (Jackson, 1962).

The samples were dried at 70°C for 48 h and powdered. Fifty mg of the powdered sample dried at 105°C for 6 h was transferred into a digestion flask and digested with 2 g of potassium sulphate, 40 mg of mercuric oxide and 2.4 ml of concentrated sulphuric acid. Gently heated the flask until frothing ceased and heating continued more strongly until the solution was cleared. After cooling 10 ml
of distilled water was added and warmed to dissolve the solute material. Blanks were prepared using reagents alone.

**Estimation**

The digested sample was transferred into the distillation flask. A quantity of 2 ml sodium hydroxide-sodium thiosulphate mixture prepared by dissolving 50 g of sodium hydroxide and 5 g of hydrated sodium thiosulphate in 100 ml of water was added and steam distilled. The liberated ammonia was collected into 5 ml of 4 per cent boric acid solution (in water), containing 2-3 drops of methyl red-bromocresol green indicator (prepared by mixing five parts of 0.2 per cent alcoholic bromocresol green solution with one part of 0.2 per cent alcoholic methyl red solution). The distillate was titrated against 0.02 N hydrochloric acid. The end point was chosen as the appearance of green colour. The blank digest was also run in the same way. Nitrogen in the sample was calculated by employing the factor, 1 ml of 1 N acid is equivalent to 14 mg of nitrogen.

3.2.2.16 *Estimation of phosphorus* (Jackson, 1962)

Phosphorus content in the samples was estimated using an auto analyser.

A quantity of 0.5 g of the sample previously dried at 105°C for 6 h was transferred into a silica dish and allowed to form ash in a muffle furnace at 500-550°C for half an hour. The dishes were allowed to cool and the ash was carefully moistened with distilled water. Adding 5 ml of 6 N hydrochloric acid, the content was digested for 1 h over a waterbath. After cooling, it was transferred to a 100 ml standard flask and made upto the mark. This solution was used for the determination of phosphorus.
The calibration curve for phosphorus was obtained using 10 and 20 ppm standards of NaH$_2$PO$_4$·2H$_2$O.

3.2.2.17 *Estimation of potassium*

Potassium was also estimated using auto analyser, using sample solution prepared in the same way for estimation of phosphorus. The quantity of K in the sample was calculated referring to a standard graph already prepared with potassium phosphate.

3.2.2.18 *Photosynthetic activity*

Carbon dioxide exchange rate was measured by a closed system of Infrared Gas Analyser (Portable Photosynthetic System, LI 6200 LICOR, Nebraska, USA). Fully expanded mature leaf was inserted to the chamber as identical to natural position and exposed to sunlight for measurement. All measurements were done between 08.30 and 09.30 A.M. in the second half of January. The ambient temperature was 27 ± 2°C. Relative humidity was approximately 60 per cent and light intensity was 1000 ± 200 μmol m$^{-2}$s$^{-1}$. After gas exchange measurements, leaves were removed from each plant and area was recorded by leaf area meter (LI 3000, LICOR).

3.2.2.19 *Nitrogenase activity* (Turner and Gibson, 1980)

*P. phaseoloides* plants grown in polythene bags were uprooted after 50 days of growth. The adhering soil particles were removed gently without damaging the root system and nodules. Roots were excised from the shoot. With intact nodules the roots were incubated in a glass container closed air tightly with a rubber stopper.
Ten per cent of the air in the container was removed with a gas tight syringe and an equal volume of acetylene was injected. The system was incubated at room temperature \((28 \pm 2^\circ C)\) for 1 h. A quantity of 0.5 ml gas sample was withdrawn and injected into a Shimadzu 9A gas chromatograph fitted with a flame ionisation detector (FID) and stainless steel column of 80-100 mesh Porapak N (column temperature \(75^\circ C\), oven temperature \(100^\circ C\)). Nitrogen was used as carrier gas. The Acetylene Reduction Activity (ARA) (ethylene production) was calculated by measuring ethylene peaks. Correction for endogenous ethylene production and admixture of any ethylene in the acetylene gas was suitably applied and the results are expressed as follows:

\[
\mu \text{ moles } C_2H_4 \text{ produced plant}^{-1} \text{ h}^{-1} = \frac{\text{Sample ethylene after 1 h}}{\text{Standard ethylene after 1 h}} \times \frac{\text{Acetylene at 0 time}}{\text{Acetylene after 1 h}} - \frac{\text{Sample ethylene at 0 time}}{\text{Standard ethylene at 0 time}} \times \frac{G_v(\text{ml}) - \text{VCF} \times \text{VPM}}{22.4 \ (T_1-T_0)h}
\]

where,

- \(G_v\) - Gas volume of the container
- \(\text{VCF}\) - Vacuum correction factor
- \(\text{VPM}\) - Ethylene concentration (Standard sample = 105)
- \(T_1-T_0\) - Difference in sampling intervals
3.3 Response of *P. phaseoloides* to VAM Inoculation at Different Intervals

Response of *P. phaseoloides* plants to VAM inoculation at different intervals of growth was studied in unsterile soil. The plants were raised in polythene bags as mentioned earlier. A set of 20 bags was inoculated with *A. laevis*, another set of 20 bags was inoculated with *G. fasciculatum* and a third set of 20 bags was treated as uninoculated controls. Five plants were maintained in each bag. The plants in the bags were arranged in RBD and they were irrigated with tap water. Plants from 4 bags in each set were harvested at an interval of 10 days, i.e., 10, 20, 30, 40 and 50 days after sowing.

Observations were made on:

1. Root colonisation
2. Shoot weight
3. Root weight
4. Nodule number
5. Nodule weight
6. Nitrogenase activity
7. VAM spore count in soil
8. Total phenols
9. OD phenols
10. Reducing sugars
11. Non-reducing sugars
12. Starch
13. Amino nitrogen, and
3.4 Studies on *P. phaseoloides* Inoculated with *G. fasciculatum* and *A. laevis* at Different Levels of Rock Phosphate Application

Plants were raised in polythene bags containing unsterile soil as mentioned earlier. Five plants were maintained in each bag. Two isolates of VAM, i.e., *G. fasciculatum* and *A. laevis* were used in this study. Phosphate in the form of rock phosphate (RP) was applied at three different levels of recommended dose (150 kg ha\(^{-1}\)). The treatments imposed are as follows.

*Treatments*

- **T\(_1\)**  No VAM inoculation and no RP application.
- **T\(_2\)**  *G. fasciculatum* inoculation without RP application.
- **T\(_3\)**  *A. laevis* inoculation without RP application.
- **T\(_4\)**  No VAM inoculation at 50 per cent recommended level of RP application.
- **T\(_5\)**  *G. fasciculatum* inoculation at 50 per cent recommended level of RP application.
- **T\(_6\)**  *A. laevis* inoculation at 50 per cent recommended level of RP application.
- **T\(_7\)**  No VAM inoculation at 100 per cent recommended level of RP application.
- **T\(_8\)**  *G. fasciculatum* inoculation at 100 per cent recommended level of RP application.
- **T\(_9\)**  *A. laevis* inoculation at 100 per cent recommended level of RP application.

Rock phosphate was added to the soil and thoroughly mixed at the time of sowing *P. phaseoloides*. There were three replications for each treatment and the bags were arranged in RBD. The plants were irrigated with tap water and sampled after 50 days.
Observations were recorded on:

1. Root colonisation
2. Shoot length
3. Shoot weight
4. Root length
5. Root weight
6. Nodule number
7. Nodule weight
8. Nitrogenase activity
9. NPK of shoot and root
10. VAM spore count in soil

3.5 Impact of *Azotobacter* sp., *Beijerinckia* sp. and *Bacillus circulans* on Root Colonisation by *G. fasciculatum* and Growth, Nutrient Content and Rhizosphere Microbial Population of *P. phaseoloides*

The VAM isolate, *G. fasciculatum* was used in this study. The bacteria used are the non-symbiotic nitrogen fixing bacteria, *Azotobacter* sp. and *Beijerinckia* sp. and the phosphate solubilising bacteria, *B. circulans*. *P. phaseoloides* plants were raised in unsterile soil in polythene bags and inoculated with VAM and other bacteria as per the treatments. Five plants per bag were maintained. The treatments imposed are:

*Treatments*

\[ T_1 \] No mycorrhizae and no associative bacteria

\[ T_2 \] *G. fasciculatum* alone
T₃ Azotobacter sp. alone
T₄ Beijerinckia sp. alone
T₅ B. circulans alone
T₆ G. fasciculatum and Azotobacter sp.
T₇ G. fasciculatum and Beijerinckia sp.
T₈ G. fasciculatum and B. circulans

Bacterial cultures in the form of broth were mixed with the soil as 10 ml kg⁻¹ of soil. Bradyrhizobium sp. treated P. phaseoloides seeds were sown in soil containing 50 per cent of recommended level of rock phosphate. The plants were irrigated with tap water. After 50 days of growth the plants were sampled and the following observations were taken.

1. Root colonisation
2. Shoot length
3. Shoot weight
4. Root length
5. Root weight
6. Nodule number
7. Nodule weight
8. Nitrogenase activity
9. NPK of shoot and root
10. VAM spore count in soil
11. Microbial population
Enumeration of microbial population

The populations of total saprophytic bacteria, fungi, actinomycetes, *Azotobacter* sp., *Beijerinckia* sp. and phosphobacteria in rhizosphere soil samples of *P. phaseoloides* in the different treatments were estimated by serial dilution method using soil extract agar, Martin’s rose bengal streptomycin agar, Kenknight’s agar, Jensen’s agar, Becking’s agar and apatite agar respectively (Annexure 1).

3.6 Screening of Plants for Mass Multiplication of VAM Fungi

Spores of *G. fasciculatum* were isolated by wet sieving and decanting technique (Gerdemann and Nicolson, 1963) and brought to pot cultures through funnel technique using sorghum plants. Further multiplication of VAM was carried out using four host plants, viz., *S. bicolor*, *Zea mays*, *P. phaseoloides* and *Pennisetum polystygon*. Vermiculite and soil mixture (1:1 ratio) were used as growing media. Five replications were maintained. Soil samples and roots of plants were collected after 40 days and spore count as well as percentage root infection were taken as outlined in 3.1.3 and 3.2.2.5.
Chapter 4

Experimental Results