MATERIALS AND METHODS

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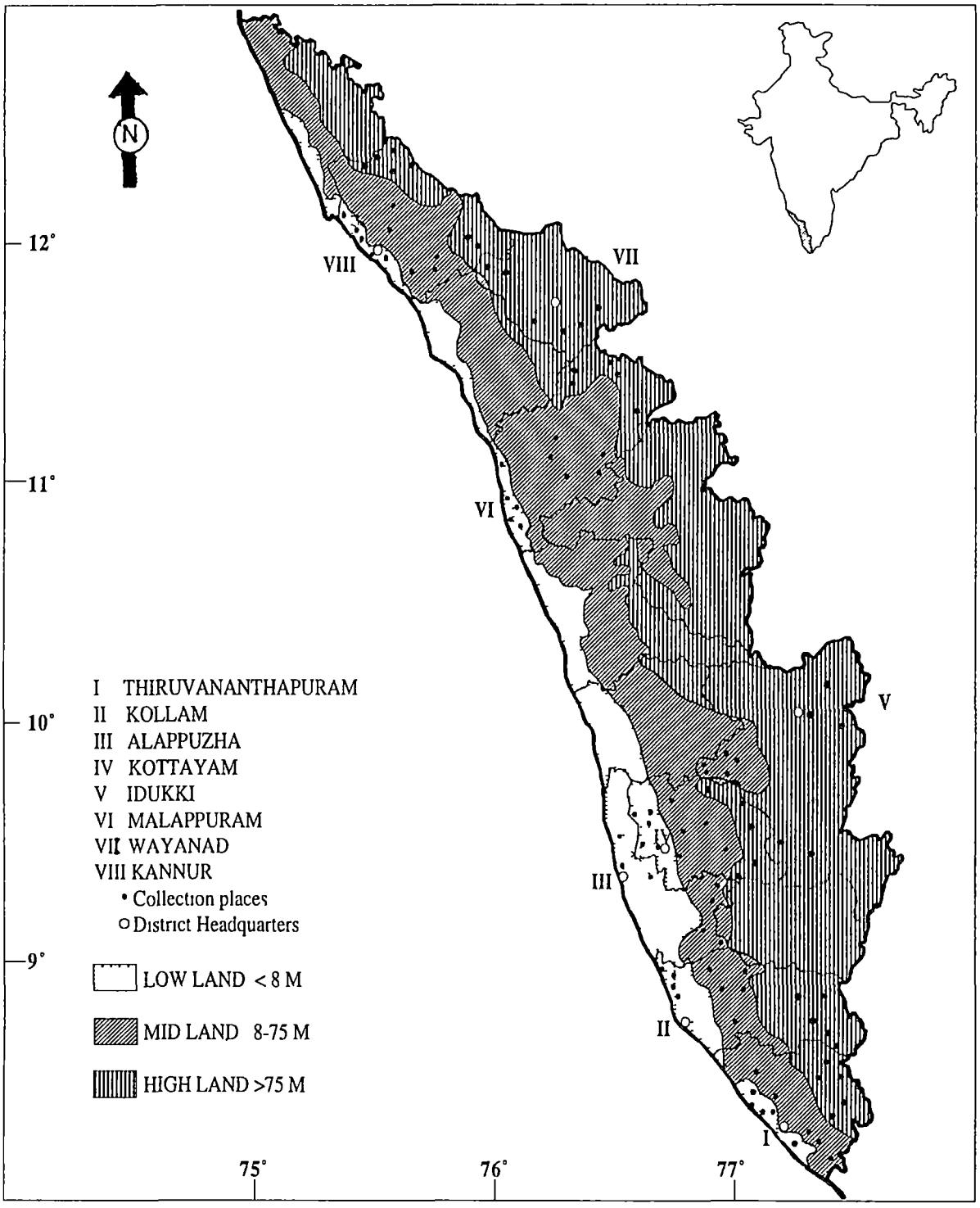
3.1. Study localities :

Eight districts of Kerala State, India namely Thiruvananthapuram, Kollam, Alappuzha, Kottayam, Idukki, Malapuram, Kannur and Wayanad (Figure-1) were selected for the study of mycorrhizal status of five different cultivars of mulberry (***Morus alba*** L.) belonging to the family of Moraceae and their colonizing VA-mycorrhizal fungi in relation to seasons. Kerala, a narrow segment in the south-western part of peninsular India, extends over a distance of 560 km along the west coast with width varying from 15 to 120 km. The physiographic regions of Kerala showing three generalised altitudinal zones namely, lowland (<8m, narrow strip lying parallel to sea coast), midland (8.75m central area with hills and valleys) and highland (>77m, eastern border comprising the high ranges of western ghats). The average annual atmompheric temperature during the years of study (1993-94) varied between 38.5°C and 18°C (max. 41°C; min. 16°C). The zone with the highest temperature was falls in the midland region. Along the coast, the temperature was moderate whereas in the east, it was low. The temperature variation due to the presence of sea in the west and high relief in the east has endowed the state with a unique agroclimate, favourable for cultivation of a wide variety of crops including mulberry.

The state of Kerala receives high rainfall amounting to an annual precipitation nearly 300cm and the major precipitation was between the months of December and January. March - May was the hottest when temperature reaches a

Figure 1 : Map of Kerala State, India.



maximum (>38°C). From June it gradually comes down due to heavy monsoon. Again an increasing trend was noticed in October and November followed by lower temperatures (<18°C) in the month of December and January (Atlas of Kerala, 1984).

3.2. Selection of sites :

Five cultivars of mulberry *(Morus alba* L.) namely MR2, Kanva 2, S_{13}, M_5 and Mysore local (Table-9; Plates-I-IV) were selected for the study of VAM fungi in relation to different seasons from three different ecosystems viz., lowland, midland and highland at eight districts of Kerala, India. At each ecosystem, five study sites were selected (Fig.1; Table 10-17). Site selection was based on the general soil characteristics. At each study site, an area of $500m^2$ was chosen for sampling. Five cultivars of mulberry plants were identifed with the help of "Genetic resources of mulberry", Environmental Resources Research Centre (ERRC), Thiruvananthapuram, Kerala.

3.3. Field sample collection - soil and root samples :

Root samples and soils from mulberry cultivars at lowland, midland and highland ecosystems in eight districts of Kerala state, India with tropical agro-climatic were sampled and analysed for the VAM fungal species in relation to four different seasons and for soil physico-chemical characteristics. At each ecosystem, five study sites were selected (Figure-1). At each study site, 3-5 vigorous appearing plants were selected for the study for collection of their roots and rhizosphere soil samples at 0-30 cm soil depth (Dickman *et al.*, 1984), during the late summer (January and February), south-west monsoon (June, July, August and September), north-east monsoon (October, November and December) and summer (March, April and May) seasons. All samples were collected by using a 13cm shovel. Each soil sample was placed in a plastic bag

that was sealed and brought to the laboratory and kept at 5-10°C (Koske and Halvorson, 1981). A portion of soil from each sample bag was utilized for the estimation of physico-chemical characteristics. Soil samples were collected at each site, mixed thoroughly and analysed for soil texture, pH, EC_{se}, N, P, K, Zn, Cu, Mn and Fe at soil testing laboratory. TRRI, Tamilnadu Rice Research Institute, Aduthurai, Tamil Nadu following standard methods (Jackson 1973; Sarma *et al.*, 1984). From the remaining portion, 100g was used to estimate VAMF spore numbers per sample bag. Root samples of five cultivars of mulberry at each site was collected, washed free of attached soil particles, cut into several small segments and fixed in FAA (Phillips and Hayman, 1970) in the field itself.

3.4. Estimation of VAMF root colonization :

The FAA fixed roots were first cut into smaller segments of 1 cm length, cleared and stained and then percent root colonization of each cultivar of plant species was estimated by the method of Phillips and Hayman (1970) as described below :

3.4.1. Trypan blue in lactophenol method :

The 1 cm long root segments were first washed thoroughly in distilled water and then placed in 10% KOH and heated to 90°C for 15-30 min. They were then washed in distilled water and immersed in alkaline 3% H_2O_2 for 5-10 min. Then, they were washed in distilled water and acidified with 5N HCl for 2-3 min. Then, the root segments were stained in 0.05% trypan blue in lactophenol for 15-30 min. and the excess stain was removed with clear lactophenol and observed.

Temporary mounts of root segments of each cultivar with gentle squashing on slides containing acetic acid : glycerol (1:1 v/v) were prepared and the coverslips

were sealed with nailpolish. They were then observed under a compound microscope using different magnifications for VAM fungal structures.

The percentage root colonization of the cleared and stained root segments were estimated (Krishna and Dart, 1984).

$$\text{Percentage of VAM root colonization} = \frac{\text{Number of VAM positive segments}}{\text{Total number of segments scored}} \times 100$$

3.5. Isolation and identification of VAM fungal spores and sporocarps :

The root-zone soils of five different cultivars of mulberry from different sites of lowland, midland and highland ecosystems of Kerala were analysed by the following method.

3.5.1. Wet-sieving and decanting technique (Gerdemann and Nicolson, 1963) :

100g of soil was suspended in 500 ml of water. The suspension was passed through a series of sieves of the following pore dimensions collecting the residue at each sieve 710, 400, 250, 106, 75 and 45 μm. The residue from 710 μm sieve was examined under a dissecting microscope for sporocarps and attached hyphae and spores on root pieces. The residues collected from 400, 250 and 106 μm sieves were also examined for sporocarps and large spores under the dissection microscope. The residues from 75 and 45 μm sieves were used for observing small and detached spores under a compound microscope. After preliminary observation, the residues from each sieve of the above mentioned sieves were collected in beakers of water and passed through circular whatman No.1 filter paper. The spores collected from the filter papers were spread on petridishes and examined under appropriate magnifications of a compound microscope for spore count. The spore number from the root-zone soil

sample of each cultivar was counted in replicates of 3-5, averaged and tabulated per 100g soil. For identification of spores and sporocarps, individual healthy ones were picked up from the filter paper, mounted on slides in lactophenol, observed under different magnifications of a compound microscope and photomicrographs were taken. The spores and sporocarps were identified using the synoptic keys of Gerdemann and Trappe (1974), Hall and Fish (1979), Schenck and Perez (1987).

3.6. Study of correlation between VAMF variables and physico-chemical characteristics of the soil :

Five cultivars of mulberry plant species identified at each ecosystem of eight study districts of Kerala was carried out. Pearson product moment correlation (Sokal and Rohlf, 1973) was estimated to examine the relationship, (a) between VAMF spore abundance and physico-chemical characteristics viz., pH, EC_{se}, percentage of organic matter, available N, P, K, Zn, Cu, Mn and Fe, (b) among the physico-chemical characteristics of the root-zone soils of five cultivars of VAM mulberry plant species. The roots were fixed in FAA, cleared and stained (Phillips and Hayman, 1970) and percent root colonization estimated (Krishna and Dart, 1984). The spores from root-zone soils were wet-sieved, decanted and counted (Gerdemann and Nicolson, 1963). All estimations done in triplicates and then averaged.

3.7. Pot experiments :

Pot experiments in sterilized soil have been reported to be valuable by providing much useful information on the role of VAMF in growth and mineral nutrition. Abbott *et al.,* (1984) by demonstrating the apparent differences in the effectiveness of VAM fungi. In this study, pot experiments using sterilized soils were performed for :

(a) bioassay-cum-inoculum production experiment and

(b) Mulberry plant growth experiments.

3.7.1. Host plants used :

For bioassay-cum-inoculum production experiment, ***Allium cepa*** L. was used as the host plant. For growth experimental studies, mulberry cultivars (***Morus alba*** L.) Kanva 2 and MR2 were used as the host plants.

Seeds of ***Allium cepa*** L. was obtained from Tamil Nadu Agricultural University (TNAU), Coimbatore, Tamil Nadu. Mulberry cultivars, Kanva2 and MR2 cuttings were obtained from sericulture unit, Thiruvananthapuram, Kerala. Seeds and cuttings were surface sterilized in 5% chloramine T for 5 min. and repeatedly washed in sterile water before sowing. Seeds were germinated in sterile petridishes between moist paper and the cuttings were rooted in moist sterile soil : sand (1:1 v/v).

3.7.2. VAMF inocula used :

For bioassay-cum-inoculum production experiment, six dominant native VAM fungal inocula were used. They were isolated from different sites of study localities.

For growth experimental studies, six native and two introduced VAM fungal inocula were used.

Indigenous VAMF isolates :

Glomus aggregatum	(Idukki isolate)
G. fasciculatum	(Wayanad isolate)
G. mosseae	(Kottayam isolate)
G. microcarpum	(Thiruvananthapuram isolate)
Acaulospora scrobiculata	(Malapuram isolate)
and *Gigaspora margarita*	(Alappuzha isolate)

Introduced VAMF isolates :

Glomus aggregatum	(Bangalore isolate)
and *Glomus fasciculatum*	(Rothamsted isolate)

The introduced isolates of ***Glomus aggregatum*** and ***Glomus fasciculatum*** were obtained from Dr. D. J. Bagyaraj, University of Agricultural Sciences, Bangalore, India. The Idukki, Kottayam, Wayanad, Thiruvananthapuram, Malapuram and Alappuzha isolates of ***Glomus aggregatum, G. mosseae, G. fasciculatum, G.microcarpum, Acaulospora scrobiculata*** and ***Gigaspora margarita***, respectively were isolated from the mycorrhizal roots of mulberry cultivars through bioassay experiments outlined below :

3.8. Bioassay for infection efficiency and confirmation of VAMF spores cum inoculum production :

In the search for efficient VAM fungal endophytes and confirmation of VAMF species, the associated dominant VAMF endophytes of mycorrhizal roots of mulberry cultivars in different localities were bioassayed in ***Allium cepa*** L. Among the VAMF endophytes associated with the roots of mulberry, only spores of ***Glomus aggregatum, G. fasciculatum, G. mosseae, G. microcarpum, Acaulospora***

scrobiculata and *Gigaspora margarita* were selected for bioassay, as these six indigenous species were found to be associated with greater number of mulberry host roots in lowland, midland and highland ecosystems of Idukki, Wayanad, Kottayam, Thiruvananthapuram, Malapuram and Alappuzha districts of Kerala. The spores of these six VAM fungal endophytes were isolated from the root-zone soils by wet-sieving and decanting technique (Gerdemann and Nicolson, 1963).

Experiment was conducted in 20cm diameter earthern-ware pots filled with 5kg of sandy loam soil : sand mixture (1:1 v/v) deficient in P (3mg P kg^{-1} soil extracted with NH_4 F and HCl, Olsen *et al.,* 1954) with a pH of 7.4. The pots containing the soils were sterilized by autoclaving twice at 1.1 kg/cm^{-3} pressure at 121°C for 1 hour on two consecutive days. Nitrogen as urea (35mg/kg soil) was applied as basal application to soil contained in the earthern-ware pots irrespective of the treatments. Uniform seedlings were transplanted to pots. Before transplanting the seedlings, mycorrhizal inoculum (5g/pot) consisted spores, hyphae and VAM plant root fragments, which was thoroughly mixed with sterile soil. The inoculum was placed 2cm below the soil surface before transplanting the seedlings. Plants without mycorrhizal inoculum served as control. The control treatments consisted of 5g per pot of inoculum extract filtered through Whatman No.1 filter paper, which was added to reintroduce the microbial inoculum population minus propagules of VAM fungi.

Ninety days after planting growth response was evaluated. Each plant was harvested, total fresh weight recorded and the dry weight was determined after drying at 70-80°C for 48 h. Mycorrhizal infection in the root was determined by clearing the roots in 10% KOH and staining with trypan blue (Phillips and Hayman, 1970). The percentage root colonization was calculated (Krishna and Dart, 1984). Mycorrhizal

spores in the root-zone soils were estimated by wet-sieving and decanting technique (Gerdemann and Nicolson, 1963).

3.9. Study of the effect of VAMF on growth and physiological parameters of mulberry cultivars :

Two set of experiments were conducted with the effect of indigenous and introduced and indigenous VAMF species on growth and nutrition of mulberry cultivars MR2 and Kanva2, respectively.

For the first growth experiment, the influence of indigenous VAMF species on growth and nutrition of mulberry cultivar MR_2 was undertaken and the second growth experiment was conducted the effect of both indigenous and introduced VAMF species on growth of Kanva2 cultivar.

Both experiments were undertaken in red sandy loam soil with moderate water holding capacity. 12 x 25 cm earthern-ware pots were filled with red sandy loam soil : sand mixture (1:1 v/v) with the pH of 7.8. The initial content of organic carbon and available N in soil as determined by standard methods (Jackson, 1973) were 0.937 (%) and 0.081 (%) respectively. The available phosphorus content of the soil as determined by the method of Olsen *et al.* (1954) was 6.6 mg Kg^{-1} soil. The experiment was laid out in a randomized block design. The pots containing the soils were sterilized by autoclaving twice at 1.1kg/cm^{-3} pressure at 121°C for 1h at two consecutive days. The experimental design was completely randomized block combination of seven different treatments. In the growth experiment, mulberry cultivar was involved MR_2 with indigenous VAM fungal species.

There were seven treatment combinations :

T1	Control (Non-mycorrhizal)	T5	***Glomus microcarpum***
T2	***Glomus aggregatum***	T6	***Acaulospora scrobiculata***
T3	***Glomus mosseae***	T7	***Gigaspora margarita***
T4	***Glomus fasciculatum***		

In the growth experiment of mulberry cultivar, Kanva2 was involved with both indigenous and introduced VAMF species. (There were seven treatment combinations :)

T1 Control (non-mycorrhizal)

T2 ***Glomus aggregatum*** (Idukki isolate)

T3 ***Glomus fasciculatum*** (Wayanad isolate)

T4 ***Glomus aggregatum*** and ***G. fasciculatum*** (Both Idukki and Wayanad isolates)

and

T2 ***Glomus aggregatum*** (Bangalore isolate)

T3 ***Glomus fasciculatum*** (Rothemsted isolate)

T4 ***G. aggregatum*** and ***G. fasciculatum*** (Both introduced isolates)

Each treatment was replicated six times. Hoagland nutrient solution (Sylvia and Schenck, 1983) without phosphorus was added for every 20 days to the plants. The plants were harvested after at 60, 90 and 120th days.

The following morphological and physiological parameters were studied in both growth experiments .

3.9.1. Morphological parameters :

Plant height, root length, leaf area and total shoot and root fresh weights were recorded and the plant dry weights were determined after drying at 70-80°C for 48h. The mycorrhizal infection in the root was determined by clearing the roots in 10%

KOH and stained with trypan blue (Phillips and Hayman, 1970). The percentage root colonization was calculated (Krishna and Dart, 1984). Mycorrhizal spores in the root-zone soils were estimated by wet-sieving and decanting technique (Gerdemann and Nicolson, 1963).

3.9.2. Estimation of chlorophyll content :

The chlorophyll content was estimated by the following Arnon's (1949) method. 5^{th} to 7^{th} standardised leaves from shoot tips were collected and washed thoroughly in distilled water and blotted dry using filter paper. They were cut into small pieces and 1g fresh leaf tissue was homogenised in 10ml of cold acquous 80% acetone. The extract was centrifuged at 1000 rpm for 5 min. and the supernatant was decanted and stored. The residue was again washed with 5.0ml of 80% acetone, centrifuged and the procedure was repeated until the pellet become colourless. The supernatants were pooled and the extract was made upto a known volume with 80% acetone. The absorbance was measured using spectronic-20 at 663 and 645 nm and the contents of chlorophyll a, b and total chlorophyll were calculated using the following formulae.

$$\text{Total chlorophyll (mg/g fresh wt.)} = 20.2 \times OD\ 645 + 8.02 \times OD\ 663 \times \frac{V}{1000 \times W}$$

$$\text{Chlorophyll a (mg/g fresh wt.)} = 12.7 \times OD\ 663 - 2.69 \times OD\ 645 \times \frac{V}{1000 \times W}$$

$$\text{Chlorophyll b (mg/g fresh wt.)} = 22.9 \times OD\ 663 - 4.68 \times OD\ 663 \times \frac{V}{1000 \times W}$$

Where OD = Absorbance at respective wavelength.

V = Volume of the extract (ml)

W = Fresh weight of the material (g)

3.9.3. Estimation of minerals :

Fifth to seventh leaves from shoot tips of each treatment were oven dried at 40°C for three days. The temperature was gradually increased to 60°C and kept constant for two days. Acid digestion of the dried and powdered sample (500 mg) was caried out using 20 ml of triple acid mixture (HNO_3 : $HClo_4$: H_2SO_4 - 7:2:1) in a Kjeldahl flask. The entire sample was digested in the digester till the extract become colourless. The colourless extract was transferred to a 100ml volumetric flask and made upto 100ml with distilled water. This sample was analysed for micro (Fe, Zn and Cu) and macro elements (N, P and K) at Tamilnadu Rice Research Institute (TRRI), Aduthurai, Tamil Nadu.

The leaf nitrogen and potassium were determined by using microkjeldahl and flame photometric methods (Jackson, 1973) respectively. The phosphorus content of leaf was determined colorimetrically by Vanadomolybdate phosphoric yellow colour method (Jackson, 1973). The micronutrients Fe, Zn and copper contents were measured in atomic absorbtion spectrophotometer (Ure, 1983).

3.9.4. Biochemical parameters :

Fifth to seventh leaves from shoot tips of each treatment were collected and washed carefully in water to remove the adhering soil particles and dried. Reducing and total sugars, proteins, free aminoacids and phenols were estimated in dried leaves, whereas the lipid content was estimated in fresh leaves and the known sample extracted with ethanal following the procedure outlined by Radhakrishnan *et al.* (1955).

Sl. No. 1.	Compound 2.	Extraction medium 3.	Experimental details 4.	Wavelength (nm) 5.	Reference 6.
1.	Reducing sugars	80% ethanol	Sample -10 ml 25% lead acetate - 1 ml 25% sodium carbonate - 1 ml Nelson-Somogi reagent - 1 ml Heated at 100°C on a water bath for 20 minutes, after cooling, 1ml of arsenomolybdate added. Total known volume made with distilled water. **Standard : Glucose.**	540	Warton and Mc Carty, 1972.
2.	Total Soluble sugars	80% ethanol	Sample - 10 ml 1N HCl - 3ml *Kept in water both* for 20 minutes. It was cooled and neutralised with 1 N NaOH - 3ml 25% lead acetate - 1ml 25% sodium carbonate - 1ml The sugar content was estimated by employing Nelson-Somogyi reaction as discribed above one. **Standard : Glucose.**	540	Warton and Mc Carty, 1972.
3.	Total proteins	Soluble in distilled water	10% TCA precipitated, centri-fuged, pellet, dissolved in 0.1 N NaOH. Sample - 0.5 ml 0 1% copper sulphate - 0.5 ml 12 5% sodium carbonate- 2.5 ml 25% folinphenol reagent - 0.5 ml Total volume made up to 5.0 ml and the reaction mixture kept in dark for 30 min. before OD measurement **Standard : Bovine serum albumin.**	660	Lowry *et al.* (1951)

1.	2.	3.	4.	5.	6.
4.	Total free aminoacids	80% ethanol	Sample - 1.0 ml 80% phenol - 1.0 ml Kept in boiling water bath for 10 min. 5% ninhydrin - 0.2 ml. Kept again in boiling water bath for 15 min. Total volume made upto 10ml with 60% ethanol. **Standard : Glycine**	570	Troll and Canan (1953)
5.	Total phenols	80% ethanol	Sample - 1 ml 20% sodium carbonate - 1 ml Folinphenol reagent : water (1:2) - 0.5 ml Kept at 100°C on a water bath for 10 minutes. Total known volume made with distilled water **Standard : Catechol**	660	Farkas and Kiraly (1962)
6.	Total lipids	Chloroform Methanol. 2:1 (v/v)	250mg of sample homogenized with extract solvent. Filtered through filter paper, filterate vortexed with sodium sulphate then it was taken in a preweighed beaker and dried by boiling. Dried extract weighed by substract in the initial weighed form, the final weight was expressed $\mu g/g^{-1}$ fresh wt.	-	Sato and Murata (1988)

3.9.4.1. Estiamation of acid and alkaline phosphatases in leaves of mulberry :

The procedure for the above estimation was outlined below :

Sl. No.	Compounds	Experimental details	Wavelength (nm)	Reference
1.	Acid phosphatase	Sample (cell free extract) - 0.5 ml 10 mM P-nitrophenyl phosphate (PNPP) in 0.1 M acetate buffer pH 4.0 - 0.5 ml 0.5 mM $MgCl_2$ - 0.1 ml Incubated for 30 min. at 30°C. Reaction terminated by addition of 5ml of 0.05 M NaOH. For control, no substrate was added. **Standard : P-nitrophenol**	405	Gianinazzi-Pearson and Gianinazzi (1976) modified by Krishna (1981).
2.	Alkaline phosphatase	Sample (cell free extract) - 0.5 ml 10 mM p-nitrophenyl phosphate (PNPP) in 0.05 M Tris-citric acid buffer pH 8.5 - 0.9 ml 0.5 mM MgCl2 - 0.1 ml Incubated for 30 min. at 30°C. Reaction terminated by addition of 5ml of 0.05 M NaOH. For control, no substrate was added. ***Standard : P-nitrophenol***	405	Gianinazzi - Pearson and Gianinazzi (1976) modified by Krishna (1981).

Note : All colorimetric readings were taken using spectronic-20 (Bauch and Lomb).

3.9.4.2. Determination of proteins qualitatively by SDS-PAGE (Sodium Dodecyl Sulphate - Poly Acrylamide Gel Electrophoresis) : (Laemmli, 1970).

Protein extraction :

The extraction procedure was performed at 4°C. 50mg of fresh leaf samples were extracted with 0.5M Tris buffer with pH 6.8. The suspension was filtered through cheescloth and centrifuged at 5000 rpm in gravity centrifuge for 5 min. Then ammonium sulphate was added (47.2g/100ml W/V yielding a 70% standard solution) as fine powder to the supernatant and stirred for 15 min. to precipitated the protein and

this was allowed to continued for further 30 min. Then the mixture was centrifuged at 10,000 rpm for 40 min. at 4°C and then poured the supernatant out then resuspended the pellet (which can be unable and is not always visible) in 0.5M tris buffer with pH 6.8, then the sample was subjected to SDS-PAGE.

SDS - PAGE reagents :-

a) Solubilizing buffer :

1M Tris-HCl, pH 6.8	- 60 µl
B-Mercapto ethanol	- 50 µl
10% SDS	- 200 µl
100% glycerol	- 100 µl
1mg ml^{-1} bromophenol blue	- 2 µl
(served as a marker dye)	

b) Seperating gel buffer :

4 x Tris-HCl	- 8.2 g
Distilled water	- 60 ml

Adjusted the pH to 8.8 with 1N NaOH make the final volume into 100ml distilled water.

c) Staking gel buffer :

4 x Tris-HCl	- 6.0 g
Distilled water	- 80 ml

Adjusted the pH to 6.8 with 1N NaOH make up the final volume into 100ml with distilled water.

d) Acrylamide stock :

Acrylamide	- 30.0g
N N' Methelene bis acrylamide	- 0.8g
Distilled water	- 100 ml.

e) Electrode buffer (stock) (or) Running buffer :

5 x Tris-HCl	- 15.1g
Glycine	- 72.0g
SDS	- 5.0g
Distilled water	- 1000ml

Diluted the stock to 1 x buffer, before used.

f) Protein stain :

Dissolved 0.25% coomassie brilliant blue - R 250 in 50% methanol and 7% v/v acetic acid.

g) Destaining solution :

Methanol	- 50ml
Acetic acid	- 7ml
Distilled water	- 43ml.

h) Storing solution :

Methenol	- 5.0ml
Acetic acid	- 7.5ml
Distilled water	- 87.5ml.

i) Standard proteins mixture :
(molecular weight markers)

Dissolved 1mg of following standard proteins in 1ml of 50mM $NaCl_2$ - 1mM sodium phospate, pH 7.0 solution

- Lactalbumins	- 14.2 kDa
Carbonic anhydrase	- 29.0 kDa
Albumin egg	- 45.0 kDa
Bovine serum ablumin	- 66.0 kDa

The slab-gel unit was thoroughly cleared and dried it. The gel plate was fixed appropriate spacers on the gel marker. To avoid the leakage, vaccum grease was applied both sides of the spacers. The volume of the gel was measured using distilled water.

The seperating gel solution was prepared (according to the volume required)

Reagent B	- 11.25ml
Reagent D	- 18.00ml
10% SDS	- 0.20ml
Distilled water	- 15.75ml
Degas the mixture and then added	
10% Ammonium persulphate (APS)	- 150 µl
NNN' N' - Tetra methyl ethylene diamine (TEMED)	- 30 µl

The above solution was poured into the plate upto the level such that 3 cm gap was allowed for staking gel. The air bubbles were removed and then added even layer of isobutanol on the top of the seperating solution, to get a flat surface on the top of the gel. Then allowed it to polymerize for 30 min. Then the isobutanol layer was removed and washed it with water.

Then the stacking gel solution was prepared (according to the volume required) by mixing of :

Reagent B	- 3.0ml
Reagent C	- 5.0ml
10% SDS	- 0.2ml
distilled water	- 12.0ml.
Degas the mixture and then added	
APS	- 100 µl
TEMED	- 20 µl

Then the comb was inserted in between the plates. The solution was poured carefully on the top of the separating gel and bubbles were removed. After 20 minutes, the bottom spacer was removed and the gel plate fixed with slab gel unit. The comb was removed and filled the well with running buffer (e). Equal volume of solubilizing buffer was added to the sample solution and boiled in a water bath for 3 min. Then about 300 µl of above sample mixture was loaded into each well and instead of sample the equal amount of standard protein mixture was loaded to any one of the well to compared the moelcular weight of the sample proteins. Then running buffer was added in anode and cathode chambers until the buffer touches the gel. The power supply was connected and applied 30V until the marker dye enter the separating gel and then increased the voltage 40 to 50. The power supply was continued until the marker dye reached the bottom of the gel. After reaching the marker dye, the power supply was disconnected and removed the slab gel set up. Then the glass plate was removed and placed the gel in coomassie brilliant blue R.250 stain (f) for 2 to 4 hours. Then the gel was destained in destaining solution (g) until clear back ground was obtained and the gel was stored in solution mixture (h).

3.10. Silkworm rearing experiment :

Mulberry leaves are the only source of food for the silkworm, ***Bombyx mori*** L. The growth and the development of larvae and subsequent cocoon production depends on the leaf quality, especially the nutrient contents. The leaf quality was assessed through silkworm rearing technique. Native mycorrhizal and non-mycorrhizal treated mulberry cultivar Kanva2 leaves were taken for the purpose of ***rearing*** performance study. The rearing trails were conducted following standard method (Krishnaswami, 1978) with LX NB_4 D_2 Silkworm ***(Bombyx mori*** L.) race with four

replicates. Observations on selected parameters of rearing viz., weight of 15 matured larvae, single cocoon weight, single shell weight and CSR value were recorded.

3.11. Microscopy and Photomicrography :

All measurements and characterization of the root squashes, spores and sporocarps were done using Carl-Zeiss (Germany) research microscope with stage and ocular micrometers. All photomicrographs including those of root squashes, spores and sporocarps were taken using Nikon optiphot microscope (Japan). Electrophoretic images were recorded in black and white (INDU *100 ASA*) and other images were recorded in colour (Konica *100 ASA*) film.

3.12. Statistical analysis :

All the results were statistically analysed and tested for critical difference at 0.05% level of significance by Duncan's Multiple Range Test (DMRT) (Gomez and Gomez, 1976). Pearson product moment correlation (Sokal and Rohlf. 1973) was estimated to examine the relationship between VAMF spore abundance and soil physico-chemical characteristics and between VAMF spore abundance and percent VAMF colonization.