BIOLOGICAL AND MOLECULAR VARIABILITY IN *GLOMUS MOSSEAE* ISOLATED FROM DIFFERENT AGRO CLIMATIC ZONES OF KARNATAKA

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BIOLOGICAL AND MOLECULAR VARIABILITY IN *GLOMUS MOSSEAE* ISOLATED FROM DIFFERENT AGRO CLIMATIC ZONES OF KARNATAKA

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CERTIFICATE

This is to certify that the thesis entitled "**BIOLOGICAL AND MOLECULAR VARIABILITY IN *GLOMUS MOSSEAE* ISOLATED FROM DIFFERENT AGRO CLIMATIC ZONES OF KARNATAKA**" submitted by **Mr. N. Mathimaran** in partial fulfilment of the requirement for the degree of **Master of Science (Agriculture) in Agricultural Microbiology** to the University of Agricultural Sciences, Bangalore, is a record of bonafide research work done by him during the period of his study in this University under my guidance and supervision and that no part of the thesis has previously formed the basis for the award of any degree, diploma, associateship, fellowship or other similar titles.

Bangalore,
October 1, 2001

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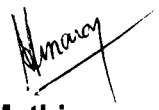

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INTRODUCTION

I. INTRODUCTION

The symbiotic associations of plant roots and fungi have intrigued many generations of biologists, and these associations were given the name mycorrhiza. It is derived from the Greek for fungus-root (Frank, 1885). Recent observations of fossil plants from the Devonian era suggest that one type of mycorrhizal association, the arbuscular mycorrhiza, existed approximately 400 million years ago, indicating that plants have formed associations with arbuscular mycorrhizal (AM) fungi since they first colonized land (Remy *et al.*, 1994).

AM fungi are distributed throughout the world in environments ranging from tropical rainforest to artic tundra (Janos, 1980). Individual species of AM fungi may be present in a diversity of habitats; they are also associated with plants grown in aquatic environment (Bagyaraj and Menge, 1978; Koske, 1987). The arbuscular mycorrhizal fungus *Glomus mosseae* (Nicol. and Gerd.) Gerdemann and Trappe, like many other AM fungi, are also known to occur globally in a broad range of dissimilar environments (Koske, 1987).

The study of AM fungi has fundamental and practical importance. First because in most environments "root biology" is actually "mycorrhizal biology", and second because of the practical importance of AM in fields as diverse as sustainable

agriculture, horticulture, silviculture and ecosystem management (Barea and Jeffries, 1995).

Glomus mosseae has been reported from citrus orchards in Florida, semi-arid shrub steppes Wyoming, forests in New Zealand and croplands in Libya (Johnson, 1977; Nemec *et al.,* 1981; Stahl and Christensen, 1982). For a species to be widely distributed in diverse environments it must be highly adaptable and species with extended geographic ranges almost always develop locally adapted populations or ecotypes (Heslop-Harrison, 1964). Ecotype differentiation has been reported for some species of AM fungi (Haas and Krikun, 1985; Hepper *et al.,* 1988), relatively little is known about geographic variation and physiological specialization in these organisms (Mosse *et al.,* 1981) Morphological differences among the isolates of AM fungi are not very clear for easier identification.

Many studies have reported that AM fungi are sensitive to environmental conditions, especially edaphic factors (Mosse *et al.,* 1981). This sensitivity seems to conflict with fact that individual species can be so widely distributed. Stahl *et al.,* (1990) provided evidence through uniform garden experiments that populations of such a broadly occurring fungus (*Glomus mosseae*) are physiologically different and adapted to local environmental conditions other studies also have indicated physiological differences among populations of *Glomus mosseae. Glomus mosseae* displays significant variation among populations for tolerance of heavy metals

(Gildon and Tinker, 1981), isozyme banding patterns (Hepper *et al.,* 1988) and mycorrhizal infectivity and effectivity (Haas and Krikun, 1985).

Molecular techniques, which provide valuable information on the magnitude of genetic variability within and between organisms of different species, have been developed. With regard to the obligate biotrophic AM fungi, which cannot be grown axenically, some of these techniques, such as conventional Restriction Fragment Length Polymorphism (RFLP) or karyotyping, appear unsuitable because of the amount of DNA required. The development of the polymerase chain reaction (PCR) provides the opportunity to work with small amounts of DNA (Erlich, 1989). One of the most successful approaches has been the analysis of the nuclear ribosomal RNA genes. These genes are found in high copy number and contain highly conserved coding regions interrupted by variable transcribed spacers. Parts of these genes have already been amplified in AM fungi using conserved, universal sequences as binding sites for the oligonucleotide primers (Simon *et al.,* 1992). Moreover, the sequencing of amplification products specific to AM fungi has allowed the design of specific primers which can discriminate between AM fungal symbionts and other fungi. However, these primers could not discriminate among related AM fungal species or different isolates of the same species (Simon *et al.,* 1992).

Plant growth response (efficiency) to mycorrhizal symbiosis depends on three major components, the plant, the mycorrhizal fungus and the soil environment.

Manipulating any of these three components can make an improvement in this efficiency. An essential requirement for the good host response to mycorrhiza is rapid colonization of roots during the early growth stages of the host plant. The fact that host plant benefits from the mycorrhizal association because of enhanced nutrient uptake is well established (Gerdemann 1968, Khan 1972). Consequently AM fungi often have positive quantitative effect on growth and vigour of their hosts (Maronek *et al.,* 1981).

The existence of variability in *Glomus mosseae* across different agro climatic zones of Karnataka has not been studied at molecular level and information available about the effect of these isolates on growth of crop plants is rather scanty. Hence, this study aims to identify the molecular variability in *Glomus mosseae* isolated from different agro climatic zones and its effect on growth of tomato with the following objectives:

1. To isolate and identify *Glomus mosseae* from different agro climatic zones of Karnataka,
2. To study the growth response of tomato to *Glomus mosseae* isolates,
3. To study the molecular variability of *Glomus mosseae* isolates using randomly amplified polymorphic DNA (RAPD) markers.

REVIEW OF LITERATURE

II. REVIEW OF LITERATURE

2.1 Mycorrhiza

Frank (1885) hypothesized that a mycorrhiza is a fungus in the root that functions to provide soil resources to the host in exchange for energy.

The majority of higher green plants and large numbers of species of fungi are involved in mycorrhiza formation. Harley and Harley (1987) found that, in the British flora, 80 per cent of the species of angiosperms, 100 per cent of the gymnosperms and 70 per cent of the pteridophytes were potentially mycorrhizal. That is, they may develop one or more kind of mycorrhiza in some circumstances.

The fungal species concerned with mycorrhiza belong to all the larger taxonomic divisions. About eight genera and more than 100 species of Endogonaceae amongst the Zygomycetes are mycorrhizal. At least 20 genera comprising several hundred species of Ascomycetes mainly Pezizales, but also Helotiales and Elaphomycetales, form ectomycorrhiza with gymnospermous and angiospermous trees; at least one species of Helotiales *(Hymenoscyphus)* forms mycorrhiza with Ericaceae. Of the Basidiomycetes, 5000 or more species of many genera of both Hymenomycetes and Gasteromycete are involved in ecto and ectendomycorrhiza of angiosperms and gymnosperms. Of the imperfect fungi, many reproductively sterile fungi, which may often be

basidiomycetous or ascomycetous, have been shown to form ectomycorrhiza, as has *Cenococcum geophilum*, which is very common and widespread on many hosts; and *Oidiodendron griseum* has been reported to form ericoid mycorrhiza (Couture *et al.*, 1983).

Majority of angiosperms, gymnosperms and pteridophytes, numerous bryophytes and a few species of algae associate with at least one quarter of the known species of fungi in non-pathogenic or mutualistic symbioses. Amongst these, mycorrhizas, although numerically fewer than the lichens in respect of the fungi involved, are very important to both green plants and fungi.

2.2 Arbuscular Mycorrhiza

Arbuscular mycorrhizas are symbiotic associations formed between a wide range of plant species including angiosperms, gymnosperms, pteridophytes, and some bryophytes, and a limited range of fungi belonging to a single order, the Glomales encompassing six genera into which 149 species have been classified (Morton and Benny, 1990). These mycorrhiza were called vesicular-arbuscular (VA) mycorrhizas on account of the characteristics intraradical structures that are formed, arbuscules being the "little tree"– like structures consisting of branched hyphae (Gallaud, 1905). Recently, there has been a concerted move to drop "vesicular" from the name, since not all of the fungal genera form vesicles (Walker, 1995). Also, emphasis on "arbuscular"

fungi is in accord with the prevailing view that the symbiotic function is due to the activity of arbuscules.

2.3 Importance of Arbuscular Mycorrhizal Fungi

Improved plant growth due to inoculation of the soil with AM fungi has been demonstrated especially under P deficient conditions (Mosse, 1973; Bagyaraj and Padmavathy, 1993). In addition to enhanced P uptake, AM fungi have been reported to improve the uptake of other elements like N and Ca (Ross and Harper, 1970), Zn and Cu (La Rue *et al.,* 1975; Krishna *et al.,* 1982) K (Powell, 1975). AM fungi are also known to be involved in increased water uptake (Allen, 1982). AM fungi also protect plants from root invading organisms, such as parasitic nematodes and phytopathogenic fungi since the invasion of root cortex by AM fungi not only reduces but also prevents colonization of that zone by pathogens (Bagyaraj, 1984).

2.4 Benefits of Arbuscular Mycorrhizal Association

In a natural ecosystem the role of AM fungi is well documented, the fact that they promote the growth of plants is overlooked. The abundance of data obtained from experiments conducted in both field and green house conditions suggest that the plant growth can be substantially increased if they possess a well-developed mycorrhizal system. Over the past three decades, observations form green house investigations reveal that AM fungi are

associated with most of the agricultural crops and enhance the plant growth in turn increase in yield (Powell and Bagyaraj, 1984)

Some of the benefits conferred by arbuscular mycorrhiza include (a) Improved uptake of through macro and micronutrients (b) Increased tolerance to abiotic stresses and (c) Beneficial alterations of plant growth regulators (Jarstfer and Sylvia, 1993). All these benefits are resultant of the complex and dynamic interactions occurring between the fungi and host roots.

2.5 Uptake of P and Other Nutrients

Studies conducted by Sanders and Tinker (1978) explained the mechanism of P uptake from soil by AM fungi. The hyphae of mycorrhizal fungi provide better distribution surface for absorbing P from the soil solution than roots alone.

The external hyphae of AM allow the root system to exploit greater volume of the soil P by (a) extending away from the root and translocating P from as far as 8 cm (Rhodes and Gerdemann, 1975); (b) by exploiting smaller soil pores as AM fungal hyphae are less than 20 per cent of the diameter of the root hairs. (c) By adding surface area to the absorptive system (Sanders and Tinker, 1978).

Inoculation of Glomus mosseae increased zinc uptake by subterranean Clover at low rates of phosphorus uptake (Pairunan *et al.,* 1980). Crops like

cowpea, Cotton and Finger millet when inoculated with AM fungus, *Glomus fasciculatum* had higher P and Zn contents as compared to uninoculated plants (Bagyaraj and Manjunath, 1980).

The results of experiments conducted with P^{32} labelled PO_4 indicate that hyphae of AM fungi obtain their extra PO_4 from the labile pool rather than by dissolving insoluble PO_4 (Raj *et al.*, 1981). According to Plenchette *et al.*, (1983), when Apple, Teak and *Tagetus patula* plants were inoculated with mycorrhizal inoculum, contained more tissue P than uninoculated plants. The arbuscular mycorrhizal hyphae have a role in the translocation of PO_4 and N from the soil zones several centimeters away from roots in cough grass or white clover (George *et al.*, 1991).

Safir *et al.*, (1972) for the first time reported the mycorrhizal effect on plant water relation in soybean plants. Mycorrhizal Soybean showed lower resistance to water transport that the non-mycorrhizal plants. Onion plants inoculated with *Glomus etunicatum* were more drought tolerant than non-mycorrhizal plants (Nelson and Safir, 1982). Allen (1982) suggested that increase in water uptake in mycorrhizal plants might be due to increase in surface area provided by the hyphae.

Pai *et al.*, (1993) conducted pot culture experiments to study the effect of moisture stress on growth and water relations of cowpea inoculated with

Glomus fasciculatum at two levels of P for non-mycorrhizal and AM plants at 3 moisture levels. The results showed that AM inoculation increased dry matter at all levels of moisture stress.

Mycorrhizal inoculation was found to reduce the root knot nematode infestation in tomato. Inoculation of mycorrhiza first followed by nematodes was found to reduce nematode infestation better than the simultaneous application of mycorrhiza plus nematodes or nematodes first followed by mycorrhiza. Mycorrhizal plants had higher quantities of phosphorus, potassium, calcium, total and reducing sugars, and amino acids phenylalanine and serine (Suresh *et al.,* 1984).

2.6 Effect of Arbuscular Mycorrhiza on Plant Growth

Mycorrhizal infection has been shown to increase flower bud production in pepper (Dodd *et al.,* 1983), and seed production in a number of species including soybean, *Abutilon theophrasti,* tomato, barley, oats and *Vulpia* (Schenk and Smith 1982; Jensen 1982,1983; Bryla and Koide 1990a; Carey *et al.,* 1992). In each case, positive effects of mycorrhizal infection on plant reproduction were associated with increases in phosphorus uptake and growth. Faster growing mycorrhizal plants may thus flower before slower growing non-mycorrhizal plants (Bryla and Koide, 1990a). Moreover, terrestrial plants are modular organisms and growth involves the reiteration of modules such as

branches. With this reiteration comes the possibility for more flowers and increased vegetative reproduction.

Increased flowering caused by mycorrhizal infection in *Abutilon theophrasti,* for example, was associated with increased branching. In addition to increasing plant fecundity, mycorrhizal infection can also increase the quality of plant offspring, both of which contribute to long-term reproductive success.

Koide and Lu (1992) showed that maternal plant mycorrhizal infections in wild oats caused offspring plants to have greater leaf areas and root shoot ratios than offspring from non-mycorrhizal maternal plants. Moreover, mycorrhizal infection of the first generation of plants increased the phosphorus concentration of seeds produced by the second generation. These persistent maternal effects of mycorrhizal infection, coupled with the mycorrhizal effect on fecundity, suggest that mycorrhizal fungi may exert a strong effect on plant fitness.

It has frequently been demonstrated that mycorrhizal infection can improve vegetative growth, but very little attention has been paid to its effect on plant reproduction. If mycorrhizal fungi do increase plant reproduction, they may control the size, rate of growth and persistence of plant populations and, therefore, the structure of plant communities. By "structure" of the community we mean the species comprising the community, their relative

abundance, and their temporal and spatial patterning within the community. In addition to the general effects of mycorrhizal infection on plant growth and reproduction, which could affect the overall density of the community, there are a number of other ways in which mycorrhizal fungi and plant communities may interact. These include differential mycorrhizal effects on the plant species comprising the community, variation in response to infection caused by variation in plant density, alteration of the outcome of plant competition caused by the mycorrhizal symbiosis, and interactions between mycorrhizal fungi and other symbionts.

2.7 Response of Tomato to AM Inoculation

Various workers have studied responses of tomato to inoculation of AM. Edathil *et al.,* (1996) have studied interaction of multiple AM fungal species on root colonization, plant growth and nutrient status of tomato seedlings. Tomato seedlings were grown in sterile, phosphorus deficient soil and inoculated with 4 species (*Glomus aggregatum, Glomus fasciculatum, Glomus geosporum and Glomus sinuosum*) of arbuscular mycorrhizal fungi in 15 possible combinations. Mycorrhizal plants exhibited a significantly higher shoot length and biomass, than non-mycorrhizal plants. AM also increased host tissue N and P concentration. Among the AM combinations, inocula containing all four

endophytes promoted markedly better shoot length and biomass than other combinations, although spore yields were lower in mixed inocula.

Silveira *et al.,* (1996) have studied various aspects of the application of VAM fungi to increase tomato productivity and their effect on plant growth and interactions with bacterial, fungal and viral pathogens, nematodes and other rhizosphere organisms. Iqbal and Mahmood (1998) have tested effect of AM fungi on tomato. *Glomus mosseae* resulted in the maximum growth of plants followed by *Glomus constrictum* and *Glomus fasciculatum.* Different combinations of the mycorrhizal fungi also resulted in varying increases in the growth of tomato plants.

2.8 Differential Host Response to Mycorrhizal Infection

The most extreme example is that the non-mycotrophic species cannot be directly benefited at all from mycorrhizal infections. Thus, mycorrhizal fungi may help to control the relative importance of species within a community. This hypothesis was first set forth by Janos (1980). It is well established that manipulation of soil phosphorus availability can alter the relative abundances of the component plant species because some species are more responsive to phosphorus inputs than others (Huenneke *et al.,* 1990). In much the same way, different plant species respond to mycorrhizal infections to different extents. This difference in the growth of a plant species with

mycorrhizal fungi as compared to non-mycorrhizal plants is known as plants mycorrhizal dependence. Variation among mycotrophic plant species in their mycorrhizal dependency is largely thought to be a function of their level of phosphorus deficiency. Those plants experiencing the greatest degree of phosphorus deficiency are generally those, which respond most favourably to mycorrhizal infection. In addition to external factors that influence phosphorus availability, variation in phosphorus deficiency can be controlled by the rate at which the plant itself can acquire phosphorus from the soil to supply its demand for growth and reproduction (Koide, 1991a). Since phosphate in the soil solution diffuses slowly, the number and length of root hairs and the total length of root systems are often good indicators of phosphorus supplying power.

Baylis (1972) indicated that variation in root hair length would be likely to lead to variation in response to mycorrhizal infection. A similar conclusion was drawn from a comparative study of several wild tomato accessions (Bryla and Koide, 1990b). A detailed study of the relationship between the degree of a plant's response to colonization by mycorrhizal fungi, also known as a plant's mycorrhizal dependency, and the rooting strategy and phenology has been carried out for 23 tallgrass prairie forbs (Hetrick *et al.*, 1992).

Using cluster analysis, the forbs could be divided into three groups based on their mycorrhizal dependence, root morphology and phenology. Stepwise regression models were constructed to predict the mycorrhizal dependence and were based largely on data of root fibrousness. The relationship between the mycorrhizal dependency and root fibrousness was relatively independent of phosphorus supply in two groups while in the other group this was affected by phosphorus supply. Thus, inherent variation in root morphological characters may contribute to variation in phosphorus supplying power and thus to variation in responsiveness to mycorrhizal infection in some cases.

2.9 Morphology and Molecular Biology of AM Spore

Asexual spores are regarded as the dominant propagules for AM fungi. Tommerup and Sivasithamparam (1990), however, observed that *Gigaspora decipiens* produced zygospores under natural and laboratory conditions, in both the presence and absence of a host plant. Their taxonomy has mostly relied on the morphology of the resting spores, the creation of the new order of Glomales (Morton and Benny, 1990) and the description of six genera and at least 130 species therein (Walker, 1992) are based principally on the spore structure.

Morton *et al.,* (1995) identified a set of hierarchically ordered morphological characters (spore wall, presence of layers and laminae, colour,

ornamentation, size) and used them to propose a model allowing the comparison of closely related groups. Spores have also allowed evaluation of molecular characters and mapping of the phylogenetic relationships between AM fungi.

Analysis of the small ribosomal subunit rRNA (SSU) from 12 isolates of Glomalean fungi corroborated the validity of the taxonomic grouping, and allowed Simon *et al.*, (1993) to construct a molecular clock and estimate the divergence times between Glomalean families and genera.

Spores of AM fungi remain in the soil for years without losing their ability to germinate and infect roots. The organization and composition of their wall offer an ultra structural key to this long-term viability. Sporopollenin is present in the spore wall of *Glomus versiforme* as a thin layer between the primary wall, characterized by parallel chitin fibrils, and the secondary helicoidal wall. The chemicophysical characteristics of this layer probably provide a substantial barrier against soil microorganisms (Tommerup, 1983).

One of the most significant features of the spores of AM fungi is their multinucleate condition, e.g., 2000 nuclei in *Gigaspora margarita* (Becard and Pfeffer, 1993). Staining with DAPI reveals many small nuclei about 2μm in diameter among the lipid globules, and often crowded near the spore wall.

Ultra structural examination shows a loose chromatin and a well-defined nucleolus.

Quantification of the nuclear DNA content by static cytofluorimetry indicates a genome size of 0.26 and 0.77 pg DNA/nucleus for *Glomus versiforme* and *Gigaspora margarita* respectively (Bianciotto and Bonfante, 1992). For these reasons, spores have also been a crucial source of DNA for the construction of genomic libraries (Franken and Gianinazzi-Pearson 1996; Zeze *et al.,* 1994).

Molecular techniques have provided new information on the genome structure: highly repeated DNA sequences have been found in *Scutellospora castanea,* where a sequence of about 1200 bp constitutes 0.24 per cent of the whole genome (Zeze *et al.,* 1996). The sequencing of ribosomal genes has led to exciting results: sequences from the same spore are heterogeneous, as many as three sequences from the same spore of *Glomus mosseae* have been found (Sanders *et al.,* 1995). A different approach was used by Longato and Bonfante (1997), who screened the genome of three AM fungi by using short repeated sequences as primers in PCR experiments, and revealed the presence of micro satellite regions already demonstrated for many other organisms.

An important step forward in the study of the conditions required for spore formation has come from the elaboration of a simple and reproducible dual culture system with *Gigaspora margarita* and hairy roots transformed by the

T-DNA of *Agrobacterium rhizogenes* (Becard and Fortin, 1988). Starting from 3 spores which were used as initial inoculum, 450 spores were harvested and demonstrated to be highly correlated with the infection units. Another important step has been the use of *Glomus intraradices* (St-Arnaud *et al.,* 1996) to achieve controlled and sustained spore production.

2.10 Molecular Markers

The prerequisite of genetic analysis is to have tools that can discriminate between biological entities with different genetically determined characters. Classically this has been done using morphological, pathogenic, mating type and physiological criteria to distinguish between species and races. With filamentous fungi, even discrimination at the species level using these traits can be very difficult and often gives erroneous results (Meyer *et al.,* 1992). At even lower taxonomic levels, among isolates of a single species from different populations, suitable distinguishing features may be difficult or impossible to find. Molecular markers, however, can be applied at these levels with great reliability, and they allow simultaneous measurement of variability at multiple loci in each individual tested. The methods for collecting molecular data for fungal evolutionary studies have been the subject of several recent reviews (Bruns *et al.,* 1991; Kohn, 1992). The nature and applications of different categories of markers are summarized below. Deciding the most appropriate

technique for addressing a particular question depends upon the extent of genetic polymorphism, the analytical or statistical approaches available, and the time and material cost of possible techniques (Parker *et al.,* 1998).

With the advent of molecular markers, a new generation of markers has been introduced over the last two decades, which has revolutionized the entire scenario of biological sciences. DNA based molecular markers have acted as versatile tools and have found their own position in various fields like taxonomy, physiology, embryology, genetic engineering etc.

The discovery of Polymerase Chain Reaction (PCR) was a landmark in this effort and proved to be unique process that brought about a new class of DNA profiling markers (Mullis, 1987). This facilitated the development of marker based gene tags, map based cloning of important genes, variability studies, phylogenetic analysis, synteny mapping, marker assisted selection of desirable genotypes etc. These DNA markers offer significant advantages with respect to increased numbers of loci detectable and mapped, overall phenotypic neutrality and the ability to score the plant and animal at any developmental stage (Caetano-Anolles and Trigiono, 1997; Joshi *et al.*, 1999).

Various types of molecular markers are utilized to evaluate DNA polymorphism (Paterson *et al.,* 1991). These are generally classified as hybridization based markers and PCR based markers like Restriction Fragment

Length Polymorphism (RFLP), Restriction Landmark Genomic Scanning (RLGS), Single Strand Conformation Polymorphism (SSCP), Variable Number of Tandem Repeats (VNTR), Randomly Amplified Polymorphic DNA (RAPD), Amplified Fragment Length Polymorphism (AFLP), Randomly Amplified Microsatellite Polymorphism (RAMPO), Sequence Characterized Amplified Regions (SCAR) have the maximum potential for tracing the genetic diversity in natural populations. These techniques are capable of resolving individual genomes and preferred methodologies for biodiversity and population genetic studies (Joshi *et al.*, 1999).

In hybridization based markers DNA profiles are visualized by hybridizing the restriction enzyme digested DNA to a labelled probe, which is a DNA fragment of known origin or sequence, PCR based markers involved in vitro amplification of particular DNA sequences or loci with the help of specifically or arbitrarily chosen oligonucleotide sequences (primers) and a thermostable DNA polymerase enzyme (Saiki *et al.*, 1988). The amplified fragments are separated electrophoretically and banding patterns are detected by different methods such as staining and autoradiography (Caetano-Anolles *et al.*, 1990).

2.10.1 Allozymes

Allozymes are alternative enzyme forms encoded by different alleles at the same locus, which can be used as informative genetic markers. In diploid organisms, most allozymes exhibit standard co dominant Mendelian inheritance. Multiple polymorphic loci can be surveyed and typically two or three alleles are detected at each locus. Randomly sampled allozyme loci are generally accepted to be of independent genetic origin. The analysis is economic and efficient for screening large numbers of isolates. When enough polymorphic loci are available for a given sample set, the technique can be a good source of useful characters for classifying organisms at population, race/type and species levels (Burdon and Roelfs, 1985a; Leuchtmann and Clay, 1989; Elias and Schneider, 1992; Damaj *et al.,* 1993). Thus, DNA-based markers are becoming a more common choice of data source for resolving genetic issues at a range of taxonomic levels.

2.10.2 Restriction Fragment Length Polymorphisms (RFLPs)

The first DNA-based markers to be developed were RFLPs. An RFLP may be the result of length mutation, and/or point mutation at a restriction enzyme cleavage site at a given chromosomal location. RFLPs can be detected by analyzing restriction digests of genomic DNA through Southern hybridization. The probes used in RFLP analysis can be generated from cloned

genomic, cDNA or mtDNA fragments, or from specific DNA segments amplified using polymerase chain reaction (PCR). Thus, depending on the probe used, RFLPs can be used to analyze mtDNA variation, ribosomal (r) DNA region variation, repetitive and single-copy sequence variations.

RFLPs are codominant markers. This makes them suitable for population genetic studies as well as for linkage map construction. By employing probes that detect multiple loci and dispersed repetitive sequences, the sensitivity of the RFLP method can be enhanced to fingerprinting resolution (Hamer, 1991; Goodwin *et al.,* 1992a; Zeze *et al.,* 1996). In addition, synthetic simple repeat oligonucleotides can also be used as fingerprinting probes (Meyer *et al.,* 1991). For the rice blast fungus *Magnaporthe grisea,* for instance, several dispersed repetitive sequences have been isolated and thoroughly characterized, and the markers derived from them have been widely used in pathotype diversity analysis and genetic mapping of the fungus (Hamer 1991; Levy *et al.,* 1991).

Amplified fragment length polymorphism (AFLP) analysis is a new method that also offers fingerprinting resolution in fungi (Majer *et al.,* 1996). RFLPs can be converted to AFLPs by ligating adaptors for PCR amplification. The method offers the potential to detect large numbers of amplification products. Although this method does not target specific areas of the genome

for marker identification, the large number of loci that can be analyzed in a single experiment greatly improves the chance of identifying markers linked to the chosen genetic locus (Vos *et al.,* 1995). In plant studies it has become a favored tool for linkage mapping of resistant genes and other traits (Keim *et al.,* 1997).

DNA fingerprinting provides a powerful tool for population studies of asexually reproducing fungi because it can be used to distinguish different clonal lineages in populations with a high degree of certainty (Goodwin *et al.,* 1992a). However, the use of DNA fingerprinting beyond the identification of individuals, such as in estimation of divergence and quantitative measures of genetic diversity and similarity, must be done with caution. The problems may arise due to potential inter-dependence among characters in pair wise comparisons among isolates, and the difficulty of ascertaining allelism among fragments (Lynch, 1990; Parker *et al.,* 1998).

DNA hybridization-based RFLP analysis requires the isolation of large amounts of purified DNA. With PCR, it becomes possible to analyze specific sequences from small amounts of tissue. The advantages of PCR-RFLP lie in its speed, sensitivity and specificity. PCR can be performed on crude DNA extracts with a pair of region-specific primers. Variation of the amplified fragment can be further analyzed by restriction enzyme digestion and

electrophoretic separation. The applications of PCR technology in fungal research are almost countless. The regions most commonly examined by PCR-RFLP are the rDNA sequences (Henson and French, 1993).

In fungi, as in other eukaryotes, rRNA genes are repeated up to several hundred times in a clustered manner. In each rDNA repeat, two internal transcribed spacers (ITS) separate the 18S, 5.8S and 28S rRNA genes. The rDNA sequences encoding 18S and 28S RNAs show slow evolutionary change and can thus be used to compare distantly related organisms (Berbee and Taylor, 1993; Simon *et al.,* 1993; Begerow *et al.,* 1997; Holst-Jensen *et al.,* 1997). The ITS region and the intergenic spacer of the rDNA repeat evolve much faster and sequence differences in these regions frequently occur between closely-related species or even between populations of the same species (Buchko and Klassen, 1990; Erland *et al.,* 1994; Lovic *et al.,* 1995). Thus, analysis of the rDNA region is very useful for comparisons over a wide range of taxonomic levels and it has a high resolving power, depending on which part of the rDNA repeat is analyzed. The rDNA sequences have been determined for a large number of eukaryotes. This allows the design of primers that are specific for a group of species, genera, or families (Gardes and Bruns, 1993; Gargas and Depriest, 1996).

2.10.3 Random Amplification of Polymorphic DNA (RAPD)

Variation within species can also be assayed using the RAPD method (Welsh and McClelland, 1990; Williams *et al.,* 1990), in which arbitrary short oligonucleotide primers, targeting unknown sequences in the genome, are used to generate amplification products that often show size polymorphisms within species. RAPD analysis offers the possibility of creating polymorphisms without any prior knowledge of the DNA sequences of the organism investigated. The patterns produced are highly polymorphic, allowing discrimination between isolates of a species if sufficient numbers of primers are screened.

The method is fast and economic for screening large numbers of samples. However, some researchers are critical of the sometimes-poor reproducibility of RAPD patterns. Inter-laboratory comparison of RAPD patterns may not always be applicable since the RAPD patterns can be influenced by many technical factors (Penner *et al.,* 1993). This implies that diagnostic RAPD markers specified purely by mobility/size may not be confirmed by another laboratory, thus posing uncertainty for data cross-referencing in race/type identifications. For any RAPD marker to be used as a diagnostic tool by a wider group of researchers, it is necessary to characterize it

more thoroughly, through isolation, cloning and sequencing to generate either probes or specific primers for future applications.

The main limitation of RAPD analysis in population studies is the dominant character of RAPD markers. In the study of diploid organisms, homozygote *AA* can not be distinguished from heterozygote *Aa*, since both will give a RAPD pattern with a band, corresponding to *A*. Thus, allele frequencies and basic population genetic parameters cannot be estimated directly. When only diploid material is available, frequencies of RAPD fragments are sometimes deduced from the frequency of the null homozygote, *aa*, assuming the population is in Hardy-Weinberg equilibrium. However, both theoretical modeling (Lynch and Milligan, 1994) and empirical data (Isabel *et al.*, 1995) have shown that this indirect approach may give very biased results, especially when the sample size is small. More individuals (2 - 10 times more), and more loci, are needed than for codominant marker analysis, to compensate for the lack of complete genotype information caused by dominance (Lynch and Milligan 1994). On the other hand, RAPD analysis is well suited to population studies in haploid organisms since there is no loss of genetic information caused by the dominant inheritance of the RAPDs.

2.11 Application of RAPD in Arbuscular Mycorrhizal Research

Studies on the biodiversity and the phylogeny of AM fungi are dependent on their clear identification and differentiation. Until recently, the evaluation has relied mainly on the morphology of the asexual spores. This is, however, dependent on environmental factors and the physiological state. Molecular methods provide an alternative for a more precise analysis that can in addition also be applied in the absence of spores. Since in the case of the AM fungi the amount of available material is very limited, most methods are based on enzymatic amplification of DNA by the polymerase chain reaction (PCR). As can be seen in the following, this allows work even with single spores or analysis of complex mixtures of molecules as in the association with the plant.

The application of PCR normally needs information of at least short sequence stretches for the design of oligonucleotides. Random amplified DNA polymorphism (RAPD) is not dependent on this, because short randomly chosen primers are used for this technique. PCR with such primers can lead to species-specific banding patterns from minute amounts of DNA as template. Wyss and Bonfante (1993) could conduct between 30 *(Glomus versiforme)* and 120 *(Gigaspora margarita)* reactions from one single spore and obtained depending on the primer choice up to 14 fragments. Based on this, they could

clearly distinguish not only between species, but also between different isolates. The disadvantage of this method is the low reproducibility and the high sensitivity for contamination. Lanfranco *et al.,* (1995) overcame this problem by cloning a RAPD fragment indicative for *Glomus mosseae* isolates. Based on the sequence, they designed primers for specific amplification of DNA of this fungus not only from spores, but also from mycorrhizal roots. So they showed that the RAPD approach could lead to clear and reproducible identification of a certain AM fungus in low amounts and complex samples of DNA. The same primer pair has been later used to quantify the degree of *Glomus mosseae* colonization of leek roots by competitive PCR using an internal standard (Edwards *et al.,* 1997). In a similar approach, two other primer pairs served for specific identification of two different AM fungal species in parallel using DNA extracted from spore mixtures or from infected roots (Abbas *et al.,* 1996).

A modification of the RAPD technique for finding polymorphic loci is the AFLP method (Amplified Fragment Length Polymorphism). This technique was originally developed to detect polymorphism in bacteria and plants, but Rosendahl and Taylor (1997) showed that it is possible to apply it also to the DNA of a single AM fungal spore. The extracted DNA was digested with a restriction enzyme; the resulting fragments were coupled to a linker and amplified with oligonucleotides binding to this linker. This mixture

of PCR products served for further amplification obtaining reproducible polymorphic patterns characteristic for each starting spore. The results showed that there is differences between single spore of a certain isolate and suggested that AM fungi reproduce clonally without showing recombination.

2.12 Population Genetic Variability

Genetic variability in a population can be measured in terms of gene and genotype diversity. Gene diversity is a function of the number and frequencies of alleles at each locus, $h = 1 - 3\ x_i^2$, where x_i is the frequency of the i-th allele (Nei, 1987). When multiple loci are sampled, mean gene diversity can be estimated across different loci. Genotype diversity is a function of the numbers and frequencies of multilocus genotypes. Genotype diversity can be quantified by a normalized Shannon's diversity index as described in Goodwin *et al.,* (1992b), $Hs = - 3\ P_i \ln P_i / \ln N$, where P_i is the frequency of the i-th multilocus genotype and N is the sample size. Values for Hs range from 0 to 1. The maximum possible value for Hs occurs when each individual in a sample group has a different multilocus genotype.

The amount of genetic variation being maintained within a population may indicate how rapidly a pathogen can evolve and adapt to changing environments. It has emerged from genetic variability analysis based on molecular markers that many populations of sexually reproducing fungi

possess a large amount of genetic variation even on a small scale. Both somatic mutation and sexual reproduction can lead to increased genetic diversity in a population, and it is difficult to distinguish between the two phenomena at marker loci (Milgroom, 1996). However, by analyzing the genetic similarity among multilocus genotypes it is possible to identify, at least tentatively, the main cause. To estimate the contribution of sexual versus asexual reproduction to population variability, genotype diversity and gametic disequilibria tests can be useful indicators (Milgroom, 1996). Sexual reproduction produces recombinant genotypes and frequent recombination causes random association of alleles at different loci. In asexual organisms, all of the loci in the asexual progeny are completely associated, a state called gametic disequilibrium. In random mating populations, where genotype frequencies are not distorted by differential asexual reproduction, gametic equilibrium is to be expected (Burdon and Roelfs 1985; Keller *et al.*, 1997).

MATERIAL AND METHODS

III. MATERIAL AND METHODS

The experiments to study the Biological and Molecular Variability in *Glomus mosseae* were conducted at the Department of Agricultural Microbiology, University of Agricultural Sciences, G.K.V.K. Campus, Bangalore. The material used and methods followed are described below:

3.1 Collection of soil samples from different agro climatic zones of Karnataka

Karnataka state is divided into ten agro climatic zones on the basis of annual rainfall, soil type, cropping pattern and other climatic conditions. The details of the zones taken for the study are given in Table 3.1 and Fig. 3.1. In order to study the biological and molecular variability in *Glomus mosseae* in the soils of different agro climatic zones of Karnataka, soil samples were collected from each agro climatic zone. Four sampling sites were selected randomly for each agro climatic zones.

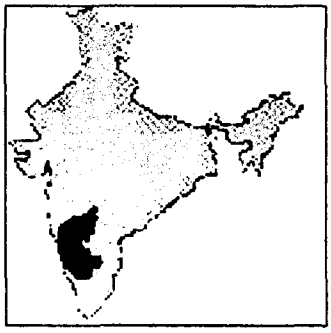
3.1.1 Soil sampling

Four soil samples of 500 grams each were collected randomly from top six-inch layer of soil from each agro climatic zone and packed in polyethylene bag. They were transferred to Department of Agricultural Microbiology, University of Agricultural Sciences, GKVK, for further study.

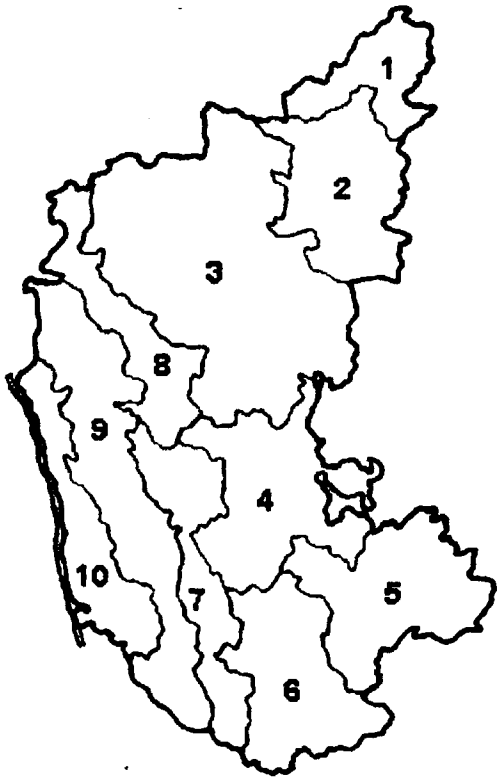
Table 3.1: Characteristics features of agro climatic zones of Karnataka taken for the study*

Zone	Name	Soil type	Rainfall (mm/year)	Temperature (Degree Celsius)	Humidity %	Sand %	Silt %	Clay %	pH	CEC Centimols
4	Central Dry Zone	Red sandy loam to Black soil	456-717	30.8-20.7	73	34.66	28.96	36.38	9.54	47.82
5	Eastern Dry Zone	Red sandy soil	674-889	29.2-18.6	71	72.97	4.93	22.1	5.31-6.21	16.5
6	Southern Dry Zone	Red sandy soil	670.6-888.6	29.1(max)	61.6	71.45	4.62	23.93	6.72	15.5
7	Southern Transition Zone	Red sandy loam	619-1300	30-19.2	81	76.17	5.86	17.94	5.8	-
8	Northern Transition Zone	Black soil	780	30.1-18.0	76	16.35	36.38	57.27	6.72-7.85	64.0
9	Hilly Zone	Red loam to Red Sandy loam	904-3695	25.2-16.6	89	69.7	20.5	9.79	5.3	20.5
10	Coastal Zone	Lateritc	4000	30.5-23.5	96.5	48.46	12.76	38.78	5.2	25.56

* Source: Department of Soil Science, UAS, GKVK, Bangalore.



(a)



(b)

Fig . 3.1: (a) Location of study area in India; (b) Agro climatic zones of Karnataka

1. North Eastern Transition Zone
2. North Eastern Dry Zone
3. Northern Dry Zone
4. Central Dry Zone
5. Eastern Dry Zone
6. Southern Dry Zone
7. Southern Transition Zone
8. Northern Transition Zone
9. Hilly Zone
10. Coastal Zone

3.1.2 Processing of soil sample

The soil samples collected from each zone were dried inside the laboratory at 28° C. Four soil samples collected from each zone were mixed well to get a pooled soil sample for a zone. Totally ten soil samples was obtained for the study. Each soil sample was sieved through 1000μ mesh to remove the bigger soil particles and debris. The sieved soil samples were used for the spore isolation.

3.2 Spore isolation

The spores from each zone were isolated by wet sieving and decanting as described by Gerdemann and Nicolson (1963) with following modifications: Twenty five grams of representative soil sample drawn from each zone was suspended in 500 ml of water and stirred thoroughly. The suspension was allowed to stand undisturbed for 15 minutes and then passed through a series of sieves of sizes 1mm, 450, 250, 105 and 45 μ m arranged in descending order of their mesh size. The spores on the bottom two sieves were transferred to a 100 ml conical flask.

3.3 Separation of spore from the debris

The conical flask containing the spore suspension was filtered through a funnel containing filter paper. The spores adhering to the filter paper in a concentric manner at the periphery were placed in a dry petriplate. The filter

paper was viewed under a stereomicroscope under 40 X magnifications. The spores were picked individually using a fine needle tip of disposable syringe. The spore(s) sticking to the needle was transferred to 1.5 ml Eppendorf tube containing about 100µL sterile water.

3.4 Mass multiplication of spores

In a plastic funnel (7 cms diameter), sterilised sand soil mixture (1:1 w/w) was filled. The funnel tip was plugged with a cotton wick (about 10 cms). The stem of the funnel was immersed into a test tube containing sterile water. Care was taken to ensure that the end of the cotton wick touches the bottom of the test tube. 25 grams of ragi seeds were surface sterilized with ethyl alcohol (70% V/V) followed by five washes with sterile distilled water. Five ragi seeds were sown in the funnel. Four replications for each zone and for control (uninoculated) were set up. A total of 44 funnels were set for the experiment (Plate 3.1). The plants were watered with sterile water and grown for a week inside the lab. Latter, the plants were shifted to glass house and maintained for 6 weeks.

3.5 Identification of the spore

In a double cavity slide, five *Glomus mosseae* spores, isolated from culture (LL3), maintained at the Department of Agricultural Microbiology, was placed using a manual pipette (2-20 µL volume). In the other cavity, one spore isolated from zone one was placed. The spore was viewed under Nomarski Interference

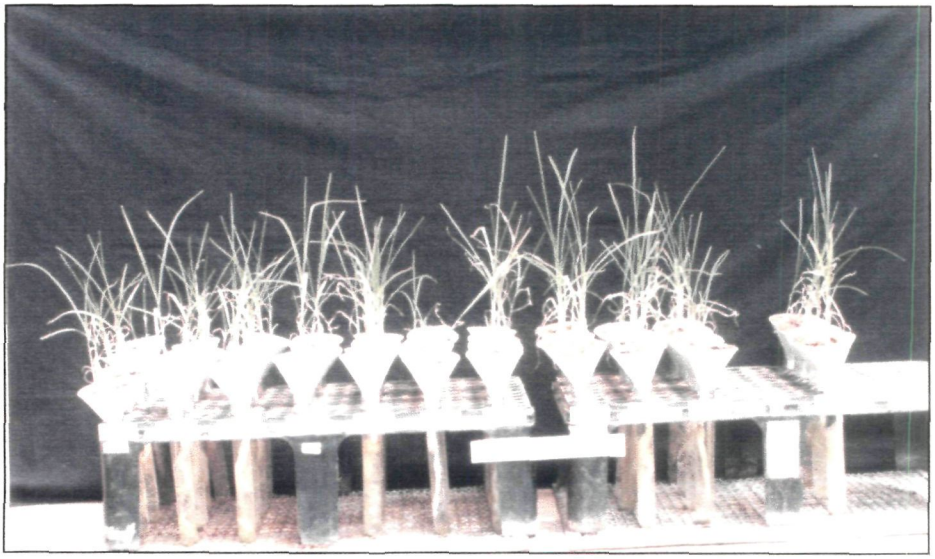


Plate 3.1: Mass multiplication of *Glomus mosseae* isolates from ten agro climatic zones of Karnataka

microscope (400X magnification) and was compared with the standard *Glomus mosseae* culture and taxonomic character as described by Schenk and Perez (1988). The spores matching with the standard culture were transferred to a sterile 1.5 ml Eppendorf tube containing 100μL sterile water. Similarly, spores, which morphologically resembled *Glomus mosseae*, were identified from all the ten zones (Plate 3.2). Thus, preliminary identification was carried out without breaking the spores in order to mass multiply it for further analysis.

3.6 Inoculation of the spore

Single spore was inoculated in the funnel containing the Ragi (*Eleusine coracana*) seedlings. Likewise, four spores from each zone were inoculated.

3.7 Effect of *Glomus mosseae* on tomato plant

3.7.1 Raising tomato nursery

Tomato seedlings were raised in a plastic tray (30x20x10cms) containing sterilized sand soil mixture (1:1 w/w).

3.7.2 Transplanting the seeds in pot

Two weeks seedlings were transplanted in to pot containing sterile sand soil mixture (1:1 w/w). About 50 *Glomus mosseae* spores isolated from zone 4, zone 5, zone 6, zone 7, zone 8, zone 9 a, zone 9b and zone 10 were mixed in the pot before transplanting. The plants were grown for 60 days and various plant growth parameters were recorded.

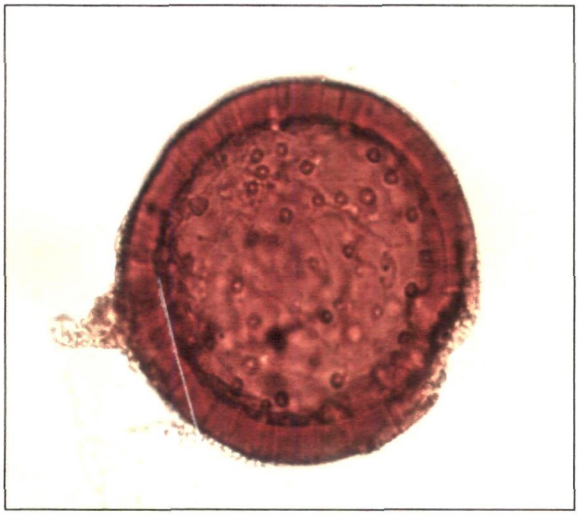


Plate 3.2: *Glomus mosseae* spore identified from mass multiplied isolates

3.7.3 Recording plant growth parameters

The observations with respect to the growth parameters including plant height, number of leaves, shoot fresh weight, shoot dry weight, root fresh weight, root dry weight, and P concentration were recorded at different periodical intervals till vegetative stage.

3.7.3.1 Plant height

The plant height was measured from the soil surface to the tip of the growing point at 15, 30, 45 and 60 days interval.

3.7.3.2 Number of leaves

The numbers of fully opened leaves were recorded at 15, 30, 45 and 60 days interval.

3.7.3.3 Plant Biomass

3.7.3.3.1 Root fresh weight

The harvested plants were weighed and then the root fresh weight were recorded and expressed as grams per plant.

3.7.3.3.2 Root dry weight

The harvested roots were dried in an oven at 60^0C for 2 days to attain constant weight and then the root dry weight were recorded and expressed as grams per plant.

3.7.3.3.3 Shoot fresh weight

The harvested plants were weighed and then the shoot fresh weight were recorded and expressed as grams per plant.

3.7.3.3.4 Shoot dry weight

The harvested plants were dried in an oven at 60^0Cfor 4 days to attain constant weight and then the dry weight were recorded and expressed as grams per plant

3.7.3.4 Phosphorus concentration of the plant

Plant phosphorus concentration was estimated colorimetrically following the vanadomolybdate yellow colour method (Jackson, 1973). The oven dried shoot and root samples was digested using 10ml of triacid mixture (Nitric acid; perchloric acid and sulphuric acid in the ratio 6:3:1 (v/v/v) in 100 ml volumetric flasks using distilled water 10 ml of this aliquot was taken and 10 ml vanadomolybdate reagent was added. The Volume was made upto 50 ml using distilled water. After shaking, the reaction mixture was made up to 50 ml using distilled water. After the shaking the reaction mixture was allowed to stand for 20 minutes. The intensity of yellow colour developed due to phospho vanadomolybdate complex was read at 420nm in a spectrophotometer (Genesys, USA) .The phosphorus in the sample was determined by comparing with a

standard curve developed using KH_2PO_4 as P Source. Per cent P was calculated using the formula given below:

$$\text{Per cent P} = \frac{\text{Graph ppm X OD value of sample X vol. of digested sample X vol. made}}{10^6 \text{ X Weight of the sample X Aliquot taken}} \text{ X } 100$$

3.7.4 Recording mycorrhizal parameters

3.7.4.1 Spore count

The spores were isolated from the pots by wet sieving and decanting as previously described counted under stereomicroscope under 40 X magnification.

3.7.4.2 Per cent colonization

Mycorrhizal root colonization was determined by the gridline intersect method outlined by Giovannetti and Mosse (1980) Fresh root samples (weighing 300mg) were cut into 1cm pieces and placed in screw cap vials. The clearing of the roots was achieved by treating them with 10 per cent KOH and leaving them overnight The KOH solution was poured off and the roots were rinsed with tap water. Then the roots were treated with 10 per cent HCL for 10 minutes to neutralize the residual effect of alkali and create an acidic environment required by the stain. Root bits were then stained with 0.05 per cent trypan blue in lactoglycerol (lactic acid, glycerol and water in the ratio (40:20:20 respectively) by boiling them at 90°C for 30 minutes. Excess stain was decanted and the root

samples were immersed in lactoglycerol for destaining (Phillips and Hayman, 1970). The stained root bits were randomly placed on a grid plate of size 10 X 10cm grid. The horizontal and vertical grid lines were viewed under a stereomicroscope at 40 X magnification to determine the total root bits and the root bits positive for mycorrhizal colonization on grid line intersection. The proportion of roots colonized by mycorrhiza was calculated by the formula:

$$\text{Per cent colonization} = \frac{\text{Total No. of intersection positive for mycorrhizal colonization}}{\text{Total No. of intersections between roots and grid lines}} \times 100$$

3.8 Statistical analysis

The data obtained form the experiments were subjected to one-way analysis of variance for completely randomized design (CRD) using MSTAT software. The treatment means were separated by Duncan's Multiple Range test (DMRT) a 5% level of significance (Little and Hills, 1978)

3.9 Isolation of spores from the funnel

From the funnel, 10 grams of the soil was collected at the root zone of the plant. Spores were isolated from each funnel by wet sieving and decanting (as described previously) was transferred to a sterile 1.5 ml Eppendorf tube. About 400 spores from each zone (100 spores from each replication) were collected.

3.10 Surface sterilization of the spores

The spores isolated from ten agro climatic zones were surface sterilized by washing twice with sterile water, twice with chloramine-T 2%(w/v), twice with 200 ppm streptomycin sulphate and final washing with five times sterile water.

3.11 Identification of *Glomus mosseae* using specific primer

3.11.1 DNA extraction from spores

DNA from the spores collected from funnel cultures of the ten zones, was extracted according to Abbas *et al.,* (1996) with slight modifications. About 50 surface sterilized spores, isolated from the funnel, were taken in a sterile 1.5 ml Eppendorf tube. The spores were crushed with 50 µL of 1X TE Buffer (pH 7. 8) using sterile forceps. The crushed spores were boiled for 10 min in a 100°C water bath. After cooling the contents were centrifuged at 10,000 rpm for 10 min. The supernatant containing the genomic DNA was transferred to another sterile 1.5 ml Eppendorf tube and stored at –20°C till further use.

3.11.2 PCR amplification with specific primers

DNA from all the isolates was amplified as described by Abbas *et al.*, (1996) with few modification. The DNA was amplified in 25 µL reactions containing 150 ng of genomic DNA, 250 µM of each dNTPs, 3 mM $MgCl_2$ and 1 unit of *Taq* Polymerase (Genei, Bangalore), and 0.5 µM of forward and reverse specific primer (Metabion,Germany; Table 3.2). Amplification was performed in

Table 3.2: Sequences of *Glomus mosseae* specific primer pairs.

Sequence	No. of bases
5' CTG CCG CCA CCC CTA TTT TAA TCT AGC 3'	27
5' CTG CCG CCA CTG TCG GAA TA 3'	20

an PTC-100 MJ Research Thermal Cycler with following programme: two cycles of denaturation, annealing and elongation of 94°C for 1 min, 40°C for 20 seconds and 72°C for 35 seconds respectively. Thirty-eight additional cycles were performed at 94°C for 1 second, 50°C for 20 sec, 72°C for 35 sec.

3.11.3 Analysis of PCR products

PCR products were separated on 1.5% Agarose gel with 0.5 μg/ml Ethidium Bromide in a horizontal electrophoresis containing 0.5 X TBE buffer (Maniatis *et al.,* 1982). The PCR products were mixed with loading buffer containing bromophenol blue dye, and loaded in the wells. λ/Hind III marker was also loaded simultaneously. The gel was run at constant voltage of 75 V for 2 hours and photographed in Alpha Innotech gel documentation system.

3.12 RAPD analysis of *Glomus mosseae* isolated from different agro climatic zones

The spores isolated from the funnel as described in section 3.9 and which have shown positive for *Glomus mosseae* specific primer were used for RAPD analysis.

3.12.1 DNA extraction from spores

Fifty spores were isolated from each zone (zone 4, 5, 6, 7, 8, 9 a, zone 9b and 10) and DNA was extracted as described previously for specific primer.

3.12.2 PCR amplification

The amplification was carried out as described by Abbas *et al.*, (1996) with slight modification. DNA was amplified in 25 μL reactions containing 150 ng of genomic DNA, 250 μM of each dNTPs, 3 mM $MgCl_2$ and 1 unit of *Taq* Polymerase (Genei, Bangalore), and 0.8μM random primer (Metabion, Germany; Table 3.3). Amplification was performed in an PTC-100 MJ Research Thermal Cycler with the following programme: two cycles of denaturation, annealing and elongation of 94°C for 1 min, 40°C for 20 seconds and 72°C for 35 seconds respectively. Thirty-eight additional cycles were performed at 94°C for 1 second, 40°C for 20 sec, 72°C for 35 sec. Totally 16 random primers were screened. However only 4 random primers showed amplification for all the 8 samples used.

3.12.3 Analysis of PCR products

PCR products were analyzed and photographed as previously described.

3.12.4 Analysis of RAPD data

Based on the presence and absence of the amplification bands, genetic differentiation of population was investigated by computing Nei's genetic distance among all pairs of populations and by partitioning the total variation into those within and among populations. Based on these distances, cluster analysis was performed following UPGMA method a dendrogram and

Table 3.3: Sequences for random primers used for the study.

Sl. No.	Sequence of 10-mer random primers	Oligo Name
1	5'-TCG CAT CCC T-3'	KIT W 10
2	5'-CTG ATG CTG G-3'	KIT W 11
3	5'-ACA CCG GAA C-3'	KIT W 15
4	5'-CCA AGC GCT C-3'	KIT W 19

dissimilarity matrix was constructed. Also effectiveness of the random primer was tested by calculating per cent polymorphism.

3.13 Standardization of DNA extraction procedure and PCR analysis from single spore.

An attempt was made to standardize the DNA extraction procedure and PCR analysis using single spore. A spore each was taken for the study from two sources i.e., are from standard *Glomus mosseae* culture (LL3) maintained in the glass house of the Department of Agricultural Microbiology, while other single spore was an isolate from zone 10. DNA was extracted from single spore using the method as described previously for 50 spores, except 20 μL of 1 X TE Buffer (pH 7.8) was used. Also, the PCR reaction mixture and the programme were same as described for 50 spores except that primer used was KIT W 13. The amplified product was resolved in 1.5 % Agarose Gel and photographed in Alpha Innotech Gel Documentation System.

EXPERIMENTAL RESULTS

IV. EXPERIMENTAL RESULTS

The results of the study conducted on "Biological and Molecular Variability of *Glomus mosseae* isolated from different agro climatic zones of Karnataka" involving glass house and laboratory experiments are represented in this chapter.

4.1 Isolation and identification of *Glomus mosseae*

The spores were isolated from ten agro climatic zones of Karnataka. Preliminary identification was done without breaking the spores. These spores were mass multiplied using funnel technique. Only spores from seven zones were found to be *Glomus mosseae* using specific primer. The details of identification using specific primer was presented in section 4.5

4.2 Response of tomato to inoculation of *Glomus mosseae* isolates

4.2.1 Plant height

The data pertaining to influence of *Glomus mosseae* isolates from seven agro climatic zones on plant height of tomato is presented in Table 4.4; Fig. 4.2 and Plate 4.3.

The plant height was found to increase steadily with number of days after inoculation. In plants inoculated with *Glomus mosseae* isolates, the height of plants remained always higher than uninoculated plants. However, the heights differed significantly among the plants inoculated with various isolates. The least plant height (31.8 cms) was recorded in uninoculated plants while maximum height (39.2

Table 4.4: Influence of *Glomus mosseae* isolates on plant height of tomato.

Zone	Plant height (cms)			
	15 DAT	30 DAT	45 DAT	60 DAT
4	16.7^{b}	24.6^{a}	32.5^{b}	38.3^{ab}
5	16.2^{b}	24.4^{a}	31.3^{c}	37.5^{bc}
6	15.6^{c}	23.5^{b}	30.4^{d}	35.6^{de}
7	12.4^{f}	22.5^{c}	28.6^{e}	33.1^{fg}
8	13.3^{e}	23.4^{b}	30.3^{d}	34.3^{ef}
9a	14.0^{d}	24.4^{a}	31.4^{c}	36.5^{cd}
9b	16.2^{b}	24.5^{a}	32.4^{b}	37.5^{bc}
10	17.6^{a}	24.5^{a}	33.4^{a}	39.2^{a}
Control	10.3^{g}	18.5^{d}	28.4^{e}	31.8^{g}

DAT: Days after transplanting
Means in the same column followed by the same superscript do not differ significantly according to Duncan's Multiple Range Test ($P<0.05$)

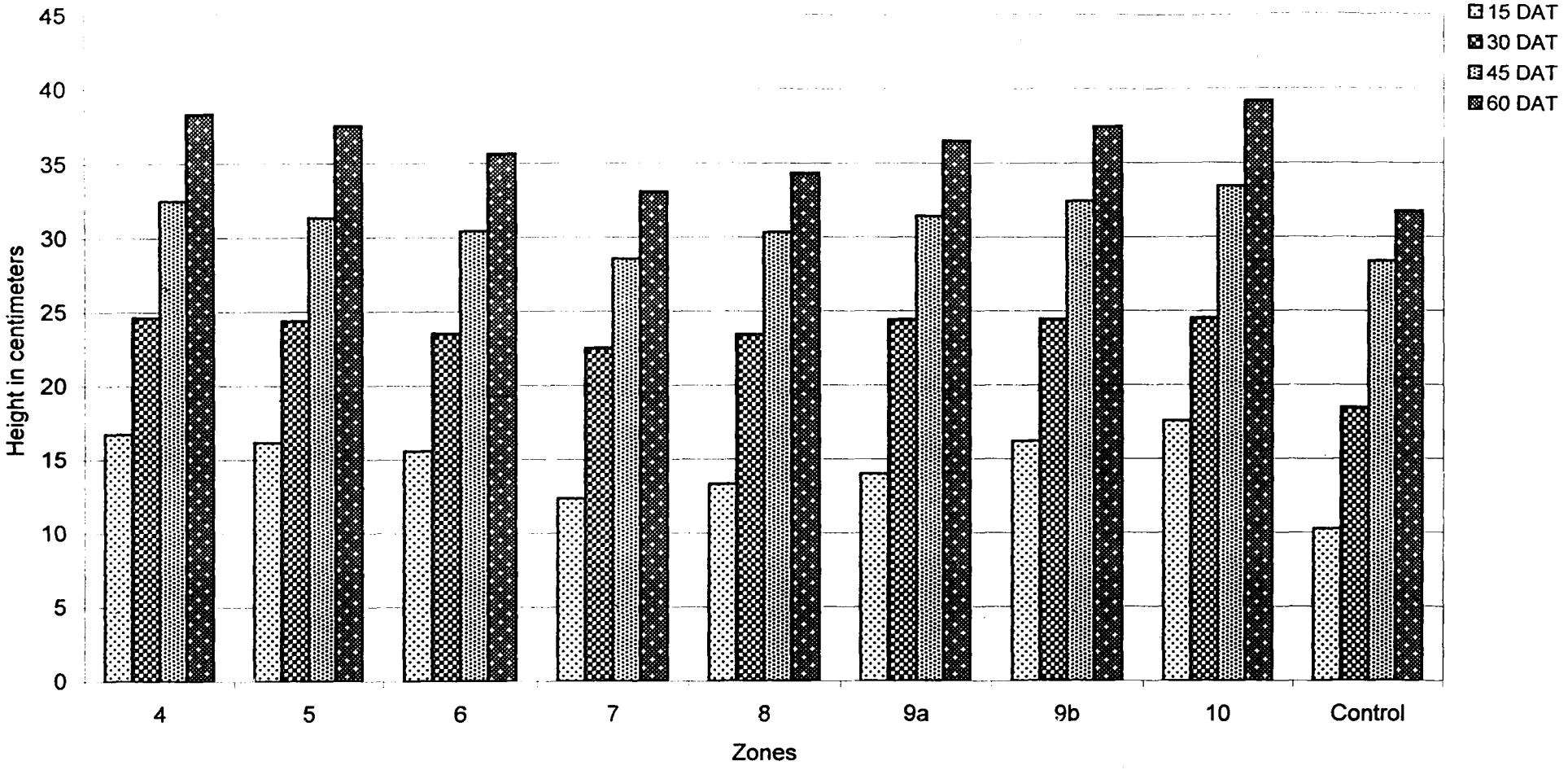


Fig. 4.2: Influence of *Glomus mosseae* isolates on plant height of tomato.

DAT- Days after transplanting

Plate 4.3: Response of tomato to isolates of *Glomus mosseae* inoculation of different agro climatic zone

cms) was recorded in plants inoculated with isolate from zone 10 at 60 days after transplanting.

4.2.2 Number of leaves

The data pertaining to influence of the isolates on number of leaves were recorded and shown in Table 4.5 and Fig. 4.3.

The numbers of leaves were found to increase constantly at different interval of time. Also found that leaf numbers in plants inoculated with isolates were always higher than the control. Highest number of leaves (10) was observed in plants inoculated with isolate of zone 4 and least number of leaves (6) were found in uninoculated plants. The number of leaves in plants inoculated with isolates from zones 4, 5 and 10 differed significantly from zones 6, 7, 8, 9a and 9b. However there was no significant difference in the number of leaves within the groups.

4.2.3 Biomass

The fresh weight and dry weight of the plants harvested 60 days after transplanting are presented in Table 4.6 and Fig. 4.4.

The total fresh weight and dry weight in the plants inoculated with *Glomus mosseae* isolates were higher than uninoculated plants.

Significant difference was observed in total fresh weight in plants inoculated with the isolates. But, there was no significant difference in the fresh

Table 4.5: Influence of *Glomus mosseae* isolates on number of leaves in tomato.

Zone	No. of leaves/plant			
	15 DAT	30 DAT	45 DAT	60 DAT
4	4.0^{b}	6.0^{b}	8.0^{a}	11.0^{a}
5	4.0^{b}	6.0^{b}	9.0^{a}	10.0^{a}
6	3.0^{bc}	5.0^{b}	7.0^{c}	9.0^{b}
7	3.0^{bc}	5.0^{b}	7.0^{bc}	8.0^{b}
8	3.0^{bc}	5.0^{b}	7.0^{c}	9.0^{b}
9a	3.0^{bc}	5.0^{b}	7.0^{c}	8.0^{b}
9b	4.0^{b}	6.0^{b}	8.0^{ab}	9.0^{b}
10	5.0^{a}	7.0^{a}	9.0^{a}	10.0^{a}
Control	2.0^{c}	4.0^{c}	5.0^{d}	6.0^{c}

DAT: Days after transplanting
Means in the same column followed by the same superscript do not differ significantly according to Duncan's Multiple Range Test ($P<0.05$)

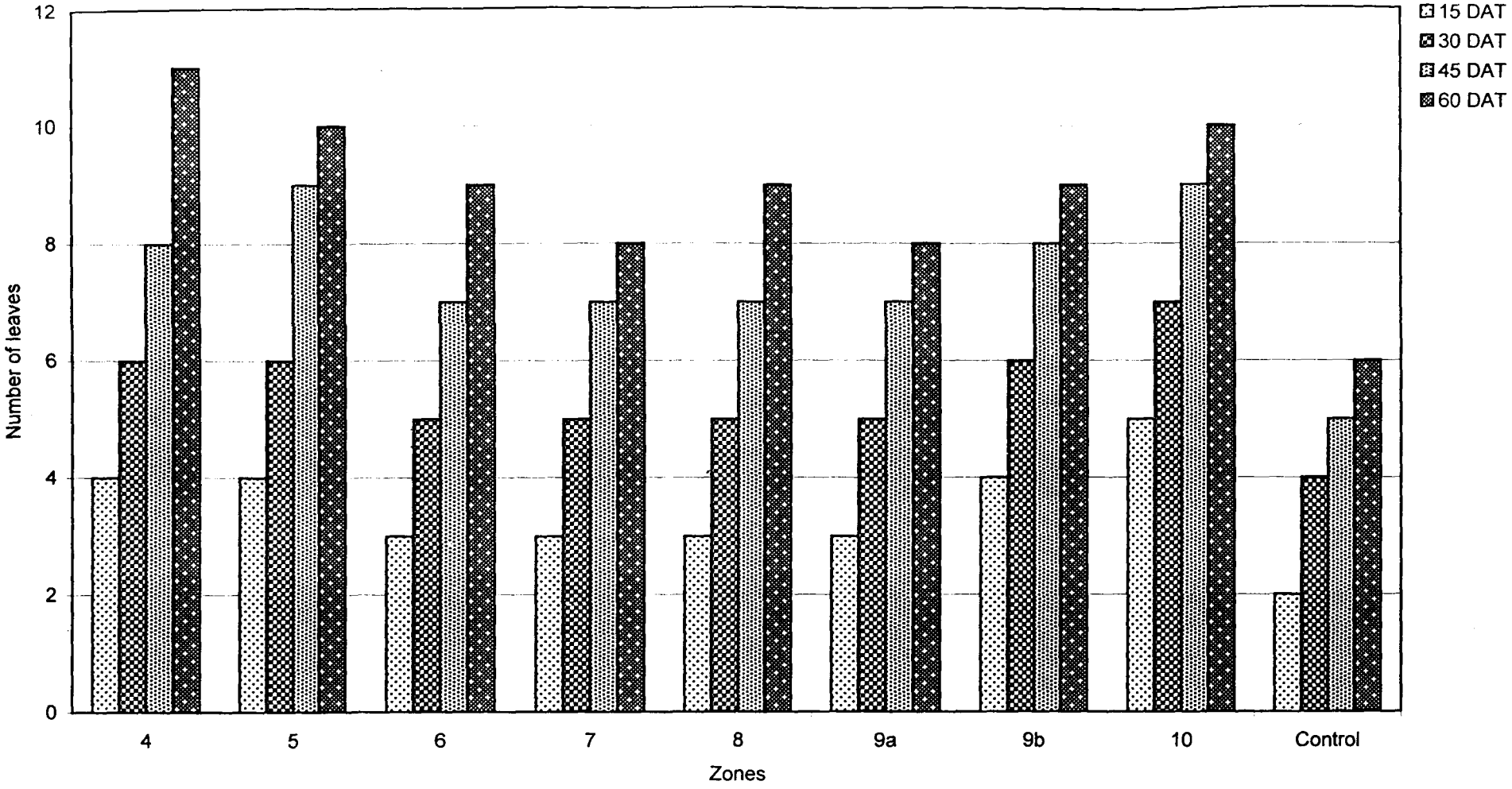


Fig. 4.3: Influence of *Glomus mosseae* isolates on number of leaves in tomato.

DAT- Days after transplanting

Table 4.6: Influence of *Glomus mosseae* isolates on biomass of tomato.

Zone	Fresh weight (g/plant)			Dry weight (g/plant)		
	Shoot	Root	Total	Shoot	Root	Total
4	17.8^{a}	15.9^{a}	33.7^{a}	4.9^{ab}	3.7^{a}	8.6^{ab}
5	17.5^{ab}	15.0^{bc}	32.5^{ab}	4.7^{abc}	3.2^{b}	7.9^{cd}
6	16.5^{bc}	15.8^{ab}	32.3^{ab}	4.1^{cd}	3.9^{a}	8.1^{bc}
7	16.6^{b}	15.2^{abc}	31.8^{b}	4.3^{bcd}	3.1^{b}	7.4^{d}
8	17.2^{ab}	15.5^{abc}	32.7^{ab}	4.2^{abcd}	3.2^{b}	7.4^{d}
9a	15.7^{c}	13.9^{d}	29.6^{c}	3.9^{d}	2.6^{c}	6.5^{e}
9b	16.5^{bc}	14.7^{c}	31.2^{b}	4.2^{bcd}	3.1^{b}	7.4^{d}
10	18.0^{a}	15.4^{abc}	33.4^{a}	5.2^{a}	3.7^{a}	8.9^{a}
Control	11.9^{d}	10.8^{e}	22.8^{d}	2.8^{e}	2.0^{c}	4.8^{f}

Means in the same column followed by the same superscript do not differ significantly according to Duncan's Multiple Range Test ($P<0.05$)

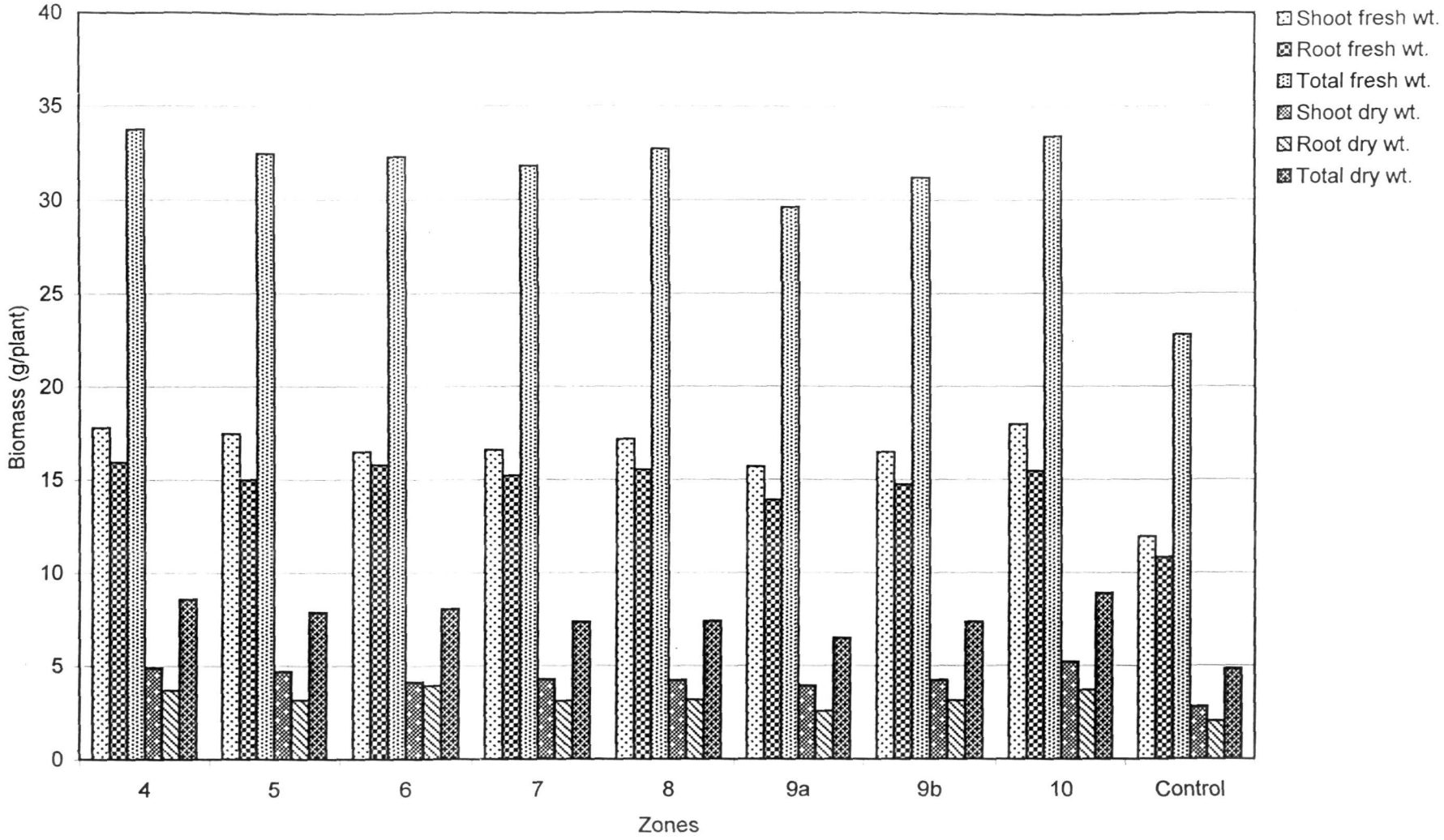


Fig. 4.4: Influence of *Glomus mosseae* isolates on biomass of tomato.

weight in the plants inoculated with isolates of zones 5, 6 and 8 as well as zone 4 and 10. Maximum fresh weight (33.7 g) was observed in the plant inoculated with isolate from zone 4 and minimum fresh weight (28.8 g) was recorded in uninoculated plants.

Maximum total dry weight (8.9 g) was recorded in plants inoculated with isolate from zone 10 and minimum (4.8 g) was recorded in uninoculated plants. No significant difference in the total dry weight of was observed in plants inoculated with isolates of zone 7, zone 8 and zone 9b.

4.2.4 Phosphorus concentration

The phosphorus concentration of shoot and root in the plants inoculated as well as the uninoculated plants was recorded and presented in Table 4.7 and Fig. 4.5.

4.2.4.1 Shoot P concentration

The shoot P concentration in the inoculated plants was found to differ significantly from the uninoculated. The shoot P concentration in the plants inoculated with isolates from zones 7, 9b and 10 as well as isolates from zones 8 and 9a were not significantly different. The maximum shoot P concentration (0.257 %) was observed in the plant inoculated with isolate of zone 5 while minimum shoot P (0.147 %) was observed in the uninoculated plants.

Table 4.7: Influence of *Glomus mosseae* isolates on shoot and root phosphorus concentration of tomato.

Zone	Shoot P (%)	Root P (%)
4	0.204^{abc}	0.198^{ab}
5	0.257^{a}	0.237^{a}
6	0.235^{a}	0.229^{a}
7	0.214^{ab}	0.188^{abc}
8	0.165^{bc}	0.161^{bc}
9a	0.161^{bc}	0.153^{bc}
9b	0.212^{ab}	0.184^{abc}
10	0.210^{ab}	0.178^{abc}
Control	0.147^{c}	0.129^{c}

Means in the same column followed by the same superscript do not differ significantly according to Duncan's Multiple Range Test($P<0.05$)

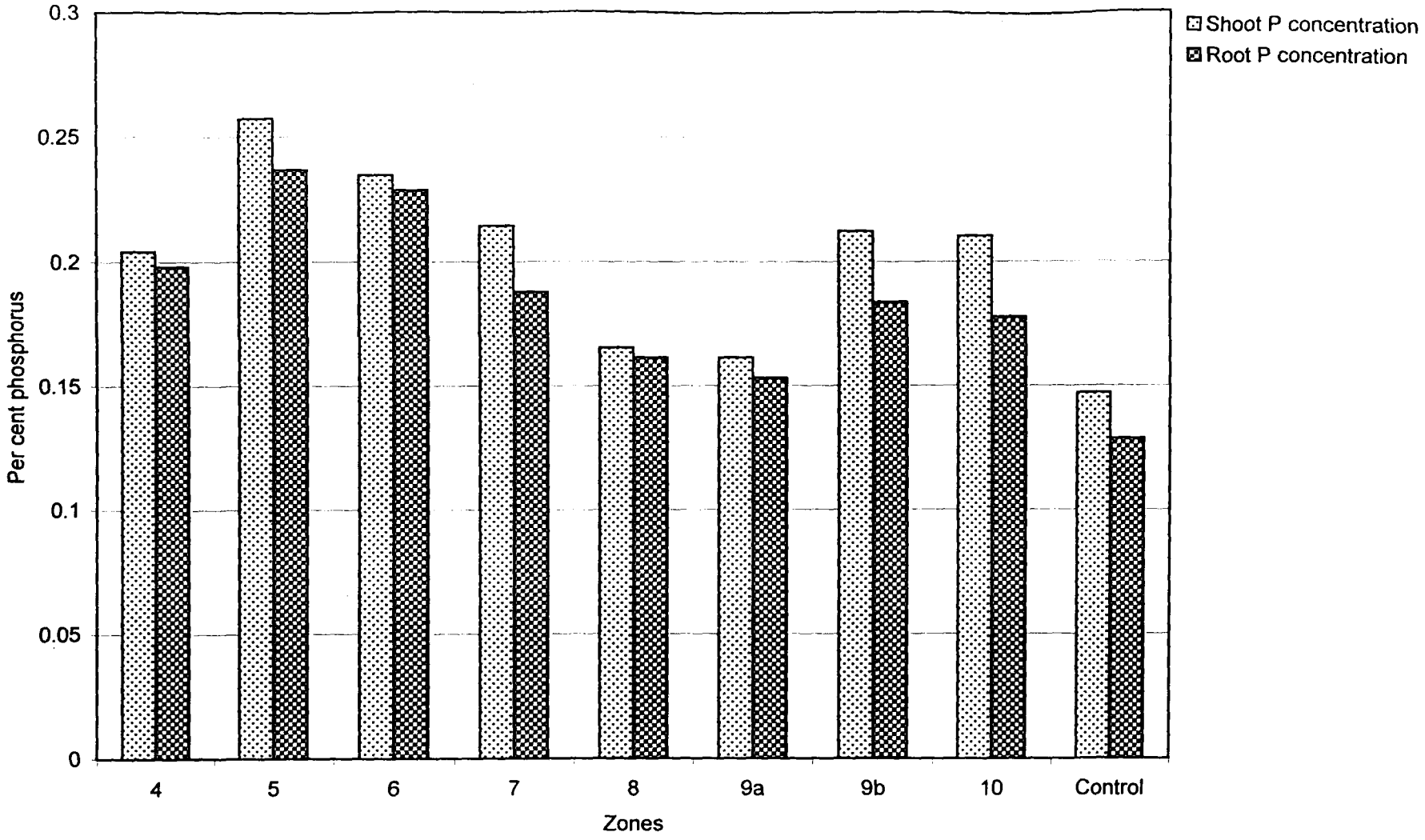


Fig. 4.5: Influence of *Glomus mosseae* isolates on shoot and root phosphorus concentration of tomato.

4.2.4.2 Root P concentration

The root P of inoculated plants was found higher than the uninoculated. There was no significant difference in the root P concentration of the plants inoculated with isolates of zones 7, 9a, 9b and 10 as well as plants inoculated with isolates of zone 5 and 6. The maximum root P (0.237 %) was found in the plants inoculated with zone 5 isolate and minimum root P (0.129 %) was observed in the uninoculated roots.

4.2.5 Spore count and per cent root colonization

4.2.5.1 Spore count

The number of spores was calculated for 50 g soil from each zone and the data was presented in Table 4.8 and Fig. 4.6.

The spore count did not vary much between the isolates. This was shown statistically that only the isolate from zone 6 differed significantly from the other isolates. In general, the spore count ranged from 140 in the soil inoculated with zone 6 isolate to 163 in the soil inoculated with zone 9a isolate. Since, sterilized soil was used for the study, no spore was found in those pots, which were uninoculated.

4.2.5.2 Per cent root colonization

There were significant differences in the per cent root colonization among the plants inoculated with isolates of zones 4, 5 and 7. Except for these isolates,

Table 4.8: Influence of *Glomus mosseae* isolates on spore numbers and per cent root colonization of tomato.

Zone	Number of spores/ 50 g soil	Per cent root colonization
4	148^{ab}	64^{bc}
5	165^{a}	62^{bc}
6	140^{b}	72^{ab}
7	157^{ab}	62^{bc}
8	142^{ab}	60^{c}
9a	163^{ab}	75^{a}
9b	149^{ab}	57^{c}
10	157^{ab}	66^{abc}
Control	0^{c}	0^{d}

Means in the same column followed by the same superscript do not differ significantly according to Duncan's Multiple Range Test ($P<0.05$)

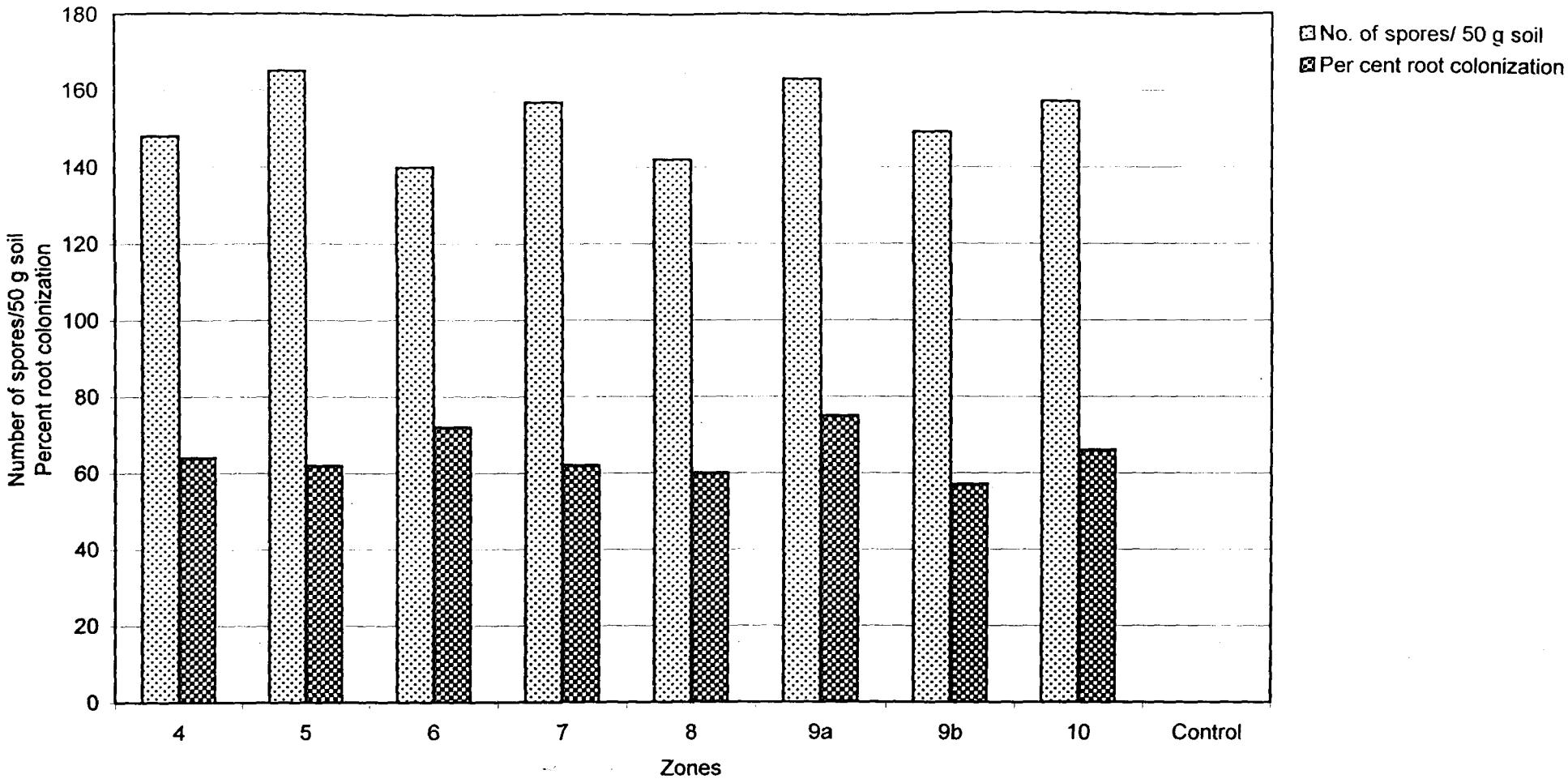


Fig. 4.6: Influence of *Glomus mosseae* isolates on spore numbers and per cent root colonization of tomato.

significant difference was observed in percent root colonization in plants inoculated with other zones isolates. The per cent root colonization ranged from 57 to 77 percent. The maximum colonization was recorded in the plants inoculated with zone 6 isolate and minimum in case of plants inoculated with zone 9b isolate.

4.3 Identification of *Glomus mosseae* using specific primer

The specific primer for *Glomus mosseae* was tested against the isolates of seven different agro climatic zones. It was found that isolates from zones 4, 5, 6, 7, 8, 9 and 10 showed presence of *Glomus mosseae* by the amplification of 0.269 kb specific DNA band (Fig. 4.7). Among the four samples of mass multiplied spores from zone 9, it was found that only replicate 1 and 2 showed presence of *Glomus mosseae*, which were named as zone 9a and 9b respectively.

4.4 RAPD analysis of *Glomus mosseae* isolated from different agro climatic zones

The spores from seven agro climatic zones, which showed specificity for *Glomus mosseae* tested with specific primer, were subjected to RAPD analysis. Among the 16 random primer screened, four random primer showed reproducible amplification product for all the seven agro climatic zone isolates including two replicates (9a and 9b) from zone 9.

The amplified product of four random primers viz., W10, W11, W15 and W19, showed considerable degree of polymorphism between them (Fig. 4.8; Fig. 4.9; Fig. 4.10; Fig. 4.11). Based on the presence and absence of the amplification

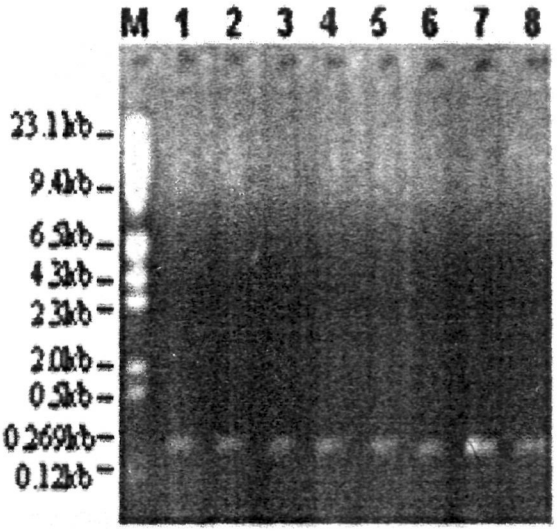


Fig. 4.7: *Glomus mosseae* specific primers tested against DNA templates from isolates of different agro climatic zones.
Lane 1, 2, 3, 4, 5, 6, 7 and 8 are zone 4, zone 5, zone 6, zone 7, zone 8, zone 9a, zone 9b and zone 10 respectively. Lane M is λ/ Hind III marker

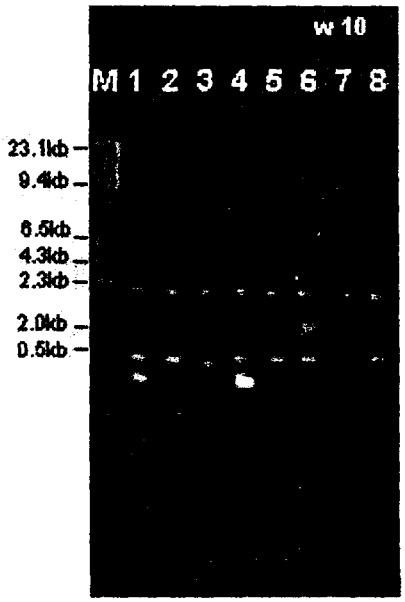


Fig. 4.8: DNA amplification products of eight *Glomus mosseae* isolates with random primer KIT W 10.
Lane 1, 2, 3, 4, 5, 6, 7 and 8 are zone 4, zone 5, zone 6, zone 7, zone 8, zone 9a, zone 9b and zone 10 respectively. Lane M is λ/ Hind III marker.

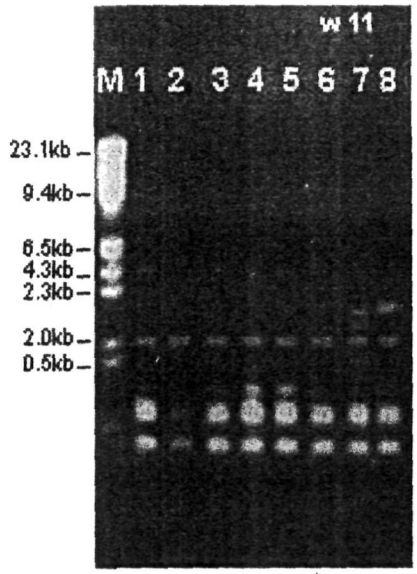


Fig. 4.9: DNA amplification products of eight *Glomus mosseae* isolates with random primer KIT W 11.
Lane 1, 2, 3, 4, 5, 6, 7 and 8 are zone 4, zone 5, zone 6, zone 7, zone 8, zone 9a, zone 9b and zone 10 respectively. Lane M is λ/ Hind III marker

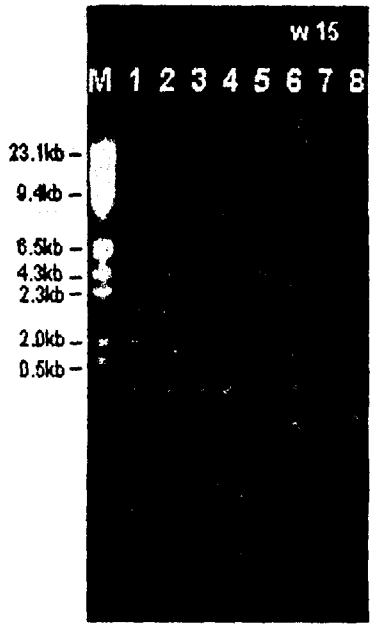


Fig. 4.10: DNA amplification products of eight *Glomus mosseae* isolates with random primer KIT W 15.
Lane 1, 2, 3, 4, 5, 6, 7 and 8 are zone 4, zone 5, zone 6, zone 7, zone 8, zone 9a, zone 9b and zone 10 respectively. Lane M is λ/ Hind III marker

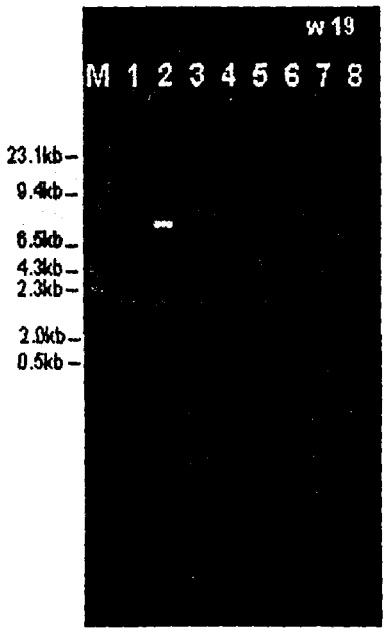


Fig. 4.11: DNA amplification products of eight *Glomus mosseae* isolates with random primer KIT W 19.
Lane 1, 2, 3, 4, 5, 6, 7 and 8 are zone 4, zone 5, zone 6, zone 7, zone 8, zone 9a, zone 9b and zone 10 respectively. Lane M is λ/ Hind III marker

products, the squared Euclidean distances as well as dendrogram was constructed (Fig. 4.12). Two clusters were clearly illustrated. The first cluster contains two groups; the first group had isolates from zone 9a and 8, while the second had isolates of zone 7 and 6. On the other hand, the second cluster had two groups, with one group having zone 10 and 9b isolates while the second group having zone 5 and 4 isolates. The dissimilarity matrix constructed based on the polymorphic bands showed that the isolates, which are clustered together, are less dissimilar (Table. 4.9). Thus, the most dissimilar ones are isolate from zone 8 and 10, while the least dissimilar are isolate from zone 6 and 7. The polymorphism among the isolates was observed as (58.33%). The average number of bands per primer is 6, while the average number of polymorphic bands per primer was found to be 3.5 (Table. 4.10).

Analysis of the clustering pattern indicates that isolates from geographically adjacent zones more closely related than isolates from farther away zone.

4.5 Standardization of DNA extraction procedure and PCR analysis from single spore.

With few modifications as mentioned in section 3.13, the protocol used for 50 spores was also found suitable even for single spore analysis. The random amplification product with W 13 showed variation between standard *Glomus mosseae* (LL3) and the *Glomus mosseae* isolate from zone 10 (Fig. 4.13).

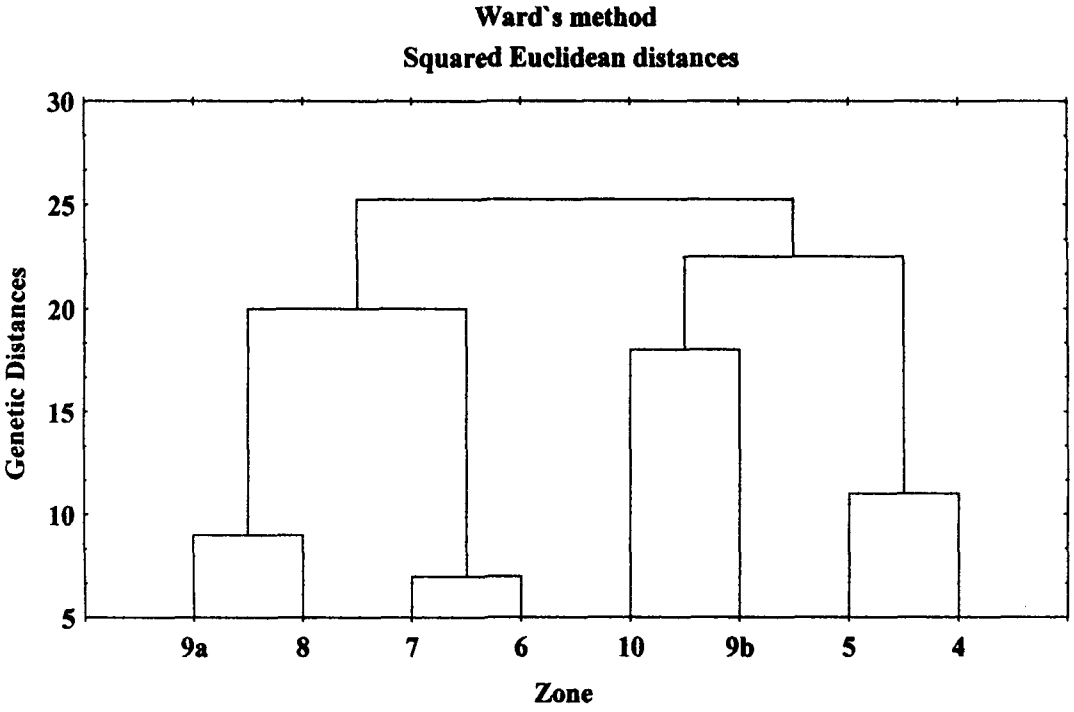


Fig. 4.12: Dendrogram of *Glomus mosseae* isolates from RAPD data using UPGMA method.

Table 4.9: Matrix of dissimilarity index of *Glomus mosseae* isolates based on RAPD analysis

Zone	4	5	6	7	8	9a	9b	10
4	0	11	18	15	19	16	18	20
5		0	17	18	16	15	17	19
6			0	7	15	12	18	14
7				0	14	15	19	17
8					0	9	19	21
9a						0	16	18
9b							0	18
10								0

Table 4.10: Effectiveness of the random primers tested in *Glomus mosseae* isolates

Random Primer No.	Sequence 5'—3'	Toal No. of Bands Amplified	No. of Polymorphic Bands	Percent Polymorphism
10	TCG CAT CCC T	6	4	66.67
11	CTG ATG CTG G	5	2	40.00
15	ACA CCG GAA C	7	4	57.14
19	CCA AGC GCT C	6	4	66.67
Total No. of Bands Amplified		24	14	58.33

Average No. of Bands/ Primer = 6

Average No. of Polymorphic Bands/ Primer = 3.5

Average Percent Polymorphism = 58.33

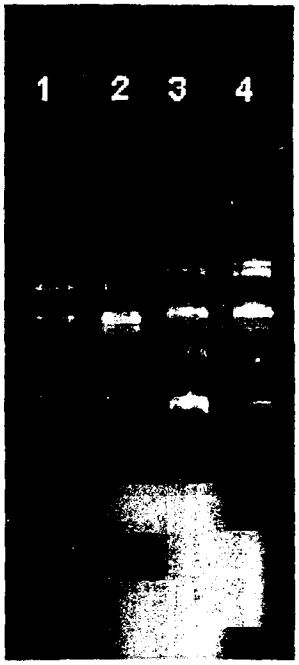


Fig. 4.13: Single spore DNA amplification using random primer KIT W 13. Lane 1and 2 are standard *Glomus mosseae* culture (LL 3) and Lane 3 and 4 are *Glomus mosseae* isolate from zone 10.

DISCUSSION

V. DISCUSSION

Arbuscular mycorrhizal fungi can be found in most terrestrial ecosystems, they dominate in temperate and tropical grassland, scrub and desert ecosystems, as well as tropical forests. Some species have been reported to occur in geographically different regions. Occurrence of variation in many glomalean fungi is probably related to their edaphic requirements. Several edaphic factors have been shown to affect spore germination, root colonization and efficiency of arbuscular mycorrhizal fungi under experimental conditions (Hetrich, 1984).

5.1 Isolation and Identification of *Glomus mosseae* spores from different agro climatic zones

Mycorrhizal fungi are usually identified on the basis of the morphological characters shown by fruiting bodies, spores, vegetative mycelia or symbiotic structures (Lanfranco *et al.*, 1995). One of the hurdles in mycorrhizal research is the difficulty in identifying the spore, which involves systematic observation of the morphological and anatomical structures of the spore. Often many mycorrhizal workers, especially beginners are led to ambiguity while identifying a particular species of interest. Similarly in this study too there were difficulties in identifying *Glomus mosseae* from the soil collected from different agro climatic zones. This is because usually the soils contain various species of mycorrhizal spores. Hence initially the spores, which had close resemblance to *Glomus mosseae*

obtained from culture, maintained at glass house was isolated and mass multiplied using funnel technique. It is necessary to break the spores to study the spore wall characters, which is one of the major criteria for accurate identification of a species. However, in the present study the spores were identified preliminarily without breaking. This is because any damage to spore would lead to failure to germinate and colonize the roots. Since, the objective of this study was to find the biological and molecular variability, it is essential to have sufficient number of pure culture spore. This can be achieved only if a single spore is multiplied. Since single spore is used as inoculum in this study too, spores were not broken for identification. Although the chance of picking an exact *Glomus mosseae* spore is less, the probability of isolating a *Glomus mosseae* from each zone was increased by having four replications, with each replication inoculated with single spore.

The mass multiplied spore from all the zones were tested by specific primer for *Glomus mosseae*. This has shown that among the ten zones, only spores from seven zones were correctly identified, with atleast one correctly identified spore among the four replications of each zone. However, it was noticed in zone 9, two spores were correctly identified as *Glomus mosseae* which is evident from the specific amplification spore DNA from replication 1 and 2, named as 9a and 9b respectively in all references in this study. It is clear that the use of specific primer would allow to identify a particular spore more accurately as well as rapidly when large samples are studied. The use of specific primer for *Glomus mosseae* was

discussed by Abbas *et al.*, (1996). Hence in the present study the specific primer was used to identify *Glomus mosseae* from different agro climatic zones.

5.2 Biological variability

Biological variability studies were conducted using tomato grown in sterilized soil, showed that in general, the plants inoculated with *Glomus mosseae* isolates showed higher growth than the uninoculated plants. The height of plant was found to be always higher in plants inoculated with *Glomus mosseae* isolates than the uninoculated plants. The increased height in the mycorrhizal plants may be due to the increase phosphorus uptake as well as other minor nutrients. However the plants differed significantly in the height in response to some isolates. Since, all the isolates belong to same species of *Glomus mosseae*, their effect on growth in terms of height may not be as significant as those usually observed in the plants inoculated with different species or genera of AM fungi or in combination with other beneficial microorganisms. Many workers have conducted studies involving growth response of plants to different AM inocula. Most of these work reported has been on the growth response of plant in response to different AM inocula or with other Plant Growth Promoting Rhizobacteria. Earlier Kandasamy *et al.*, (1986) reported a similar result in Mulberry. Iqbal and Mahmood (1998) found that tomato plants inoculated with *Glomus mosseae* resulted in the maximum growth of plants followed by *Glomus constrictum* and *Glomus fasciculatum*. Different combinations of the mycorrhizal fungi

also resulted in varying increases in the growth of tomato plants. later Sumana and Bagyaraj (1999) also reported higher plant growth response in terms of plant height in Neem.

Increase in number of leaves in the mycorrhizal plants over the non mycorrhizal plants have shown that mycorrhizal plants has more growth rates in terms of increased leaf production, which may result in increased photosynthesis rate. This upholds the observation made by Thanuja (2000) who has reported increased number of leaves in *Adhathoda* and *Datura* inoculated with different AM fungi.

Increase in biomass in terms of fresh weight and dry weight has been noticed in mycorrhiza inoculated plant than the non-mycorrhizal plant. This may be due to the increased plant height as well as the leaf number. Earanna *et al.*, (1999) in their study has recorded significantly higher plant biomass in two medicinal plants *Coleus aromaticus* and *Coleus barbatus* on inoculation with *Glomus fasciculatum*. Mamatha, (2000) reported increased plant growth and nutrition in Papaya raised in polybags.

Khaliel and Elkhider, (1987) has conducted detailed study on response of tomato to inoculation with AM fungi and found that greater dry weight and higher percentage of survival in plants inoculated with *Glomus mosseae*. They also

found that number of nodes; lateral branches and leaves per plant were almost doubled in mycorrhizal transplants.

Phosphorus is most studied element in mycorrhizal symbiosis. Higher P content in mycorrhizal plants than the nonmycorrhizal plants has been well documented by many workers. Since P is crucial nutrient for better plant growth, any change in the P content will reflected in the plant growth and reproduction. AM fungi play a significant role in the P uptake by plants. Hence, any variation in the efficiency to transport P by AM fungi to the plants has direct role in the increased P content in shoot and root. In the present study P content of both root and shoot was found to higher in inoculated plants than the uninoculated. This clearly establishes role of AM fungi in P uptake by mycorrhizal plants. Higher P content in mycorrhizal fungi was also recorded by Thanuja (2000) in *Adhathoda* and *Datura* inoculated with *Glomus fasciculatum*. Earanna *et al.*, (1999) has also recorded similar results in *Coleus aromaticus, Coleus barbatus* and *Rawolfia serpentina.*

AM fungi show variability in their sensitivity to phosphate additions to soil can negatively affect the capacity of some species to infect roots and therefore their ability to multiply and survive. There are many reports of inter and intraspecific differences in the effectiveness of arbuscular mycorrhizal fungi for plant growth and protection, but the response range can be affected by host plant or soil conditions and the physiological bases of variations.

Investigations into this concern almost entirely growth responses caused by increased supply of P to the plant by the fungus. Rapid and extensive infection of the root system is crucial for enhancing phosphorus absorption, but uptake rates may vary between fungi even when the extent of root colonization is similar (Graham *et al.,* 1982). Furthermore, considerable variations can occur between fungi in the amount of external hyphae produced in the soil, without there being any clear relationship to plant growth responses (Sanders *et al.*, 1977).

Important marked variability in the capacity of arbuscular mycorrhizal fungi to increase P uptake into the plant are differences in the actual rate of hyphal development, in the spatial distribution of hyphae in the soil and in the P transport capacity of the hyphae themselves. Variations in the P uptake between the symbionts may be at the basis of mechanisms by which some arbuscular mycorrhizal fungi are more efficient than others.

The spore count and per cent root colonization vary in plants inoculated with different isolates. However, there was no significant difference between the isolates in the spore count and percent root colonization. Such increased root colonization levels in plants inoculated with AM fungi have been observed earlier by Bagyaraj *et al.*, (1988). As expected, there was no spore as well as root colonization in the soil that is not inoculated. This is due to the reason that the native mycorrhizal fungi are eliminated by sterilization of the soil. Increased spore

count and root colonization was noticed by Edathil *et al.,* (1994) in tomato inoculated with AM fungi.

5.3 Molecular variability

The RAPD analysis has shown that all the seven zone isolates form two major clusters. The two major clusters in turn are grouped in to two minor clusters with varied genetic distances. The first major cluster includes isolates from zones 6, 7, 8 and 9a within which the isolate from zone 6 and 7 forms a minor cluster and isolates of zone 8 and 9a forms another minor cluster with. The second major cluster consists of isolates from zones 4, 5, 9b and 10. This major cluster includes isolate from zone 4 and 5 as a minor cluster and isolate from zone 9b and 10 as another minor cluster. The four minor clusters differ in their genetic distance with least genetic distance noticed between zone 6 and 7 while the maximum genetic distance was noticed between the isolates of zone 10 and 9b. It is interesting to note from the cluster analysis that, the isolates from adjacent zone has clustered together. This is evident from the dendrogram constructed using UPGMA method. The results uphold the study conducted by Wyss and Bonfantae (1993). Using short arbitrary primers to amplify genomic DNA from *Glomus mosseae,* isolates from different geographical location show intra specific variation. Similarity in banding patterns is maximum in spores of different lines of the same isolate, while it decreases between isolates and is minimum in spores of different species. They have also shown that isolates form close geographical

regions shared 60-70 percent of the amplified fragments, whereas, isolates from different geographical regions only shared 44-64 percent of the bands. The percent polymorphism observed among the isolates is 58.33 percent. This seems to be quite high within the species. However, Wyss and Bonfante (1993) have reported similarity between the *Glomus mosseae* isolate from different geographic region could be as low as 46 percent. This suggests that natural AM fungal populations exhibit unexpectedly high genetic diversity, despite the assumption that diversity in these seemingly asexual fungi could be low (Varma, 1999).

Molecular techniques based on DNA analysis offer a wide range of possibilities to develop specific probes for arbuscular mycorrhizal fungi, not only for phylogenetic relationships and studies of their biodiversity at different levels of organizations (ecosystem, population, species, isolate), but also for their accurate identification in soil and in plant. Based on randomly amplified polymorphic DNA (RAPD), specific primers were designed to identify *Glomus mosseae* isolates (Lanfranco *et al.,* 1995).

Routinely Chelex–100 resin is commonly used for the extraction of DNA from AM spore. However on modification of the protocol of (Abbas, 1996), it was found that sufficient quantity and quality of DNA can be extracted in the absence of Chelex-100 resin, which can provide as template in the RAPD analysis. This new method adopted in the study enables not only rapid isolation of DNA from AM spore for PCR analysis but also could save the cost and time of analysis.

Thus the DNA extraction method followed in the present study is simple and rapid. It was found that the DNA extraction procedure adopted for 50 spores also proves efficient for single spore, with slight modification. Hence, this protocol can be widely used for any PCR based analysis of AM fungi for future studies. It can be concluded that even in the absence of Chelex-100 resin, genomic DNA from AM spore could be amplified provided the time and duration of the PCR programme are standardized. RAPD is a useful diagnostic tool to analyze isolates (or populations) of individual species (Khon, 1992) and possibly to distinguish among species if enough primers are used.

Stahl and Christensen (1990) conducted a series of experiments in the field, green house and laboratory to investigate population variation among three geographic isolates of the widely distributed AM fungus *Glomus mosseae*. Uniform garden experiments were used to determine whether these populations from dissimilar environments are physiologically different ecotypes or phenotypic variants. Using *Melilotus officinalis* as the host, comparisons were made of the mycorrhizas formed spore production, spore germination, propagule density and the influence of the endophytes on host growth and physiology, large disparities were observed in the responses of the three fungal populations. Analysis of variance indicated highly significant differences among the *Glomus mosseae* populations in terms of amounts of mycorrhizas formed, spore production and their effect on biomass production, shoot phosphorus concentration and water

relations of *Melilotus officinalis*. These results support the hypothesis that populations of *Glomus mosseae* from dissimilar environments are genetically different races or ecotypes and suggest that there may be significant genetic and physiological diversity within this morphologically defined taxon.

An early example of variability among populations of AM fungus was provided by Daniels and Duff (1978) who observed disparities in germination and spore morphology in four isolates of *Glomus mosseae*. Powell *et al.*, (1982) reported substantial different effects on host plant growth and phosphorus uptake among isolates of *Glomus tenue*. Hass and Krikun (1985) reported large intraspecific variation in effects on host plant growth between *Glomus macrocarpum* isolates from different soils and within a soil. These evidences indicates that intraspecific differentiation is and important means of adaptation in AM fungi. The above papers confirm the broad distribution and variability of *Glomus mosseae* in widely different habitats. Similar results were observed in the present study. Diverse environment existing in different agro climatic zones could have contributed to the biological and molecular variability in *Glomus mosseae*, which was evident in this study.

SUMMARY

VI. SUMMARY

The arbuscular mycorrhizal fungus, *Glomus mosseae* (Nicol. and Gerd.) Gerdemann and Trappe, like many other AM fungi, have received considerable attention because they are known to occur globally in a broad range of dissimilar environments. There are many reports of inter and intra specific differences in the effectiveness of arbuscular mycorrhizal fungi for plant growth and protection, but the response range can be affected by host plant or soil conditions and the physiological bases of variations.

A study was undertaken to find the biological and molecular variability in *Glomus mosseae* isolated from different agro climatic zones of Karnataka with three major objectives: (a) to isolate and identify *Glomus mosseae* from different agro climatic zones of Karnataka, (b) to study the growth response of tomato to the inoculation of the isolates and (3) to study the molecular variability of isolates using RAPD analysis.

With the above major objectives, the *Glomus mosseae* spores from different agro climatic zones were isolated, identified and confirmed using specific primer for *Glomus mosseae.* The identified spores were mass multiplied and using funnel technique. These spores were used for plant growth response studies and RAPD analysis. In the plant growth response studies using tomato as host and sterilized soil the following results were obtained: The heights of plants were found to

increase steadily with number of days. In plants inoculated with *Glomus mosseae* isolates, the height remained always higher than uninoculated plants. However, the height differed significantly among the plants inoculated with various isolates. The numbers of leaves were found to increase constantly at different interval of time. The leaf numbers in plants inoculated with isolates were always higher than the control. Highest number of leaves was observed in plants inoculated with isolate of zone 4 and least were found in uninoculated pants. The total fresh weight and dry weight in the plants inoculated with *Glomus mosseae* isolates were higher than uninoculated plants. Significant difference was observed in total fresh weight in plants inoculated with the isolates. There was no significant difference in the fresh weight in the plants inoculated with isolates from zones 5, 6 and 8 as well as zones 4 and 10. Total dry weight did not significantly differ in plants inoculated with isolates from zones 7, 8 and 9b.

The spore count did not vary much between the isolates. This was shown statistically that only the isolate from zone 6 differed significantly from the other isolates. There were significant differences in the percent root colonization among the plants inoculated with isolates of zones 4, 5 and 7. Except for these isolates, significant difference was observed in percent root colonization in plants inoculated with other zones isolates. The maximum root colonization was recorded in the plants inoculated with zone 6 isolate and minimum in case of plants inoculated with zone 9b isolate.

The shoot P concentration in the inoculated plants was found to differ significantly from the uninoculated. The shoot P concentration in the plants inoculated with isolates from zones 7, 9b and 10 as well as isolates from zones 8 and 9a were not significantly different. The root P of inoculated plants was found higher than the uninoculated. There was no significant difference in the root P content of the plants inoculated with isolates from zones 7, 9a, 9b and 10 as well as plants inoculated with isolates of zones 5 and 6.

The specific primer for *Glomus mosseae* was tested against the isolates of seven different agro climatic zones. It was found that the, isolates from zones 4, 5, 6, 7, 8, 9 and 10 showed presence of *Glomus mosseae* through the amplification of 0.269 kb DNA band. Among the four replicates of zone 9, it was found that replicate 1 and 2 also showed specificity for *Glomus mosseae*, which was named as 9a and 9b respectively. A modified method of isolating DNA from spores without the use of Chelex-100 was standardized, as well as isolating DNA from a single mycorrhizal spore, this enables one to save time and cost.

The spores from seven agro climatic zones, which showed specificity for *Glomus mosseae* tested with specific primer, were subjected to RAPD analysis. Among the 16 random primer screened, four random primer showed reproducible amplification product for all the seven agro climatic zone isolates including two replicates (9a and 9b) from zone 9. The amplified product of four random primers viz., W10, W11, W15 and W19, showed considerable degree of

polymorphism between them. Based on the presence and absence of the amplification products, the Squared Euclidean Distances as well as dendrogram was constructed. Two clusters were clearly illustrated. The first cluster contains two groups; the first group had zone 9a and 8 isolates while the second had isolates of zone 7 and 6. On the other hand, the second cluster had two groups, with one group having zone 10 and 9b isolates while the second group having zone 5 and 4 isolates.

The percent polymorphism observed in the isolates is (58.33 %). This seems to be quite high within the species. This suggests that natural AM fungal populations exhibit unexpectedly high genetic diversity, despite the assumption that diversity in these seemingly asexual fungi could be low.

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VII. REFERENCES

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APPENDIX

APPENDIX

1. Vanadomolybdate reagent for phosphorus estimation

Solution A was prepared by dissolving 25 g of ammonium molybdate in 400 ml of distilled water. Solution B was prepared by dissolving 1.25 g of ammonium metavanadate in 300 ml boiling water. Solution B was cooled and then 250 ml concentrated HNO_3 added. Finally solution A and B were mixed and the mixture was diluted to 1 litre.

2. Buffers and Reagents used for Agarose Gel Electrophoresis

A. DNA Extraction Buffer for AM spore

1 X TE buffer

10 mM Trs-HCl pH 7.8

1 mM EDTA

B. TBE Electrophoresis Buffer

a. 10 X stock solution

108 g Tris base

55 g boric acid

40 ml 0.5 M EDTA, pH 8.0

Final volume made up to 1 liter with double distilled water.

b. 1 X working solution

89 mM Tris base

89 mM boric acid

2 mM EDTA

C. EDTA (ethylenediamine tetraacetic acid), 0.5 M

Dissolve 186.1 g $Na_2EDTA.2H_2O$ in 700 ml H_2O. Adjust pH to 8.0 with 10 mM NaOH (~ 50 ml). Make up the volume to 1 liter.

D. Ethidium bromide solution

1000 X stock solution, 0.5 mg/ml:

50 mg ethidium bromide

100 ml water

Working solution, 0.5 μg/ ml

Dilute stock 1: 1000 for gels or stain solution

Protect solutions from light. **Caution**: Ethidium bromide is a mutagen and must be handled carefully.

E. Loading buffer

20 % (w/v) glycerol

1 mg/ml (w/v) bromophenol blue

1 mg/ml (w/v) xylene cyanol

Store in aliquots at – 20°C and discard after 3 months.